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Medications Development: Drug Discovery, Databases, and Computer-Aided Drug Design

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Medications Development: Drug Discovery, Databases, and Computer-Aided Drug Design

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FOREWORD

We are at a point in the development and application of new technologies in medications development that is expanding at an incredible rate. This meeting brings together the various disciplines associated with new drug design, all of which make use of cutting edge technologies.

Receptor characterization through computer modeling techniques and the cloning and application of such clones in *in vitro* screening is greatly advancing the power of discovery programs for new pharmacotherapies. New modeling techniques, in particular the new means to use theoretical models as a basis for rapidly searching large 3D SAR databases for the discovery of pharmacophoric entities among previously synthesized compounds, is an area of technological advancement that changes almost on a daily basis. These changes are being spurred by rapidly decreasing costs and increasing sophistication in the hardware and software available.

Another new technological area of rapid growth is the mass synthesis and screening of peptides, where thousands of unique peptide structures can be prepared, screened, and new active individual peptides identified in a very short time. These techniques in combination with receptor clones, themselves resulting from characterization based on ligands prepared with new modeling techniques, are rapidly altering the way we search for new pharmacotherapies, both in the drug abuse area and in the pharmaceutical industry in general. Limited resources can now be focused in a discovery approach that is much more effective and efficient than was ever conceived of a decade ago.

This monograph provides an overview of some of these new techniques and approaches, particularly as they apply to pharmacotherapeutic development for drug abuse, and will hopefully serve to stimulate new ideas and their application and integration in the effort to solve the drug abuse problem.

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Strategies for Drug Discovery

Michael Williams

INTRODUCTION

From vaccines that have reduced many fatal diseases to the realm of history, to psychoactive agents that have permitted many people to live normal lives, to antihypertensive drugs that prolong and improve the quality of life, to contraceptives that have permanently changed sexual mores, the products of the ethical pharmaceutical industry have had a major and irrevocable impact on the social fabric of society. Yet many disease states still remain untreated or are currently treated by agents whose side-effect profile leaves significant cause for concern. The aging of the population, partly a result of the availability of more effective medications, has presented additional challenges in therapeutic agent development, with an urgent need for drugs to treat arthritis, cancer, neurodegeneration, and cognition impairment.

However, the process of drug discovery is complex and costly (Jack 1989; DiMasi et al. 1991), and innovative science is especially so. But drug discovery is more than the application of basic research knowledge and technologies (Black 1986); it involves many facets of project management and focus (Klimstra and Rafael 1992; Roussel et al. 1991; Jacques et al. 1992). To precisely define the drug discovery process and the parameters crucial to success is a difficult task. For each successful drug there are many accounts, both corporate and scientific, as to the process and the visionaries and facilitators involved in bringing the compound to the marketplace. Overviews of the drug research and development (R&D) processes (Sneader 1985, 1986; Williams and Malick 1987; Hesp and Resch 1987; Spilker 1988; Hamner 1990; deStevens 1991; Gilbert 1992) have been complemented by insightful case studies for several important drugs (Maxwell and Eckhardt 1990). Given the proprietary nature of the drug discovery process, the failures, the blind alleys, and the persistence that are such an important part of the drug discovery environment and that can account for up to 80 percent of the corporate research effort are rarely documented. Thus, the database from which to derive lessons that may aid in the implementation of new projects is limited. The commercial focus of the drug discovery process equates the success of a project with a product and rarely with the successful testing of a hypothesis (via the use of a new compound) that results in the

advancement of scientific knowledge. Given that discovery is a process of "learning things not already known" (Roussel et al. 1991), success is equally tenable when a project is terminated for technical or competitive reasons, resulting in the avoidance of further "sunk costs" (Staw and Ross 1989).

In this chapter, some integral elements of the drug discovery process are discussed, focusing on newer technologies and the scientific and business environments that dictate the feasibility of bringing a needed therapeutic entity to the marketplace.

THE DRUG DISCOVERY PROCESS

A uniform strategy for the drug discovery process has remained elusive despite considerable analysis. Like the science on which the drug discovery process is based, the approach to a problem involves many paths, with considerable trial and error. Strategies for drug discovery are dependent on interrelated factors that include:

- Corporate, research, and marketing department cultures
- Individual scientists working within a company
- Synergies among various research disciplines
- Morale and quality of research management
- R&D leadership
- Individual and corporate experience
- Extent to which the management of a company is accustomed to risk
- Serendipity
- A particular company's market franchise (the therapeutic areas in which it markets drugs)

Inevitably, many drugs are described in terms of the individuals who were associated with championing the research effort, often against considerable scientific and corporate odds. Although making for interesting (and often exciting) reading (Sneader 1985; Parnham and Bruinvels 1983; Teitelbaum 1989; Schwartz 1989; deStevens 1991; Djerassi 1992; Duncan, in press), drug discovery is inevitably a team effort requiring constant iteration based on experimental findings, planning, and synergies across many different scientific, development, and marketing disciplines.

The drug discovery and development process can be divided into distinct steps that interface with each other. The first step involves deciding on a given therapeutic target, which entails an iterative process involving the current state of the understanding of disease etiology, scientific knowledge and available technology, unmet medical need, and commercial opportunity. In diseases such as hypertension, for which there are many effective medications already in the marketplace, new programs directed at this target must focus on significant additional benefits. For agents being developed for diseases such as Alzheimer's disease (AD) or cancer, for which there are currently no effective or safe treatments, the decision to target the disease state is an easier one to justify even though the science involved represents a significantly greater risk.

Once a decision has been made to target a particular therapeutic area (e.g., cardiovascular, anti-infective), diseases within the given area can be evaluated and prioritized based on the existence of potential molecular targets, existing technologies (both in-house and external) and scientific expertise, competitive aspects of the marketplace, project maturity (and time to market), unmet need, ease of clinical trials, and so on. After this prioritization has been done, the process of drug discovery can begin.

From a historical perspective, drug discovery as a distinct scientific discipline is very much a 20th century endeavor that has undergone continuous evolution as the scientific skill bases that support the process have increased in sophistication.

Through the early 1940s, much of the process of drug discovery was dependent on plant sources and serendipity (Sneader 1985, 1990; Mann and Plummer 1991). The technology base was synthetic chemistry, often based on dyes, with qualitative testing of compounds as anti-infective agents, in whole animals, with microbiology and limited biochemistry to support compound evaluation. Among the drugs from this era were the fungal antibiotics.

A second phase of drug discovery emerged with advances in enzymology and protein biochemistry. Many of the biological pathways and processes were identified as enzymologists and pharmacologists defined new enzymes, receptor ligands, and their functions. As a result, although serendipity was still a major factor, drugs were now directed toward distinct molecular targets, The evolution of this phase was rapid, with increased Federal support of the biomedical sciences, growing sophistication in computational processing, and the availability of the personal computer.

The third phase of drug discovery is one represented by the popular vision of computer-driven discovery and development of compounds that not only treat

the symptoms of the disease but also lead to its cure. The potential for the Intellectually rigorous targeting of therapeutic agents to molecular targets whose genetics, structure, function, and pathophysiology are well understood is a noble goal. Unfortunately, this somewhat naive view of several promising yet still emerging technologies presumes more than biomedical science has learned to date. Although there are superior medications to treat hypertension, asthma, schizophrenia, and anxiety, for example, their etiology remains unknown, leading to a major dependence on hypothesis testing.

Interestingly, the drug discovery wheel has come full circle as fermentation, plant, marine, and invertebrate sources have again emerged as important sources of novel therapeutic entities. Among the compounds identified in the past decade from such sources are the cholecystokinin antagonist asperlicin (Chang et al. 1985) and the immunosuppressant FK 506 (Schreiber 1991).

Once a disease has been targeted and the appropriate test protocols have been assembled, it is still uncertain whether a particular approach will result in a drug. When little is known about the disease, to the extent that molecular targets are unknown or sufficiently ill addressed by the available compounds, test procedures usually depend on animal models. Such models are usually empirical, relying on either the inducement of disease-like symptoms via the use of exogenous agents or surgical procedures or on the expression of behaviors that have been defined for compounds known to be clinically efficacious. In the former instance, asthma can be induced by allergens in guinea pig and new compounds used to relieve the symptoms. However, this process does not define the causative factors for asthma in humans but does address the symptoms. Similarly, anxiolytic drugs are currently tested in several variations of a passive avoidance situation. In such a paradigm, an animal is taught to perform a behavior for a reward. It is then exposed to aversive stimuli so that it does not perform the behavior. When given a compound with antianxiety actions, disinhibition of the aversion behavior occurs so that the animal will seek rewards in spite of the aversive stimuli. Although the benzodiazepines and N-methyl-D-aspartate antagonists are active in this paradigm, its relationship to human anxiety is empirical. Identification of compounds in such paradigms is a useful indication of *potential* human activity, When a molecular target can be selected, there is still uncertainty as to whether stimulation or inhibition of that target will be effective in a given disease state. Thus, to a major extent, the design, synthesis, and testing of new molecular entities selective for a given target frequently provide the tools by which a hypothesis can be evaluated. If the hypothesis proves to be in error, information is added to the scientific literature but no drug is found. This is the working approach to drug discovery that is frequently unappreciated and unreported. If drug targets and the design of their ligands were as simple as the application of new technologies, more than 20 percent of the effort in drug discovery would reach fruition in the identification of clinical candidates. As it stands, 80 percent of the effort is valuable, if ultimately nonproductive, hypothesis testing.

EMERGING TECHNOLOGIES IN DRUG DISCOVERY

As noted, a major target in the drug discovery process is to develop a high degree of "rationality." This would represent an intellectually rigorous approach incorporating computer-assisted molecular design (CAMD), limited but sophisticated chemical synthetic effort, and highly focused biological assays. To many, rational drug design suggests that it is now possible, with the knowledge of the three-dimensional (3-D) structure and sequence of various drug targets, to design new compounds, by iteration, on a computer. Thus, the drug design process may become significantly less of a risk and quantally more resource efficient.

However, many of the enabling technologies that support the rational design approach are still in the emergent stage; that is, they have theoretical promise usually based on their use in the retrospective analysis of known compounds. With the exception of the enzyme thymidylate synthase (Appelt et al. 1991), molecular modeling techniques have yet to be generally used in a predictive manner.

It is important to recognize that individual technologies, however sophisticated, may become self-limiting if there is insufficient vision in their appropriate application and integration into the mainstream drug discovery effort. A goal-oriented discipline such as drug discovery is dependent on the coherent, focused, and resource-efficient use of necessary technology rather than technology for technology's sake.

Using a building as an analogy, the architect represents the individual who provides the global vision of the final product. In the process of building, he or she makes use of various technologies provided by experts such as carpenters, electricians, stonemasons, and plumbers in a highly integrated and focused manner. To allow the electrician, the artesan of what was high technology in the late 19th century, to drive the building process based solely on what electrical wiring could do would not be considered a particularly wise approach to completing the building project. To continue the analogy, the architect of the drug discovery process is by necessity the pharmacologist, who, because of training in a hierarchical systems approach, orchestrates the biological testing technologies to the desired endpoint.

Receptor binding—the use of radiolabeled ligands to "tag" drug targets, receptors, and enzymes-has revolutionized the ability to determine compound structure-activity relationship (SAR) in a rapid, cost-effective manner (Williams 1991). This technology resulted from early work on characterizing the insulin receptor by Roth and Cuatrecasas (Cuatrecasas 1974). In its present form (and diversity), receptor binding was driven by the work of Snyder and coworkers such

that binding assays for nearly 100 receptors or enzymes have been developed. The technology has also been used in the identification of new receptors and recepto subtypes.

Advantages of the technology include a direct analysis of the interaction of a ligand with a receptor (or substrate with an enzyme), the use of small amounts of material, and rapid throughput, Thus, as little as 3 mg of compound (sufficiently little to permit the analysis of intermediates from a synthetic pathway) can be run in 30 to 40 assays in 2 to 3 weeks at an estimated cost per assay of approximately $200. This allows for a binding profile to be developed, which can then be used as a potential predictor of diverse activity in a compound. The binding approach can be contrasted with more classical functional assays where as much as 2 g of material was used and 3 to 4 weeks elapsed before data on a compound were available. Although the cost in this instance was typically in the range of $1,500 to $3,000, such assays also provided information on whether a compound was an agonist or an antagonist at the receptor target. Using binding as prescreen, researchers can reduce the number of compounds put into more complex and time-consuming functional assays.

Targeted screening (Burch 1991; Williams and Jarvis 1990) involves the use of the receptor-binding technique to evaluate large numbers of compounds—20,000 to 50,000 per year—in multiple assays to identify new pharmacophores. Compound sources include herbal, marine, and bacterial fermentations as well as chemical compound libraries, The latter include dissimilar pharmacophores from chemical companies, compounds synthesized as part of a directed chemical effort, and novel structures with no known biological activity.

Targeted screening is an iterative process dependent on a finite availability of compounds and binding assays. Ideally, as newer targets are identified and assay systems for them are developed, compounds should be reevaluated in a continuous manner.

Molecular modeling, or CAMD, is an emerging technology that makes use of knowledge of the steric and electronic aspects of the receptor/ligand, enzyme/substrate interaction to identify pharmacophores or aid in their design or both (Marshall and Naylor 1990; Blaney and Hansch 1990; Snyder 1992). The target/ligand interaction can be studied from three vantage points: (1) knowledge of the SAR within a series and among series of pharmacophores, in effect approaching the receptor or enzyme from the drug perspective; (2) knowledge of the structure of the receptor or enzyme, approaching the problem from the receptor viewpoint (Hollenberg 1990); and (3) information regarding the receptor/ligand, enzyme/substrate interaction derived by 2- or 3-D nuclear magnetic resonance (NMR) (Fesik 1991), x-ray crystallographic, or other structural protein analysis methods.

Each of these approaches has inherent limitations. The compound SAR approach is limited in that the protein target (receptor or enzyme) is normally configured on a computer database in a minimal energy configuration with an approximation of water content. This approach has traditionally assumed that the protein and ligand have limited degrees of flexibility, a constraint that reflected the computational power available. With supercomputers such as the Cray 2, the protein/ligand interaction can now be assayed in real time with increased flexibility in the programing assumptions. It recently was noted that the receptor/ligand interaction can involve more than a single step (Saunders and Freedman 1989) and that receptors can induce changes in ligand conformation (Wuthrich et al. 1991) features that had been known for enzymes for a number of years. These facets of the receptor/ligand interaction present additional dimensions to the CAMD process.

Knowledge of the structure and 3-D conformation of the protein target provides an opportunity to identify the amino acid sequences and conformations that are responsible for ligand recognition and efficacy. These can be derived by knowledge of the primary sequence and, for a receptor, knowledge of which transmembrane helices are involved in ligand recognition. The interaction of various pharmacophores and compounds within a pharmacophore series can be used to identify the critical amino acids, When molecular biology is used to change these critical amino acids as point mutations, their importance in defining the ligand recognition parameters can be assessed (Hollenberg 1990).

Additional information regarding the biophysical aspects of the protein/ligand interaction in real time using NMR (Fesik 1991) can then be used to hierarchically integrate information from the previous two approaches to gain a more concise understanding of those properties of a molecule that impart selectivity, activity, and efficacy at a given protein.

CAMD is frequently a visually attractive technology based on colorful and complex computer images. From a theoretical perspective, it has the potential to significantly enhance the drug design process, As a technology, it is limited by several necessary assumptions already noted and by limitations in database construction. Information from biological assays cannot yet be downloaded into CAMD programs while knowledge on protein structure is currently being built. In addition, the CAMD process is limited because few biological data are available. Activity and efficacy represent two compound properties that are amenable to existing technology. Bioavailability and metabolism have yet to be addressed by this important technology. Consequently, to assume that CAMD is presently at a stage where it can be used to design, iterate, and select new chemical structures independently of biological testing and intuition is to devalue the technology by overstating its present-day capabilities.

Molecular biology is the use of recombinant DNA (rDNA) technology to express biologically important proteins and peptides in prokaryotic and eukaryotic cell systems (Schoepke 1988). Although the phrase "molecular biology," or "biotechnology," has become a broad descriptor for many facets of modern biology, including molecular biology, biochemistry, and immunology, it also includes the venture capital-driven revolution in biomedical research, which is discussed further below. Included in the technique of molecular biology are gene cloning, splicing, and expression and polymerase chain and ligase chain technologies (Koshland 1991).

The drugs produced by molecular biology, proteins or peptides known as biologics, were initially considered as replacements for the small molecules produced by synthetic chemistry. Because biologics were "natural" drugs, they were considered, in the absence of data, to be essentially free of the side-effect profiles of more traditional drugs (Buell 1988). Although this has not proven to be the case (Williams 1990; Williams et al., in press), many useful therapeutic agents such as insulin, insulinotropin, human growth hormone, tissue plasminogen activator, erythropoietin (EPO); various cytokines, including interleukin 2 and colony stimulating factors; and other growth factors and interferons, monoclonal antibodies, vaccines, and blood products (Factor VIII and Factor IX) have proven the usefulness of biologics as therapeutics. The majority of biologics are systemically active replacement therapies for hormones and other blood-borne autacoids. Their effectiveness is then a function of their being administered systemically and acting at sites proximal to the blood supply. Although useful in certain circumstances, biologics in their present forms cannot replace traditional drugs, which cover a broader spectrum of activity and tissue specificity and have superior pharmacokinetic properties.

The role of molecular biology in the drug discovery process has been increasingly enhanced as a tool that, when properly integrated, can offer major benefits to the drug hunter. The cloning, transfection, and expression of receptor and enzyme genes can be used to prepare mammalian cell lines specific for a given drug target, These cell lines can then be used instead of animals to evaluate the activity and efficacy of new compounds free of the complexity of cells derived from animal sources, Deliberate alterations in the genetic material can be used to develop point mutations in amino acid sequences to assess the importance of various substitutions in the expressed protein as well as to prepare chimeric receptors (Kobilka et al. 1988) that aid in the understanding of receptor recognition and the transduction processes. Complementary DNA technology can be used to identify new proteins and, using tissues from patient populations, to determine the genetic bases of various diseases.

Such information can then be used to transfect laboratory animals to make transgenic animals that have the genetic code for human diseases. These

animals can be used to study the etiology of the disease and the effects of new compounds on disease progress. Transgenic models of various cancers, AD, and diabetes are being developed. In addition, the transgenic approach is being used to produce biologics in cow's milk.

In the AD area, models of cognitive impairment involve either chemical lesions of the basal forebrain cholinergic system or the use of aged rats or primates. In both instances, the experimental paradigms are artificial. Although it is known that the cholinergic system undergoes degeneration in AD, there are major deficits in other neurotransmitter systems as well as plaque and tangle formation associated with amyloid deposition (Selkoe 1991). Although ablation of forebrain cholinergic systems is an approach to an animal model of AD, it does not reflect the nuances of the human disease state. Similarly, in aged animals, the cognitive impairment is not necessarily reflective of AD. A primary focus in the past year by at least four groups has been to transfect rodents with various amyloid precursor protein constructs with the expectation that overexpression of amyloid protein will lead to AD pathophysiology. To date, the results of these transgenic studies remain controversial (Marx 1992).

Peptide combinatorial library technology is an emerging technology that involves the production of many millions of different peptide sequences to enhance the discovery of pharmacophores for peptide receptor targets. The technology exists in varying forms as exemplified by the Affymax (Fodor et al. 1991), Selectide (Lam et al. 1991), and Iterex/Houghten Pharmaceuticals (Houghten et al. 1991) approaches. Given the attractiveness of peptide receptors, adhesion molecules, and so forth as drug targets and the paucity of agonist pharmacophores identified by conventional peptide chemistry approaches, the peptide library approach represents an important new tool for drug discovery.

Antisense technology encompasses the use of synthetic oligonucleotides as potential drugs. This technology has been a primary focus in venture capital companies and has been somewhat modestly described as the "first revolution in drug discovery since the discovery of the receptor" (Crooke 1991). Proponents of the approach view traditional drugs as molecules acting on cellular proteins (enzymes and receptors). These are the product of mRNA expression, By selectively blocking events at the ribosomal or nuclear levels, antisense ligands can potentially prevent the expression of aberrant proteins at a much earlier stage "downstream" and theoretically may be more precise drugs than those blocking the function of the expressed protein. The technology involves oligonucleotides of 15 to 18 bases that act at three levels (Crooke 1992; Riordan and Martin 1991): (1) as agents that bind to mRNA to selectively prevent protein synthesis, (2) as triple helix (triplex) agents that block DNA promoter regions, and (3) as aptamers, randomly coiled oligonucleotides that interact with conventional protein targets (receptors and enzymes). Limitations to this technology are bioavailability and selectivity. How does an antisense drug make its way, not only through the gut

and liver, but also inside the cell to reach the nucleus? And if this transition can be attained, how is selectivity implied?

Intracellular *receptors or hormone-responsive elements* (HREs) represent newer drug targets. These entities, nuclear receptors for the steroid receptor superfamily (Evans 1988; O'Malley 1990), are present on DNA and act as transcription factors that modulate promoter activity to stimulate or inhibit gene expression. Several putative receptors belonging to the steroid receptor family have no known ligands and have been designated as orphan receptors (Denner et al. 1990) whose activity may be modulated by phosphorylation in the absence of ligand. One such orphan receptor, the chick ovalbumin upstream promoter transcription factor, is activated by dopamine (Power et al. 1991), providing an unusual example of an intracellular HRE that is responsive to an extracellular ligand. The HRE technology has been commercialized by Ligand Pharmaceuticals and is being used by a number of companies on a contract basis as **a** targeted screening approach for their in-house chemical libraries.

Technology Acquisition

The acquisition and incorporation of new technologies into a research organization can be an expensive, continuous, and, frequently, incremental process. Maintaining a competitive edge requires "technological literacy" whereby a research organization has a complete and viable repertoire of techniques necessary for the advancement of drug discovery projects within the organization.

Keeping up with new technologies requires the addition of new staff with expertise in the necessary disciplines or the retraining of existing personnel (Williams et al., in press). The delay in the latter can be 1 or 2 years and may represent a self-defeating catchup process. No sooner do existing staff members become proficient in the new technology than the needs become redefined. In addition, the transitional nature of the retraining process creates a negative impact on the ongoing research effort, inasmuch as those individuals being retrained are no longer able to perform their previous duties. Although the addition of new staff requires incremental head count, the technology acquired is typically available within 3 to 6 months of recruitment and does not involve the transfer of staff from ongoing research efforts. Nonetheless, the issue of redundant technology is not addressed, although retraining can occur when a newer technology replaces an existing one.

Another approach to technology acquisition is contract research. A pharmaceutical company can pay a university laboratory to evaluate a compound or series of compounds in a test procedure ongoing in that laboratory. Similarly, a commercial screening laboratory can also be used to "hire" technology without the need for in-house retraining or increasing head count.

As technologies have become increasingly sophisticated and proprietary via the formation of biopharmaceutical startups, research collaborations have become the means to acquire cutting-edge technology in a highly focused and cost-effective manner. By investing in smaller, high-tech companies, the larger pharmaceutical companies are able to acquire "turnkey" technology in an area without the necessary commitment of increasing staff in-house. This investment can provide a short- to medium-term solution to recruiting, which may be limited by staff, space, and capital equipment availability, at the same time allowing feasibility assessments of new technologies or research areas. A company could provide seed money to four or five biopharmaceutical companies involved in different technologies or approaches to a therapeutic area and follow the relevant research activities over a 2- to 3-year period before investing heavily in one particular approach. This strategy can provide the means to enhance the amount of exploratory research a company can fund, while avoiding the problem of investing in such research in a new technology or area only to find that it is not relevant or practical and then having to find alternative projects for the staff members who were hired.

ORGANIZATIONAL ASPECTS OF THE DRUG DISCOVERY PROCESS

The establishment of the project team necessary to integrate the various complex technologies involved in the drug discovery process is highly dependent on the structure of the discovery organization. Three main types of organization exist within the pharmaceutical industry.

The *functional or technical line* organization (figure 1) is made up of distinct, technologically based departments such as chemistry, biochemistry, and pharmacology. Support groups for these major drug discovery disciplines would include structural biology, CAMD, formulation, drug metabolism, and so on. A project would function across these line organizations, with individuals from each of the major disciplines involved in the ongoing project. Reporting responsibilities would be through the technical directors, who typically would work together to allocate and prioritize resources. The advantage of this type of organization is that it permits technological specialization with an emphasis on "cutting-edge" science. In addition, resource prioritization is highly flexible in that head count can be moved to higher priority projects as necessary. In addition, there is a central vision as to where the organization is directing its efforts. The disadvantages are that the individual projects are superimposed onto the technical line organization and that the directors of each of the technologies (or the research director) decide on priorities. This situation can often lead to friction between different projects and the technical department. The aggregate resources requested are viewed by the technical director as in excess of his or her resources, whereas the project members view resources used in maintaining cutting-edge technology as negatively affecting their project. Another disadvantage is a perceived diffuseness in the responsibility

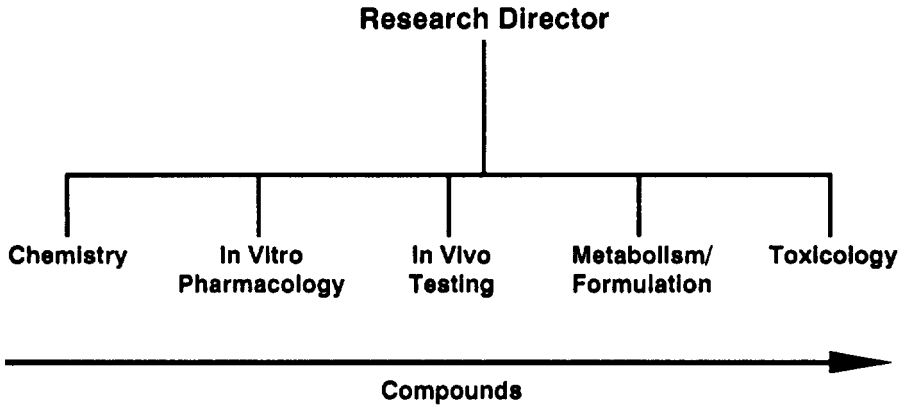

FIGURE 1. *Functional line organization*

for project goals because most individuals will be working on two or more projects.

The second type of organization is that of the *dedicated project team* (figure 2). In this type of organization, the technical disciplines necessary for drug discovery are contained within a single unit. Thus, a group of chemists and biologists focus their efforts exclusively on the project. The reporting relationship is to a project leader. The major advantages of this system relate to the focus on a single project with shared therapeutic commitment and a lack of conflicting pressures. The disadvantages are a lack of flexibility, which makes it difficult to effectively reallocate resources without major changes in global priorities; a diminished focus on enabling technology development; and a narrow focus on the project, which has the potential to overlook scientific advances outside the needs of the project.

The third type of organization is that of the *project matrix* (figure 3). This is a mixture of the previous two types and involves a basic functional line organization involving technical specialties with a project team cutting across the organization. Individuals have line reporting relationships to a technical director but are also responsible to a project leader. The advantages of this system reflect the best of the previous two as well as an accentuation of the negative. Thus, the matrix is flexible and technology is current, but reporting priorities are complex. Unless there are sufficient resources, technical directors perform a balancing act between the needs of the various projects while providing time for their reports to maintain their technical skill base. The matrix organization can on the one hand be very dynamic and creative and on the other the source of considerable friction.

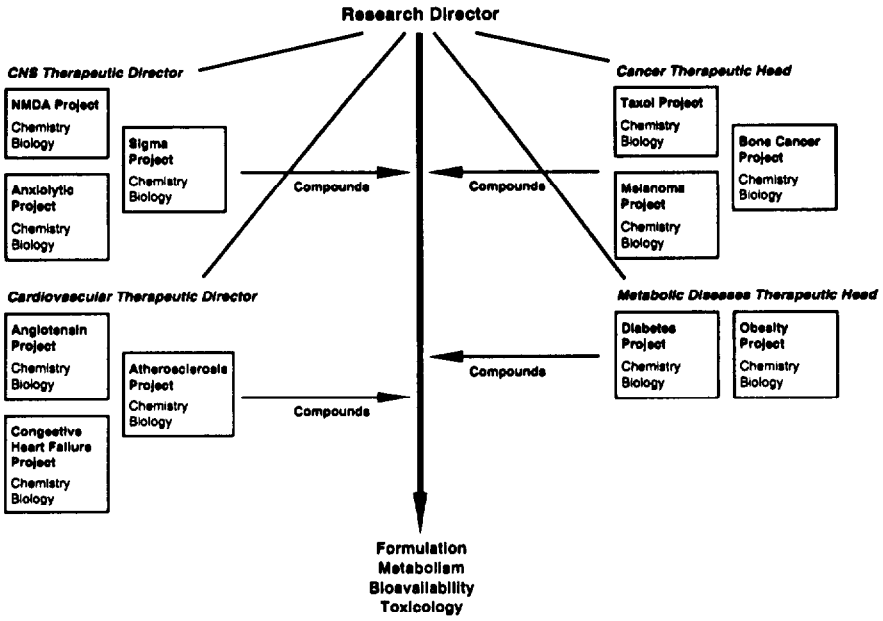

FIGURE 2. *Dedicated project team organization*

Variations on these three major themes abound. Some organizations have line organizations based on therapeutic areas, and others superimpose a therapeutic matrix on a line organization, Flexibility in some companies is achieved by having chemistry "swat teams." These function independently of the mainstream and are used by the organization to push projects forward rapidly by injecting a bolus of chemical resources. Such entities are extremely valuable when the project is highly competitive and lead structures among several companies are similar. Discovery organizations (that part of the R&D organization conducting research) may undergo further modification via the nature of their interface with the Development organization, the complementary component of the R&D process involved in clinical evaluation of new components.

The design of the line Discovery organization follows, for the most part, organizational structure in academia. Deviations from this in terms of the various forms of therapeutic and project matrices as well as the mode of interfacing with Development frequently arise as the result of the evaluation of an existing organization by a management consultant group. Spilker (1988) has described such groups as "lack[ing] adequate knowledge about the drug industry [and] drug development." Nonetheless, management consultant groups are often used by companies in an effort to increase research

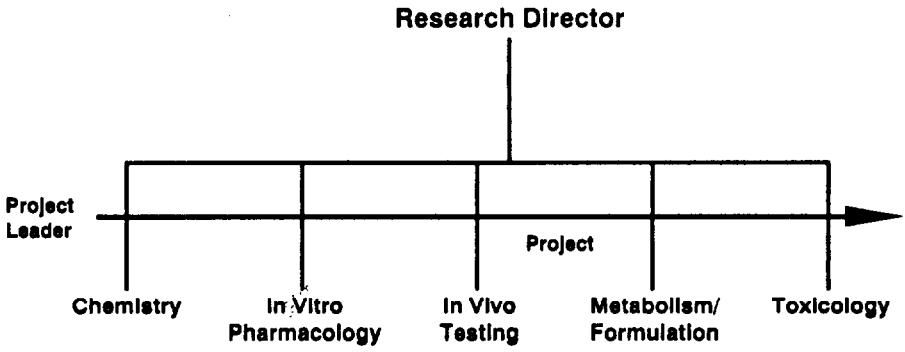

FIGURE 3. *Project matrix organization*

productivity. This effort usually results in a reorganization of the research department, either functionally or from a personnel perspective. For many in the industry, this reorganization has become a seemingly continuous process that in itself is an effective barrier to productivity. On the positive side, constant reorganization does provide gainful employment for management consultant groups.

Project management represents an important adjunct discipline to the project team approach. Inevitably, irrespective of the type of project team or the organizational backdrop, there is a need for the objective prioritization of resources to meet project needs. A typical midsize pharmaceutical company will have 12 to 25 research projects all with their own constituency and resourcing needs that probably exceed those of the organization by at least twofold. For the research director, the ability to prioritize the project needs via a project management team that is independent of the individual projects with a prime focus on compound flow can be a major benefit.

SCIENTIFIC SKILL BASES

Organizations dedicated to the discovery of new therapeutic entities are multidisciplinary in nature, involving chemistry, biology, and computational and physical sciences. Many subspecialties exist within the major disciplines, reflecting the complexity of the drug process and the specialization of modern-day science. Because an individual is unlikely to have more than passing familiarity with these various disciplines, the drug discovery process has become increasingly dependent on the team approach (Drucker 1988; Roussel et al. 1991).

Included in the discipline of chemistry are organic, medicinal, physical, process, theoretical, and computational chemists, each of whom represents a subspecialty of chemical synthesis with different experiences and techniques. As an example, the chemical goals involved in the novel synthesis of milligram quantities of a new compound are very different from those involved in bulk drug manufacturing for commercial sale. In the first case, the synthetic pathway is developed to reach a new molecule as rapidly and easily as possible. There is little consideration for economies of scale, cost of starting materials, number of steps, yield, timeframe, or synthesis. As a result, synthetic costs per gram of compound at this stage can be as much as $50,000. At the stage of bulk drug manufacturing, synthesis is carried out in line with the U.S. Food and Drug Administration's (FDA) Good Manufacturing Practices conditions. The focus is on cost reduction, ease of synthesis, yield, and quantity of material. Consequently, there is a very different focus on how the compound is made. When kilogram quantities are made, the cost per gram moves progressively downward from about $1,000 to $5.

Although the discipline of chemistry is primarily involved with compound synthesis (deStevens 1991), there is a recent trend toward training "hybrid" chemists with skills encompassing both compound synthesis and molecular biology. Although one benefit from melding the two disciplines has been to more effectively understand the interactions between compounds and their protein targets, it has also augmented an increasing shortage in chemistry graduates with interest and experience in organic synthesis.

Biological disciplines are considerably more diverse and encompass biochemistry (including enzymology); pharmacology (including both in vitro and in vivo aspects of cell, tissue, and whole animal function); molecular biology; cell biology; pharmacokinetics; drug delivery; and drug formulations. It is probably a truism that the biological sciences involved in the drug discovery process are considerably more dynamic than the chemistry skill bases, because change occurs much more rapidly in the biological disciplines and at an ever-increasing pace.

Computational, physical, and organizational disciplines affecting the drug discovery process should not be overlooked. These include CAMD, NMR, and x-ray crystallography, which have already been discussed. Underlying these disciplines are support services, such as research computing, that provide a great deal of programing skill and assistance to benefit computers and the other sophisticated instrumentation that drive these technologies forward.

Information scientists make a major contribution by providing the mechanisms to maintain currency with the scientific literature as well as by designing and supporting laboratory information management systems and databases. Tracking compounds, their activities, and archival storage is an underestimated technology that has a major impact as organizations mature and their information bases

expand. Patent activities, an increasingly complex facet of the biomedical research arena, can also be included under this aspect (Grubb 1987; Vieillefosse 1990; Yevich 1991).

However, drug discovery should not be viewed as a technology-driven process, a trend that unfortunately has emerged throughout the industry in the past decade but, rather, as a technology-facilitated one. Technologies represent the tools by which to move the discovery process forward. However, they are fragmented parts of a whole and need a unifying discipline and vision to integrate their contributions and drive the process toward goals that are more global. The discipline that provides this focus is pharmacology. As a technology, pharmacology has had a major role in establishing the pharmaceutical industry (Swann 1988); as a discipline, it seeks the technologies necessary to discover and characterize new drugs rather than abstract uses for the technologies themselves. Although pharmacology is not as widely practiced a discipline as it was 20 years ago and is not widely taught within the U.S. university system, its rightful reemergence as the driving force for drug discovery is reflected in the increasing numbers of pharmacologists from European universities being hired by US. drug companies,

THE PROJECT TEAM APPROACH

Ideally, a project team should include the necessary skill bases, resources, and decisionmaking authority to ensure that the project proceeds in an expeditious, timely, and resource-effective manner (Jack 1983). The project team should be viewed as a dynamic organization driven by goals rather than technology. Thus, the project team process can be viewed in distinct stages as illustrated in figure 4.

The initiatory stage involves the validation of the concept, which may be taken from existing work from the literature and require internal repetition or may involve an original idea that has to go through the same process of systematic evaluation as any scientific project. In either event, other than the chemistry required for small amounts of reference compounds, this stage of the project would involve a nucleus of biological disciplines. The staff needs of such a project have been estimated at between 8 and 12 scientists, Once the concept has been validated and accepted by research management, additional resources would be added and the project would then move to a *planning* stage. This would involve the development of the appropriate screening procedures and their validation in regard to compound flow; the sequence would be incorporated as the project flowchart. At this point, dedicated chemical effort would be added to begin the search for lead compounds. Project resources would increase to about 16 scientists. Once a lead series had been identified, the chemical effort would be enhanced to effect a *realized* project—the fully resourced effort involving chemistry and biology with appropriate milestones, compound target dates,

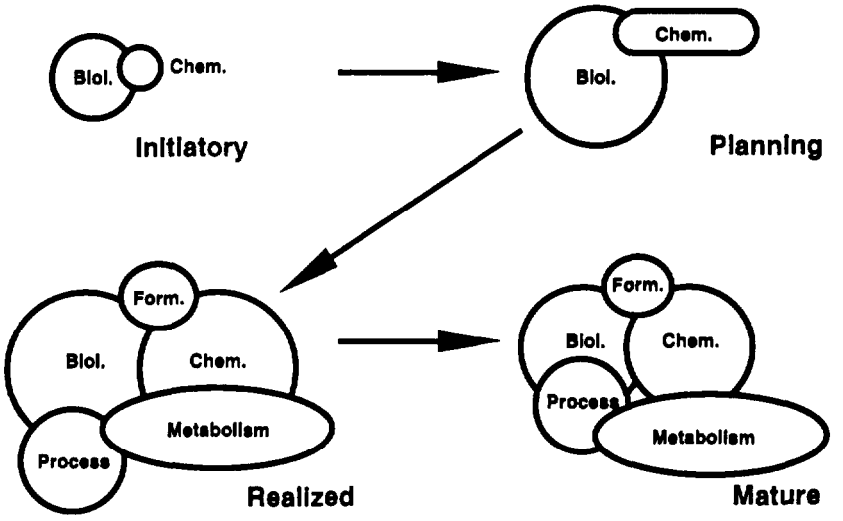

FIGURE 4. *Schematic of the stages of a Discovery project*

KEY: Biol.=biology; Chem.=chemistry; Form.=formulation; Process=process chemistry

and corporate visibility. The size of the project at this point would be about 25 people, with additional support in terms of formulation, drug delivery, and process chemistry to begin planning for the scaleup of synthesis of the compound to the larger quantities required for toxicology and clinical testing. With the emergence of a compound candidate for toxicological evaluation and the identification of a backup compound, the project can be reduced in size, releasing resources for new initiatory efforts. At this stage the project would be termed *mature* and, although still involving formulation, drug delivery, and process chemistry, would be reduced in size.

The timing for termination of effort on a given project has been the topic of considerable debate. One school of thought would argue that a lead compound and a backup compound distinguished by a different chemical structure with related properties would be sufficient. Given that the time when a compound is identified to its progress to marketing approval is in the range of 5 to 8 years, others have argued that a gatekeeping effort should be maintained until the compound reaches the marketplace. If unexpected problems occur, the project then can be revived relatively easily to continue the search for a better compound.

The number of individuals on a project obviously affects the rate of progress toward lead compound status. Clearly, increasing resources, to a point, can facilitate the objectives of the project. Inevitably, irrespective of the organizational structure, there is considerable competition for both finite and incremental resources.

Some projects in larger pharmaceutical companies have been rumored to have 100 to 150 people working on them to accelerate the pace toward compound identification. Some companies use chemistry "swat teams" to move resources from one project to another to ensure critical mass at crucial points.

The compound flowchart (figure 5) offers a convenient focal point for project team efforts. In it the individual test paradigms are identified with appropriate criteria established on an ongoing basis as compound properties are improved. By this means, every member of the project is familiar with the immediate goals of the project, when a compound has satisfied the criteria, and whether it should continue forward in testing. Structuring the various test procedures used for compound evaluation into a flowchart is a highly effective facilitatory mechanism to enhance efficiency and support for project goals.

COMPOUND IDENTIFICATION AND OPTIMIZATION

A drug is a chemical entity that by interacting with a specific molecular target effects a change in cell and tissue function to ameliorate the effects of trauma or tissue pathophysiology. Such compounds are usually small molecules with molecular weights in the range of 250 to 700 daltons and, when racemic, are usually developed in their enantiomeric form unless additive or synergistic benefit can be demonstrated for the individual components of the racemic mixture. Although agonists are effective as drug entities, especially in the area of rDNA-derived drugs (biologics), the majority of effective agents are antagonists acting to reduce the actions of endogenous agonists that contribute to the disease state.

Optimal characteristics of a drug are that it be (1) pure, preferably as a single enantiomer if chiral; (2) efficacious on repeated administration; (3) safe in terms of its beneficial actions; (4) affordable within the context of patient use and cost of discovery and development; and (5), from a commercial viewpoint, patentable, either by composition of matter or by use. Safety issues are highly dependent on the disease or condition being targeted. For example, for an appetite suppressant for use on a daily basis for reducing food intake, the safety margins in terms of unwanted side effects would have to be extremely high. For an agent to be used in the acute treatment of stroke, safety issues, although present, are balanced against the brain damage resulting from the reduction in blood flow to the brain. Irrespective of the nature of the disease

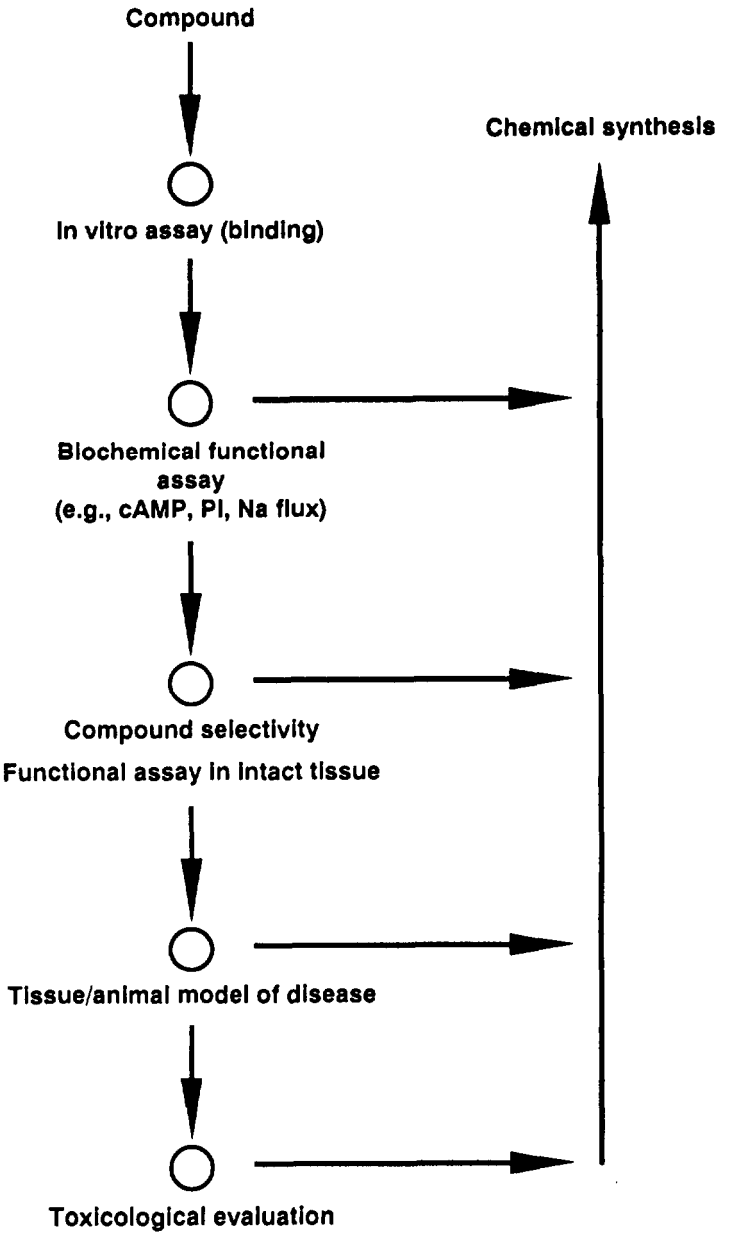

FIGURE 5. *Compound flowchart*

Each circle represents a predetermined decision point with appropriate criteria. PI=phosphatidyl inositil; Na=sodium.

state, however, a compound with fewer safety aspects than existing medications and no significant, additional benefits is unlikely to be a clinical entity.

The lead compound directed toward a disease target may arise from two sources, by chemical modification of a natural receptor ligand or enzyme substrate (Black 1989) or by the screening of diverse compound sources against a molecular target Williams and Jatvis 1990).

Once an active lead compound is found, it is refined by further synthetic effort to develop an SAR for a number of properties of the molecule, including:

- Activity: a measure of the interaction of a ligand with its molecular target, expressed as a K_i (inhibitory constant) for a ligand-receptor interaction or K_m (Michaelis-Menten constant) for an enzyme.

- Efficacy: the ability of a ligand once bound to effect a change in cell and tissue function. This refers to whether a compound is an agonist or an antagonist at a receptor or a substrate or an inhibitor for an enzyme. For a receptor ligand, intrinsic efficacy is the relative ability of a ligand to produce a functional effect, biochemical and/or physiological, compared with a reference agonist. A full agonist has an intrinsic efficacy of unity, whereas an antagonist, which lacks efficacy, has no agonist activity and has an intrinsic efficacy of zero.

- Selectivity: a measure of the ability of a ligand to interact predominantly with a single molecular target. The concept of selectivity often involves considerable semantic issues. Thus, selectivity may be anywhere between a 10-fold and a 1,000-fold delineation between the activity or efficacy at the desired target compared with its ability to interact with other targets, In vivo, selectivity—as defined by the ability of a compound to produce a preclinical action thought to reflect the therapeutic action of an agent compared with unwanted side effects—is the therapeutic index.

- Bioavailability and pharmacokinetics: crucial, yet frequently elusive, properties of a compound series. The susceptibility of a compound to glucuronidation, sulfation, and so forth, and its ability to cross the gut and blood-brain barrier are difficult parameters to define. In vivo estimates are compounded by a multiplicity of factors contributing to the pharmacodynamic actions of the compound. These include the enzymic modification of the compound, especially when given orally; its lipophilicity, plasma levels, and half-life; and in the case of central nervous system (CNS)-active agents, the relationship between plasma and brain concentrations and the retention of the compound in the brain.

Species differences in metabolism, rat vs. primate vs. human, become a major confounding feature of bioavailability and pharmacokinetic studies. Compounds may be 90-percent bioavailable in one species and less than 10 percent in another. Predicting what the situation might be in humans becomes very difficult. Extensive efforts in defining such properties using in vitro study approaches (e.g., human liver slices) have yet to prove predictive (Kaphegian and Traina 1990).

Compound Status

As seen in the flowchart (figure 5), the establishment of project norms for identifying compounds of interest can greatly enhance communication within a project and avoid misunderstandings as to the priority of a compound. With an extensive synthetic effort, many hundreds of compounds may be made in a year. Although each of these is a well-known entity to the chemist who made it, the biologist, especially one working in a tunctional line organization where he or she is involved in several projects, needs additional descriptors for the compounds beyond their company numbers. A system used by companies is a list of new compounds according to their biological activity and evaluation status. One such system involves designating compounds by a numerical system. A category 4 compound is one that has reached a predetermined cutoff point for activity in the primary assays of the flowchart. When selectivity and function have been established, the compound becomes a category 3 compound. With further evaluation in whole animal tests, bioavailability and half-life determination, and preliminary toxicological evaluation, a compound would achieve category 2 status and high visibility within the R&D organization. At this time it would undergo full review as a potential drug candidate and further workup in terms of formulation as a toxicological candidate. This would lead to the synthesis of a sufficient amount of compound for toxicological as well as stability testing. On entering toxicology, the compound would be designated as category 1. On completion of the toxicology required for the Notice of Claimed Investigational Exemption for a New Drug (IND) submission, the compound shifts into a similar prioritization system within Development. The advantage of such a scheme is that each compound, when of sufficient interest to a project team to be elevated to a given status, achieves instant recognition within the organization so that it can be appropriately prioritized.

Compound Value

The lifeblood of any drug company is reflected in the proprietary compounds it has patented. Patents are of two major types: composition of matter patents, which cover the molecules synthesized, and use patents, which cover specific uses for these entities. Typically, within a drug company both composition of matter and use are contained within a single patent series. In some instances, however, where unexpected data regarding the biological activity of a compound have been derived, a use patent can be obtained. This can extend the

proprietary life of a compound beyond that of the initial patent. This strategy is useful when the patent applicant in both instances is the same but can become complex when a second party has a use patent on a compound owned by another party. For a further discussion of patents, the reader is referred to articles by Grubb (1987), Vieillefosse (1990), Yevich (1991), and Hueni (1992).

Compound Safety

Once a compound has been found to meet criteria for potential use as a drug, its safety must be extensively evaluated to ensure that the potential risk-to-benefit ratio is such that a drug will not produce significant damage under specific conditions of use. Toxicity should be clearly dissociable from efficacy (Cavagnaro and Lewis 1987).

After preclinical evaluation, a potential new drug is evaluated in acute (14 to 20 days), subchronic (28 to 90 days), and chronic (3 to 24 months) toxicity paradigms. Acute studies are performed in rodents of both sexes using oral and parenteral routes of administration. Such studies are required before Phase I studies may be conducted in humans. Dose-ranging studies are performed to identify a dose that is safe in normal subjects and that can be used as a basis for efficacy testing in the target population in Phase II trials. Subacute mutagenicity studies (Ames test) represent the first test in which a new pharmacophore is assessed before recommendation for indepth toxicological evaluation.

The duration of initial toxicity testing is very dependent on the type of drug and the plans for Phase I trials. In the case of an antibiotic that will be used for the acute treatment of opportunistic infections, the data required for human trials are significantly less than those required for a contraceptive pill for which usage is measured in years. Carcinogenicity, mutagenicity, teratogenicity, and behavioral toxicity testing occurs in more lengthy trials as a compound is moving through Phase I and Phase II trials in humans. For further information on toxicological evaluation, refer to Cavagnaro and Lewis (1987).

DRUG DEVELOPMENT

Research/Discovery and Development may be separate units, organized along therapeutic lines reporting to separate heads of R&D. Alternatively, the R&D organization can exist in a self-contained therapeutic organization with a single head often known as a Strategic Business Unit. In either event, the relationship between R&D in terms of compound movement is a crucial one. Without synergy, Research may bring forward compounds for which there is no interest in Development. This can be of special concern in the present business environment where there is a continuing need for new products. For a Development organization charged with a well-balanced, temporally

sequential product pipeline, compounds can be taken from the internal organization or in-licensed from other companies. Resources committed to the latter usually affect Research resources and the ability to develop internal candidates. It is therefore important that communication between the two groups is optimal as well as flexible, with Discovery responding to Development needs and Development actively responding to high-risk, innovative approaches to drug therapy.

Given the nature of such innovation, the enthusiasm of the Development organization for a totally novel compound is crucial to its introduction to the clinic and the risk taking necessary to establish a novel therapeutic target. This is exemplified in Glaxo's portfolio of serotonin receptor antagonists (Waldholz 1991), which were novel compounds in search of diseases for which no known treatments existed. A popular example of perceived shortsightedness in developing new medications is that of the antiulcer medication, the histamine H_2, blocker cimetidine (Slack 1989; Duncan 1990).

When this compound was developed in the 1960s treatments for gastric ulcer encompassed antacids and surgery. The leap of faith in developing a totally new approach to ulcer therapy in light of existing effective medications was considerable. Although the retrospective viewpoint suggests that the mechanistic approach of blocking H_2, receptors was obvious, there was considerable risk in bringing cimetidine into the clinic with the long lead time to the marketplace. Inevitably, development and marketing are forecasting the medical need for a new compound 8 to 10 years in the future. For an area in which there are existing medications, there are known criteria against which new compounds can be compared. In new areas, or in areas where a totally new mechanistic approach is being targeted, the value of the innovation can be assessed only in the clinic. Yet before a compound reaches this stage, the momentum to the marketplace takes considerable faith and an element of risk taking that is not always present within large corporations. As an example, hypertension was effectively treated in the mid-1960s with diuretics and β-adrenergic blockers. The vision of Ondetti, Cushman, Horowitz, and their colleagues in the 1970s led to the discovery of the first angiotensin-converting enzyme inhibitor, captopril (Cushman and Ondetti 1980). This compound was considered by many to be not only superior to existing treatments for high blood pressure but also the definitive drug for this indication. Statements were made at the time that further medications for the treatment of hypertension were unnecessary, this in the face of a good percentage of "nonresponding" patients, especially in the African-American population. In the 1990s however, renin inhibitors and angiotensin-II antagonists represent newer approaches to the regulation of blood pressure that may have additional indications related to congestive heart failure and atherosclerosis, two complications of cardiovascular function that are in need of improved approaches,

The synergies between R&D have been the subject of numerous articles and have assumed the status of a research project on their own. This should not result in the trivialization of the interaction or the impact that this relationship has on a technology-based organization (Roussel et al. 1991). Frequently, a productive Research organization has less than optimal channels of communication with the Development organization. In the pharmaceutical industry, the nature of the science is such that there is a large need for buy-in from the Development organization to ensure that a compound with high potential receives appropriate prioritization.

A lack of synergy between R&D can result in compounds appearing as toxicological candidates taking the Development organization by surprise. Another facet of the R&D interface, which operates more in the management of R&D, relates to the balance in needs and resources. A highly productive Discovery group can present the Development function with more compounds than the Development organization can handle. Management is then left with establishing priorities, either by increasing Development resources, usually at the expense of the Discovery effort, or putting compounds on the shelf and thus raising the question of the need for urgency in the Discovery process. In the past, not a few R&D operations have disbanded Discovery efforts as compound candidates were produced, the logic being that the product pipeline was full and that further compounds from Discovery could not be accommodated. Because attrition is a major feature of the various stages of the Discovery process, the loss of compound candidates caused by unexpected findings in clinical trials with no Discovery organization to find replacement candidates made such organizations extremely vulnerable within the global marketplace.

CLINICAL TRIALS

Clinical evaluation of a new compound is divided into discrete stages designated by FDA and comparable organizations in the European Economic Community (EEC). Before a drug can be tested in humans, an IND package containing information on the safety, activity, and chemical properties of a new compound is filed with FDA. In EEC, this process is known as the Clinical Trial Certificate (CTC).

FDA is obligated to respond to the IND within 30 days, at which time, if there are no objections from FDA, a company may proceed with clinical trials. Phase I studies focus on the evaluation of compound safety in normal human volunteers using dose-ranging studies to determine safety and a therapeutic window. Side effects as well as human pharmacokinetics are established at this stage. A dose in excess of that at which a therapeutic response is anticipated must also be established. Phase II studies involve open-label, single- and multiple-dose studies in the patient population. Efficacy is

determined using placebo controls. The therapeutic dose range is established as well as the route and frequency of compound administration. There is a major trend in relating compound efficacy to plasma levels rather than to dosage (Peck et al. 1992) because there are many individual patient variables that affect the amount of drug that reaches the bloodstream. Phase III studies expand on the initial patient population, involving multicenter trials to establish uncommon (<2 percent) side effects and drug interactions. The data derived from Phase III studies are used as the basis for the new drug application (NDA), the data required for registration of the drug with a regulatory agency for approval for sale. In EEC, the NDA is known as the Product License Application. Phase IV studies are postmarketing studies to further elucidate common side effects, to focus on patient populations not adequately covered in Phase III trials, and to gather definitive data on additional indications for the compound.

The data derived from clinical trials and for which a company seeks regulatory approval relate to the uses of the drug and its side effects and safety. The instrument to define these parameters is the package insert, the documentation accompanying a prescription drug. In seeking regulatory approval, a company should have data that will support the claims made in the package insert. Thus, the claims are the driving force in determining clinical trials and the interactions of a company with FDA. Rather than an isolated, two-stage (IND, NDA) process, frequent meetings with FDA to seek advice, opinions, and clarification can significantly accelerate the movement of a compound from the laboratory to the pharmacy.

Clinical Markers of Drug Action

The predictivity of animal and biochemical test procedures is unclear because, as discussed above, many models are highly empirical. The ability to diagnose disease states on the basis of blood or tissue pathology and to use such tests to determine compound efficacy is a major goal within the pharmaceutical industry. For 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (lovastatin [Mevacor]) that prevent cholesterol formation, clinical endpoints were based on pharmacoepidemiological studies linking elevated cholesterol levels to atherosclerosis and other disorders of the vascular system. In the clinic, therefore, determination of efficacy is related to a decrease in plasma cholesterol levels produced by lovastatin rather than direct effects on atherosclerotic pathophysiology.

CNS DRUG DISCOVERY—AN OVERVIEW

The pressures and complexity of modern society have increased the use of psychotropic drugs and the incidence of anxiety and depression, creating a need for medications that are more efficacious and free of side effects than those currently available. The need for effective treatments to treat addictive disorders

(e.g., substance abuse, obesity, alcohol and nicotine addiction) is a high priority in neuroscience research because of their high cost and negative impact on society.

The unmet needs in CNS disease treatment (Subcommittee on Brain and Behavioral Sciences 1990) have led the U.S. Congress to declare the 1990s as the Decade of the Brain, a major initiative (Judd 1990; National Advisory Neurological and Communicative Disorders and Stroke Council [U.S.] 1989; Williams 1992) to facilitate the discovery and development of new medications for a variety of CNS disease states.

CNS drug discovery may be conveniently divided into three phases. The first, the age of serendipity, occurred from the early 1950s through 1975 and involved the clinical discovery of compounds originally targeted at other disease states that had unexpected CNS actions. Iproniazid, chlorpromazine, imipramine, and L-dopa were products of this era (Sneader 1985). A second phase began in the early 1970s with the search for second-generation compounds with improved efficacy and reduced side effect liability compared with those agents discovered in the first phase. The use of receptor binding and targeted screening led to the discovery of antagonists for the cholecystokinin (CCK-B) MK 329 (Chang et al. 1985) and substance P receptors CP 98,345 (Snider et al. 1991) and RP 67580 (Garret et al. 1991). Despite the increased emphasis on molecular pharmacology, drugs discovered in this period were, to a large extent, incremental in nature or, like buspirone (New 1990) dependent on serendipitous evaluation in the clinic. The antipsychotic clozapine (Bonate 1991) and the antidepressant fluoxetine (Fuller et al. 1991) were major therapeutic agents from this era.

Advances in molecular biology and molecular pharmacology have resulted in the identification of new drug targets: receptor subtypes and enzyme isoforms. These permit elucidation of receptor/enzyme function with the development of ligand/substrate SAR and compound selectivity. Such advances, fueling the third phase of CNS drug discovery, also have the potential to facilitate disease diagnosis and the understanding of disease etiology.

The emerging computer-based structural technologies, such as CAMD and NMR, have been limited in the CNS area because many potential drug targets have yet to be crystallized and thus require approximations for structural analysis,

LIMITATIONS IN CNS DRUG DISCOVERY

Present-day understanding of brain function is limited and has become overly reductionist with minimal focus on the four principal levels of research: molecular, cellular, systems, and behavior (Shepherd 1988; Bloom 1991). Objectively integrating and interpreting data obtained at these various levels are crucial to make allowances for the complexity of function that distinguishes the brain from

other organs within the body. Although considerable emphasis is placed on the density of receptors within the brain (usually 10-fold to 100-fold greater than found in other tissues), the possibility that these add to functional complexity is rarely taken into account. Similarly, although glial cells are an important component of nervous tissue, they are frequently ignored in defining hypotheses related to drug action. It also has been noted that "neuroscience stands . . . today [1991] where atomic physics was in 1919 . . . or . . . molecular biology . . . in 1944" (Ridley 1991). A lack of knowledge regarding CNS disease etiology and the descriptive nature of current psychiatric diagnosis (Baldessarini, in press) are other contributing factors that affect progress in CNS drug discovery.

Another critical issue relates to the predictive animal models. These have been highly empirical and have not always been useful in moving a compound into the clinic. In the area of depression, for instance, there are a number of biochemical and behavioral models (e.g., β-receptor down-regulation, muricidal rat, swim test) in which classical antidepressants have effects, These are not especially robust test procedures, and few are on the critical path for compound characterization. A limitation to such tests has been that research in the area of animal behavior as related to human CNS disease states, both in terms of determining efficacy and side effect potential, has undergone a significant deemphasis in the past decade in favor of more molecular approaches (Williams 1992). Transgenic animals have been heralded as a more useful approach to disease pathology, but their use, to date, has been limited in the CNS. Various mouse strains (e.g., DBA) have been used in anticonvulsant testing, yet these also are empirical, rather than based on definitive genetic defects related to defined CNS function.

The limitations in behavior become self-defeating as research becomes more focused on molecular approaches. To judge the potential of behavioral paradigms based on current models, which are being improved in a very limited fashion because of funding constraints, is impractical. New knowledge regarding disease etiology cannot readily be integrated because of a paucity of researchers and the complexity of test paradigms. It is crucial that a greater focus on animal models of CNS diseases be part of the Decade of the Brain initiative.

MANAGEMENT OF THE DRUG DISCOVERY PROCESS

The need for innovative research to find drugs that allow the treatment or more effective treatment of human disease is a sine qua non for the pharmaceutical industry. Yet understanding of the scientific approach and what factors motivate scientists is not always a given within a research-based organization (Roussel et al. 1991). The R&D organization is frequently viewed as an unpredictable, uncontrollable entity, a black hole into which vast sums of money are poured in hope that products will emerge. Communication among scientists, research

management, and corporate management is not always optimal (Williams and Neil 1988; Spilker 1988; Cuatrecasas 1990; Roussel et al. 1991) so research may often be perceived as a necessary evil rather than the lifeblood of an organization. To the necessarily pragmatic businessperson, the abstractly focused scientist may on one level be an icon representing the dynamics and vision of the R&D-based commercial enterprise. On another level this individual may represent a challenge to be molded and redirected into a more goal-oriented, productive path. The challenges of managing and motivating scientists are formidable (Roussel et al. 1991; Williams and Stork, in press) and are in need of attention in today's changing scientific marketplace.

The numbers of qualified scientists entering industry have been dramatically reduced in the past decade, and this is already leading to major shortages in the areas of chemistry and pharmacology. In a highly controversial public debate, some participants have focused on the inferior quality of education in the United States as a major causative factor of the shortages (Shakhashari 1991) whereas others have directed attention to more lucrative career alternatives in investment banking and the legal profession. Whatever the reasons, there has been an exponential increase in the hiring of foreign nationals because of an absence of qualified U.S. scientists.

With such shortages and considerable in-house investments in the continued training of research staff, the effective management, motivation, and retention of scientists are as much a part of an effective strategy for drug discovery as the acquisition of enabling technologies. Many research organizations are "flat," having few managerial levels (Drucker 1988). As a result, career advancement has become limited, placing a major focus on job satisfaction. For a research organization to provide this requires an understanding of motivational factors within the scientific arena.

Classic scientific training, especially at the graduate level, places a major premium on independent thought, on the dissemination of research results through the global scientific community, and on peer respect and acceptance. These qualities usually undergo modification within the organizational context of a corporation that places a high premium on teamwork and loyalty. However, such qualities do not disappear completely and must be factored into the research environment. This is of special concern when project priorities change and scientists have to refocus their efforts on different activities.

Ideally, scientists in industry should have the opportunity to work on interesting projects, to make their own individual contributions to research projects, to be part of the process of new project initiation, to be rewarded for their efforts in drug discovery, to publish original research findings, to attend important scientific meetings, and to have clear career goals. The assimilation of an individual into a Research organization should be an active process, melding

with the culture of the Research organization in a synergistic manner from both a technical and a personal viewpoint (Badawy 1985). Most large Research organizations have both managerial and scientific hierarchies to enable scientists to align their careers with science rather than having to compete for limited managerial positions for which they may not be suited. Active recognition and support from upper management for scientific excellence is a major aspect of the culture in many pharmaceutical companies. Some scientists may align their careers with a project or a therapeutic area so that when priorities change, they are more inclined to seek other opportunities than to realign with corporate needs. Such individuals are highly focused, dedicated, and often very entrepreneurial in outlook and in their ability to motivate others. Fitzgerald (1990) has described these "drug hunters" as "having a broad knowledge of corporate science, . . . being non-compliant, disliking the status quo, risk taking, having strong convictions which they forcefully express, and being ambitious for drugs rather than themselves." Several major drugs have been discovered because of the efforts of such "swashbuckling" individuals, Although their activities were not always appreciated at the time, they have since become both corporate and industry folk heroes,

As the 21st century approaches, the urgency of scientific research has increased considerably. Capable scientists are changing companies more frequently to enhance their career prospects. This urgency has been fueled by venture capital interest in pharmaceuticals (Klausner and Rodgers 1990; Hamilton 1992) and by the global consolidation within the pharmaceutical industry (Mergers and other alliances . . . 1991) and driven to a major extent by a short-term focus on research (Poste 1990; Mergers and other alliances . . . 1991; Williams 1992) that has led to a number of mergers and takeovers with staff realignments, downsizing, and cultural dissidence. Fifteen years ago, a scientist in the pharmaceutical industry could expect a well-paid, secure career with one company and a comfortable retirement (Williams and Neil 1988). Today, such security is relative, even though many scientists, as evidenced by events at Genentech, Amgen, and so on, can become millionaires by (re)aligning their careers with biopharmaceutical/biotechnology startups.

The continuing high premium placed on innovative, focused biomedical science has thus provided an increasing number of career options for experienced, productive drug hunters. The high element of risk in the venture capital arena with the attendant high rewards presents a challenge to the major pharmaceutical companies to be competitive in hiring and retaining their scientific personnel. In the era of major expansion in industry research from 1976 to 1989, career needs could be met as organizations expanded. When organizations stabilize or even shrink, career opportunities become fewer. Additional dynamic factors are imposed on the Research organization as technologies and goals change.

A major challenge to research management for the future will be to provide the leadership and the culture that effectively mesh the needs of the individual scientist with those of the Research organization. In this context, Cuatrecasas (1990) has noted that "few, if any, large organizations have ever come close to adjusting their operational models to encourage creativity and invention. Most R&D organizations tend to be bulky, awkward, regimented, controlling, inflexible, 'conformist,' bureaucratic, formalized, overstructured, and intolerant." Against such a litany of negatives, one wonders how any technology reaches the marketplace. The imperative in upward communication (Roussel et al. 1991) is exceedingly high.

CLOSING COMMENTS

The drug discovery process is a complex human endeavor involving many different scientific disciplines and personalities. The potential benefits to society in terms of quality of life represent a major goal of knowledge building in the biomedical sciences. Although issues regarding the economic benefits resulting from new medications are ongoing (Vagelos 1991; O'Reilly 1991), society continues to seek relief from the pain and suffering of disease, aging, and trauma. Whereas the scientific basis of present-day drug discovery has changed considerably from the plant medicines of the 15th century, the expectations remain the same.

The promise for biomedical research in the 21st century is to develop drugs that not only will treat disease symptomatology but also will be either prophylactic or curative. This hope places considerable expectations on the biomedical community as well as the pharmaceutical industry. It also requires that society prioritize its efforts in providing health care to the maximum number of people. Concerns continue to emerge regarding the quality and cost of health care to elderly persons (Callahan 1987, 1990), the need for medications that will effectively treat drug abuse (Johnson and Vocci 1991), and the realization that the highly effective products of the drug industry represent only 5 to 7 percent of the health care dollar (Pharmaceutical Manufacturers Association 1991).

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Mass Ligand-Binding Screening Strategies for Identification of Leads for New Drug Discovery

Ronald M. Burch

SCREENING METHODS

Historically, screening for new drug leads has involved primarily studies in animals and isolated tissues. Recently, cultured cells as well as isolated enzyme preparations and receptor-binding preparations have been used for assays. Screening in animals has the advantage of most relevant feedback. If a reasonable animal model can be used to demonstrate the desired effect, it provides a largely proven bioavailability and biological effect. This type of screening is so successful that Beyer (1977) has noted that in the search for novel diuretic agents in the 1950s while many laboratories searched for carbonic anhydrase inhibitors and synthesized very potent agents, his group, which relied on screening in animals, synthesized and developed a new class of diuretic agents, the thiazides.

However, there are several drawbacks to the use of animals in screening. The use of animals tends to be slow and requires large quantities of experimental compounds. The use of animals is also very labor intensive, and together these disadvantages make such screening an expensive proposition. In addition, if the animal model is not chosen carefully, nonspecificity may result. For example, if inflammatory reactions are initiated in animals, then, if the animal becomes ill with the experimental agent, the inflammatory reaction generally will not occur.

Screening in isolated tissues or cultured cells generally requires less compound than in animals and often is less labor intensive. In addition, screening at this level of complexity allows the determination of whether an agent is an agonist or an antagonist. As with animals, inhibition of a response may reflect toxicity rather than efficacy.

The use of receptor binding requires the least amount of compound. In many laboratories, a single disbursement of 1 mg of compound may be sufficient to perform screening in as many as a dozen assays in different receptors.

Receptor binding is also rapid. In the author's small laboratory, as many as 1,000 compounds often are screened per day in a given receptor assay. In receptor-binding assays, specificity often may be readily determined by assaying the compound in preparations of several different receptors. However, receptor assay screening gives little or no clue of bioavailability and ultimate efficacy in an animal. In addition, it is impossible to determine from a single receptor assay whether a compound will be toxic to an animal.

The theory of ligand-binding assays is quite simple. A preparation of the receptor is prepared using either tissue homogenates or intact cells and incubates replicate tubes with a radiolabeled ligand. Certain tubes contain saturating concentrations of unlabeled ligand to allow the determination of nonspecific binding (figure 1). It is possible to determine potency of a competitor by adding various concentrations of competitor to different tubes and determining the amount of ligand bound. Then, one can either determine percent inhibition of binding or the IC_{50}. value for inhibition of binding by the competitor. In mass ligand-binding screening, a single concentration of competitor is often chosen for testing. Any compounds that inhibit binding a certain specified percentage are further evaluated to determine IC_{50}. This approach dramatically speeds the process because very few compounds will generally be shown to be active.

CHARACTERISTICS OF THE IDEAL RECEPTOR ASSAY

Optimization of a receptor-binding assay requires attention to two primary components, the tissue and the ligand. The goal is to achieve the highest signal-to-noise ratio possible, that is, to maximize specific binding and minimize nonspecific binding. The ideal tissue will have a high density of binding sites, Often, there are 10,000 to 50,000 binding sites per cell in a tissue. Tissues with only a few hundred binding sites per cell tend to have very low signal-to-noise ratios. In addition, the tissues should express a pure receptor subtype. For example, to simply homogenize brain and then attempt to perform dopamine binding, it is necessary to deal with distinguishing among binding to dopamine D1, D2, D3, D4, D5, and D6 subtypes. With currently available ligands, this would be impossible. Evaluating compounds against one of these dopamine receptor subtypes would require the identification of a tissue that expresses only one subtype or would require access to a cell line that stably expresses a cloned subtype. Stably transfected cell lines expressing only a single receptor subtype are becoming more common and will probably be the norm within the next few years. Finally, an ideal tissue must be readily obtained in quantity.

A ligand must be highly specific for its receptor. Norepinephrine, for example, would make a poor candidate for a binding ligand because it binds to virtually all subtypes of α- -and β- adrenergic receptors. When novel receptor subtypes are screened, a specific ligand rarely will be available. Generally, the goal of the

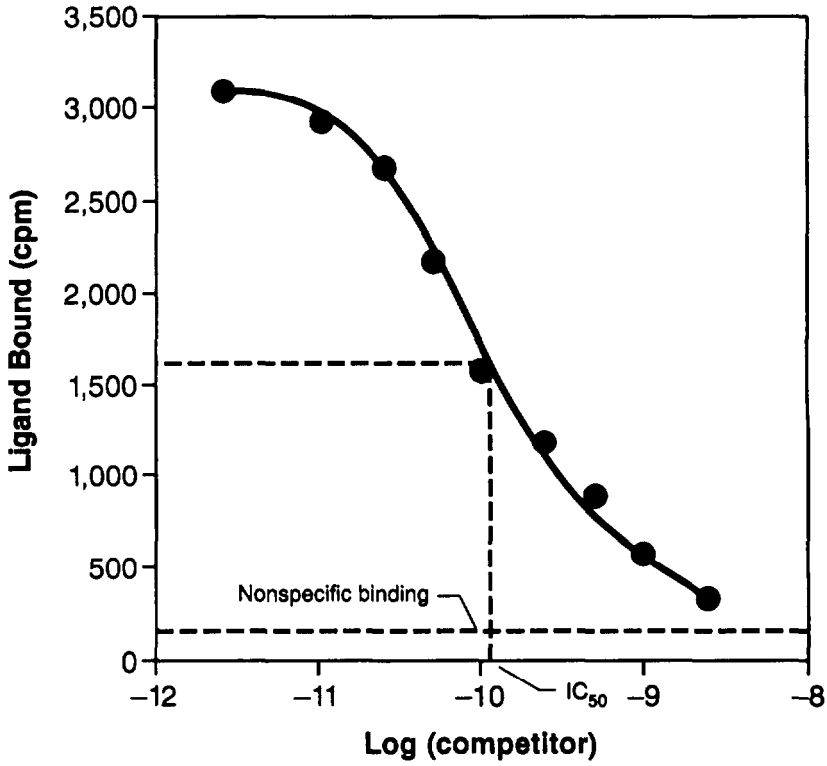

FIGURE 1. *Theory of ligand-binding assays*

screening exercise will be to discover such ligands. Thus, in practice the option is to choose a tissue that expresses only a single subtype of receptor to achieve this specificity. In addition, a ligand must be of as high specific activity as possible to evaluate binding. ^{125}I-labeled ligands are preferable to tritiated ligands because, after completion of the binding experiment, processing of samples is minimized. Tritiated ligands may require solubilization in liquid scintillation cocktail, which greatly increases the labor involved in a binding assay because of filling and capping vials. However, because ^{125}I has a relatively short half-life, in practice tritiated ligands tend to be more stable and easy to use.

In choosing a ligand, an antagonist is often better than an agonist. In particular, affinity shifts for agonists in G protein-coupled receptors in response to ions make for increased nonspecificity when dealing with unknown competitors. Of course, for the reasons mentioned above, an agonist may have to be used because discovery of an antagonist is often the goal of the exercise.

Receptors for many biogenic amines and many peptide hormones are expressed at 10,000 to 50,000 per cell. This translates to about 10^{12} sites per gram of tissue wet weight and allows the evaluation of 200 to 300 experimental tubes per gram of tissue. In contrast, many cytokines are expressed at 200 to 3,000 sites per cell. This often necessitates the use of 10^6 cells per tube or more for experimental assays. For those receptors that are expressed in the least abundance, as few as 2 to 5 tubes may be assayed using a 10 cm^2 dish for contact-dependent cells or perhaps only 10 to 20 tubes per T-75 flask for contact-independent cells. Increased receptor densities may often be obtained in transfected cell lines expressing cloned receptors in which the expression is commonly as high as 50,000 to 100,000 receptors per cell. Alternatively, transformed cells may overexpress certain receptors. For example, certain leukemia cell lines express as many as 50,000 to 100,000 interleukin-1 (IL-1) receptors per cell compared with 200 per cell in nontransformed lines.

Assays with high signal-to-noise ratios make screening a much simpler process. For example, binding of bradykinin to B_2, receptors in guinea pig ileum exhibits about 95-percent specific binding. If in a given tube total binding is 3,000 disintegrations per minute (DPM), then nonspecific binding is about 150 DPM, leaving a window of 2,850 DPM over which to observe competition. Fifty-percent competition at this site would result in observing 1,575 DPM. If errors in addition of experimental compounds, ligand, and tissue, together with counting error, total 10 percent, this would amount to about 300 DPM in an assay, which would change apparent percent inhibition by only about 10 percent.

In contrast, the binding of ligands to the thromboxane A_2 receptor in human platelets shows about 50-percent specific binding. In such an assay, total binding might be 2,000 DPM per tube and nonspecific binding 1,000 DPM. In this example, the window over which to observe competition is reduced to 1,000 DPM, and 50-percent competition would amount to 1,500 DPM being observed in a tube. With such low specific binding, a lo-percent error in addition of all reagents would change the apparent percent inhibition of binding by 20 percent. In many of these low-signal assays, all the experimental compounds are often assayed at least twice to reduce the number of false negatives and false positives.

DISCOVERY OF A NEW LEAD FOR A SINGLE RECEPTOR

Screening efforts may be set up on a variety of levels-from those in which all parts of the assay are performed manually by the binding technicians, to those in which teams of technicians subdivide the various tasks and in which computerization of all steps in the process leads to very high throughput and generation of extensive databases. In the manual approach, a single technician harvests and prepares the tissues, sets up and terminates the assays, calculates results, and places them into a database. Using this type

of system, it is often not possible to perform assays more than 2 days in a given week. In such a system, a single technician should be able on a given day to set up and assay twelve 48-tube racks or six 96-tube racks. This amounts to 576 tubes. Each rack has duplicate total and nonspecific binding tubes, and a standard compound is run each day. Therefore, about 520 tubes are available for test compounds. In this scenario, a single technician disburses compounds. Each disbursement technician can weigh and solubilize about 250 compounds per day. Thus, in a given week, somewhat more than 1,000 compounds may be solubilized and assayed by these two people. If such an approach is used to screen a library of 10,000 compounds, then about 10 weeks, or 20 technician-weeks, would be required (figure 2).

Considerable time and cost benefits can accrue using a high-throughput approach. In this scenario, two disbursement technicians are required for each binding technician. Each binding technician performs an assay each day. Therefore, about 2,500 compounds are assayed per week. The disbursement technicians weigh bar-coded compounds, and the computer attached to the balance notes how much diluent to add to each vial. Scintillation counters also are accessed by the computer, which calculates percent inhibition by the test compounds. Before commitment of data to a database, the binding technician quickly calls up the assay for examination to determine that total and nonspecific counts are within normal values and that the standard compound inhibited with the expected potency. Using this kind of approach,

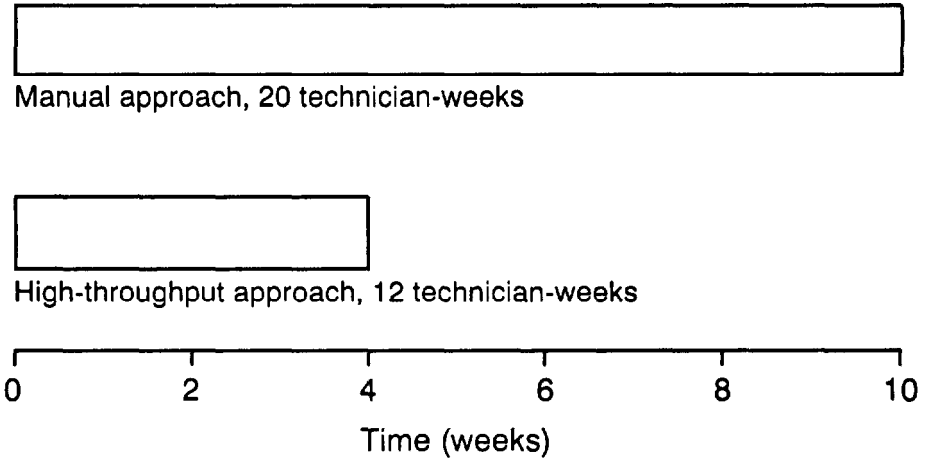

FIGURE 2. *Time required to screen a library of 10,000 compounds through a single receptor assay*

the 10,000-compound library can be screened through one receptor assay in about 4 weeks (figure 2), requiring only about 12 technician-weeks.

This high-throughput approach can be extended to other receptor assays with minimal cost. If a single batch of compound is disbursed, it may be stored in a cold room in dimethyl sulfoxide or other appropriate solution and used for up to several dozen different assays. Low signal-to-noise assays require more time because each compound is assayed multiple times.

DEFINING SUCCESS

Depending on the definition of an active compound or a "hit," success may be great or little. Certain assays tend to have many apparent hits (false positives), whereas others have very low hit rates. Receptor-binding assays for excitatory amino acids tend to have hit rates of 10 to 20 percent in libraries of "random" compounds, when hit is defined as 50-percent inhibition at a concentration of 10 μm. In contrast, in the IL-1 receptor-binding assay in fibroblasts in the author's laboratory, the hit rate is less than 1 in 5,000 using the same criteria. Given these examples, to avoid being swamped following up false positives, inclusion criteria must be strict. Of course, if criteria are too restrictive, then promising leads may be missed. In the author and colleagues' work, an example of the weakest compound in a bradykinin receptor-binding assay that can be shown to be an antagonist in a functional assay is NPC 361, which has a K_i, of 400 nm. The prototype cholecystokinin antagonist, asperlicin, had a K_i of 600 nm in a binding assay (Chang et al. 1985). For either of these examples, even our relatively strict inclusion criteria of 50-percent inhibition of binding at a concentration of 10 μm would have tagged these compounds as active. However, Wong and colleagues' (1988) angiotensin II antagonist lead had a K_i, of 40,000 nm in their assay. Such a compound would have been excluded in any of the assays in the author's laboratory as inactive.

If such weakly active compounds are included as hits, then 50-percent inhibition at 100 μm would be required. Unless the assay is very "clean" (as described above for IL-l), a method is required to make certain that most nonspecific (false positive) compounds are weeded out quickly. This is done by running the compounds in several different binding assays and excluding those that are hits in multiple assays. In situations in which compounds are routinely screened at only a single site, all apparent hits are collected and run in additional binding assays. Thus, a "receptor profile" or a "cross-selectivity report" is obtained.

In the cross-selectivity mode, an attempt is made to determine K_i, values in each assay. Concentrations to be evaluated are chosen by noting the apparent K_i, in the assay of interest, then bracketing that value. In these studies, two compounds are evaluated per 48-tube rack (11 duplicate concentrations of each test compound plus duplicate total count and nonspecific count tubes).

Each binding technician can perform determinations for 24 compounds per day. For the specialized laboratory that performs screening in only its target of interest, K_i, determinations in multiple assays may be contracted commercially with several companies.

FUNCTIONAL SCREENING

Functional screening is the vital next step after a hit is identified in a binding assay. The functional screen is necessary to determine whether a given compound is an agonist or antagonist. Functional screens also serve to provide an idea of selectivity or, at times, toxicity. When determining whether a compound has agonist activity, one must always evaluate the compound in an assay alone, even if the antagonist assay suggests that the compound does reduce the activity of an agonist. It must be remembered that a partial agonist will appear to be an antagonist when assessed in an antagonist assay.

The first choice in deciding on a functional screen is whether to use an isolated tissue response (e.g., smooth-muscle contraction) or a second-messenger assay (e.g., increase in intracellular inositol phosphates [IP_3] or cyclic AMP [cAMP]). The second-messenger assays often lend themselves to very high throughput. For example, using a radioreceptor assay for IP_3, the author's laboratory cultures fibroblasts in 24-well plates. Cells are then stimulated with bradykinin to determine whether test compounds inhibit bradykinin-induced IP_3 accumulation. This assay also lends itself to determination of affinity by determining K_b, or pA_2 values. Such assays may be used to assess selectivity of test compounds. In the assay just cited, the cells also express receptors for angiotensin II, vasopressin, a,-adrenegic agonists, and bombesin, all of which increase the accumulation of IP_3. Similarly, the cells express β_2--adrenergic and adenosine receptors, whose activation increases the accumulation of cAMP. These, too, can be used to assess selectivity and may be preferred, because assessing the effect of a compound on a second transduction system adds an additional chance to detect nonspecific effects.

The utility of a functional screen is increased if it measures a "positive activity" rather than a "negative activity." In a negative-activity assay, an attempt is made to block the ability of an agonist to elicit a response in a resting cell or tissue. This type of assay often is used and represents the common agonist-induced muscle contraction or agonist-induced IP_3 accumulation. Here, the test compound is assessed for its ability to inhibit the response. A problem that often arises when assessing unknown compounds is that, if the compound nearly kills the cell, usually an agonist will not cause increased IP_3, accumulation, appearing to confirm activity of the test compound as an antagonist. Thus, the negative-activity assay may be prone to generating false negatives. In the positive-activity assay, the agonist does something to decrease the function of the cell or tissue, and an antagonist restores a normal state. For example,

tumor necrosis factor (TNF) induces cytotoxicity in many cell types. If a test compound blocks this effect, then the cell, rather than dying in response to exposure to TNF, continues to act normally. In this type of assay, a toxic compound tends to augment the ability of TNF to kill the cell rather than make the cell function more normally. Thus, nonspecifically toxic compounds will tend not to appear as active receptor antagonists.

PROCESS TECHNOLOGY

Process technology refers to throughput methodologies. Many laboratories spend extraordinary amounts of money and time on automation of assays. In my laboratory I find automation largely wasteful. Given hand-held automatic pipetting devices, multiple-sample filtration manifolds, and dry scintillant pads, my laboratory always outperforms automated laboratories. Often, a library of compounds may be screened in the time it takes to reprogram all the automation devices. The only situation in which automation *may* be arguably worth the bother and expense is when a library of many thousands of compounds will be screened.

I find that the real rate-limiting step in mass ligand-binding screening is tabulation of data. To this end, all aspects of the process have been computerized. Compounds are bar coded with molecular weights and structural formulas. Bar-code readers are used to track compounds through the computerized weighing procedure, and disbursements are easily solubilized to the correct stock concentrations using computer-calculated printouts. Disbursed compounds retain bar coding, and assays are set up in standard fashion in the racks so that the computer accesses the scintillation or)y-counter data and calculates results. Before ordering the computer to commit the data to the database, the binding technician looks at the onscreen display of the assay and ensures that the standard compound acted properly in the assay. This data-handling system has been previously described in some detail by Burch and Kyle (1991). Beyond simple lead identification, this online system can search the database for hits (the criteria for which can be specified with a few key strokes) and, because the structures are also present, perform one of several structural search paradigms for identification of pharmacophores for given activities.

CONCLUSION

Mass ligand-receptor binding has proven useful in the identification of structural leads in several instances where no prototype candidates were available. The keys to success with the approach are acquisition of diverse and large libraries of compounds, diversity being at least theoretically more important than size,

and constant attention to selectivity, both in other binding assays and in functional assays.

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Antibody- and Peptide Structure-Based Drug Design

Manfred E. Wolff

INTRODUCTION

For more than a century, the search for new drugs has been rooted in an organic chemistry-driven technology based on the synthesis and biological evaluation of large numbers of candidate compounds (Burger 1980). Highlighting the inefficiency of this process is the fact that US. pharmaceutical companies were estimated to have spent $7.3 billion on domestic research and development in 1991 (Pharmaceutical Manufacturers Association 1991) to achieve U.S. approval of only 11 new chemicals (Scrip Review Issue 1992).

Hoping to improve the success rate in drug discovery, today most major pharmaceutical companies and several startup companies have organized efforts in rational drug design. Several distinct strategies are employed, including approaches based on computer modeling (DesJarlais et al. 1988) of data obtained from binding assays and enzyme crystallography as well as methods depending on bioorganic chemistry (Walsh 1984). These applications of rational drug design thus require at least the purification and sometimes the crystallization of targets that are central to each disease process. Yet many prospective drug targets are membrane-bound macromolecules that cannot readily be crystallized or even identified. For example, in a recent inhibitor design study involving the crystal structure of the RNase H domain of human immunodeficiency virus-1 reverse transcriptase (Davies et al. 1991), only an inactive RNase H fragment could be obtained as diffraction quality crystals.

Antibody and peptide structure drug design (Wolff and McPherson 1990) is aimed at avoiding these pitfalls. In the antibody approach, the remarkable power of the mammalian immune system is first harnessed to access the three-dimensional (3-D) structural information contained in pharmacological receptors, enzymes, and viruses without the need for special purification or isolation efforts. Then, the pharmacophoric information embedded in the resulting monoclonal antibody is read out by one of several methods. From the data obtained, peptide agents are produced whose structure is used as the basis for the computer-assisted design of orally active nonpeptide peptidomimetic drugs with receptor subtype specificity for many therapeutic applications. In those cases where the

structure of a lead peptide, such as a natural mediator, is known, the process can begin without the creation of the antibody, which serves only as an informational intermediate. These steps are considered briefly in the next sections.

ANTIBODY TECHNOLOGY

Antibody-directed drug design begins with the selection of a drug target, which can be a pharmacological receptor, an enzyme, or a virus. Through monoclonal technology, idiotypic antibodies (Ab-1) to key drug action sites in such receptors, enzymes, and viruses can be selected and produced in any desired amount without the need to isolate the target (figure 1). The production and selection of antibodies to drug targets represents a screening of a repertoire of several billion different antibody structures generated by the immune system and a fine tuning of these structures through somatic cell mutation (Tonegawa 1983).

Antibody-directed drug design requires the antibodies to the drug target to have functional (druglike) activity. Clearly, an antibody that binds to a nonfunctional area of the target, or one that binds in some nonfunctional manner, will be useless as a prototype for drug design. A list of functional antibodies that have been obtained to targets such as peptide hormone receptors, autocoid receptors, and enzymes is given in table 1 and is an impressive array of the potential of this technology.

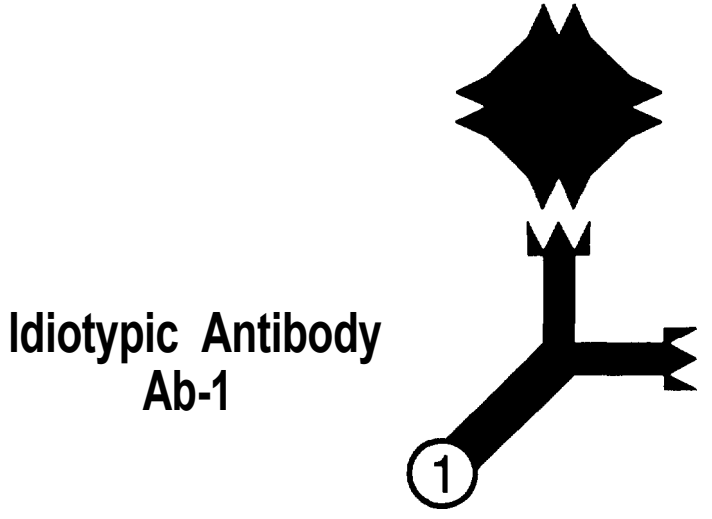

FIGURE 1. *Raising the antibody to the drug target*

The idiotypic antibody is a mirror image of the target site, both in a spatial and an electronic sense. For example, Conti-Tronconi and colleagues (1990) have mapped the binding site of the nicotinic acetylcholine receptor a-subunit with several monoclonal antibodies raised against native Torpedo receptor. The binding of these antibodies to the receptor is inhibited by all cholinergic ligands, and it was found that the antibodies bound to specific amino acid clusters positioned discontinuously within the receptor sequence 181-200.

By using the idiotypic antibody as an antigen, an anti-idiotypic antibody (Ab-2) (Linthicum and Farid 1988) can be produced as a positive image of the target site (figure 2). Again, selection criteria exist for anti-idiotypic antibodies that are faithful internal images of the original antigen (Ertl and Bona 1988).

COMPOUND LIBRARY SCREENING

Idiotypic antibodies have high affinity both for the antigens that were used to raise them as well as their corresponding anti-idiotypic antibodies. Because the interaction of these pairs can be observed conveniently using conventional or sandwich enzyme-linked immunosorbent assay (ELISA) methodology, it is possible readily to screen large numbers of compounds (compound libraries) for their ability to inhibit this interaction. In this way, potentially useful compounds that bind to either receptors or their anti-idiotypic images can be identified.

X-RAY CRYSTALLOGRAPHY IN ANTIBODY-DIRECTED DRUG DESIGN

Important drug design information is obtained from antibodies, their fragments, and complexes and from peptides by crystallization using conventional protein crystallization techniques (McPherson 1982) (figure 3). After x-ray diffraction data are collected, the crystal structure is solved using the conventional multiple isomorphous replacement technique requiring heavy atom derivatization of the crystals. An alternative approach is molecular replacement, which depends on some degree of knowledge of the structure of the antibody, the antigen, or both.

As an example, the 3-D structure (Sheriff et al. 1987) of an antilysozyme Fab-lysozyme complex is illustrated in figure 4. A closeup view (figure 5) shows the spatial and electronic complementarity of the interacting surfaces of lysozome residues 41-46 and light chain residues 55-59 that contribute to the hydrophobic, electrostatic, and hydrogen bonding accounting for the nanomolar affinity of the two ligands.

It must be emphasized that these crystal structures may or may not represent a bioactive conformation. There is abundant evidence that only certain conformations of peptides are bioactive (Sarantakis et al. 1973), and the conformation that exists in the crystal may not be one of these. Moreover,

TABLE 1. *Biologically functional antibodies*

Receptor	Antibody Type	Action	Reference
β1 -adrenergic	Ab-1 to receptor	Stimulates cAMP	Chapot et al. 1987
Muscarinic acetylcholine receptor	Ab-1 to receptor	Guinea pig myornetrium contractions	Leiber et al. 1984
Nicotinic acetylcholine receptor	Ab-1 to receptor	Blocks agonist-induced ion flux	Donnelly et al. 1984
Morphine	Ab-2 to morphine	Guinea pig ileum contraction	Ng and Isom 1985
Morphine	Ab-2 to β- -endorphin	Blocks enkepthalin effect on cAMP	Gramsch et al. 1988
Morphine	Ab-1 to HEAPI peptide	Suppresses cAMP	Carr et al. 1989
Insulin	Ab-1 to receptor	Stimulates 2-deoxyglucose uptake	Soos et al. 1989
Fibrinogen	Ab-1 to gpII-IIIa	Inhibits thrombus formation	Coller et al. 1988
Rat growth hormone	Ab-2 to hormone	Increases body weight	Gardner et al. 1998
LFA-1	Ab-1 to receptor	Blocks C5A-induced leukocyte adhesion	Argenbright et al. 1991
IL-I	Ab-1 to receptor	Inhibits thymocyte proliferation	C. Escobar, personal communication. January 1992
IL-4	Ab-1 to receptor	Inhibits B-cell proliferation	Maliszewsky et al. 1990
Angiotensin II	Ab-2 to hormone	Binds to receptor in situ	Berlove and Piekut 1990
Renin	Ab-1 to enzyme	Antihypertensive in monkeys	Lykegaard 1980; Wood et al. 1989
Human neutrophil elastase	Ab-1 to enzyme	Enzyme antagonist	E. Skaletsky, personal communication, February 1998

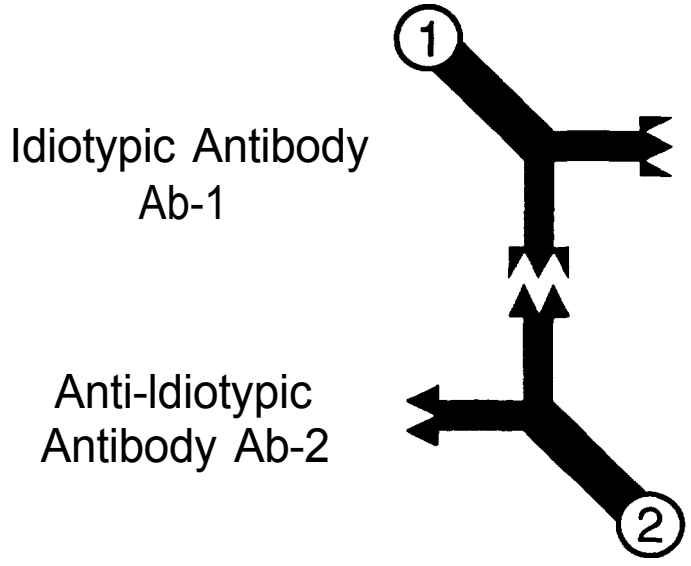

FIGURE *2. Creating specific internal image anti-idiotypic monoclonal antibodies*

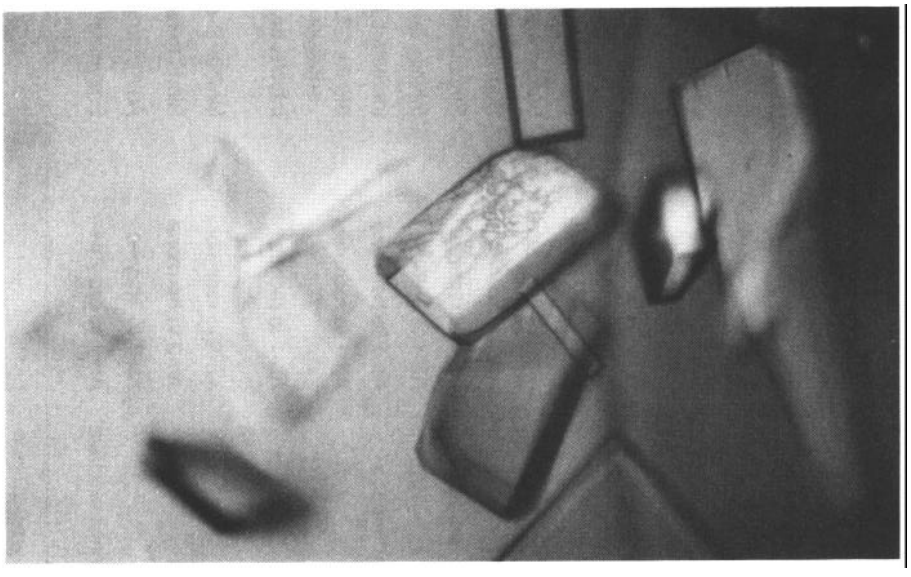

FIGURE 3. *Crystals of therapeutic lymphoma antibody*

NOTE: See Harris et al. 1992 for the solution to this crystal structure.
SOURCE: ImmunoPharmaceutics, Inc.

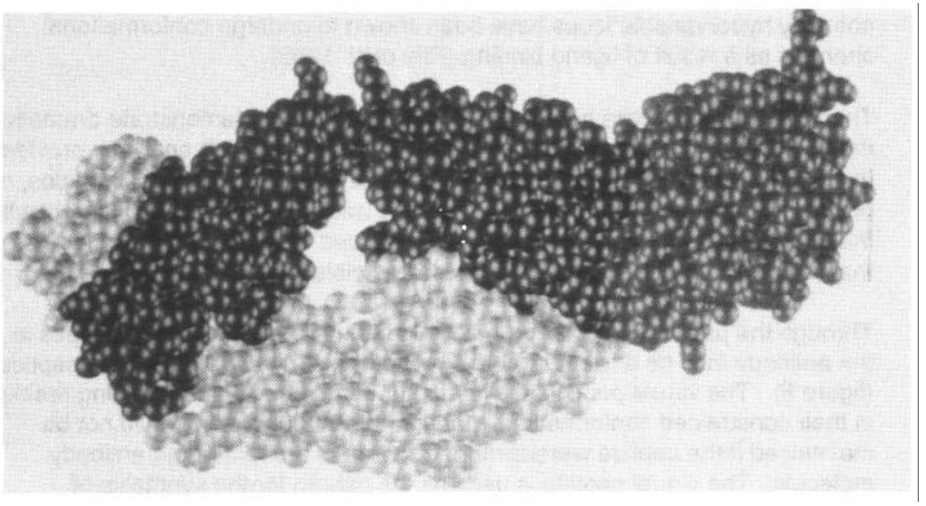

FIGURE 4. *Computer-generated Corey, Pauling, Koltun (CPK) model of lysozyme-HyHEL-5 complex from coordinates in the Protein Data Bank*

SOURCE: V.N. Balaji and U.C. Singh, personal communication, December 1989

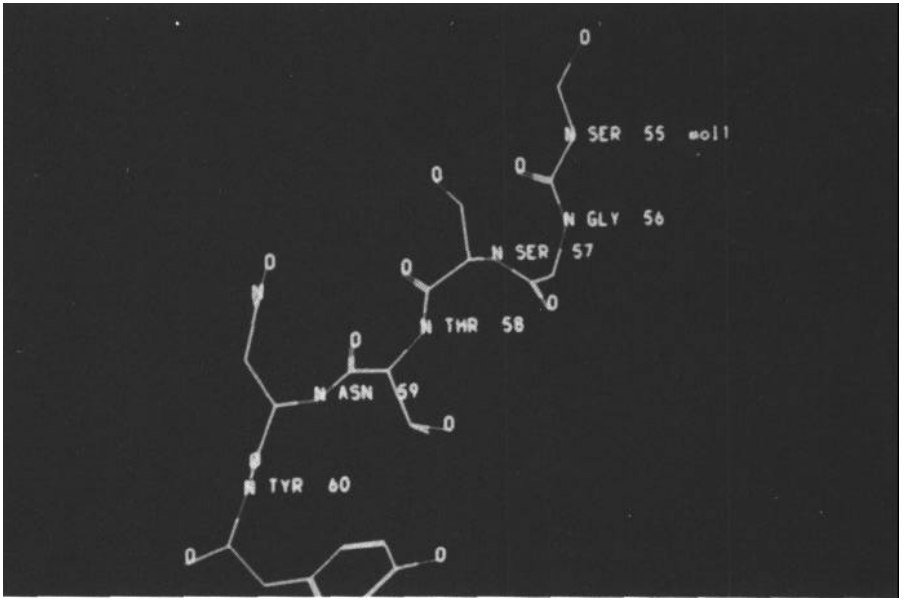

FIGURE 5. *Stick model closeup view of the contact residues (4Å) of figure 4*

SOURCE: V.N. Balaji and U.C. Singh, personal communication, December 1989

antibody hypervariable loops have been shown to undergo conformational changes as a result of ligand binding (Rini et al. 1992).

The specific interactions between antibody and antigen demonstrate dramatically the potential of the approach for directed drug design. Such analyses provide an unequalled structural basis for the creation of synthetic molecules, peptides, or peptidomimetics that share with the idiotypic antibody the ability to interact with the antigen. Related information can be deduced for anti-idiotypic antibodies that are replicas of receptors or other inaccessible macromolecules.

Through the use of molecular graphics algorithms, the interacting residues in the antibody may be excised from the rest of the structure as a "virtual peptide" (figure 6). The virtual peptide is a computer construct of the interacting residues in their constrained conformation, although this conformation would not be maintained if the peptide were synthesized free of the rest of the antibody molecule. The virtual peptide is used as the pattern for the synthesis of constrained peptidomimetic drugs as outlined in a later section.

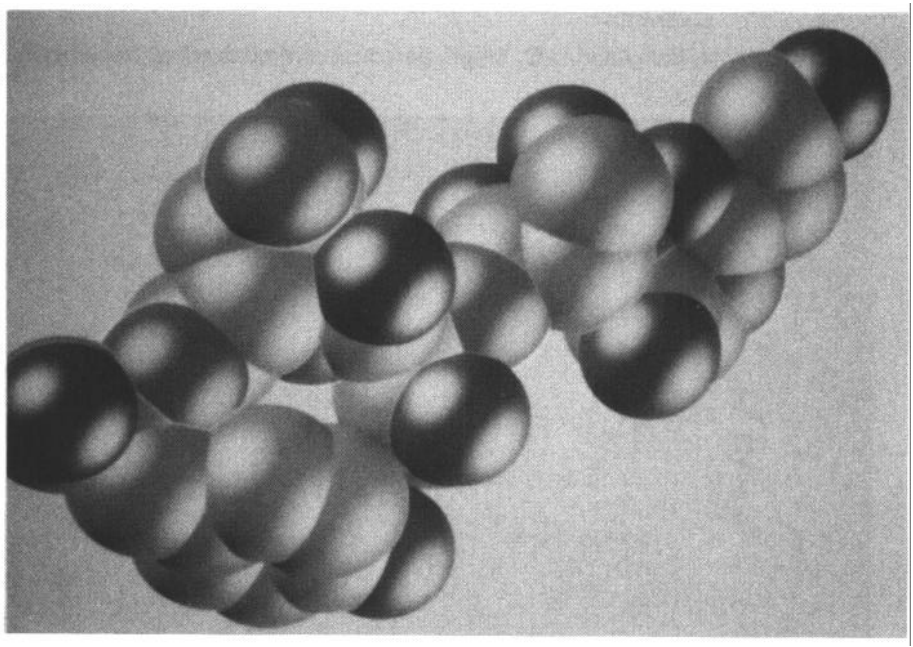

FIGURE 6. *Computer-generated CPK model of the virtual peptide of the enzyme ligand in figure 4*

SOURCE: V.N. Balaji and U.C. Singh, personal communication, December 1969

EXTRACTION OF INFORMATION

In addition to x-ray crystallography, three other techniques are used to read out structural information from the embedded pharmacophore in the antibody or from the anti-idiotypic antibody.

First, if specific leader sequences (Orlandi et al. 1989) are used in the polymerase chain reaction, the amino acid sequence of the hypervariable loops of the antibodies can be determined. With the knowledge of these sequences and of the x-ray crystal structure of representative antibodies, a combined knowledge-based and ab initio algorithm can be applied to model the structure of the hypervariable loops (Martin et al. 1989). In this way, structural information similar to that which can be obtained from x-ray crystallography is at hand without the need to resort to the crystallographic methodology.

Second, peptides that are complementary to the surface of anti-idiotypic receptor surrogates can be selected from libraries of filamentous phage clones, each displaying one peptide sequence on the virion surface (Scott and Smith 1990). A properly selected peptide that is complementary to a receptor surrogate results in a receptor antagonist.

Last, evidence for the location of key amino acid sequences in the hypervariable loops of anti-idiotypic antibodies can be adduced from their homology with sequences in the initial antigen (Williams et al. 1988).

Derivatives of these peptide sequences, chemically constrained to preserve their spatial characteristics, have been evaluated as drugs in their own right. For example, a synthetic mimetic derived from an omega loop structure that mimics a prominent, exposed series of eight residues in interleukin-1 (IL-1) was evaluated for its ability to bind to IL-1 receptors (Sarabee et al. 1991).

PEPTIDOMIMETIC SYNTHESIS

The use of peptides as design templates for peptidomimetic drugs is the key step in the implementation of antibody- and peptide structure-based drug design, In the preceding process, the end product is the design of a peptide drug that may be an enzyme inhibitor, a receptor antagonist (or sometimes an agonist), or an antiviral. Alternatively, the end product may be an antibody containing the embedded pharmacophore for any of these activities. Because both peptides and antibodies typically have short biological half-lives and poor oral bioavailability, it remains to synthesize a peptidomimetic (Kemp 1990) based on the peptide structure to afford a bioavailable, orally active drug.

The design of peptidomimetics (Morgan and Gainor 1990) requires two different applications of computer-assisted drug design techniques. First, an estimate of the bioactive conformation of the peptide must be obtained from x-ray or nuclear magnetic resonance results and from modified data obtained from primary sequence information through the application of computational chemistry algorithms (Gibson and Scheraga 1987) such as those of V.N. Balaji and U.C. Singh (personal communication, December 1989). This information is used to interpret the structure-function studies on the molecule. For example, it has been shown that the fragment Phe^{7}-Trp^{8}-Lys^{9}-Thr^{10} is essential for growth hormone release inhibitory activity by somatostatin (Vale et al. 1978; Verber et al. 1979) and that a ß II-type turn and a ß-sheet are also required for activity (Van Binst and Tourwe 1992). Based on the predicted or observed 3-D spatial and electronic characteristics of the peptide, a database and an algorithm of peptidomimetic surrogate elements (V.W. Balaji and U.C. Singh, personal communication, December 1990) are used to design candidate drugs for synthesis. A measure of conformational flexibility is required in these compounds to allow for the conformational changes that occur on binding to the target. Docking routines may be used to optimize complementarity in those cases where the 3-D structure of the receptor surrogate has been obtained.

A recent example of this approach involved the synthesis of a synthetic p-loop structure that mimics the second complementarity-determining region of a monoclonal antibody, which recognizes the cell surface receptor for reovirus type 3 (Reo3R) (Saragovi et al. 1991). The synthetic organic mimetic, termed the 87.1 mimetic, is a water-soluble, 10-membered macrocycle containing amide bonds. Evidence was obtained that indicates the 87.1 mimetic binds efficiently to Reo3R.

Antibody- and peptide structure-based drug design offers wholly new insights and approaches in the design and optimization of new lead compounds for therapeutic application, and risk is minimized by the multiplicity of available strategies. As these methods are developed further, even greater savings in time and effort will be at hand in the search for new drugs,

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The Use of Cloned Human Receptors for Drug Design

Paul R. Hartig

INTRODUCTION

Cloned human receptors are increasingly used by pharmacologists and medicinal chemists for the development of novel, site-specific drug therapies. This approach can be expected to produce many new drugs with improved efficacy and fewer side effects. The availability of sets of transfected human receptors makes it possible to target drugs to single human proteins from the inception of a drug design project. A critical requirement of this technology is that human genes must express a human pharmacology when expressed in the host cell line chosen for transfection. Studies on the human serotonin 5-HT_2, and 5-HT_{1D}/5-HT_{1B} receptors demonstrate that the gene sequence appears to determine the expressed receptor's pharmacological properties, with only a minor role played by the cellular environment in which the receptor is expressed. The cloning and characterization of the 5-HT_{1B} receptor also demonstrate another emerging principle of molecular pharmacology: the equivalent G protein-coupled receptor gene in different species can encode proteins with strikingly different pharmacological properties. Another important issue is the relationship between agonist and antagonist binding sites. Studies comparing agonist—1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM)—and antagonist binding sites of the human 5-HT_2 receptor demonstrate the strong differences exhibited by these two binding states of the same receptor protein, Finally, the fact that receptors of the G protein-coupled or 7TM (7 transmembrane) receptor superfamily exhibit many properties in common allows receptor homologies to be used to predict certain drug-binding properties. Examples from the serotonergic and adrenergic receptor families are presented.

A MOLECULAR PHARMACOLOGY

Cloned human receptors, conveniently expressed in transfected mammalian cell lines, now make it possible to approach drug design from a truly molecular perspective. In the past several years, the amino acid sequences of many receptor genes have been determined. Most of these cloned receptors are members of a closely related superfamily of genes known as the G protein-coupled receptors (or 7TM receptors), because of their characteristic single

subunit structure with 7TM-spanning segments. Similar cloning successes are now being reported for multisubunit ligand-gated ion channels, including the $GABA^A$ receptor, the 5-HT_3 receptor, and the N-methyl-D-aspartate receptor. Molecular pharmacologists are beginning to integrate this new information into their views of physiological and pharmacological processes and are introducing cloned human receptors into the drug development process, often from the very start of a drug design effort.

Expression of cloned human receptors in mammalian cell lines allows pharmacologists to develop assay systems that individually express each receptor subtype important to a drug design project. Host cell lines can be chosen that are devoid of any related receptor sites, producing clean, unambiguous assay systems. These subtype-specific human receptor assays will allow medicinal chemists to design drugs with high affinity for the desired site of action and low affinity for those receptor subtypes that may induce side effects. In many cases, this design strategy can be expected to produce more potent medications with fewer side effects because of an improved molecular targeting of the drug. Even in cases where a blended drug possessing a spectrum of receptor activities may be the desired endpoint, pure human subtype assays provide a drug design team with an important advantage: unambiguous assays of the affinity of the drug candidate at each human receptor site of interest.

The second advantage of the cloned receptor approach is that low abundance sites, which have been difficult to study in tissue preparations (e.g., autoreceptors), can now be isolated and expressed at high density for use in ligand screening and basic science investigations. The third, and perhaps'most important, advantage is the fact that many new receptor subtypes have been and continue to be discovered by receptor cloning efforts, which are providing a rapid increase in the number of potential drug target sites and can be expected to lead to several new medications in the future.

SPECIES DIFFERENCES IN RECEPTORS

The pharmacological binding properties of receptors are often similar for the same receptor subtype in different species, although many exceptions do occur. When species differences do exist, they dictate that great care be used in the choice of receptor assays for the purpose of human drug design. The best situation is found when human receptors can be used to screen for drug activity and selectivity. Cloned human receptors are making human receptor screening possible. These clones are commonly expressed in mammalian or sometimes in bacterial expression systems (Chapot et al. 1990) for use in drug-binding assays. This leads to the important question of what influence the cell-line will have on the pharmacological properties of the human receptors that they express, For the purpose of drug screening, host cell lines must be chosen

so that cloned human receptors will properly reproduce the binding properties of native human tissues.

The influence of the cell-line host on the pharmacological binding properties of cloned human receptor subtypes has been studied in some detail. The binding properties of the serotonin 5-HT_2, receptor have been known to differ in different species, especially for certain ergot compounds. For example, mesulergine exhibits approximately thirtyfold higher affinity for rat cortical membranes than for comparable human tissue (Pazos et al. 1984). Transfection of a cDNA clone encoding the human 5-HT_2, receptor subtype (Hartig et al. 1990) into mouse fibroblast cells leads to expression of a serotonin receptor whose binding properties match that of human rather than rat cortical membranes (table 1). The largest drug-binding differences are seen for mesulergine, which binds to both the transfected human receptor and to human cortical membranes with an apparent affinity of approximately 150 nM, thirtyfold weaker than its affinity for the rat cortex 5-HT_2, receptor. This species difference also is seen for ritanserin, which belongs to an entirely different chemical class (table 1). In both cases, the transfected human receptor exhibits binding affinities in close agreement with human cortical tissue, even though the human gene has been expressed in a rodent, nonneuronal cell line. Together, these observations suggest that

TABLE 1. *Ligand-binding properties of a human 5HT_2 receptor gene expressed in mouse fibroblast cells. A cDNA clone encoding the human 5-HT_2 receptor (Hartig et* al. *1990) was expressed in mouse fibroblast L-M(tk-) cells. Membrane preparations were labeled with [^{3}H]5-HT, and apparent dissociation constants were determined as described in Branchek and colleagues (1990). Comparative values from human cortex and rat cortex assays (Hoyer et al. 1986) are also provided.*

Apparent Dissociation Constant (K, in nM) for Displacement of [^{3}H]Ketanserin Binding

Drug	Human Clone	Human Cortex	Rat Cortex
Ritanserin	1.1 ± 0.16	1.3	7.2
Cyproheptidine	2.9 ± 0.10	6.3	1.8
Mesulergine	129±7.9	151	4.7
5-HT	598 ± 252	174	79
Quipazine	2,111 ± 295	3,802	1,549
5-CT	7,790 ± 50	8,130	21,878

SOURCE: Part of the data in this table was previously described in Hartig and colleagues (1992).

it is the amino acid sequence of the receptor (nature), rather than the cellular environment in which the receptor gene is expressed and processed (nurture), that determines the species-specific pharmacological properties of the receptor. Further studies are needed to determine whether this will prove to be a general property of neurotransmitter receptors. For most cases examined so far, it appears that transfection of a human 7TM receptor gene into mammalian cell lines has produced ligand-binding properties in good agreement with previous binding assays in human brain tissue preparations. Furthermore, the use of different cell lines as transfection hosts was not found to substantially affect the pharmacological binding properties of a series of human G protein-coupled receptors that the author and colleagues at Synaptic Pharmaceuticals have been investigating. This information provides a welcome degree of freedom in the choice of host cells for transfection, which can then be chosen based on ease of transfection, complement of native G proteins, or other desirable criteria.

A similar result was observed in the case of the serotonin 5-HT_{1D} receptor, which illustrates an extreme case of the variations that can occur in the pharmacological properties of homologous genes in different species. The 5-HT_{1D} receptor is not present in rat cortical membranes: however, the rat brain contains a homologous receptor that exhibits such different pharmacological properties that it has been named a separate serotonin receptor subtype (5-HT_{1B}). Recently, a gene encoding the rat 5-HT_{1B} receptor was isolated and shown to be highly homologous to the human 5-HT_{1D} receptor gene (Adham et al. 1992). This demonstrates that a previous suggestion that the 5-HT_{1B} and 5-HT_{1D} receptors are essentially the same receptor subtype (Hoyer and Middlemiss 1989) was correct. This suggestion was based on the fact that both receptor subtypes show similar distribution in the basal ganglia, similar coupling to adenylate cyclase inhibition, and similar roles as terminal autoreceptors on raphe neurons (Waeber et al. 1990). When a human 5-HT_{1D} receptor clone was expressed in the same mouse fibroblast cell line used to express the 5-HT_{1B} receptor, this human receptor exhibited the binding properties appropriate for the human origin of its gene, rather than the mouse origin of its cell host (Branchek et al. 1991). It appears that no counterexample has been described where the host cell line dictates the pharmacological binding properties of a transfected gene. Protein processing and glycosylation patterns will differ from cell to cell, which may influence receptor kinetics, desensitization phenomena, receptor turnover, and the membrane compartment distribution of the receptor. Nevertheless, it appears that the pharmacological binding properties of 7TM receptors are determined primarily by their gene sequences with a much smaller role (if any) played by their cellular environments.

THE RELATIONSHIP BETWEEN AGONIST AND ANTAGONIST BINDING SITES

G protein-coupled receptors cycle through a complex series of G protein- and ligand-binding affinities. These binding states induce different affinity states for agonist ligands. The availability of transfected cell lines expressing single receptor subtypes now makes it possible to examine these binding states in much greater detail. Two recent studies (Branchek et al. 1990; Teitler et al. 1990) have resolved a longstanding controversy in the serotonin receptor field, namely, whether the serotonergic binding site for the agonist 4-bromo-2,5-dimethoxyphenylisopropylamine (DOB) and other related compounds is the high affinity agonist binding state of the 5-HT_2 receptor, which binds antagonist ligands such as ketanserin, or is a separate, closely related receptor subtype. Both interpretations have been advanced (Lyon et al. 1987; Pierce and Peroutka 1989) but the existing data now appear to strongly support the two-site rather than the two-receptor interpretation, as two studies utilizing transfected human (Branchek et al. 1990) and rat (Teitler et al. 1990) 5-HT_2 receptor clones have shown. Both studies reached the same conclusion: Transfection of a single cDNA clone into host mammalian cells produced two distinct binding sites (distinct [^{3}H]DOB and [^{3}H]ketanserin binding sites). Addition of guanine nucleotides to these systems reduced the number of agonist high-affinity binding sites with no change, or a slight increase, in the number of antagonist binding sites. Thus, it appears that [^{3} H]DOB and [^{3}H]ketanserin binding sites are distinct ligand-affinity states that exist at different times on the same 5-HT_2 receptor protein. A summary diagram of these affinity states, based on a review article by Freissmuth and colleagues (1989) is provided in figure 1.

The scientific community's increasing molecular understanding of this ligand affinity cycle needs to be better integrated into investigations of receptor function and into drug design programs. Because a complex interaction cycle involving two separate proteins and several forms of guanine nucleotides is involved in agonist binding, the model systems used as templates for drug design must be carefully chosen and carefully adjusted. They must properly mimic the natural processes occurring in those regions of native human brain that are chosen as a target for drug design. Because the types of G proteins, the relative receptor excess (spare receptors), and the amounts of intracellular guanosine diphosphate (GDP) and guanosine triphosphate (GTP) may vary widely in different brain regions, this complexity should be dealt with from the start of a drug design effort. Fortunately, the great freedom of choice of cell hosts and transfection densities that is possible when using cloned human receptors allows just the type of experimental freedom needed to address these issues. It also must be kept in mind that two distinct, but partially overlapping, sets of conformational states are involved in antagonist and agonist binding, and it is important to be sure that the proper mix of the proper states is present in biological screening models.

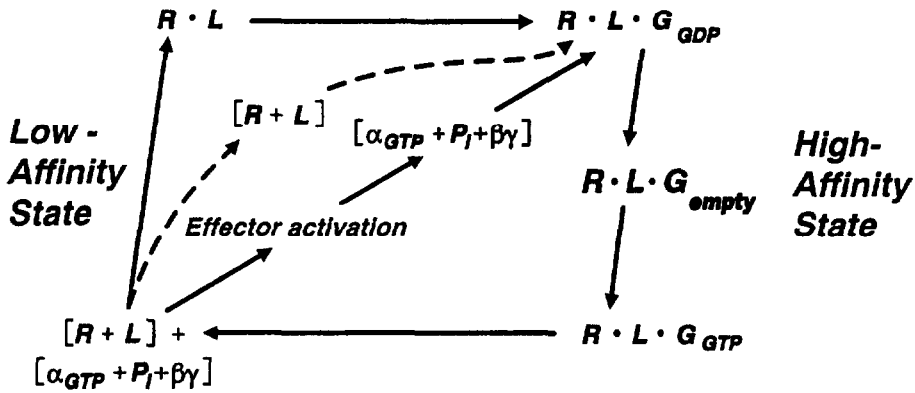

FIGURE 1. *Model for the functional activity cycle of G protein-coupled receptors (derived from Freissmuth and colleagues [1989]). Binding of an agonist ligand (L) to the receptor (R) produces a receptor-ligand complex (R•L), which binds a G protein complex. Agonists induce dissociation of guanosine diphosphate (GDP) from this complex, resulting in a "G empty" state, which is the high-affinity agonist-binding state. The G protein can then bind guanosine triphosphate (GTP), which induces dissociation of the α-subunit with GTP bound. This α_{GTP} complex can activate effector mechanisms (such as adenylate cyclase or phospholipase C) until such time as the intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP, starting the cycle again.*

AMINO ACID AND PHARMACOLOGICAL HOMOLOGIES BETWEEN RECEPTORS

Receptor researchers have traditionally organized their research groups around a specific neurotransmitter or hormone that activates a set of closely related receptors. For example, serotonin clubs and histamine meetings tend to segregate away from neurokinin researchers, even though all three of these biological mediators activate a group of 7TM receptors with many properties in common. Recent advances in molecular understanding of receptor structures suggest that this approach should change. Receptors of the 7TM superfamily are much more closely related in amino acid sequence and function than had been recognized previously. In addition, Hartig and colleagues (1992) have found that 7TM receptors of the same family (e.g., serotonin receptors) are often less closely related to each other than they are to other 7TM receptors. For example, the serotonin 5-HT_{1A} and 5-HT_{1D} receptors are both more homologous to several adrenergic receptors than they are to either the serotonin 5-HT_2 or 5-HT_{1C} receptors (Hartig et al. 1992). Interestingly, this relationship is also

reflected in the pharmacological binding properties of these clones. Many compounds that are classically recognized as adrenergic compounds exhibit moderate-to-high affinity for the 5-HT_{1A} receptor, and a similar relationship holds for the 5-HT_{1D} receptor. For example, yohimbine, which is a classic α_2 antagonist, exhibits a 25-nM affinity for the 5-HT_{1D} receptor (Branchek et al. 1991). This value is only sixfold weaker than its affinity at the α_{2A} site (Kobilka et al. 1987).

These molecular homologies have implications that extend beyond nucleotide sequence comparisons into the field of pharmacology. As is true for all proteins, the form of a neurotransmitter receptor determines its function. As a consequence, the pharmacological binding properties of 7TM receptors often cross family lines in ways that are reflected in the amino acid sequences of these receptors. In general, neurotransmitter receptors that display significant amino acid sequence homologies often display some overlap in the chemical structures that they will bind. Thus, the existence of greater than 50-percent transmembrane amino acid sequence homology should alert researchers to the possibility that receptors from different families may be more closely related in their binding properties than had been previously appreciated. Increased attention to these similarities in sequence and pharmacology could greatly aid the medicinal chemist in efforts to find high-affinity compounds for each receptor subtype and will help establish a much more directed approach to drug design.

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Synthetic Peptide Combinatorial Libraries May Be Used To Determine Peptide Ligands for Opioid Receptors

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INTRODUCTION

Isolation of naturally occurring peptide sequences from biological sources often requires years of laborious extraction, purification, and identification. This was true for the naturally occurring opioid peptides methionine and leucine enkephalin (Hughes et al. 1975). Optimization of these peptide sequences over the past 17 years has involved the synthesis and screening of thousands of analogs of the original biologically active sequences (Schiller 1990). Although thousands of analogs have been prepared and tested, they represent only a small fraction of the possible pentapeptides (20^5 for the L-amino acids alone). Existing methods for the synthesis and screening of many peptides are severely limited in their ability to generate and screen such numbers (i.e., millions) (Merrifield 1963; Geysen et al. 1984; Houghten 1985) and/or in their ability to generate unmodified free peptides in quantities capable of interacting in solution at concentrations appropriate for relevant in vitro assays (Fodor et al. 1991; Geysen et al. 1986; Scott and Smith 1990; Devlin et al. 1990; Cwirla et al. 1990). Therefore, Houghten and colleagues (1991, 1992) and Pinilla and colleagues (1992) have developed synthetic peptide combinatorial libraries (SPCLs) composed of mixtures of free peptides that in total exceed 50 million hexapeptides and in quantities that can be used directly in virtually all existing assay systems. Thus, in the example presented here, a library composed of 52,128,400 hexapeptides along with an iterative selection process was used to confirm the utility of the SPCL approach for the identification of active sequences in existing radio-receptor assays.

METHODS

Preparation of Synthetic Peptide Combinatorial Libraries

An SPCL consisting of six-residue peptide sequences having free N-terminals and amidated C-terminals was synthesized in which the first two amino acids in each peptide were individually and specifically defined, whereas the last four

amino acids consisted of equimolar mixtures of 19 of the 20 natural L-amino acids. (Cysteine was omitted from this library.) This synthesis generated 400 different peptide mixtures, each represented by the formula $O_1O_2XXXX\text{-}NH_2$ [O_1O_2=AA, AC, AD, AE, etc., through YV, YW, YY at positions O_1 and O_2; each X represents an equimolar mixture of the 19 amino acids] and containing 130,321 combinations ($1\ 9^4$), This library contains 400x130,321=52,128,400 hexapeptides.

The SPCL was assembled using the solid-phase approach (Merrifield 1963) on methylbenzhydrylamine (MBHA) polystyrene resin in combination with simultaneous multiple peptide synthesis (Houghten 1985) using t-Boc protected amino acids. The XXXX-resin was prepared using a process of division, coupling, and recombination (DCR) of individual resins, which ensures the equimolarity of each peptide within the XXXX-resin (Houghten et al. 1991). For this procedure, 19 equally weighed portions of resin (and, therefore, equal numbers of milliequivalents) were placed into porous polypropylene packets and then coupled to each of the protected N-*a*-t-Boc amino acids. Completion of coupling was determined by Kaiser's ninhydrin test (Kaiser et al. 1970). The resins were combined and thoroughly mixed. The resulting mixture (X-resin) was then divided into 19 equal portions, the N-a-t-Boc protecting groups were removed, and trifluoroacetic acid salts were neutralized. Each individual portion was then coupled to completion using one of the 19 different activated amino acids to generate 361 dipeptide resin combinations (XX-resin). The DCR process was repeated twice more to yield a XXXX-resin, representing an equimolar mixture of 130,321 tetramers. This XXXX-resin was then divided into 400 equal portions, and the two defined positions, O_1, and O_2 were coupled using the solid-phase multiple synthesis method (Houghten 1985). Amino acid analysis confirmed the expected equimolarity (±10 percent). Following deprotection and cleavage from the resins, each of the 400 peptide mixtures was extracted with water to yield a final peptide concentration of 1 to 3 mg/mL, which ensures that there is a sufficient concentration of each individual peptide within a mixture for use in standard in vitro assays (i.e., receptor/ligand interactions, antigen/antibody interactions, and enzyme/substrate interactions). At 1.0 mg/mL, each of the 130,321 peptides within each peptide mixture is present at a concentration of approximately 10.3 nM. Mixture concentrations as high as 5.0 mg/mL can be utilized readily.

Radioreceptor Assay

Particulate membranes were prepared using a modification of the method described by Pasternak and colleagues (1975). Rat brains frozen in liquid nitrogen were obtained from Rockland, Inc. (Gilbertsville, PA). The brains were defrosted, the cerebella were removed, and the remaining tissue was weighed. Each brain was individually homogenized in 40 mL Tris-HCl buffer (50 mM, pH 7.4, 4 °C) and centrifuged (16,000 rpm) for 10 minutes. The pellets were

resuspended in fresh Tris-HCl buffer and incubated at 37 °C for 40 minutes. Following incubation, the suspensions were centrifuged as before, the resulting pellets were resuspended in 100 volumes of Tris-HCl buffer, and the suspensions were combined. Membrane suspensions were prepared and used in the same day. Protein content of the crude homogenates ranged from 0.15 to 0.2 mg/mL as determined using the method described by Bradford (1976).

Binding assays were carried out in polypropylene tubes. Each tube contained 0.5 mL of membrane suspension, 8 nM [^{3}H]-DAGO (specific activity 36 Ci/mmol 160,000 cpm), 0.08 mg/mL peptide mixture, and Tris-HCl buffer in a total volume of 0.65 mL. Assay tubes were incubated for 60 minutes at 25 °C. The reaction was terminated by filtration through GF-B filters. The filters were subsequently washed with 6 mL Tris-HCl buffer, 4 °C. Bound radioactivity was counted on an LKB Beta-plate Liquid Scintillation Counter and expressed in counts per minute (cpm). To determine interassay and intra-assay variation, standard curves in which [^{3}H]-DAGO was incubated with a range of concentrations of unlabeled DAGO (0.13 to 3,900 nM) were included on each plate of each assay (using a 96-well format). Competitive inhibition assays were performed as above using serial dilutions of the peptide mixtures. IC_{50} (the concentration necessary to inhibit 50 percent of [^{3}H]-DAGO binding) values were then calculated using the software GRAPHPAD (ISI, San Diego) and were found to be consistent in three determinations.

RESULTS

Screening and Selection of Peptide Ligands

There are at least three known subclasses of opioid receptors: μ, δ, ,and κ (Schiller 1990). Enkephaiins, the natural ligands, bind to each of these binding sites with differing affinities. For this study the authors used an analog of met-enkephalin that is known to bind specifically to the μ-binding site, [^{3}H]-DAGO. Each of the 400 different peptide mixtures of the SPCL (O_1O_2,XXXX-NH_2) was assayed to determine its ability to inhibit binding of [^{3}H]-DAGO to the μ-receptor.

IC_{50}s were determined for each of the most effective peptide mixtures. YGXXXX-NH,, with an IC_{50}=3,452 nM, was found to be the most effective inhibiting peptide mixture (figure 1, table 1).

An iterative process was then carried out in which the subsequent X positions of YGXXXX-NH_2 were defined with each of the 20 natural L-amino acids. The peptide mixture sequences at each step of the screening and selection process, along with their respective IC_{50},s, are illustrated in table 1. Twenty new peptide mixtures were synthesized in which the third position of the peptide mixture YGXXXX-NH_2 was defined (YGOXXX-NH_2, i.e., YGAXXX-NH_2, YGCXXX-NH_2,

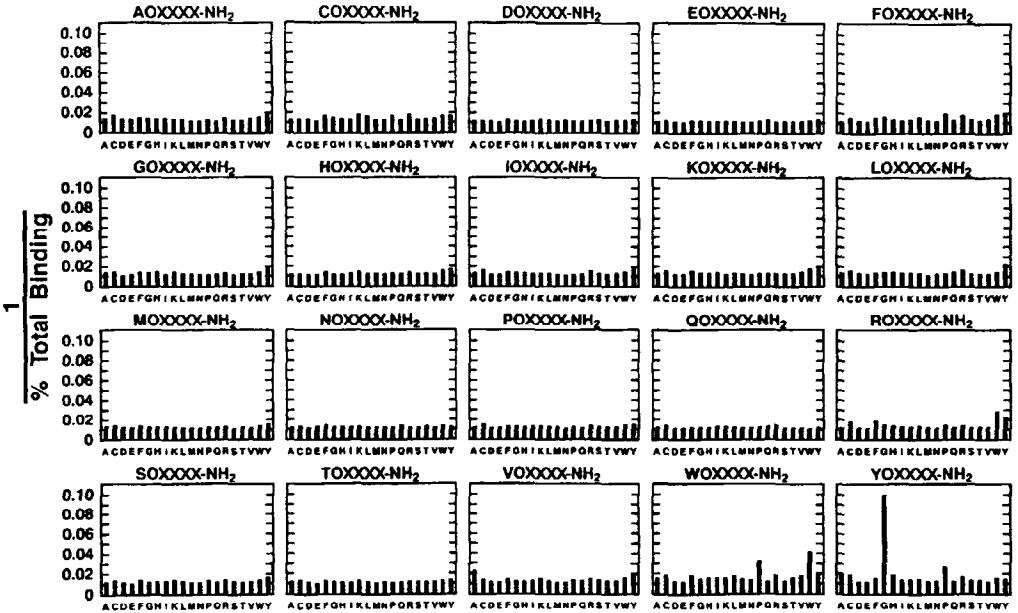

FIGURE 1. *Initial screening of the SPCL (O_1,O_2XXXX-NH_2) for ability to inhibit binding of [^{3}H]-DAGO to crude rat brain homogenates. Individual bar graphs are segregated by the first amino acid O_1 with individual bars in each graph representing the 20 amino acids making up the second position (O_2). The Y-axis represents the reciprocal of percent rota/ bound. Peptide mixtures (0.08 mg/mL) were incubated with [^{3}H]-DAGO (8 nM), 0.5 mL rat braii homogenate (0.15 to 0.2 mg/mL protein), and Tris-HCl buffer pH 7.4 at 25 °C for 60 minutes.*

TABLE 1. *Binding inhibition of [^{3}H]-DAGO by peptide mixtures in the iterative process*

Peptide Mixture	IC_{50} (nM)	Peptide	IC_{50} (nM)
(a)		(e)	
YGXXXX-NH_2	3,452	YGGFMA-NH,	28
YPXXXX-NH_2	6,005	YGGFMN-NH,	28
YCXXXX-NH_2	15,365	YGGFMX-NH,	28
YLXXXX-NH_2	16,055	YGGFMM-NH,	30
YWXXXX-NH_2	16,400	YGGFMS-NH,	34
YHXXXX-NH_2	16,770	YGGFMG-NH,	35
		YGGFML-NH,	35
(b)		YGGFMH-NH,	37
YGGXXX-NH,	3,254	YGGFMY-NH,	39
YGXXXX-NH,	3,452	YGGFMQ-NH,	40
YGFXXX-NH,	4,037	YGGFMT-NH,	41
YGNXXX-NH,	7,265	YGGFMK-NH,	41
YGTXXX-NH,	8,689	YGGFMR-NH,	46
YGSXXX-NH,	8,718	YGGFMP-NH,	57
		YGGFMI-NH,	80
(c)		YGGFMV-NH,	107
YGGFXX-NH,	153	YGGFMD-NH,	167
YGGWXX-NH,	292	YGGFME-NH,	204
YGGXXX-NH,	3,254	YGGFMF-NH,	223
YGGYXX-NH,	5,190		
YGGRXX-NH,	11,860		
YGGCXX-NH,	13,415		
(d)			
YGGFMX-NH,	28		
YGGFFX-NH,	57		
YGGFIX-NH,	67		
YGGFLX-NH,	74		
YGGFYX-NH,	119		
YGGFXX-NH,	153		

NOTE: IC_{50} values for the most effective inhibitory peptide mixtures at each iterative step are illustrated for (a) peptide mixtures from the initial screening of the SPCL, (b) the third position (YGOXXX-NH_2), (c) the fourth position (YGGOXX-NH_2), (d) the fifth position (YGGFOX-NH_2) and (e) the sixth position (YGGFMO-NH_2). The IC_{50} value of the peptide mixture of the previous iterative step is boxed for comparison.

and YGDXXX-NH_2). Of these, YGGXXX-NH_2 (IC_{50}=3,254 nM) was found to be the most effective inhibiting peptide mixture. The next best case, YGFXXX-NH_2, was approximately 50 percent less effective; all other substitutions in the third position were found to have activities much less than YGGXXX-NH_2 (table 1).

The iterative process was repeated for the remaining three positions, On defining YGGOXX-NH_2, the 2 most active cases, YGGFXX-NH_2 (IC_{50},=l53 nM) and YGGWXX-NH_2 (IC_{50}=292 nM), were substantially more active than the other 18 cases, and they were 34- and 18-fold better than the third most active case YGGYXX-NH_2 (IC_{50}=5,190 nM) and 21- and 11-fold better than YGGXXX-NH_2. This illustrates the specificity of the fourth position in the enkephalin sequence. When the fifth position of YGGFXX-NH_2 was defined (YGGFOX-NH,), it was found that YGGFXX-NH_2 was the most effective inhibiting mixture (IC_{50}=28 nM) and YGGFLX-NH, was the fourth most effective inhibitor (IC_{50}=74 nM). The first five residues of the peptide sequence mixture YGGFMX-NH, (IC_{50}=28 nM) exactly match the sequence of naturally occurring enkephalins found in earlier studies to recognize this receptor (figure 2). The second and third best cases at this position were YGGFFX-NH_2 (IC_{50}=57nM) and YGGFIX-NH_2 (IC_{50}=67 nM). When the final position (YGGFMO-NH_2) was defined, little improvement was found, and YGGFMA-NH_2, the most effective peptide

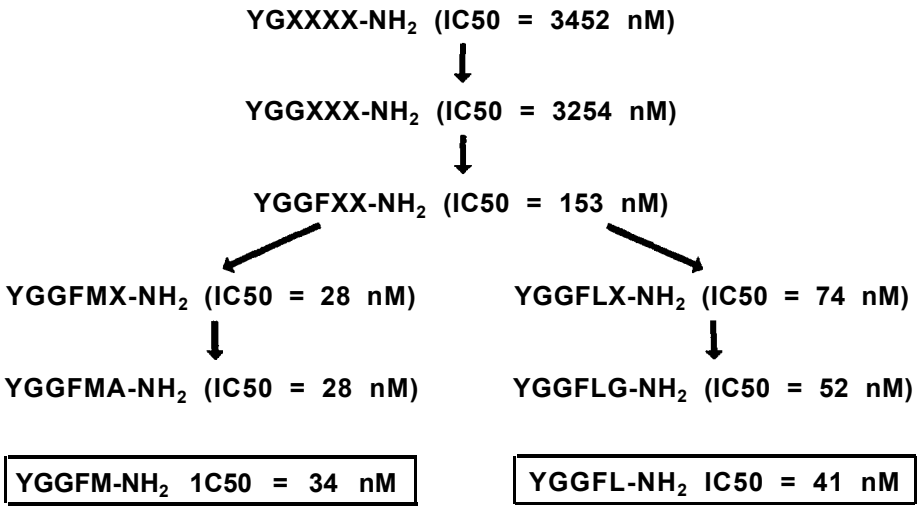

FIGURE 2. *A summary of the most active peptide mixtures found at each step of the iterative process; IC_{50} values obtained for the pentameric enkephalin sequences are boxed for comparison.*

(IC_{50}=28 nM), was only tenfold greater than the least effective peptide YGGFMF-NH_2 (IC_{50}=223 nM), which illustrates the redundancy of this position.

Using an SPCL composed of more than 52 million hexapeptides, combined with an iterative selection and enhancement process, the authors were able to determine a series of specific peptide sequences that inhibited binding of [^{3}H]-DAGO to its receptor. The equimolar representation of all possible individual peptide sequences in the SPCL enables precise identification of peptide ligands. It should be noted that no information about the sequence of the binding ligand is required to carry out determinations of this kind. We used a library that was not acetylated at the N-terminal because the enkephalins are known to need a free N-terminal tyrosine. Although this example yielded the expected sequences of leucine and methionine enkephalin as the first five residues in the hexapeptide sequence, the same process carried out with an N-acetylated hexapeptide library yielded sequences with no obvious relationship to the known enkephalins. This work will be reported elsewhere.

CONCLUSION

We have employed SPCLs in a variety of other assay systems, including the use of competitive enzyme-linked immunosorbent assay to study the binding of synthetic peptide antigens to monoclonal antibodies in which the correct antigenic determinant was identified (Houghten et al. 1991), in microdilution assays for the development of novel antimicrobial peptides (S. *aureus, P. aeruginosa, C. albicans)* (Houghten et al. 1992), and for potential antiviral peptides in plaque inhibition assays (HIV-1 and HSV) (manuscripts in preparation). SPCLs can be readily applied to the identification of peptide ligands in the majority of existing assay systems.

Because large numbers of highly active, defined peptide sequences can be readily generated through the use of SPCLs for any receptor system of interest, we believe that information derived from SPCLs will complement existing methods currently used for the detailed study of peptide/receptor interactions, such as x-ray crystallography, nuclear magnetic resonance, and computer modeling. We have found that SPCLs are of broad, general utility and greatly facilitate basic research and drug discovery involving peptides.

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Preparation of Large Peptide Libraries With One Peptide per Bead and Their Use for the Discovery of Peptides That Bind to Acceptors

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INTRODUCTION

Most biological processes are controlled and modulated by intermolecular interactions. These interactions can occur with a change in the covalent structure of one or more of the participants (e.g., an enzyme substrate interaction) or with no change in covalent structure (e.g., an antibody antigen interaction). In the latter case, in general, the three-dimensional structures of both the ligand and the acceptor molecule(s) change as a consequence of the interaction, which in turn can lead to a change in the physical and chemical properties of the acceptor molecule(s) in a way that modulates cellular structure and function. Thus, these interactions are critical requirements for the conformational, dynamic, and stereoelectronic changes that are central to the "chemistry of life" in biological systems. In this regard, peptides and proteins and their posttranslationally modified derivatives constitute the most widely used "molecules of life" and can serve many functional roles (e.g., as antibodies, enzymes, growth factors, immunomodulators, hormones, neurotransmitters, and receptors) and the many other functions that are critical to cellular function and to intercellular communication in complex living systems. It is no accident that nature uses proteins as the major functional molecules in living systems, which undoubtedly is related to the enormous physical and chemical diversity that is possible for these compounds. For example, if one utilizes only the 20 standard amino acid residues found in proteins and examines the number of distinct chemical species available, millions of molecules already are possible at the pentapeptide stage (3.2×10^6); that number becomes 1.28×10^9 at the heptapeptide stage. Thus, for even a small protein of 100 amino acid residues, there are an astronomical number of possibilities. It is not clear how many of these possibilities nature has explored, but it is clear that only a small fraction of those possible are used.

There is a great opportunity in the possibility for utilizing enormous chemical diversity to obtain a better understanding of how nature works and for the discovery of novel lead molecules that can serve as starting points for the development of agonist or antagonist drugs with specific biological effects. It also is a challenge to develop a simple method to prepare such large and diverse chemical libraries in a cost-effective manner that will allow examination of the binding properties of a particular species in the library and at the same time have each chemical species present in sufficient quantities for structure determination.

The authors recently proposed and developed a methodology for preparing large, statistically diverse peptide libraries (10^4 to 10^8 individual species) in a short time and for examining their ligand-binding activities with acceptor macromolecules such as antibodies and enzymes (Lam et al. 1991, 1992). This chapter briefly reviews the basic approach in this development and discusses some of the possible applications for the future.

RESULTS AND DISCUSSION

The creation of a large peptide library creates several problems. The most important is that of scale. The basic question posed was how much of each peptide is needed so that, on the one hand, sufficient concentrations are available to screen for binding and, on the other hand, sufficient amounts are available for structure determination. After examining several approaches, the authors devised a method that maximizes chances for success with the minimum amount of peptide. This method exploits the idea of one peptide for one bead. The synthetic methodology used is the Merrifield method (Merrifield 1963) in which the peptide of interest is synthesized on a solid support. This method has revolutionized peptide chemistry (and synthetic chemistry in general) in that it greatly simplifies the multistep synthetic processes of preparing a peptide. The growing peptide chain is assembled on a resin bead, and as a consequence, the solvents and reagents required for the synthesis can be removed by the simple expedient of washing. This methodology provides two other previously unexploited advantages. First, depending on the substitution level and size of the bead (in standard Merrifield syntheses, the substitution level is generally 0.2 to 1.0 mmol/g resin, and the bead size is generally from 50 to 200 microns), a high local concentration of peptide can be present on each bead. Calculations suggest that the local concentration of peptide on a single bead is in the millimolar to micromolar range and that the amount of peptide present would be from 50 to 300 pmol, more than sufficient to determine the sequence, because modern sequencers can sequence in the 2 to 5 pmol range. Second, a bead provides a unique surface (like a cell) for interaction of the ligand present on the surface of the bead with a macromolecule in the surrounding

medium. Such a surface should be excellent for large-scale screening in a minimum volume but with a large surface area.

The next question posed was, given the above considerations, how to prepare a large, chemically diverse peptide mixture in which each peptide in the mixture would be localized to a single resin bead and each bead would possess a single peptide and in which each peptide in the library would be present in approximately the same concentration. The answer to the latter part of the question is to have each bead in the mixture approximately the same size and substituted to the same extent. To ensure that each bead has a unique peptide and only that peptide, it is necessary that each bead be exposed to only a single coupling reaction at a time and that the reaction be driven to completion. The approach is similar to that presented by Furka and colleagues (1988, 1991), who, however, did not recognize the potential of having a unique peptide sequence on every bead. Finally, for diversity, one must turn to statistics. For example, if the 20 standard amino acids are used for the construction of a library, by dividing the resin into 20 equal parts, adding each amino acid to only one of these parts, then thoroughly mixing all the parts, by dividing into 20 equal parts again, adding each amino acid to only one of these parts, and so forth, one can rapidly develop a library of millions of peptides on millions of beads. Because a gram of standard-size solid-phase resin contains about 1 million 100-micron beads, very large libraries are feasible using Merrifield solid-phase methodology.

It is important to recognize two important synthetic issues in the construction of such a large, diverse peptide library. First, different amino acid residues have very different chemical reactivities. For example, activate N^{α}-Fmoc-alanine is much more reactive than N^{α}-Fmoc-isoleucine. Second, and even more important, when a large peptide library is constructed, the peptide products become very diverse chemically and hence differ greatly in their reactivity (nucleophilicity). Therefore, every reaction must be driven to completion. As a practical matter, we found that using a threefold to fourfold excess of the amino acid, a 1- to 2-hour reaction time in a minimum volume, and optimized Merrifield synthetic procedures generally leads to completion of the reaction even for difficult sequences such as Val to Val-peptide coupling. To check on the fidelity of the methodology for producing single peptide moieties on a bead, we examined a large number of single resin beads chosen both randomly and from our binding assay for their structure and for the synthetic fidelity of the synthetic peptide on the bead using a Pulsed Liquid Automatic Peptide Sequencer to determine the sequence and quantitate the amount of peptide on a single bead and then used modern preview analysis to determine the fidelity of the peptide on a single bead. These studies showed that each bead contains between 50 and 300 pmols of peptide as predicted, and preview analysis demonstrated that the peptides on each bead were homogeneous, with the percentage of a single peptide species being between

97 to 99.9 percent in a pentapeptide library of more than 2 million peptides (Lam et al., in press). The results of such an analysis for several pentapeptide beads that interact with streptavidin (Lam et al. 1991, 1992) are given in table 1. These results demonstrate that the synthetic methods used, optimized to maximize completion of reactions on solid phase resins, can provide large peptide libraries in which each peptide produced is present in enough yield and each bead present has on its surface and throughout the bead a unique peptide sequence. The realization that a single solid-phase bead could contain enough peptide in sufficient purity for use in a screening process was a critical element in the development of this methodology.

Next, these libraries were utilized to screen for binding to acceptor macromolecules. For this purpose, the authors prepared the libraries to leave the peptide attached to the bead, but with all the protecting groups (amino terminal and side-chain moieties) removed. Thus, it was necessary to develop a synthetic strategy so that all side-chain protecting groups could be removed following construction of the peptide library while the peptide remained on the resin and, in addition, to ensure that the peptide on the surface of the bead was accessible for binding to the macromolecular acceptor by construction of a spacer of suitable length that is "biocompatible." One approach is to use polyamide resins that have been modified with a spacer arm that consisted of a β-alanine, an ε-amino caproic acid moiety, and an ethylenediamine moiety attached to the polyamide polymer. In this strategy, Fmoc protection is used for N^{α}-amino groups, and tert-

TABLE 1. *Examination of the amount of peptide and preview analysis for individual pentapeptide beads that interact with streptavidin*

Sequence Determined	Peptide Recovered (pmol)	Percent Preview for Cycle 5
HPQGP	60	0.35
HPQAG	53	1.5
FHPQG	72	0.23
QHPQG	60	2.3
GHPQG	250	0.44
REHPQ	112	0.56
IQHPQ	192	1.8
GNHPQ	222	0
WMHPQ	257	2.7
TPHPQ	158	0
WNHPM	59	2.5
MHPMA	140	0.31

butyloxycarbonyl, tert-butyl esters, and tert-butyl ether type side-chain protections are used (N^{α}-Boc protection in conjunction with Fmoc-based side-chain protecting groups also can be used). The N^{α}-Fmoc groups were removed with piperidine, and following construction of the peptide library, the side-chain protecting groups were removed by 90 percent trifluoroacetic acid in N,N-dimethylformamide (v/v) containing 1 percent anisole and 0.9 percent ethanedithiol. The peptide-resin beads were then neutralized with 10 percent diisopropylethylamine in N,N-dimethylformamide and thoroughly washed with several solvents (including water) before use in screening. Utilizing this methodology, we obtained a number of peptide libraries suitable for rapid screening of the type described below.

To screen a library composed of millions of different chemical species requires careful consideration. Among the most common problems for consideration are the signal-to-noise (background signal) ratio, nonspecific chemical reactions, unexpected chemical reactions, and the usual problems of sensitivity and specificity. To overcome these problems, the authors developed a rapid screening method that utilizes an enzyme-linked immunoassay method in which the acceptor molecule (e.g., a monoclonal antibody) is coupled to the enzyme alkaline phosphatase, which can then catalyze a reaction in which those beads that interact with the monoclonal antibody become colored as a result of the enzyme-catalyzed reaction. These beads can be easily distinguished from those that do not react because they are colorless. The reacting beads (those that are colored) can be removed easily from the incubation medium by a micromanipulator under a low-power dissecting microscope as previously discussed (Lam et al. 1991, 1992).

The colored beads are then washed, the bound monoclonal antibody-enzyme complex removed by treatment with denaturing reagents that can solubilize proteins (e.g., 6M guanidine hydrochloride), and the single bead subjected to sequence analysis. In this way, unique new structures can be discovered that bind with high affinity (micromolar to nanomolar) to acceptor molecules. Ordinarily, we separately synthesize the binding peptides discovered by these procedures and examine their binding to the acceptor molecules by standard radioreceptor binding assays. Generally, there is a good correspondence between the binding seen on the bead and that obtained in the solution binding experiments (Lam et al. 1991, 1992, in press).

SUMMARY AND OVERVIEW

The authors developed a simple, highly reproducible, and synthetically clean method for preparing large, statistically diverse peptide libraries that can be used to discover ligands that will bind to acceptor molecules. Several other

methods were developed using both biological and chemical methods to provide polypeptide diversity for a variety of applications (Scott and Smith 1990; Geysen et al. 1984; Fodor et al. 1991; Cwirla et al. 1990; Houghten et al. 1991; Oldenburg et al. 1992; Devlin et al. 1990; Scott et al. 1992; Cull et al. 1992; Brenner and Lerner 1992). These methods, and others to be discovered and developed, offer a number of opportunities to examine in new ways the fundamental issues of molecular recognition and the diversity of ways in which molecular recognition problems can be solved. With the availability of large, diverse peptide libraries, it is likely that, for the same acceptor site on a macromolecule, several different peptide structures will bind specifically to the same site with an affinity within a few orders of magnitude of each other.

From these and other fundamental studies, it will be possible to address issues that could not be addressed before the availability of these libraries. For example, there are the basic questions of whether a small peptide of 5 to 15 residues can mimic a protein in its activities, whether a small linear peptide can serve as a mimotope for a discontinuous epitope on an antibody, or how big an area an inhibitor needs to recognize to be able to block the binding of the native ligand. The last question is an especially critical question for protein-protein interactions, where fairly large surface areas appear to be involved in molecular recognition. Can a small peptide serve as a competitive inhibitor for one of the sites of interaction to block the entire interaction? The answer is not clear.

Another major issue is the difference between a peptide agonist and a peptide antagonist. It was suggested many years ago that peptide agonists and antagonists, even competitive ones, bind differently to the acceptor molecule (Meraldi et al. 1977). In this case, a peptide antagonist had greatly reduced flexibility relative to the agonist, and the specific induced rigidity was critical for antagonist bioactivity (Meraldi et al. 1977). Perhaps this effect of rigidity explains why small, relatively rigid peptidomimetics generally are antagonists of ligands for macromolecular acceptors.

Another issue is how many different ways a particular molecular recognition problem can be solved. For example, it already has been noted that a particular recognition favors a particular primary sequence, called a consensus sequence, and that this sequence is conserved throughout evolution. The question is whether this sequence is truly “essential” or whether some other sequence can serve the same purpose. This question now can be explored by constructing a large library of peptides (millions or more) that do not possess this consensus to determine whether one or more new sequences can accomplish the same binding interactions and whether a new consensus sequence” will be developed. Another extension of these methods is molecular

recognition processes involving nonpeptides, such as sugars, polysaccharides, nucleotides, and lipids. Again, some very critical issues can be addressed.

Many other possibilities exist for exploiting molecular diversity, and acceleration can be expected in the development of assay methods, chemical methods, and physical methods to examine complex diverse mixtures. In this regard, it is likely that the development of new synthetic methods to prepare large, diverse chemical structures will challenge the current methods of structural elucidation.

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Computer Design of Bioactive Compounds Based on 3-D Properties of Ligands

Y.C. Martin

INTRODUCTION

The computer design of bioactive molecules is now a reality (for a review, see Martin 1992). The design is based on the observed or postulated three-dimensional (3-D) properties of the ligand as bound to the target macromolecule. Single crystal x-ray crystallography of the ligand macromolecule complex can supply the necessary information. However, an x-ray structure may not be available, at least at the beginning stages of a medicinal chemistry investigation. As an alternative, ligand structure-activity relationships are a sensitive probe of the shape and chemical properties of the binding site on a macromolecule of unknown 3-D structure. This structure-activity relationship can be used to map the binding site from the inside out instead of from the outside in as one would do with protein crystallography (Martin 1991).

The resulting pharmacophore maps are 3-D summaries of the structure-activity relationships on which they are based. Beyond this, they provide the necessary information for computer techniques that design new compounds to meet the 3-D criteria (Martin 1992) and for those that forecast the potency of compounds suggested by people or computer (Cramer et al. 1988). Thus, there are three aspects of the structure-activity-based molecular design strategy: pharmacophore mapping, 3-D searching and molecular design, and 3-D quantitative structure-activity relationships (Martin 1991). Each of these pieces forms a part of the strategy to convert two-dimensional (2-D) structure-activity information into a novel bioactive molecule by consideration of the 3-D properties of the molecules.

PHARMACOPHORE MAPPING

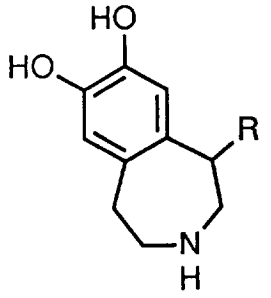
Description

The goal of pharmacophore mapping is to establish the bioactive conformations of the ligands and how to superimpose these conformations. For pharmacophore

mapping, one needs structure-activity relationships of structurally diverse and conformationally informative molecules. From this information, one first proposes a pharmacophore, that is, the chemical nature of the groups required for bioactivity and the geometric relationships between them. The structure-activity relationships of the ligands establish the required groups, answering the question, Activity is destroyed by removal of what group? Conformationally constrained compounds that are also active may help establish the bioactive conformation of all the compounds. The molecules are superimposed, in their proposed bioactive conformations, over the atoms of the pharmacophore or their projected binding points on the macromolecule. The union of the volumes occupied by the active compounds as superimposed suggests the regions in space that can be occupied by any newly designed active ligand. In addition, new regions in space occupied by compounds that meet the pharmacophore requirements but are inactive define "forbidden regions" that, if occupied, destroy activity.

Application to D_1 Agonists

Martin and colleagues' work (1991) started with the observation that the phenyl group of SKF 38393 (I in figure 1) increases the affinity for the D_1 dopaminergic receptor by 100X compared with II. The goal was to find potent D_1 agonists that have little D_2 activity. Distance geometry generation of conformations (Crippen and Havel 1988) and energy minimization revealed that the axial and equatorial conformations shown in figure 2 are approximately equal in energy and are the lowest found. To establish which is the bioactive conformation, we synthesized several compounds that placed an added phenyl in an axial or equatorial position. Only those with the phenyl in the equatorial position show the phenyl boost. Thus, the bioactive conformation of I is equatorial.



I. R = C_6H_5
II. R = H

FIGURE 1. *The structure of SKF 38393 (I) and its analog*

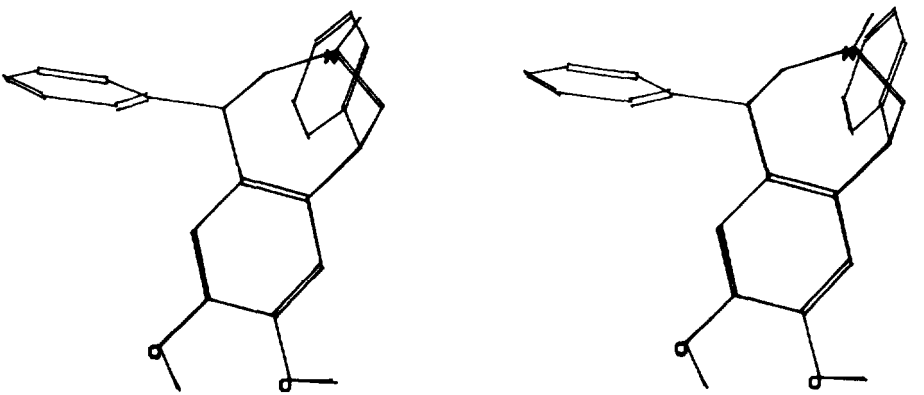
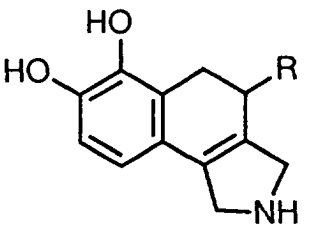


FIGURE 2. *The two low-energy conformations of I. The equatorial conformation is shown in fine lines and the axial conformation in heavy lines.*

The first potent mimic of I was designed by using molecular graphics of the pharmacophore model. In screening, III (figure 3) had been identified as a potent but nonselective dopaminergic agonist. Examination of the 3-D structure of this molecule (figure 4) showed that a phenyl should be placed as in IV (figure 3). IV is a potent and selective D_1 agonist (table 1).

UTILITY OF 3-D DATABASES IN MOLECULAR MODELING

To organize the pharmacophore modeling of many compounds by the small-molecule group at Abbott Laboratories, Martin and colleagues (1988) developed



III. R = H
IV. R = C_6H_5

FIGURE 3. *The lead compound (III) and the designed compound (IV)*

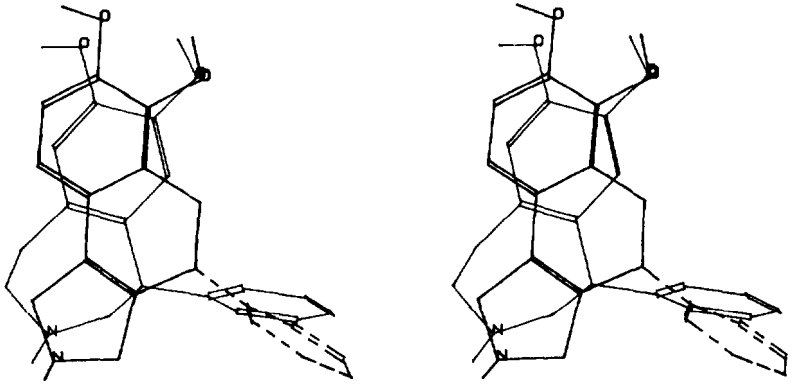


FIGURE 4. *The 3-D structures of III (fine lines) and IV (dashed fine lines) over the bioactive conformation of I (heavy lines)*

a 3-D database system based on the Daylight Chemical Information System. The coordinates and charges as well as literature and project biological properties are stored. In addition, the compounds can be searched in the database by substructure. An advantage to using a chemical information database is that all sets of coordinates of a compound are together regardless of their original source.

Abbott Laboratories' small-molecule molecular graphics program reads and writes directly to these 3-D databases (Martin et al. 1992). This permits the graphics program to be used to display the bioactive conformation without the user's knowing its dataset name, to display all stored conformations of a molecule, or to display all members of a group used to derive a pharmacophore or 3DQSAR model. Because the bioactivity associated with each bioactive conformation is identified, the database also can be asked whether the same conformation is responsible for different bioactivities.

3-D SUBSTRUCTURE SEARCHING BACKGROUND

The discovery of IV created a problem. How could I design compounds that mimic the arrangement of phenyl groups in the bioactlve conformation of I or II while maintaining the proper geometric relationships between the pharmacophore N and O atoms? As I pursued this goal at the molecular graphics screen, my enthusiasm turned to frustration. No imagined molecule matched well enough, and energy minimization often changed conformations that looked promising on the screen. By the time I had designed three compounds, I lacked more ideas.

TABLE 1. *Receptor affinity of dopaminergic agonists studied*

Compound	Source	-Log Affinity		Log Selectivity
		D_1	D_2	
I	Literature	7.19	5.16	2.13
II	Literature	5.16	5.60	-0.44
III	Screening of Abbott Laboratories compounds	5.89	5.53	0.46
IV	Pharmacophore mapping plus molecular graphics of III	7.24	<4.0	>3.24
V	3-D searching of Abbott library of compounds based on pharmacophore mapping	4.88	<4.0	>0.88
VI	Pharmacophore mapping plus molecular graphics of V	6.82	5.19	1.63
VII	Traditional medicinal chemistry and molecular graphics based on VI	8.52	6.11	2.41
VIII	Traditional medicinal chemistry and molecular graphics based on I, IV, VI, and VII	7.50	5.89	1.61

SOURCE: J.W. Kebablan, R. MacKenzie, and R. Schoenleber, unpublished observations

On reflection, it became apparent that many of the structures I tried were not built from scratch. Rather, I made them from molecules that I had studied earlier and for which a shape, as remembered, resembled the design target. In other words, I had searched my mental database of 3-D structures for those with the desired features. Because coordinates were already being stored in a chemical information database, a computer program could be written to do this searching properly. We did so in the program ALADDIN (Van Drie et al. 1989).

Gund and colleagues (1974) and Gund (1977) had suggested 3-D searching much earlier but had created no 3-D database. Esaki (1982) followed this work with another prototype system. Simultaneously with the author and colleagues' work, Jakes and Willett (1986), Jakes and coworkers (1987), Brint and Willett (1987), DesJarlais and colleagues (1988), Sheridan and Venkataraghavan

(1987), Lewis and Dean (1989*a,* 1989*b),* Bartlett and coworkers (1989), and Sheridan and colleagues (1989) were addressing the same problem from different viewpoints. The field has grown so much that there are now six 3-D database-searching systems available to researchers other than that of the original developer (Martin 1992). Dozens of laboratories around the world are doing research on the topic. The impetus for this work is the increasing availability both of computer hardware for 3-D molecular modeling and of 3-D protein structures.

Description

The aim of 3-D substructure searching is to find or design molecules that meet 3-D criteria by examining the 3-D structures of hundreds or thousands of molecules (Martin et al. 1990; Martin 1992). In contrast to pharmacophore mapping, a 3-D search needs no existing structure-activity relationships. However, the more information used, the more likely the correct search question will be posed.

There are three main types of searches. Geometric search questions deal with the intramolecular relationships between geometric objects (points, lines, and planes) calculated from a structure. They are independent of the enantiomer of the compound and its orientation in space. Similarity searching asks how similar a database molecule is in 3-D properties to the reference molecule. In contrast, steric searches ask whether a candidate structure can fit into a particular volume. The result of a steric search is different for enantiomers of a molecule. The allowed orientations of the molecule in the target space are determined by some steric searching programs or specified by the user in others.

Uses of 3-D Searching

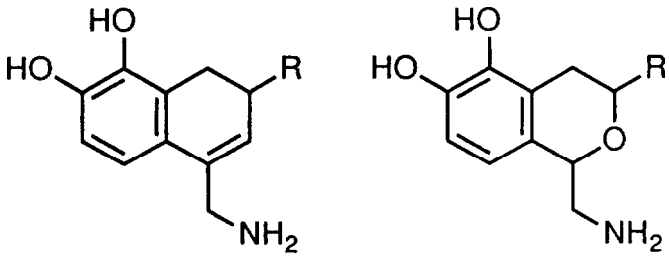
3-D searching has three distinct applications in medicinal chemistry. First, 3-D searches may recognize known compounds that meet 3-D criteria (new bioactivities in old molecules). Second, 3-D searches may test a proposed pharmacophore. Only if the search identifies all active molecules is the pharmacophore correct. Third, 3-D searching may help design new compounds that mimic one or another of the low-energy conformations of an active molecule. As described above with I (Martin et al. 1991), the synthesis and testing of such a set of compounds may identify which is the bioactive conformation. Note that 3-D searching may help design new compounds that mimic the bioactive conformation of a molecule. These may show improved features such as improved bioavailability, selectivity, ease of synthesis, or patentability.

Sources of 3-D Structures To Search

Of course, one needs 3-D structures to search. In our original work, the only coordinates available were in databases of modeled compounds, Somewhat later the CONnectivity to COoRDinates (CONCORD) program, which generates a 3-D structure of a molecule in a few seconds, became available.' We used CONCORD to build 3-D databases of our corporate and commercially available compounds (M. Bures, unpublished data). Sometimes it would serve no use for coordinates to be in a database; thus, files of 3-D structures also can be searched with ALADDIN. Recently, we added the option to generate the structure with CONCORD as part of the 3-D searching process (Y.C. Martin and E.B. Danaher, unpublished data).

Design of Novel D_1 Agonists With 3-D Searching of Existing Compounds and Molecular Modeling To Suggest Analogs

ALADDIN was used to search Abbott's small-molecule modeling group's database of compounds modeled for various projects to find existing compounds that might have dopaminergic activity (Martin et al. 1991). This search identified V (figure 5) as well as several other compounds that were later shown to be active. Molecular graphics of V (figure 6) showed where to add the phenyl group to impart D_1 selectivity, compound VI. Table 1 summarizes the D_1 and D_2 binding affinity of the compounds.



V. R = H
VI. R = C_6H_5

VII. R = C_6H_5
VIII. R = adamantyl

FIGURE 5. *Structures of the database compound V and molecules designed from it*

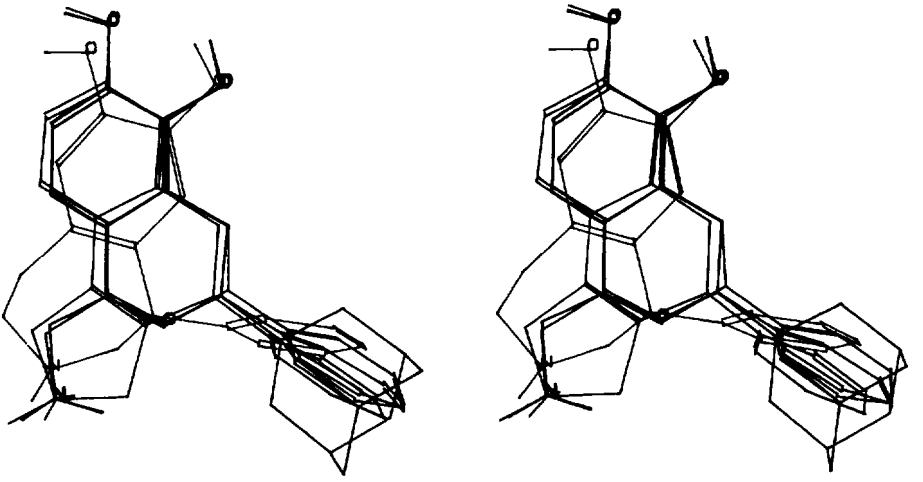


FIGURE 6. *The superposition of the bioactive conformations of I (solid heavy lines), IV (fine lines), VI (fine lines), VII (fine lines), and VIII (fine lines)*

Traditional medicinal chemistry of this new lead led to a more potent analog, VII (DeNinno et al. 1991). This compound is the most potent and selective D_1 dopaminergic agonist known. Figure 6 shows this compound compared with the other D_1 selective compounds. Notice that the pharmacophore N and meta OH groups are in roughly the same geometric relationship in these active dopaminergic compounds. The pendant phenyl group occupies slightly different regions in space in the different compounds. This suggested that the phenyl might be replaced with other bulky groups. Accordingly, the adamantyl analog VIII was synthesized. It is also a potent agonist. Thus, we successfully applied the strategy of finding weakly active compounds with a 3-D search and using molecular graphics and traditional medicinal chemistry to suggest more potent and selective analogs (Martin et al. 1991).

Strategy To Design Novel Compounds With 3-D Searching for Templates and Computer Transformation of Structures

3-D searching also can design novel compounds that match a pharmacophore. Table 2 shows the strategy (Martin 1990). The core of this strategy is to identify molecules that match the pharmacophore in geometric relationships but not atom types, The appropriate atoms are then mutated into those required for the pharmacophore. Thus, the search covers a database of molecules included for their geometric properties only. Notice that we use CONCORD to generate the 3-D structures of the designed molecules. The process shown in table 2 represents the computer invention of new molecules.

TABLE 2. *Steps in the de novo design of bioactive compounds using 3-D searching*

Step in the Desian	Process	Result
Map the pharmacophore	Computer investigation of active compounds to suggest a bioactive conformation and superposition rule	Geometric model of pharmacophore
Search for geometric templates	3-D search based on geometry of the pharmacophore	Molecules that match pharmacophore geometry
Change the templates into the 2-D structures of the proposed compounds	MODSMI transformation of 2-D structures of the templates	2-D structures of new molecules that might match the pharmacophore
Generate 3-D structures of designed molecules	CONCORD generation of 3-D structures from 2-D	3-D structures of new molecules that might match the pharmacophore

KEY: MODSMI = MODify SMIles

To implement the strategy, we invented a language (MODify SMIles [MODSMI]) that is used to tell the computer how to change the structure of the database molecule into that expected to be active (Martin and Van Drie 1990). This MODSMI language transforms the SMILES (Weininger and Weininger 1988) description of the 2-D structures of the database molecules into the target 2-D structures. The four verbs of MODSMI are “nibble,” to remove an atom: “replace,” to change one atom into another; “axe,” to break a bond or change its bond order; and “join,” to make a bond between two atoms. Because the atoms to be transformed into the pharmacophore atoms are identified in the SMILES of the database molecules, the transformations can be applied only to certain atoms (Martin and Van Drie 1990).

The MODSMI language was used for two purposes in the design of potential dopamine agonists (Martin 1990; Martin and Van Drie 1990). First, the pharmacophore atoms were added to the appropriate places in the structure. Second, geometrically irrelevant substituents were removed from the structures, and nonpharmacophore heteroatoms were changed into carbons, This

prevents the computer suggestion of a series of close analogs. Figure 7 shows the transformation of three database molecules into potential dopaminergic agonists. Both the removal of irrelevant substituents and substructures and the addition of the pharmacophore atoms are shown.

Application of the Strategy to the Design of Novel Dopaminergic Agonists

To evaluate this procedure, I tested its ability to design dopaminergic agonists (Martin 1990). Compounds IX through XVII (figure 8) are the essential substructures of known dopaminergic agonists in which ail bonds between the N and O are in a ring.

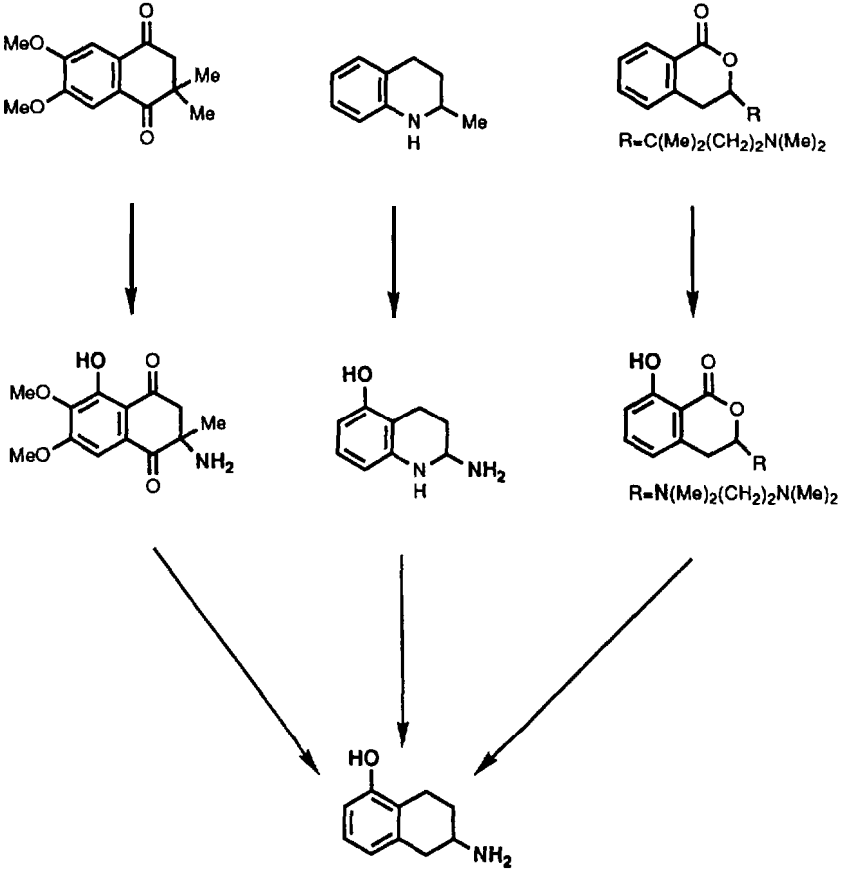


FIGURE 7. *The transformation of three database molecules into the same molecule suggested for synthesis*

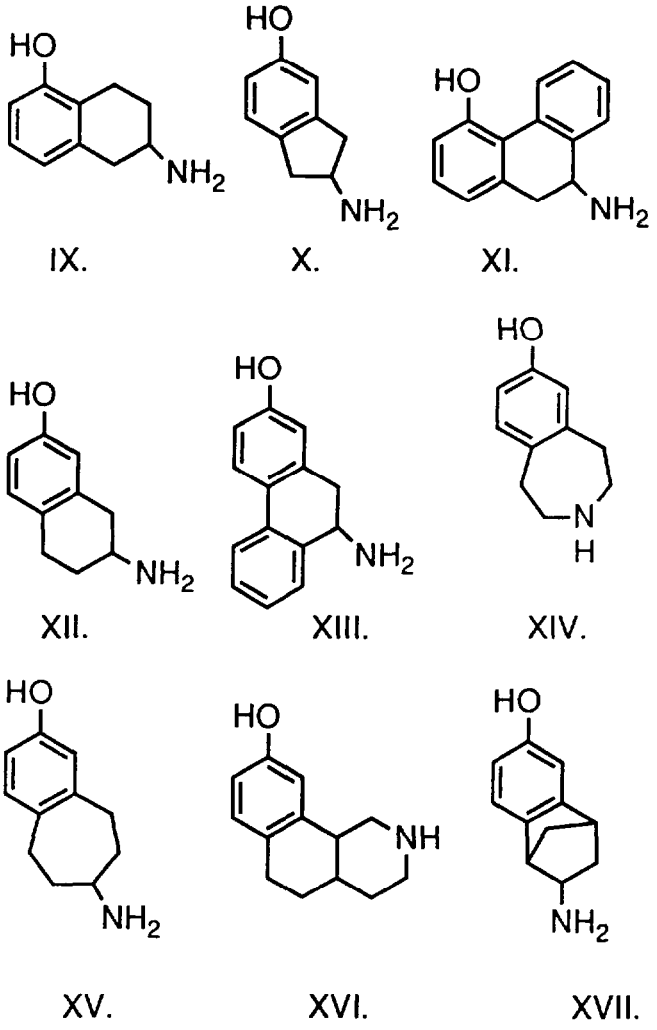


FIGURE 8. *Essential structures of known dopaminergic agonists*

I searched three databases: (1) the Fine Chemicals Directory of 80,000 commercially available compounds, (2) the Pomona College Medicinal Chemistry Project database of 27,000 compounds with measured solvent-water partition coefficients (the 3-D structures in these databases were generated with CONCORD), and (3) the Abbott small-molecule modeling group's database of carefully modeled compounds, 9,000 3-D structures from 3,000 compounds,

Compounds XVIII through XXI in figure 9 are examples of the types of compounds suggested. Figure 10 shows one of the designed compounds superimposed over apomorphine. There is a strong resemblance between the 3-D structures of the two compounds.

Does this strategy design the known molecules? Yes, it suggested eight of nine known classes of fused-ring dopaminergics. Does it design new molecules? Yes, it designed 75 novel series compounds in which all atoms between the O and N of the pharmacophore are in a ring (a total of >300 molecules). It also designed more than 100 other compounds with one rotatable bond in the path for a total of 508 molecules.

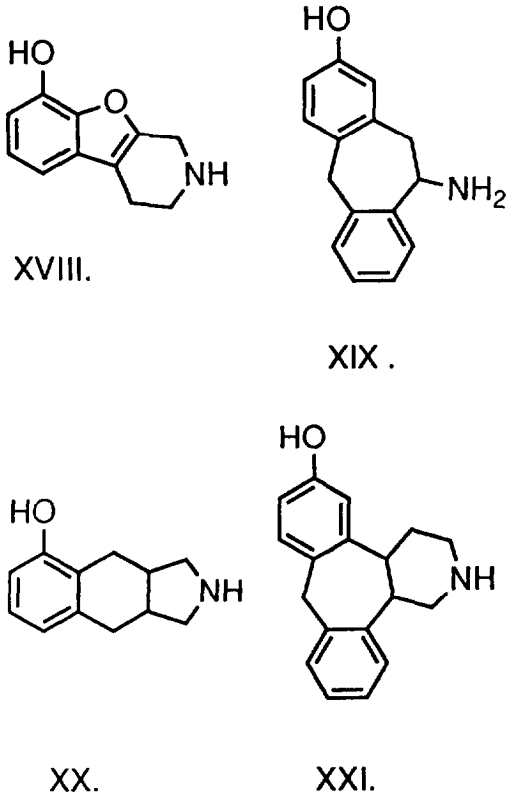


FIGURE 9. *Examples from the >500 potential dopaminergics designed by ALADDIN*

SOURCE: Martin 1991

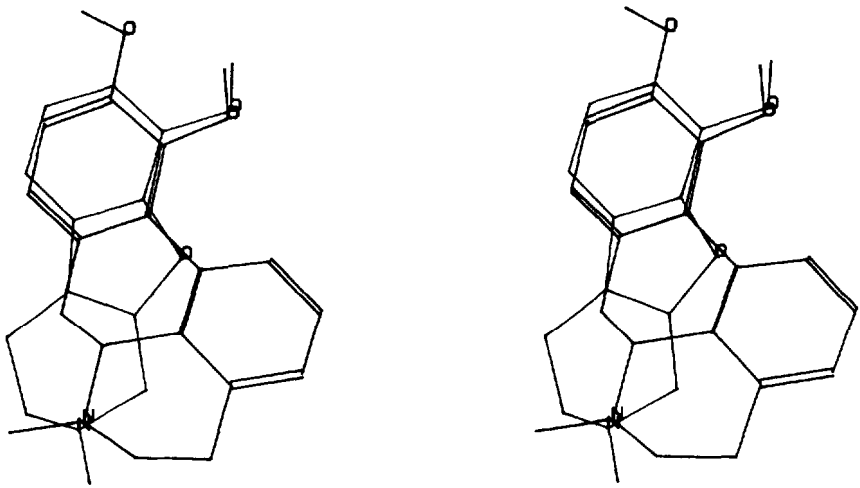
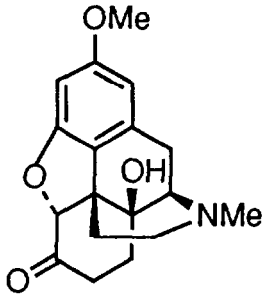


FIGURE 10. *The superposition of XVIII (heavy lines) and apomorphine (fine lines)*

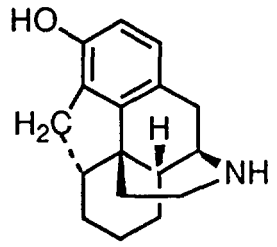
Evaluation of the Strategy by the Design of Morphine Mimics

Similar strategies can be used to design mimics of peptides in their bioactive conformations (Bartlett et al. 1989). If one does not know the bioactive conformation, one can use these methods to design mimics of the various low-energy conformations. Similarly, one can use 3-D searching to design compounds that probe the direction of binding of heteroatoms to a macromolecule.

I decided to test the strategy by using the bioactive conformation of enkephalin for the search criteria and asked whether morphine is designed (Y.C. Martin, unpublished data). To not bias the results with structures we had modeled or tested at Abbott, we searched the Fine Chemicals Directory for 3-D templates and transformed them as had been done in the dopaminergic example. The search criteria included an unsubstituted aromatic atom to which the required OH would be added. Thus, morphine would not meet the search criteria. However, the search identified XXII (figure 11), from which the morphine mimic XXIII was designed with MODSMI. Thus, the computer would design morphine from enkephalin distances.



XXII



XXIII

FIGURE 11. *A database molecule, XXII, that led to the design of a morphine mimic, XXIII*

QUANTITATIVE FORECAST OF POTENCY OF SUGGESTED MOLECULES USING COMFA 3-D QUANTITATIVE STRUCTURE-ACTIVITY ANALYSIS

It is not sufficient to design novel compounds. These compounds also must be potent. Thus, the third aspect of the computer design of bioactive molecules involves the understanding of the 3-D quantitative structure-activity relationships, We have successfully used a special implementation of comparative molecular field analysis (CoMFA) (Cramer et al. 1988) for this purpose (Martin et al. 1992).

Description of CoMFA

For CoMFA one needs to have a biological potency measured for at least 15 molecules, In addition, one needs the superposition rule and bioactive conformations derived from pharmacophore mapping. A CoMFA starts with calculating fields around each molecule at intersections of a 2Å lattice. The fields are calculated using traditional potential energy equations. Typically, energies are calculated for at least 1,000 points. In the analysis, high positive steric energies are truncated to a more modest value (4 to 25 kcal/mol, for example). Lattice locations with low standard deviations are discarded from the calculations. The relationship between the remaining fields and potency is evaluated using partial least squares, a variant of principal components. Because there are more energy values per compound than compounds, cross-validation is an essential aspect of the method. In cross-validation, the potency of each compound is forecast from a model from which it had been deleted.

The result of a CoMFA analysis is an equation that describes the contribution of each lattice energy value to potency. Such equations are used for forecasting the potency of additional compounds, For ease of understanding, one displays contours of the coefficients such as those shown in figure 12.

Evaluation of CoMFA

The Abbott implementation of CoMFA (Martin et al. 1992) differs from that of Cramer and colleagues (1988) in that the molecular fields are calculated using the program GRID (Boobbeyer et al. 1989; Goodford 1985).

Martin and colleagues (1992) showed that CoMFA performs as expected. First, CoMFA fits and forecasts physical properties such as pKa's and linear free energy sterlc constants. Also, It provides a good fit for biological data that previously had been shown to be correlated with octanol-water logP. For these three situations, we used different properties of the probe atoms. For pKa's, a positively charged probe with no hydrogen bonding character was used: for steric effects, a neutral probe with no hydrogen bonding character was used: and for logP, a neutral probe that is both a hydrogen-bond donor and an acceptor was used.

We also showed that CoMFA steric parameters correctly describe the structure of the binding site. For this we derived a CoMFA for a set of quinones that bind to the bacterial photosynthetic reaction center. The locations of the CoMFA coefficients agree well with the 3-D structure of the protein determined by single crystal x-ray crystallography.

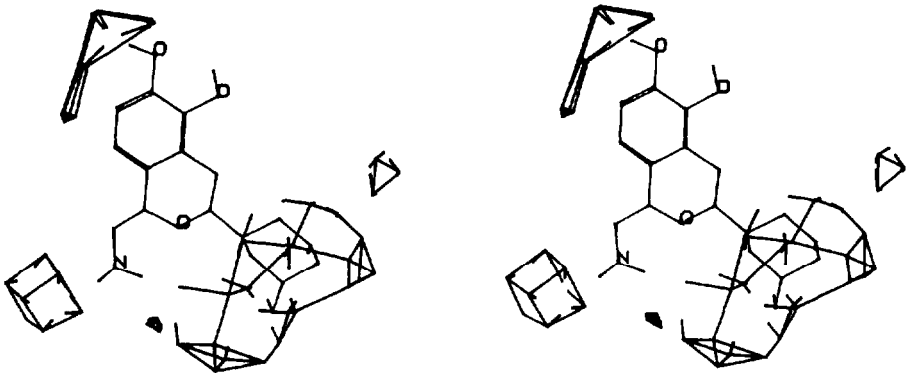


FIGURE 12. *The positive CoMFA contours for the D_1 dopaminergic binding model in fine lines and VIII in heavy lines*

Last, we showed that CoMFA relationships correctly forecast the potency of compounds not included in its derivation.

INTEGRATION OF 3-D SEARCHING AND 3-DQSAR

We added an option to ALADDIN so that it now reads a set of CoMFA coefficients, a coordinate set that was part of the CoMFA, and the superposition rule (Y.C. Martin and E.B. Danaher, unpublished data). ALADDIN orients the designed molecule (and its enantiomer if requested) and marks atoms of the molecule that hit negative CoMFA coefficients. These atoms can be removed with the MODSMI routines.

As noted above, ALADDIN designed 508 compounds that match the D_2 pharmacophore. Of these, 110 did not hit negative D_1 steric contours. Thus, only 22 percent of the compounds designed to match a pharmacophore were forecast to be active. MODSMI was used on the remaining 398 compounds to remove the atoms that hit a negative D_1 CoMFA steric contour. Of these redesigned compounds, 132 did not hit negative contours, and 26 of these were not in the first 110. Thus, ALADDIN designed 136 compounds that fit into the D_1 steric contours.

The characteristics of the series of compounds tested will determine the information gained from a CoMFA analysis. Does the computer design a better set of compounds for CoMFA analysis than do chemists? A series of 20 chemist-designed D_2 agonists were composed with a series of the same size selected from those designed by the computer (Lin et al. 1990). Shape was described by the steric energies as used in CoMFA. To choose the series of computer-designed compounds, a cluster analysis based on the 25 highest principal components of the steric fields was used. Compared with the chemist-designed series, the computer-designed series shows a larger variation in steric properties and has less correlation between steric properties. it explores all space explored by the former series. The mean and range of the forecast D_1 and D_2 dopaminergic receptor-binding affinities for the two series are not different. Thus, multivariate statistical methods based on energy fields provide the tools to choose a good series of molecules for a CoMFA analysis. These methods also reduce a large set of suggested molecules to one that is reasonable to synthesize.

SUMMARY

3-D database searching has many uses for a medicinal chemist. It can aid in the design of compounds to probe or to mimic the bioactive conformation of a natural ligand or to fit a hypothetical or experimental structure of a binding site. It also can identify existing molecules that meet these criteria—new uses for old

molecules. If one has a database of active compounds, 3-D searching can validate or refute a pharmacophore hypothesis.

The CoMFA method of 3DQSAR can be used to forecast the potency of the designed analogs. Also, the integration of CoMFA and 3-D searching concepts provides a framework for the design of a good series for CoMFA. In addition, CoMFA 3DQSAR coefficients provide a model of the binding site to facilitate the design of compounds that fit the pharmacophore and do not hit stericaiiy unfavorable regions.

NOTE

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- J. DeLazzer—Programed much of the molecular graphics.
- K. Kim—Collaborated in CoMFA development and evaluation.
- T. Lin—Implemented PLS and collaborated in CoMFA development and evaluation.
- P. Paulik—Modeled compounds for the D_1 pharmacophore and CoMFA.
- R. Schoenleber—Synthesized the D_1 dopaminergic compounds to elucidate the bioactive conformer of I and supervised the synthesis of IV, VI-VIII.
- J. Van Drie—Programed the initial version of ALADDIN, designed and programed MODSMI.

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Toward the Design of New Inhibitors: A Computer Modeling Approach

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INTRODUCTION

There has been increased interest in the use of databases for the discovery of potential new lead compounds (Sheridan and Venkataraghavan 1987; Sheridan et al. 1989; Martin 1990). An excellent example of this effort is provided by ALADDIN (Van Drie et al. 1989), a computer program that searches a database of three-dimensional structures for the design of new compounds with interesting biological properties. The author believes it is important to try to use more than one method in combination with a database of crystal structures for the design of new inhibitors. If one candidate is found with more than one method, this would enhance the possibility of its being a good template. This process is a distinct feature of this study. Initially, I focused my work on searches based mainly on shape properties, and thus I chose the following independent methods:

1. Docking techniques
2. CAVEAT analysis
3. QUEST programs

The Cambridge Crystallographic Database (CCD) version 4.5 of July 1991, which contains 90,296 entries, was used in this work. What follows gives a succinct description of each of the methods used in combination with the CCD.

Docking Techniques

I have used DOCK versions 1.0 and 2.0 by DesJarlais and colleagues (1988). These programs use spheres to describe the active site of an enzyme, the structure of which is known from, for example, x-ray crystallography. The "negative" image of this receptor site is then used to test out compounds from the CCD. Using a score to rank these molecules docked onto the receptor site, one usually gets a family of compounds that fit well onto the receptor site. Although many compounds of interest are rigid, this program has been used for flexible molecules (DesJarlais et al. 1986). An example of the use of DOCK for

the design of inhibitors is haloperidol, a compound that inhibits the human immunodeficiency virus 1 protease (DesJarlais et al. 1990).

CAVEAT Analysis

The program CAVEAT (Bartlett et al. 1990) makes use of the CCD in a different way. For every molecule in the database, it stores the intramolecular bonds as vectors. This new database of vectors is stored independently from the original one, which contains atomic coordinates, and is then searched for vector matching. The approach is to start with the crystal structure of an inhibitor in complex with an enzyme and then search for new templates based on the spatial arrangements of the bonds of this known ligand. The user is then allowed to choose up to three vectors belonging to this ligand. The natural choice for these vectors are those along the bonds that make important chemical interactions with the target enzyme. It is then possible to search for molecules that could have a similar relative orientation of their bonds. Figure 1, panel A, shows two vectors chosen along two bonds of a peptide of known structure. Figure 1, panel B, shows the structure of a hit, a compound that satisfies the search, superimposed on the starting peptide. This method has been applied to tendamistat, a 74-amino acid inhibitor of α-amylase (Bartlett et al. 1990).

QUEST Programs

These programs supersede the querying codes that belong to older versions of the CCD (Allen et al. 1979). In particular, a similarity search (Willett et al. 1986) and a powerful connectivity search are now an integral part of this package. It is also worth noting that a pharmacophore model for an inhibitor can be built easily using QUEST. The pharmacophore model, which is the set of distances and angles of a substructure that is important for a certain biological activity, can also provide new ideas for new templates (Sheridan et al. 1989).

SYSTEMS STUDIED

This chapter's emphasis is on two systems—papain and thrombin.

Papain

Cysteine proteases have been implicated in various pathological conditions, such as inflammation and malignancy. Papain belongs to the family of cysteine proteases, and it is isolated from the latex of tropical papaya fruit. Cathepsin B also is a cysteine protease found in, for example, human liver. Mechanistic data gathered for cathepsin B suggest that it shares a common basic enzymatic mechanism with papain (Khouri et al. 1991). Papain, which

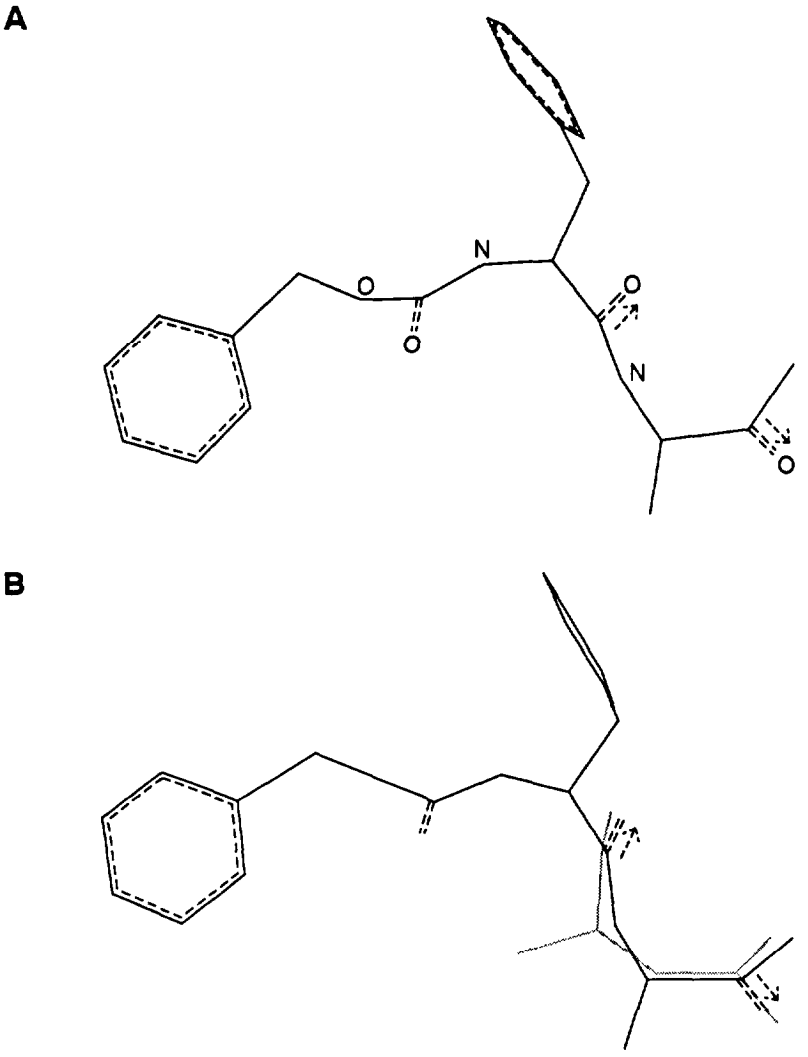


FIGURE 1. *(A) Peptide used to define a pair of vectors, which are indicated by dashed arrows. (B) A hit, that is, a compound with a similar spatial arrangement of its bonds, is shown superimposed on the original peptide.*

has been extensively studied, can therefore serve as a template for the design of inhibitors aimed at related cysteine proteases of pharmacological importance such as cathepsins B, H, and L. From the known crystal structure of Bzo-Phe-Ala-CH_2Cl, an inhibitor, in complex with papain (Drenth et al. 1976),

three vectors were chosen along bonds that make, for example, hydrophobic interactions with papain. The choice of vectors is important because the basic assumption of this method is that the shape of a compound can be described reliably by vectors along its bonds. This will, in turn, allow choosing other templates that interact with papain and, after some chemical modifications (e.g., introduction of a chloromethylketone group), show inhibitory properties. Using the CAVEAT analysis, I found two main classes of compounds.

The first family of compounds obtained with CAVEAT, shown in figure 2, panel A, had been suggested earlier by DesJarlais and colleagues (1988) in an attempt to find good inhibitors of papain using docking techniques. This is an interesting result because it shows that, by using different methods, families of compounds with similar geometrical features can be identified. The second class of compounds found using CAVEAT, shown in figure 2, panel B, had also been obtained independently by me with a similarity search available as part of QUEST.

This similarity search was based on a known weak inhibitor of papain, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (Perez and Niemeyer 1989), also known as Dimboa. Figure 3, panel A, shows the structure of Dimboa, and figure 3, panel B, gives the input data corresponding to the similarity search based on Dimboa's connectivity.

Furthermore, within QUEST it is possible to search the CCD database using a model for the pharmacophore (see figure 4, panel A). This application has provided some ideas for new lead compounds, one of which is shown in figure 4, panel B. This is a very promising candidate, not only because it is similar to the starting compound, but also because it has many hydrogen-bonding donor (acceptor) atoms. We at the Biotechnology Research Institute are in the process of synthesizing new compounds based on this template.

Thrombin

Thrombin is an important enzyme that initiates blood coagulation. Fibrinogen, thrombin's natural substrate, interacts strongly with two sites of thrombin, namely, the active site and the exosite. Because thrombus formation inside the vessels, started by the hydrolysis of fibrinogen, may lead to thromboembolic diseases, many scientists have been searching for new thrombin inhibitors to control blood coagulation. One of the most potent inhibitors of thrombin is hirudin, a 65-residue peptide from the salivary glands of the leech *Hirudo medicinalis* (Walsmann and Markwardt 1981). A potent thrombin inhibitor based on hirudin's sequence has been synthesized (DiMaio et al. 1990) and characterized (Yue et al. 1992) at the Biotechnology Research Institute. The high affinity and specificity of hirudin as a thrombin inhibitor are the result of its bivalent binding mode. In other words, in addition to blocking the active site,

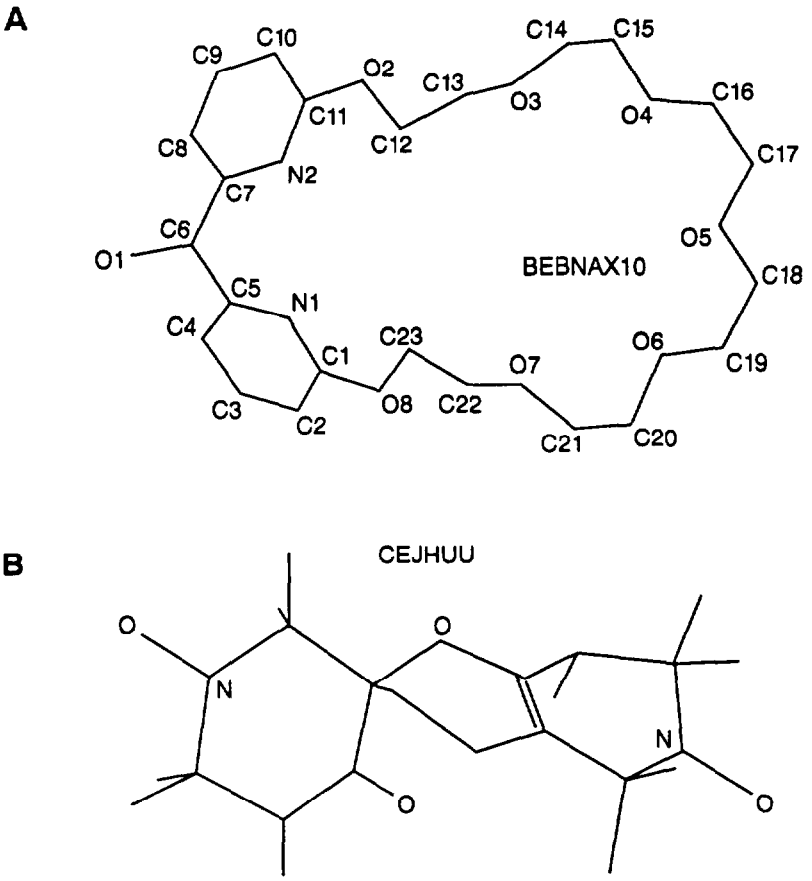
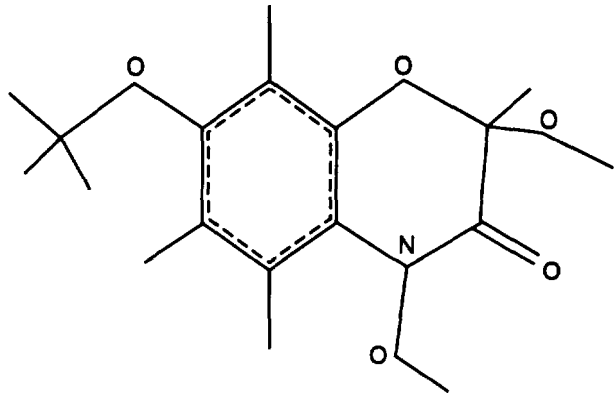


FIGURE 2. *Families of compounds obtained using CAVEAT analysis based on a known inhibitor of papain, Bzo-Phe-Ala-CH_2-Cl (Drenth et al. 1976). The six (or eight) letter refcodes are from CCD. (a) BEBNAX10 (Newkome et al. 1984). (b) CEJHUU (Shevyrev et al. 1983).*

hirudin interacts with the exosite via its C-terminal undecapeptide fragment. Because the C-terminal hirudin fragment is available to digestion by many enzymes (Chang 1983) and its intactness is necessary for antithrombotic activity, a search for new analogs of the C-terminal fragment of hirudin, which are resistant to proteolysis, is necessary. I have concentrated on the thrombin exosite because it has been shown that blocking this site disrupts the cleavage of fibrinogen. Using docking techniques, I have been able to design a cyclic form of a hirudin analog. It is known that cyclic peptides may enhance not

A **DIMBOA**



B

SIMILAR TANI

at01 N 3	at09 C 3	bo 1 2	bo562
at02 C 3	at10 C 2	bo 1 6	bo 5 10
at03 C 3	at11 O 1	bo 1 13	bo 6 7
at04 0 2	at12O 1 E	bo 2 3	bo782
at05 C 3	at13 0 1	bo2122	bo89
at06 C 3	at14 0 2	bo 3 4	bo 9 10
at07 C 2	at15 C 1	bo 3 11	bo 9 14
at08 C 2	at16 C 1	bo 4 5	bo 14 15
	end		

FIGURE 3. *(A) Structure of Dimboa (Perez and Niemeyer 1989), a weak inhibitor of papain. (B) input for a similarity test available within QUEST, based on the connectivity of Dimboa (Perez and Neimeyer 1989).*

only the biological activity (Al-Obeidi et al. 1989) but also the biological stability (Sham et al. 1988; Szewczuk et al., In press). Between residues Phe56 and Ile59 (hirudin's numbering is used), there is space to accommodate extra groups. Through the use of docking programs, many aromatic compounds were successfully placed in this region. This suggests that aromatic rings in this position are well accepted by thrombin. This fact prompted me to design new inhibitors with an aromatic motif between the two residues mentioned above. The aromatic ring between these two residues was approximately in

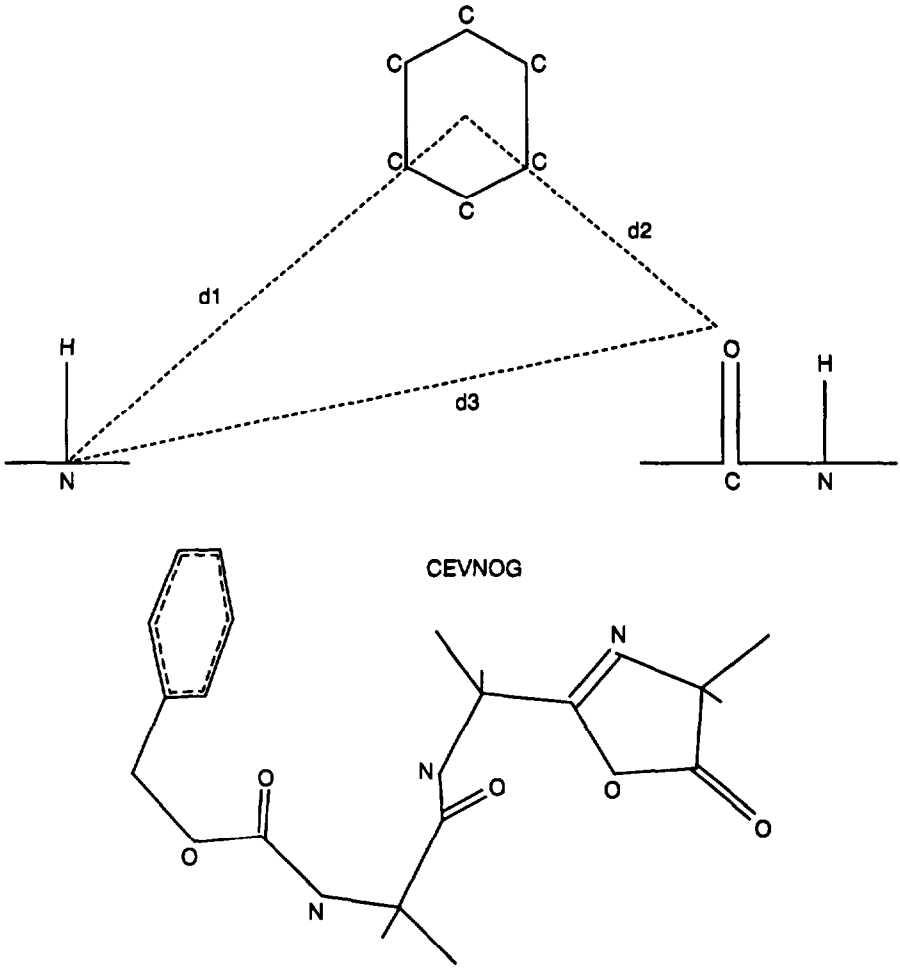


FIGURE 4. *(A) A pharmacophore model for papain based on the known structure of Bzo-Phe-Ala-CH_2-C1. (B) The structure of CEVNOG (Toniolo et al. 1983) was retrieved with this pharmacophore model, and it is presently under investigation.*

the same position as the best docked candidate extracted from the CCD. This constraint should allow the peptide to adopt the biologically active conformation even in solution, resulting in an increase of the activity because of the entropic gain during the interaction with thrombin. This compound, shown in figure 5, is being synthesized at the author's institute, and it will be reported elsewhere.

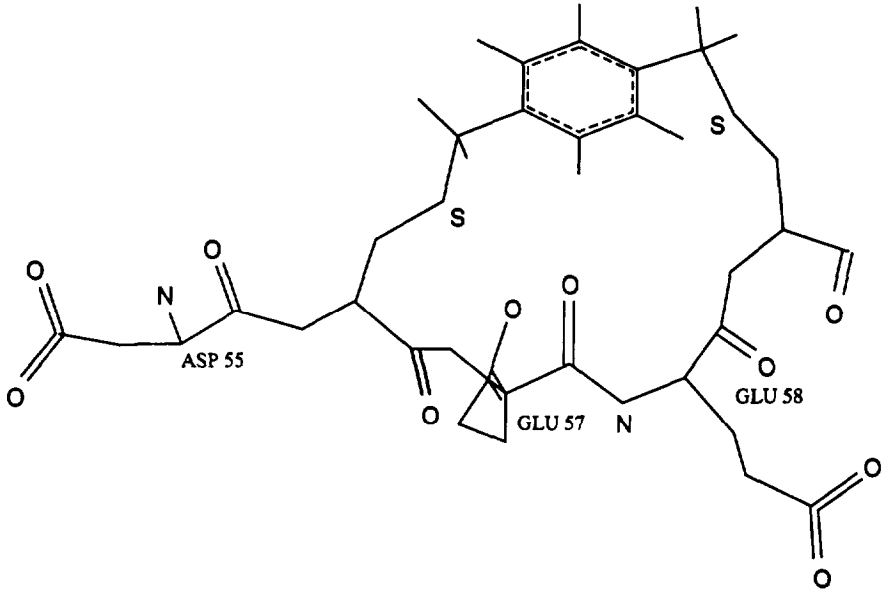


FIGURE 5. *A cyclic analog of the C-terminal fragment of hirudin currently being synthesized. Hirudin's numbering scheme has been kept for the natural amino acid residues.*

CONCLUSIONS

This work has shown that, using three different techniques in combination with the CCD, I could suggest new compounds for specific target enzymes. Furthermore, it is very encouraging that some of these compounds (or parts of them) were obtained by two independent methods. It is worth mentioning that for both papain and thrombin I retrieved well-known inhibitors using different vector triplets with CAVEAT. Thus, this method has successfully withstood an important test. This project was undertaken to assess the potential of computer modeling, used in combination with a database of small molecules, for the design of new lead compounds. Work is in progress at the institute to carry out biological assays to test the compounds synthesized based on the templates suggested by computer modeling. I emphasize the interplay between design of new compounds using databases and biological assays. The latter will always be the acid test for any suggestion obtained with computer modeling. For compounds that are very flexible, I will have to include not only the single conformation stored in the CCD but also other conformations that are compatible with the active site. There have been some attempts in this direction using the commercial computer modeling package CHEM-X (Murrall and Davies 1990).

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Efforts To Improve the Utilization of Protein Sequence and Structure Databases

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INTRODUCTION

For the past several decades knowledge of cellular function has been growing rapidly. This rapid growth was caused by the introduction of many technological advancements in biology, which not only opened up a new field, biotechnology, but also brought an information explosion. To illustrate this growth of bioinformation, consider the basic constituent of all living organisms, a cell. Vast amounts of information have been gathered on various types of cells, their chromosomes, genes, nucleic acid, and protein. Most of this information has been accumulated as published literature, generated by different laboratories around the world. As this information began to flow, it was compiled into various databases that were circulated among only a few individuals. When the size of these databases started to increase, the need for quick archiving and retrieval became critical. This need was filled by the advent of fast computers and disk drives with large storage capacity, which in turn paved the way for the birth of a new field called bioinformatics. This field addresses the issues of access, update, and manipulation of various types of computer-based biological information.

The biological databases contain a wealth of information for understanding the structure and function of cells. The number of databases illustrates the current status of bioinformatics. The database Listing of Molecular Biology Databases (LiMB) contains various databases available in the field of molecular biology. There are more than 3,000 cultured cell lines available in the database maintained by American Type Culture Collection (ATCC). DNA and protein sequences are maintained, respectively, by Genbank and National Biomedical Research Foundation (NBRF) (George et al. 1986). A protein three-dimensional (3-D) structural data bank is managed by Brookhaven National Laboratory (BNL) (Bernstein et al. 1977). When the sources of these databases are examined, it is clear that each database is managed by a different scientific agency.

An era of biological research is beginning in which scientists must rely on more than one of these databases. A major problem confronting modern research in bioscience is the availability and ease of utilization of the biological databases. There is a need for either a certain uniformity among these databases, special tools to access the information across different databases, or both. Some earlier attempts in this direction were undertaken in Europe; OWL and SWISS-PROT are the outcomes of this attempt to integrate protein and nucleic acids sequence databases. It should be noted that a few in-house molecular modeling programs (Blundell et al. 1988; Claessens et al. 1989; Thornton and Gardner 1989; Bryant 1989; Unger et al. 1989; Jones and Thrirup 1986) are available to automatically search and extract the sequence and structure information from the Protein Data Bank (PDB). Often these programs are found to be closely integrated with large onsite graphics or molecular modeling programs and are not available for public use because of cost and hardware limitations.

Advancements in the fields of protein sequencing and genetic engineering will permit the addition of a large number of protein sequences to the Protein Identification Resource (PIR) sequence database. In addition, many 3-D structures of natural and genetically engineered proteins also will be determined to atomic resolution, Thus, a direct link between the primary sequence database (PIR) and the 3-D structure database (NBRF/PDB) becomes increasingly important in understanding the structure-function relationships of proteins. Prior to the author and colleagues' work (Namboodiri et al. 1988; Pattabiraman et al. 1990) these two important databases containing information about the structure and function of protein molecules did not have a direct link. Therefore, the authors created a sequence-structure database called NRL_3D derived from protein entries in PDB and searchable within the PIR environment.

As the number of databases pertaining to a certain scientific area increases, every effort should be made to cross-check and retrieve information. The most significant effort to integrate biological databases started with the establishment of the National Center for Biotechnology Information (NCBI) (Boguski et al., in press) under the National Library of Medicine at the National Institutes of Health. In July 1990 NCBI announced a new database, GenInfo Backbone Sequence Database (New molecular sequence . . . 1990). This new and integrated database includes the MEDLINE records that correspond to protein or nucleic acids sequences. GenInfo will maximize the use of standard nomenclature and gene names from original sources. It is the first major database to use International Standard Organization format (Abstract Syntax Notation, ASN.1) for data representation, GenInfo is implemented as a relational database offering a variety of advantages over other database systems. For the past few years, the authors have been interested in integrating databases on protein molecules. This chapter describes and gives examples of our efforts to improve the utilization of the protein sequence database (PIR) and the 3-D structure data bank (PDB).

DESCRIPTION OF A SEQUENCE-STRUCTURE DATABASE, NRL_3D

PIR's protein sequence database contains most of the published protein primary sequences (George et al. 1986). PIR also provides a variety of software tools for protein sequence manipulation, sequence similarity searches, and sequence alignments. Figure 1 shows a histogram of the number of protein primary sequences added every year from 1983 through 1991 (D. George, personal communication, September 1992). It is clear from the histogram that the growth of this primary sequence database is exponential.

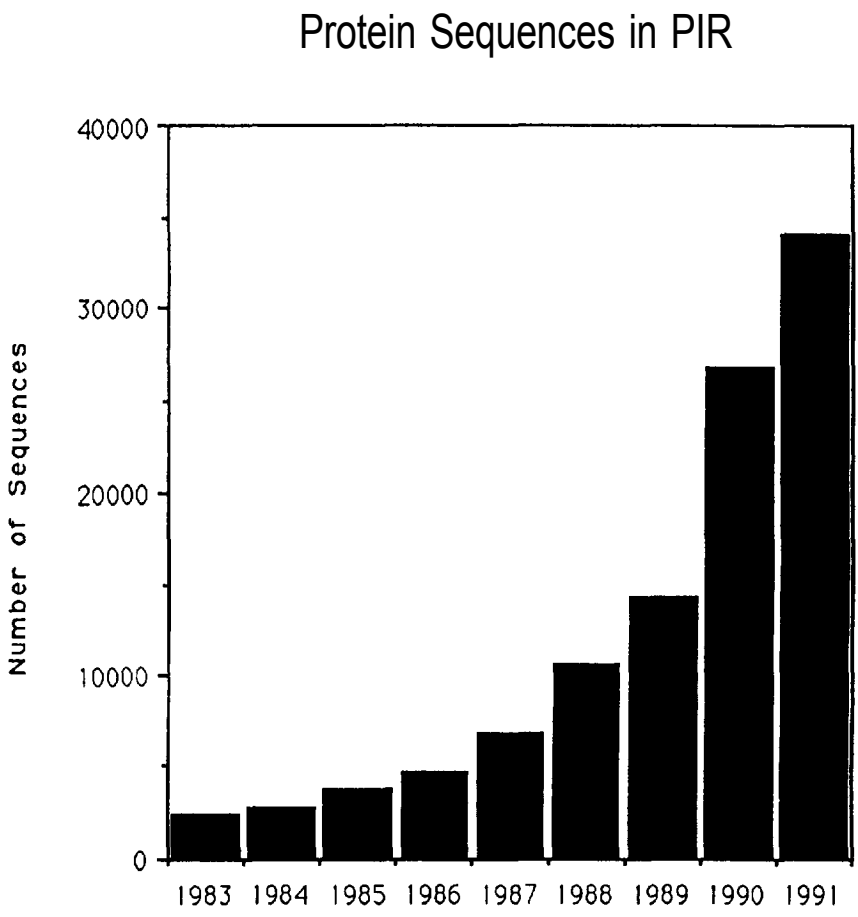

FIGURE 1. *Histogram showing the exponential growth of protein primary sequences deposited over a period of 9 years with PIR*

BNL's PDB (Bernstein et al. 1977) contains coordinates of 3-D structures of several proteins and other related biomolecules obtained using single crystal x-ray diffraction data. Figure 2 shows a histogram of the number of entries added to PDB every 6 months from January 1988 through September 1991 (E. Abola, personal communication, September 1992; Bernstein et al. 1977). Again, the growth is clearly exponential. In addition to x-ray diffraction data, PDB also contains coordinates obtained from nuclear magnetic resonance, modeling, and neutron diffraction data. A typical PDB entry for a biomolecule contains not only the 3-D atomic coordinates but also information such as sequence, bibliography, quality of the reported data, and secondary structure. However, because PDB uses a rigid, cryptic, and flat-file format for the distribution of these data, the general utility of this vast treasure of information has been restricted to a few scientists.

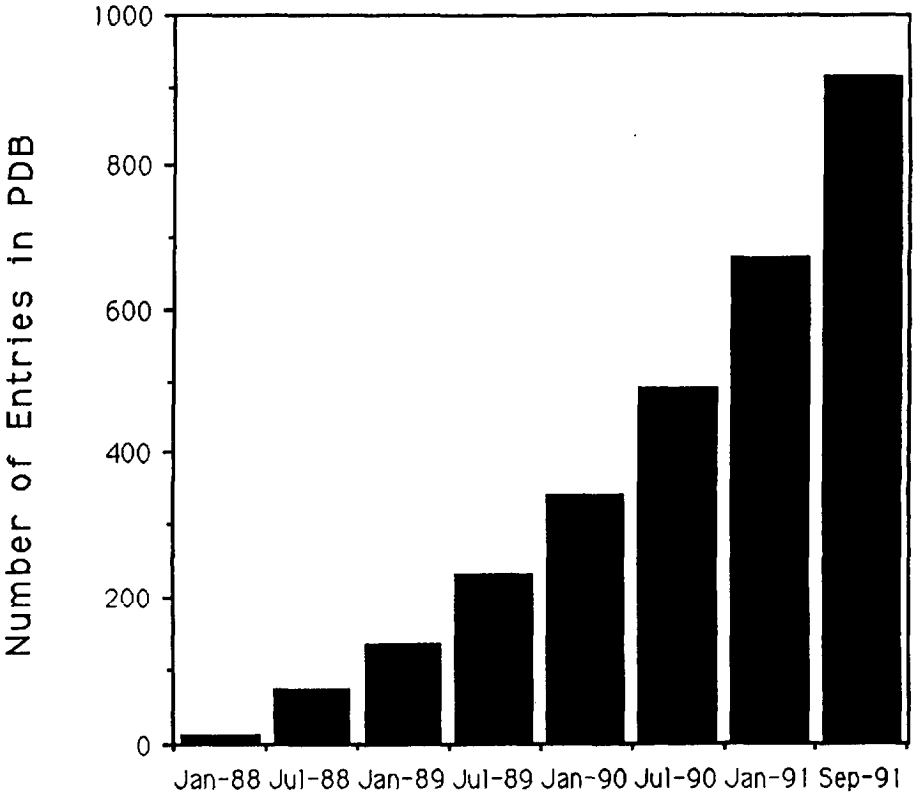

FIGURE 2. *Histogram showing the exponential growth of number of entries deposited over a period of 4 years with BNL*

in each PDB entry, a variety of protein structure information is stored as records using a flat-file format, Each record is identified by a record identifier. For example, the primary sequence of the protein is stored under the "SEQRES" record identifier. It may appear simple to construct a new sequence database similar to PIR by extracting the PDB protein sequences from these "SEQRES" records and then performing sequence similarity searches on them. However, this process is not straightforward for the following reasons. In PIR entries, the N-terminal residue is always the first residue and is identified by the number 1. The following residues are numbered contiguously. In PDB entries, the residue numbers (residue identification scheme) may not be identified by contiguous numbers starting with 1 from the N-terminal residue. in some cases, the residue numbers also contain certain letters. The reasons for following this residue identification scheme in PDB are as follows. The residue identification was assigned according to the previously studied homologous protein. In cases where there is an insertion of amino acids, the sequence numbers of the inserted residues are the same as those of the homologous protein. in addition, an alphabetical designator (e.g., A, B, C) is attached to the sequence numbers of these inserted residues. For example, in table 1b residues 15A and 15B are inserted between residues 15 and 16. If there is a deletion of amino acids, the residue numbers before and after the deletion will be the same as that of the homologous proteins. in other words, the residue numbers at the deletion site are not contiguous. In table 1b, there is a deletion between residues 19 and 31, which are linked by a peptide bond. Finally, because of the quality of x-ray data and the nature of the structure refinement, it may not be possible to trace the backbone atoms of some of the residues. Hence, the 3-D coordinates for these residues are not reported in the PDB entry. Thus, using the primary sequence database derived from the "SEQRES" records alone, one cannot extract the 3-D coordinates for a given sequence. In short, when PDB sequences are reformatted, one has to keep track of the identification for each residue given in the "ATOM" record.

In the development of the NRL_3D database, Pattabiraman and colleagues (1990) extracted the sequence information from the "ATOM" records, based on the distance between two consecutive C^{α} atoms. The sequences were then formatted according to PIR sequence database. In addition, the sequence identifications were extracted from the "ATOM" record, and a separate database file was created. Table 1 shows NRL_3D database files for the alpha-lytic protein (PDB code is 2alp [Fujinaga et al. 1985]). In the current setup the NRL_3D database can be used interactively within PIR using the SCAN and MATCH commands (George et al. 1986; Pattabiraman et al. 1990). The authors and collaborators also developed a computer program (PRENRL_3D) in C language (Kusunoki et al. 1991) that automatically creates NRL_3D database files from PDB protein entries.'

TABLE 1. *Typical examples of NRL_3D database files*

a. NRL_3D.SEQ file

>P1;2ALP1
Alpha-lytic protease (E.C. number not assigned-(lysobacter enzymoqenes)
ANIVGGIEYSINNASLCSVGFSVTRGATKGFVTAGHCGTVNATARIGGAVVGJAARVFPGNDRAWVSLT
SAQTLLPRVANGSSFVTVRGSTEAAVCRSGRTTGYQCGTITAKNVTANYAEGAVRGLTQGNACMGR
GDSGGSWITSAGQAQGVMSGGNVQSNGNNCGUPASQRSSLFERLQPILSQYGLSLVTG*

b. NRL_3D.NUM File

>p1;2ALp1
Alpha-lytic protease (E.C. number not assigned-(lysobacter enzymogenes)

15A	1 5 B	16	17	16	19	31	32	33	34	35	36	38
39	40	41	42	43	44	44A	45	46	47	48	48A	48B
48C	49	50	51	52	53	54	55	56	57	58	59	59A
59B	60	61	62	64	65	66	67	81	62	83	84	85
67	88	88A	89	90	91	94	95	100	101	102	103	104
105	106	107	108	109	110	111	112	113	114	119	120	120A
120B	120C	120D	120E	120G	12OH	120I	120J	120K	121	122	123	124
125	129	130	131	132	133	134	135	136	137	138	139	140
141	142	143	156	157	158	159	160	161	162	163	164	165
166	167	168	169	170	171	173	174	175	176	177	178	179
180	161	162	183	184	165	189	190	191	192	193	194	195
196	197	196	199	200	201	201A	202	207	208	209	210	211
212	213	214	215	216	217	218	219	219A	219B	219C	219D	220
220A	221	222	222A	222B	222C	223	224	225	226	227	228	229
230	231	232	233	234	235	236	237	238	239	240	241	242
243	244	245	*									

c. NRL_3D.REF file

NRL_3D-Entry. 2ALP1
Alpha-lytic protease (E.C. number not assigned-(lysobacter enzymogenes)
PDB-entry 2ALP 07-MAR-85 NBRF-entry TRYXB4
HEADER. Hydroiase (serine proteinase)
RESOLUTION: 1.7 Angstroms. R-value: 0.131
PDB-depositor(s)=Fujinaga, M.; Delbaere, L.T.J.; Brayer, G.D.; and James, M.N.G.

APPLICATIONS OF NRL_3D IN PROTEIN MODELING

The authors' laboratory has been interested in the structure-function relationships of phospholipase A2 (PLA2) molecules. PLA2 catalyzes specifically the hydrolysis of the ester bond at the C2 position of 3-sn-phosphoglycerides. PLA2 is a biologically important molecule involved in arthritis and wound healing. In addition, the activities of PLA2 found in snake venoms may be responsible for pharmacological effects such as myotoxicity, anticoagulant properties, and hemolytic effects (Waite 1987). PLA2 includes a class of low molecular weight proteins composed of approximately 120 amino acids. In the current version of PIR, 53 primary sequences of PLA2 from different sources have been included (Waite 1987). Six crystal structures of PLA2 are available in PDB. Figure 3 shows a C^a backbone tracing of bovine pancreas PLA2. This was the first molecule of this class whose structure was determined (Dijkstra et al. 1978). Secondary structures such as a-helix and β, -strand are held together by six disulfide bridges, which are shown as dashed lines (figure 3). The structure of the PLA2 molecule is rigid because of these disulfide linkages.

Because of the linkage of PIR and PDB via the NRL_3D database, it is now possible to align the sequences of PLA2 of known 3-D structure with those of the sequences of unknown structure. The alignment of the sequences of pig, horse, and a snake venom PLA2 (C. *Atrox)* with that of bovine pancreas PLA2 (Waite 1987), whose 3-D structure is known, is shown in figure 4. A star in the aligned sequences denotes that the amino acid is the same as that of the bovine pancreas PLA2. Shown within boxes of solid lines are the stretches of amino acids for which the aligned residue in the bovine PLA2 structure is an a-helix. Similarly, the β -helices are represented by boxes with dashed lines. This gives an idea of possible secondary structures for the aligned sequences. Also, in the bovine pancreatic PLA2, the residues that are not solvent accessible are circled. This surface is computed by rolling a spherical "solvent" molecule around the exterior of the protein molecule, maintaining contact with the van der Waals' surface of atoms near the exterior. The locus of the points on the surface of the solvent molecule as it touches the protein during this journey generates the molecular surface (Connolly 1983). The molecular surface of the protein was calculated using the program MS (Connolly 1983). This information will help to identify those residues that are buried inside the protein. The secondary structure and the solvent-accessible information described here will be useful to molecular biologists in their mutation experiments.

Now, let us examine another application of NRL_3D in the design of de novo metal-binding proteins. Arnold and coworkers (Suh et al. 1991) have studied the characterization of His-X_3-His sites in α -helices of synthetic metal-binding bovine somatotropin. Craik (1990) has demonstrated that changing the residue 97 in trypsin to a His and adding copper causes the activity of the protein to be shut

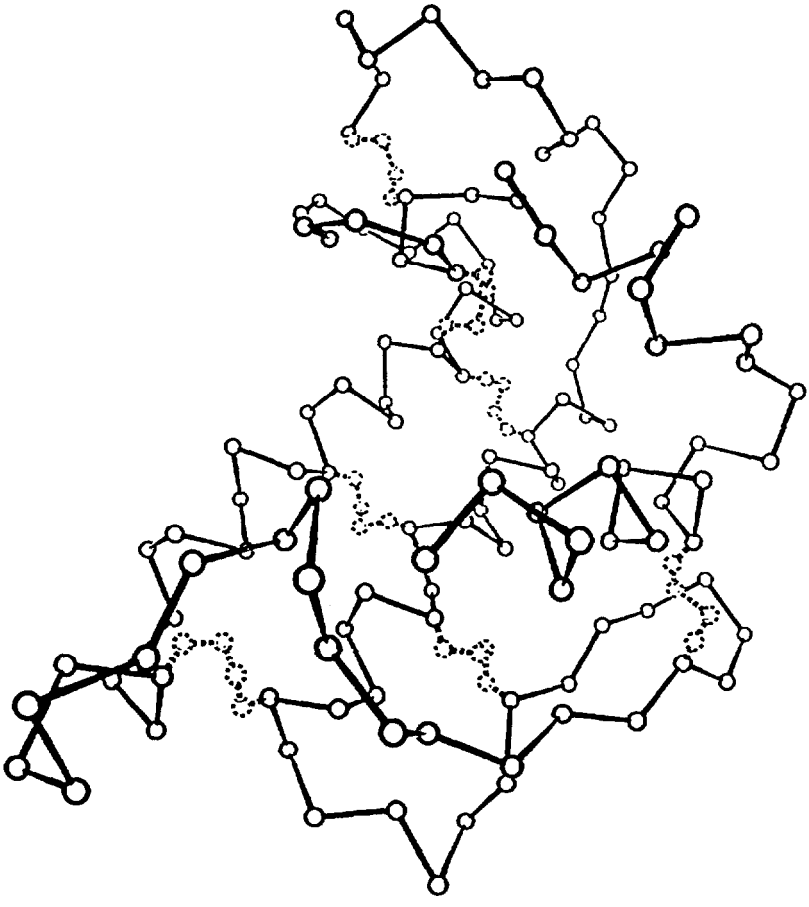

FIGURE 3. *C^{α}-backbone atoms tracing (solid lines) of bovine pancreatic PLA2. The six disulfide bridges are shown as dashed lines.*

down. In this process, a Cu^{+2} ion chelates to the residues His-57 and the nearby mutated His-97. To bind the metal, His-57, which is involved in the protein catalytic activity, must undergo a conformational change. Because of this metal-induced conformational change of His-57, the activity of the protein is lost. Once the CU^{+2} ion is removed, the catalytic activity can be restored. This is referred to as the active site "metal switch."

We were interested in deactivating the PLA2 enzyme using the metal switch mechanism as it was demonstrated in trypsin. In PLA2, His-48 is important for the activity of the protein. If one of the active site residues in PLA2 is replaced

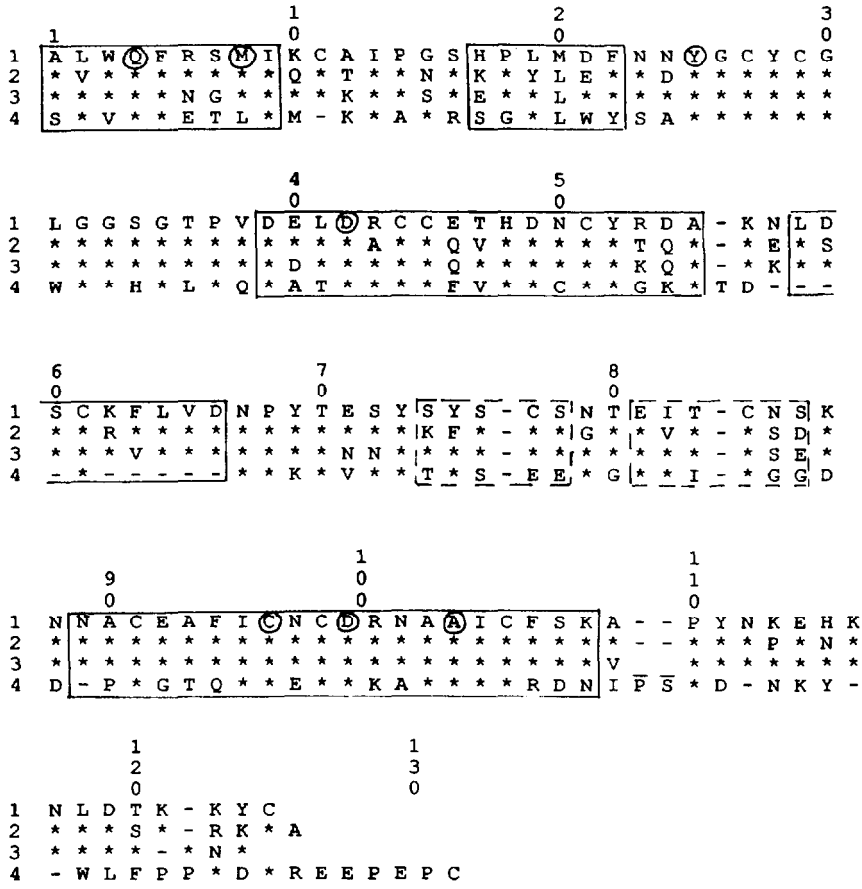

FIGURE 4. *Comparison of PLA2 sequences from pig (2), horse (3), and C. Atrox (4) with that of bovine pancreas PLA2*

with a His, the modified residue and His-48 may bind to a Zn^{+2} ion. In PLA2, the residue His-48 is in the a-helix region. Hence, to create a metal binding site, there should be another His four residues away from His-48. To test this hypothesis, the NRL_3D database was used to extract sequences and 3-D coordinates corresponding to HXXXH (where X is any amino acid). The output of our search results are summarized in table 2. Listed here for the matched sequences are the PDB code, the name of the protein, the sequence, and the sequence number range. Eleven fragments (a through k) containing the sequence HXXXH from the structures in PDB were extracted. The

TABLE 2. *Sequence search results for HXXXH*

Fragment	PDB Code	Protein Name	Sequence	Sequence Number Range
a	1MB5	Myoglobin (carbonmonoxymyoglobin) (neutron study)—sperm whale (physeter catodon)	HATKH	93-97
b	3GAP1	Catabolite gene activator protein-cyclic AMP complex (cap)-(Escherichia coli)	HCHIH	17-21
c	1FC11	FC fragment (IGG1 class)—human (Homo sapiens) pooled serum	HEALH	429-433
d	3TLN1	Thermolysin (E.C. 3.4.24.4)-(Bacillus Thermoproteotyticus)	HELTH	142-146
e	2CAB1	Carbonic anhydrase form B (carbonate dehydratase) (E.C. 4.2.1 1) —human (Homo sapiens) erythrocytes	HGSEH	103-107
f	1PYP1	Inorganic pyrophosphetase (E.C. 3.6.1.1)—Baker's yeast (sacchromyces cerevisiae)	HGYIH	86-90
g	1HMQ1	Hemerythrin (met)--sipunculid worm (themiste dyscritum)	HKKAH	73-77
h	3DFR1	DihydrofoLate reductase (E.C. 1.5.1.3) complex with NADPH and methotrexate—(lactobecillus casei). dichloromethotreate-resistant strain	HLPWH	18-22
i	4RHV1	Rhinovirus 14 (HRV14)—human (Homo sapiens) virus grown in HELA cells	HRAKH	245-249
j	2CPP1	Cytochrome p450cam(camphor monooxygenase) (E.C. 1.14.15.1) With bound camphor—(pseudomonas putida)	HVPEH	17-21
k	2MHR1	Myohemerythrin—sipunculan worm (themiste zostericola) retractor muscle	HKKMH	73-77

extracted coordinates for the fragments were displayed and analyzed using MIDAS (Ferrin et al. 1988).

In figure 5, a ray-traced Corey-Pauling-Koltan surface of the 11 fragments is shown (a through k). The darker spheres are the atoms of the side chains of the His residues. It is clear from figure 5 that several conformations are available for this sequence (HXXXH). The coordinates of C^{α} atoms of the fragments were superimposed on the corresponding C^{α} atoms of residues 48 to 52 of PLA2.

Fragments d, g, and h have a backbone conformation similar to that of residues 48 to 52. For fragment d, which was extracted from thermolysin crystal structure (Holmes and Matthews 1982), the His residues at the N- and

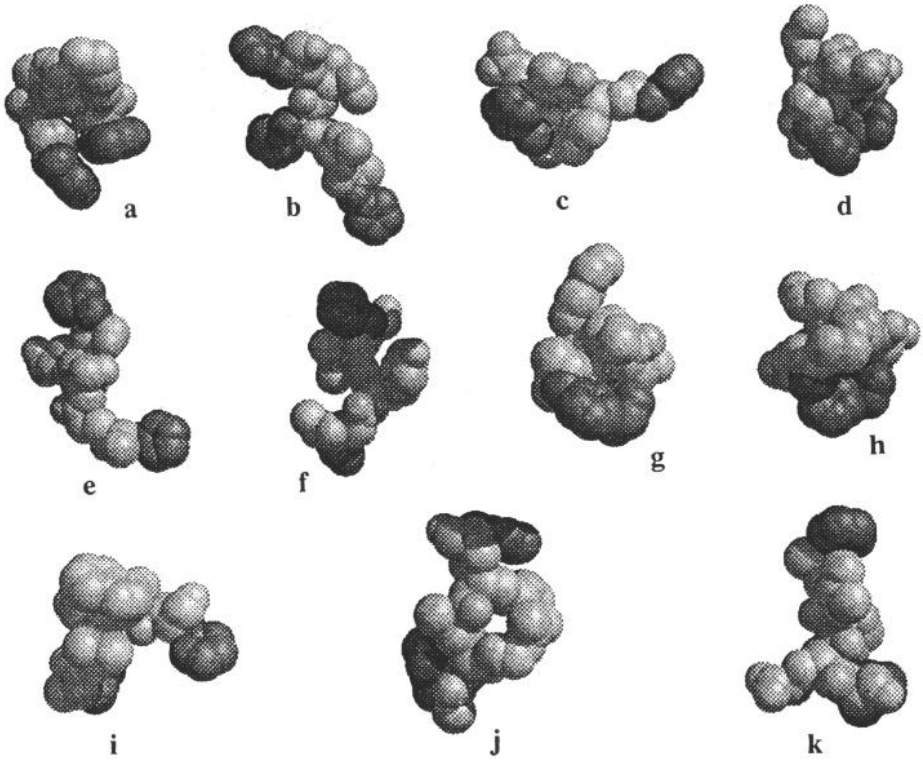

FIGURE 5. *Ray-traced CPK molecular surface of 11 HXXXH fragments extracted from PDB (refer to table 2 for identification)*

C-termini are found to interact with a zinc ion. With the use of this fragment, a zinc-binding site in PLA2 was built by replacing Tyr-52 with a His. In this model, the conformation of His-48 had to be significantly changed to chelate the Zn^{+2} ion. Figure 6 shows the C^{α} atoms tracing of all the residues of this mutated PLA2. All the atoms and bonds for residues His-48 and His-52 are shown in the figure. Because of the change in the conformation of His-48, we propose that the activity of PLA2 will be lost if a zinc ion happens to Interact wlth this modified protein. Mutation and metal-binding studies are needed to verify this model and to confirm the possibility of zinc-induced metal switching In PLA2.

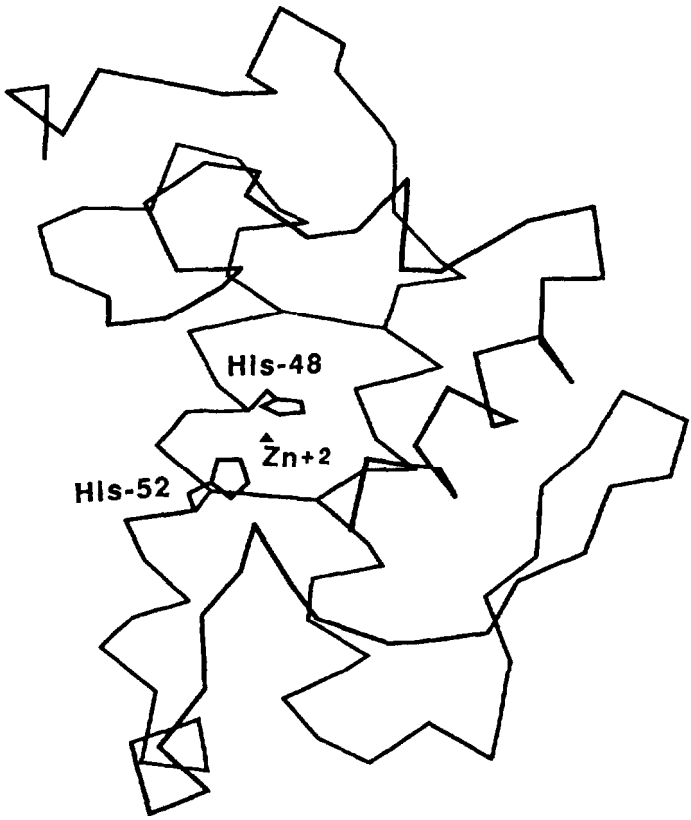

FIGURE 6. *C-carbon backbone atoms tracing of PLA2. Residues His-48, His-52 (substituted for Tyr), and zinc ion are shown.*

CONCLUSION

This chapter discusses some issues concerning various databases used in biology. Most of these databases are growing exponentially. To improve the utilization of these databases in the fields of protein engineering and drug design, efficient database management systems as well as database integration efforts are critical. We describe in detail various aspects regarding the linkage of two important databases (PIR and PDB) for proteins through the sequence-structure database NRL 30. A few useful applications in protein modeling under PIR environment using NRL_3D are discussed.

The linkage of these two protein databases is only the first step toward the goal of integrating all other biological databases. As mentioned earlier, the GenInfo backbone database (Boguski et al., in press) is designed to be an integrated database for several biological databases. Recently, we have started a collaboration with NCBI to develop a sequence-structure database under the GenInfo backbone database. This proposed 3-D module of GenInfo will be similar to NRL_3D and will contain not only proteins but also other biomolecular structures in PDB. A program in C language has been written to parse all the PDB entries and convert them into various object modules based on structural and other pertinent information. This conversion will enable us to manipulate and extract various structural information from PDB entries as well as to produce files that are compatible with ASN.1 format. The details of this work will be published elsewhere.

NOTE

1. The PRENRL_3D program and the NRL_3D database can be obtained from NBRF in Washington, DC. PDB can be obtained from BNL in Upton, NY.

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Use of Computer Graphics in Drug Design

G. W.A. Milne and M.C. Nicklaus

INTRODUCTION

A common approach to chemotherapeutic control of disease involves intervention in a biochemical pathway by exogenous chemicals or drugs (DeVita 1982). Compounds that block metabolic steps in this way usually do so by occupying a specific enzyme site, which is therefore denied to the normal substrate. For this reason, they are called enzyme inhibitors (Santi and Kenyon 1980). Many of the drugs that have been used in cancer treatment inhibit DNA biosynthesis; some examples are shown in figure 1. In the normal cellular biosynthesis of DNA by DNA polymerase, all four 2'-deoxynucleotide triphosphates are required (Kornberg 1980). Prior to its use by DNA polymerase, 2'-deoxycytidine triphosphate (dCTP), for example, is produced by the two major routes shown in figure 1, The de novo pathway can be blocked at different stages by different compounds, each of which thus has potential for the control of the growth of tumor cells. The inhibitors shown here-hydroxyurea, 3-deaza-UTP, PALA (phosphonoacetyl aspartate), and pyrazofurin-all are in use in cancer chemotherapy, although they all represent compromises because they fail to block the pathway to dCTP from deoxycytidine and, as a result, are not totally effective.

A variation on this strategy is the use of “fraudulent” precursors (Yarchoan et al. 1988). Here, the chemotherapeutic agent is similar enough to the natural chemical to be assimilated into the normal biochemical elaboration and to proceed through early steps of biosynthesis of a needed compound, such as DNA. Because of its structural differences, however, it fails a subsequent step. An example of this is shown in figure 2. In the course of reproduction of the human immunodeficiency virus (HIV), viral RNA is used as a template by reverse transcriptase to build a new viral DNA from cellular nucleoside triphosphates. A dideoxynucleoside such as 2',3'-dideoxyadenosine (ddA) (1) (figure 3) will be incorporated into the growing DNA, but because it has no 3'-hydroxyl group, growth will terminate at this point. If sufficient quantities of such dideoxynucleosides are in the HIV-infected cell, they can cause a shutdown of reverse transcription and the cell will die (Mitsuya and Broder 1986). Dideoxynucleosides have shown some success in treating

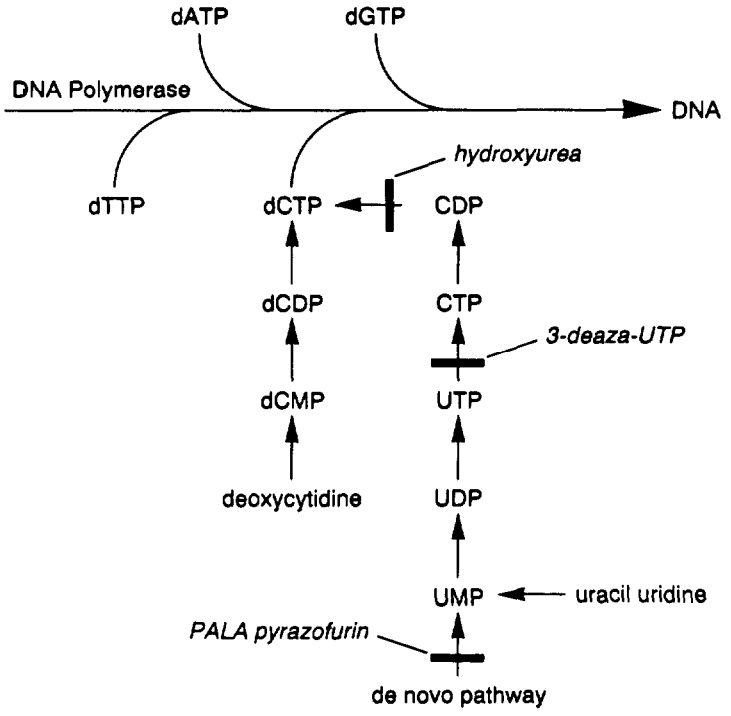


FIGURE 1. *Role of chemotherapeutic agents in blocking steps in DNA biosynthesis*

acquired immunodeficiency syndrome (AIDS); the only anti-AIDS drugs available in the United States, AZT (2) and 2',3'-dideoxyinosine (ddI) (3) (the major metabolite of ddA) (figure 3), are in fact both members of this family (Mitsuya and Broder 1986; Balzarini et al. 1986; Mitsuya et al. 1985).

Design of potential enzyme inhibitors and fraudulent substrates is an active area of medicinal chemistry, and some of the authors' and colleagues' recent work at the National Cancer Institute is discussed in this chapter.

ENZYME-SUBSTRATE INTERACTIONS

Much of our effort in molecular modeling has been devoted to a detailed study of the interactions that take place between an enzyme and a substrate. Enzymes typically possess sites where substrate molecules can reside while being chemically transformed. These sites, which can be likened to a workbench or a jig, are highly structurally specific and often will accommodate

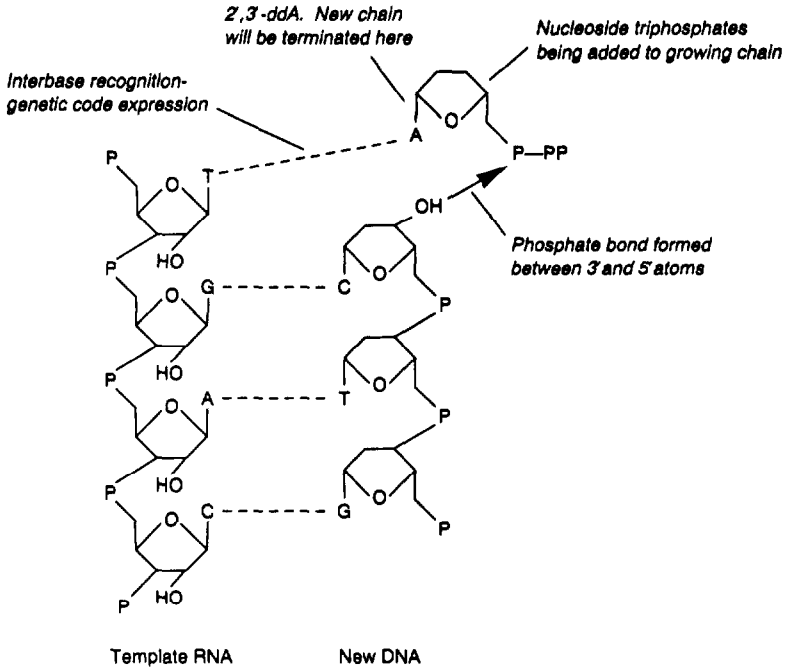


FIGURE 2. *Reverse transcription of viral RNA into a new DNA*

only a very few specific chemical structures. Provided that it has the correct geometry, a substrate approaching a site will be able to enter and will be held in place by several hydrogen bonds between its atoms and atoms in the enzyme. If the geometry is not optimal or the hydrogen bonds are not all completed, the molecule will fail to lodge in the site and will not serve as a substrate to the enzyme. Some examples of enzyme-substrate relationships are shown schematically in figure 4. A putative enzyme cavity is shown with three attachment atoms. The first substrate shown can enter the cavity but is too small to bind effectively, and the second is too big to enter the cavity. The third one is the correct size and shape to get into the cavity and stay there.

In practice, the details of an enzyme cavity, or active site, are rarely revealed as clearly as depicted in figure 4. The best source of such data is x-ray diffraction measurements made on the crystalline enzyme—or better, the enzyme-substrate complex. However, such data are difficult to measure; proteins cannot be crystallized easily, and the diffraction patterns they afford are extremely complex. The Cambridge Crystallographic Database (Allen et al. 1983) contains nearly 100,000 crystal structures of organic molecules, but the Protein Data Bank (Abola et al. 1985), which is maintained at Brookhaven

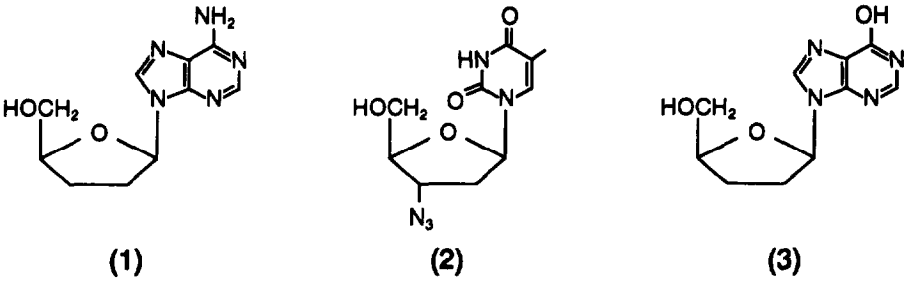


FIGURE 3. *Protein kinase C (PKC) inhibitors*

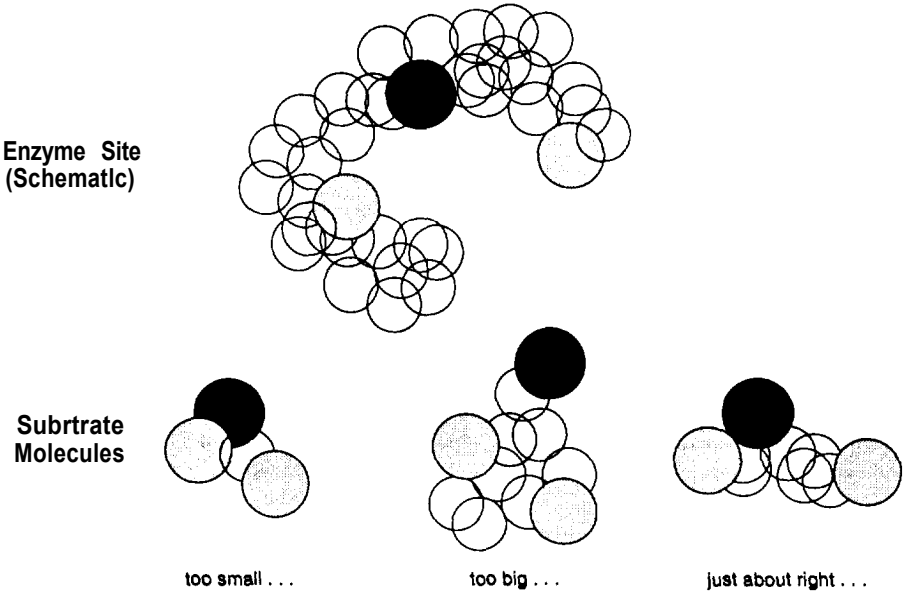


FIGURE 4. *Schematic view of substrates and an enzyme site*

National Laboratory, contains only a few hundred examples of protein structures that have been solved by x-ray diffraction. A well-known example of the latter type, the structure of dihydrofolate reductase containing a known inhibitor, methotrexate (Oeffner et al. 1988), is shown in figure 5.

However, even data of this sort are not easy to take advantage of because they show only the positions of the atoms of the substrate and the enzyme; the important bonds that hold the substrate in place have to be identified deductively by means of energy calculations. This identification can be made,

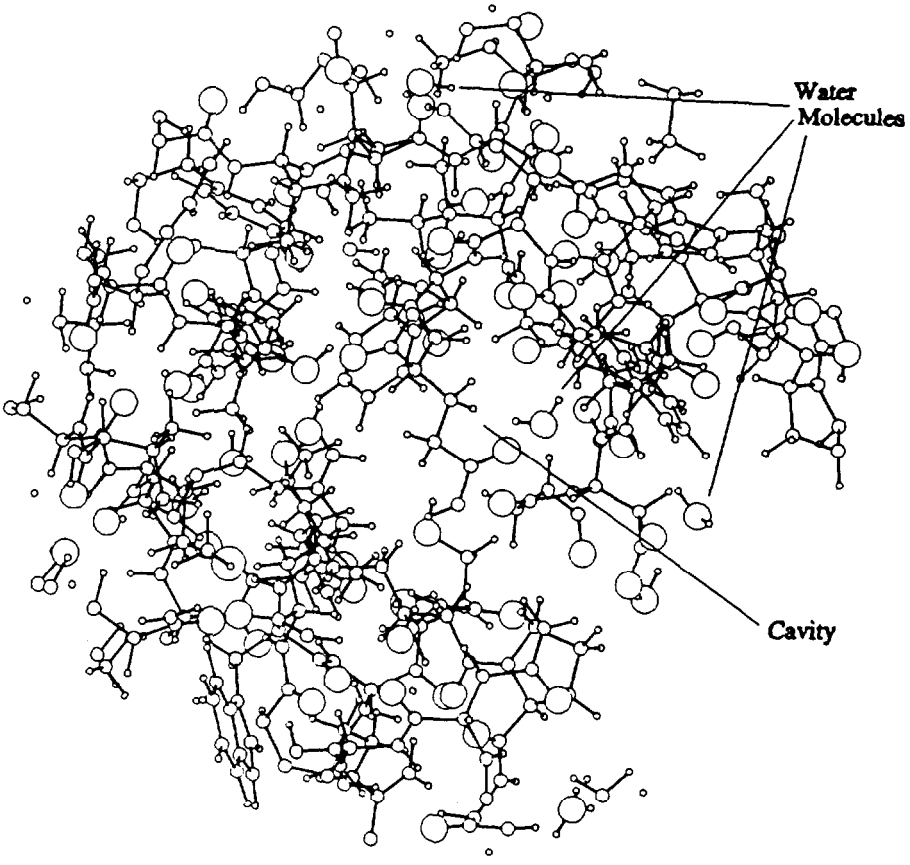


FIGURE 5. *Crystal structure of dihydrofolate reductase*

and the results are shown in figure 6 where the six hydrogen bonds that hold the methotrexate in the active site of dihydrofolate reductase can be seen. This reveals that, to bind in this site, a substrate must have, at certain specific points in space, hydrogen bond acceptors such as carbonyl oxygen or amino nitrogen, or hydrogen bond donors such as hydroxyl groups. This information can be used in the design of a substrate that may compete with the natural material for that enzyme site; some examples of this approach, as it has been employed in our laboratory, are given in the next sections.

A more common situation is one in which there is knowledge, not only of natural substrates for an enzyme, but also of one or more inhibitors, which have been discovered empirically. With this information it is often possible, by comparison of these molecules, to determine which parts of each of them are necessary for

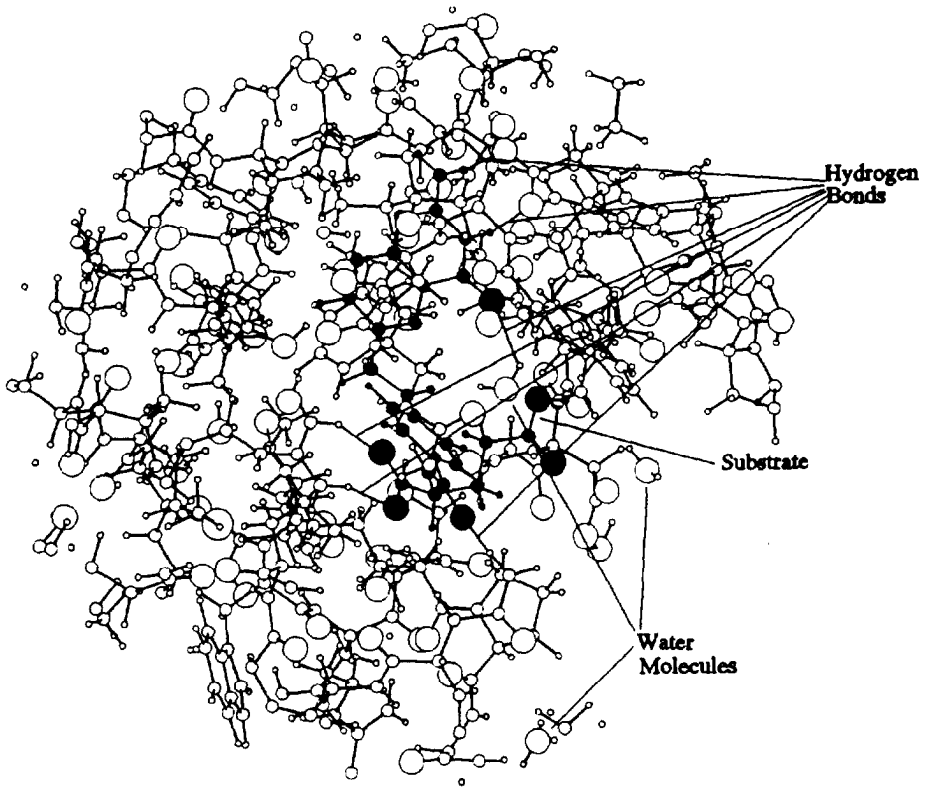


FIGURE 6. *Crystal structure of dihydrofolate reductase with a merhotrexate molecule*

successful binding to the active site. The enzyme uridine phosphorylase, which cleaves uridine to uracil and ribose-1 -phosphate, as shown in figure 7, is inhibited by many uracil derivatives with the general structure shown (Baker and Kelley 1970). It is clear from this that the uracil ring is what the enzyme recognizes; if this ring is part of a uridine molecule, the expected cleavage is observed, but if it is part of a 1-alkyl uracil, the enzyme is inhibited. It is fairly straightforward to take this sort of information and deduce precise details of the active site, including its shape and size and its binding points, and this is the procedure that will be followed in the examples described here.

Anti-HIV Agents: Reverse Transcriptase Inhibitors

As mentioned earlier, ddA (**1**) possesses anti-HIV activity (Mitsuya and Broder 1986); and there is considerable information available concerning its mode of

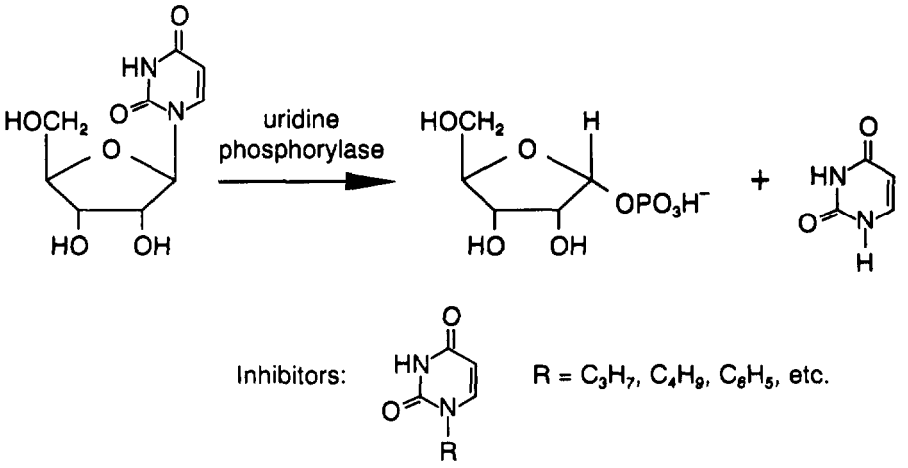


FIGURE 7. *Inhibitors of uridine phosphorylase*

action. In mammalian systems, ddA is rapidly converted to ddI (**3**); this is the pharmacologically equivalent form that now is administered to AIDS patients. Both ddA and ddI are phosphorylated to their monophosphates (Johnson and Fridland 1989), then phosphorylated again to the triphosphates (Ahluwalia et al. 1987), which enter into and ultimately terminate the reverse transcription step of DNA synthesis in the HIV-infected cell.

Acceptance of these molecules by either of the phosphorylating enzymes is known to be contingent on their molecular geometry because other, quite similar compounds are not phosphorylated in this way. However, the microbial metabolite oxetanocin A (compound 4 in figure 8) (Shimada et al. 1986; Nakamura et al. 1986) (**4**) is known to inhibit reverse transcriptase (Hoshino et al. 1987; Seki et al. 1989), and molecular modeling reveals that significant dimensions in this Compound are very close to those in ddA, as shown in figure 9. The 3'-hydroxymethyl derivative (**5**) of ddA also has these same dimensions, but in the 2' isomer (**6**) the sugar ring adopts a different "pucker." As a consequence, the critical dimensions shown in figure 9 are quite different. In fact, like ddA and oxetanocin A, the 3'-hydroxymethyl isomer does provide a triphosphate that interferes with reverse transcriptase (Tseng et al. 1991). The 2'-isomer, on the other hand, does not inhibit reverse transcriptase. This experience suggests that molecular modeling may be a legitimate means with which to predict the shape of a molecule or an active site and whether there may be an interaction between the two, and the authors attempted to exploit the interaction in a study of the PKC problem.

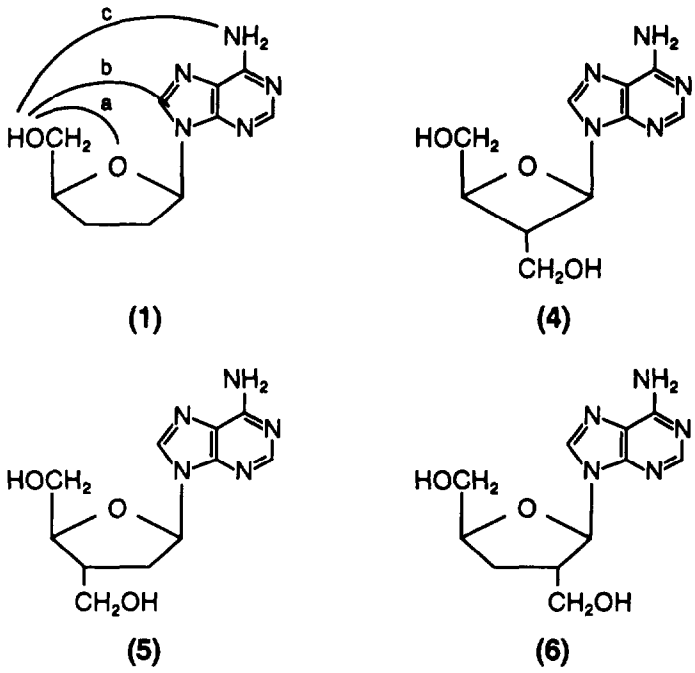
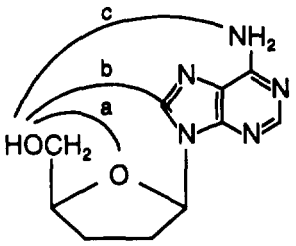


FIGURE 8. *Oxetanocin A, ddA, and derivatives*

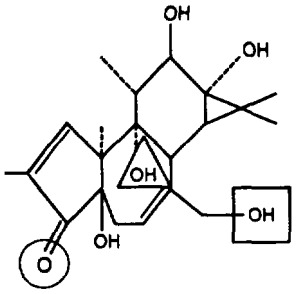
Protein Kinase C Inhibitors

PKC is of great interest because it controls transmission of signals through the cell membrane (Berridge 1987). An event outside the cell can, through a PKC-mediated mechanism, lead to consequences within the cell, and it is thought that many cellular events, in particular cell growth, are regulated in this way. The normal endogenous substrates for PKC are S-l ,2-diacylglycerols (Boni and Rando 1985; Young and Rando 1984) such as S-1-oleyl-2-acetylglycerol; however, several natural materials, such as phorbol (Wender et al. 1986) (**7**) (figure 10) and aplysiatoxin (Nakamura et al. 1989) (**8**), are known to bind to PKC more irreversibly than the diacyl glycerols with various consequences, such as tumor promotion. The normal substrate, S-1 ,2-diacylglycerol, has five oxygen atoms that could interact with the active site of PKC, and it is thought (Nakamura et al. 1989; Brockerhoff 1986) that three of these, the two carbonyl oxygens and the oxygen of the primary hydroxyl group, are necessary for activity. It was hoped that this information could be used to design a PKC inhibitor that could block the binding of diacyl glycerols while avoiding the undesirable effects of the tumor promoters, phorbol and aplysiatoxin.

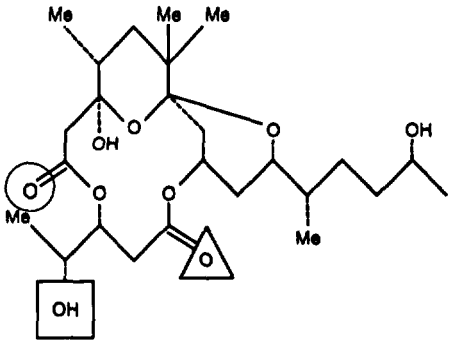


Compound	a (Å) $O_5'O_4'$	b (Å) O_5-N_9	c(Å) O_5-N_6
ddA (1)	2.91	3.94	7.45
Oxetanocin A (4)	2.84	3.51	6.95
3'-ydroxymethyl ddA (5)	2.87	3.68	6.96
2'-Hydroxymethyl ddA (6)	2.77	3.82	7.72

FIGURE 9. *Dimensions of ddA, oxetanocin A, and analogs*



(7)



(8)

FIGURE 10. *Dideoxynucleoside used in chemotherapy of AIDS*

Molecular modeling suggests that the conformation of S-1 ,2-diacylglycerol is as is shown in figure 11. However, the molecule is extremely flexible, and it is difficult to decide on the most stable configuration. The structures of phorbol (Brandt et al. 1971) and aplysiatoxin (Moore et al. 1984), on the other hand, have been determined by x-ray diffraction. They are relatively rigid, and because they both can fit into the active site of PKC, they can be used to deduce the characteristics of this active site. The active form of phorbol is

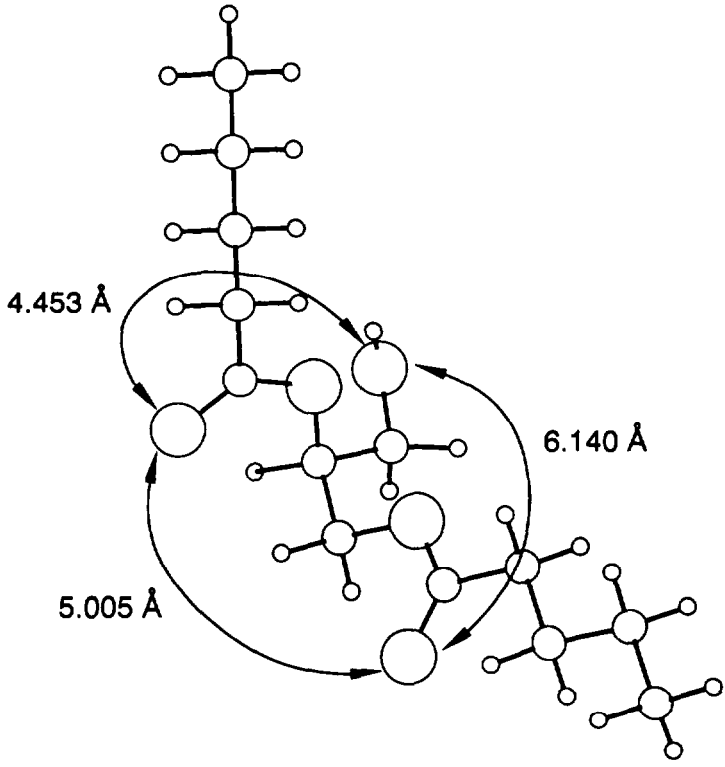
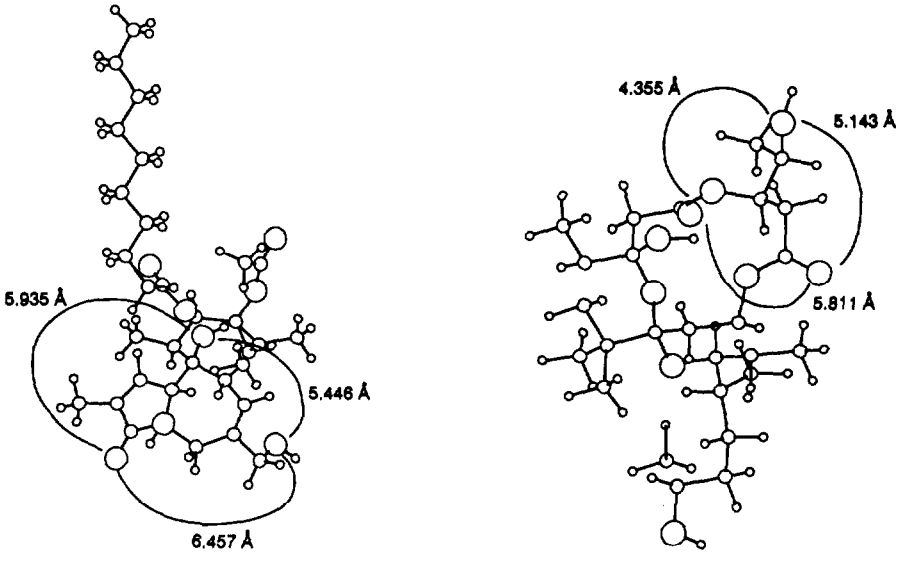


FIGURE 11. *Minimum energy conformation of 1,2-diacylglycerol*

its 12-acetyl-13-myristyl ester, and the model shown in figure 12 was built using the x-ray diffraction data of phorbol as a starting point. In the case of aplysiatoxin, a C_6O side chain was added to C-11 of the x-ray-based model of debromoaplysiatoxin, and the resulting model also is shown in figure 12. Studies (e.g., Nakamura et al. 1989) with analogs of phorbol have shown that the three atoms that are involved in the binding to active sites of PKC are the primary hydroxyl at C-20, the C-S hydroxyl oxygen, and the C-3 carbonyl group in ring A, as shown in (**7**). Likewise, in aplysiatoxin (**8**), the two ester carbonyl groups and the hydroxyl group that lies between them have been implicated in PKC binding (Brandt et al. 1971). In phorbol, the carbonyl oxygen and the C-9 hydroxyl oxygen serve as hydrogen bond acceptors, whereas the third, the primary hydroxyl group, is a hydrogen bond donor. In aplysiatoxin, the two carbonyl oxygens behave as hydrogen bond acceptors and the hydroxyl group is the donor. Finally, the location of the hydrocarbon side chain must be important because, when it is absent, binding is noticeably diminished;



(7) **(8)**

FIGURE 12. *Molecular models of phorbol (7) and aplysiatoxin (8)*

furthermore, if only three points were involved in the binding, optical isomerism in the substrate would be irrelevant. It is known that this is not so; only one of the isomers of 1,2-diacylglycerol, for example, binds efficiently to PKC. Accordingly, in studies of the fit of substrates to PKC, the location of the hydrocarbon side chain of the substrate was taken into account, along with the three-atom fit,

Several carbohydrates related to the diacyl glycerols were modeled. These all contained the diacyl glycerol skeleton but were cyclic and thus relatively rigid. They included ribonolactones and their ring-expanded analogs, all of which are shown in figure 13. The molecular models of all these compounds were examined to determine how well each would fit into an active site that accommodates phorbol acetyl myristate or the aplysiatoxin derivative. On a three-point basis, most of the lactones fit this active site fairly well, but when the substrate's hydrocarbon side chain was required to overlay that of the phorbol or aplysiatoxin esters, it became clear that compounds (**9**) and (**12**) offered the better fits. When tested for their ability to compete with phorbol for the active site in PKC, compounds (**9**) and (**12**) did in fact bind to this site better than their isomers. The model of the binding of the *trans*-ribonolactone (**12**) to the aplysiatoxin derivative in shown in figure 14.

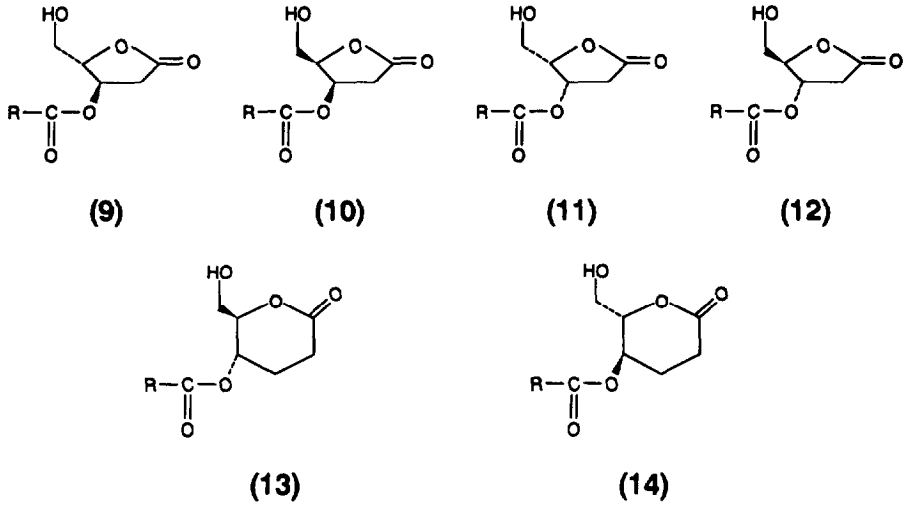


FIGURE 13. *Possible PKC inhibitors*

The results obtained in this way suggest that molecular similarities between known substrates and new molecules can be used to determine whether the new molecules have the capability of binding to the active site. Other considerations such as solubility, stability, and transport are also important, but a fit to the receptor site is a necessary, if insufficient, condition for inhibition. Modeling pharmacophores in this way seems to be a valid approach to defining effective inhibitory molecules.

Three-Dimensional Databases

The approaches outlined above require, as a first step, that a molecule containing the appropriate pharmacophore be defined. It then can be modeled and, if promising, synthesized and tested. The requirement that a candidate molecule be defined is a very limiting requirement, and it would be much more useful if one could work with the precise pharmacophore, searching databases in some way to find all compounds that contain the specified polyatomic arrangement. This possibility quickly became clear to many in the field in the late 1980s and much work went into developing such databases and search systems.

The conventional substructure search systems operate in two dimensions and deal in terms of topology, That is, they are concerned with what atom is connected to what other atom, not with how close two atoms are together in a spatial, three-dimensional (3-D) sense. Replacing *connectivity* with *interatomic*

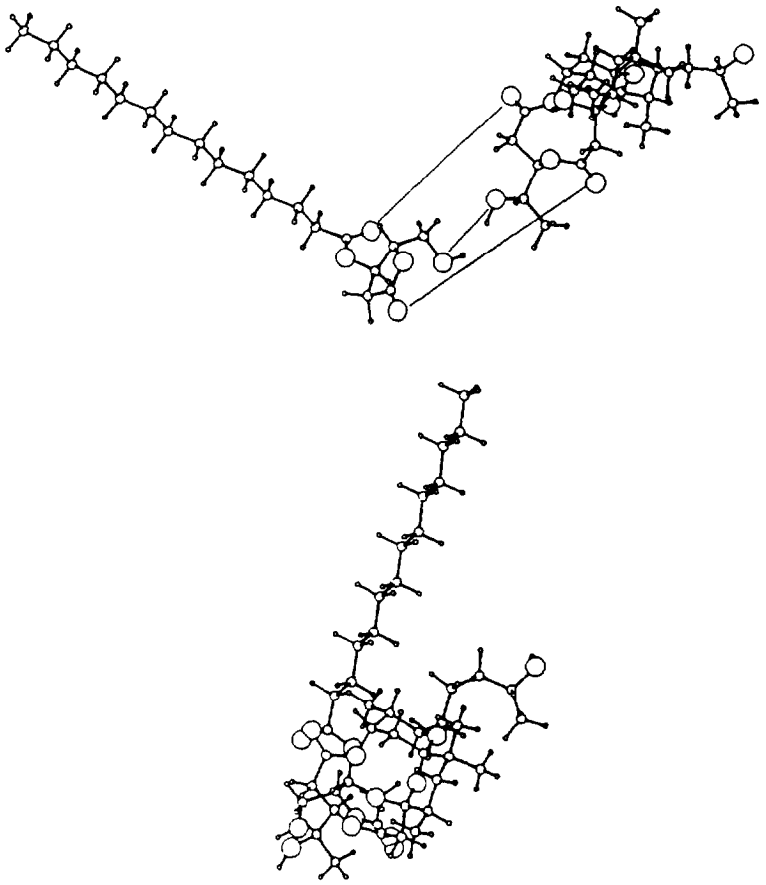


FIGURE 14. *Fit of aplysiatoxin to the trans-ribonolactone (12)*

separation in these search systems is conceptually easy; in practice, methods to do this have already been worked out if not yet perfected.

If a structure contains n atoms, then, counting all possibilities, there are n^2 interatomic distances. When all duplicates and zero separations are removed, there will remain½n(n+1) distances, and it is thus clear that the 3-D database will be considerably larger than its two-dimensional precursor. The precise nature of the atom at either end of such an interatomic separation can be appended in each case to the number representing this distance, and the collection of all these interatomic distances over a whole group of chemical structures becomes the new "3-D database." With such a database, it is possible to search for all compounds containing a pharmacophore like

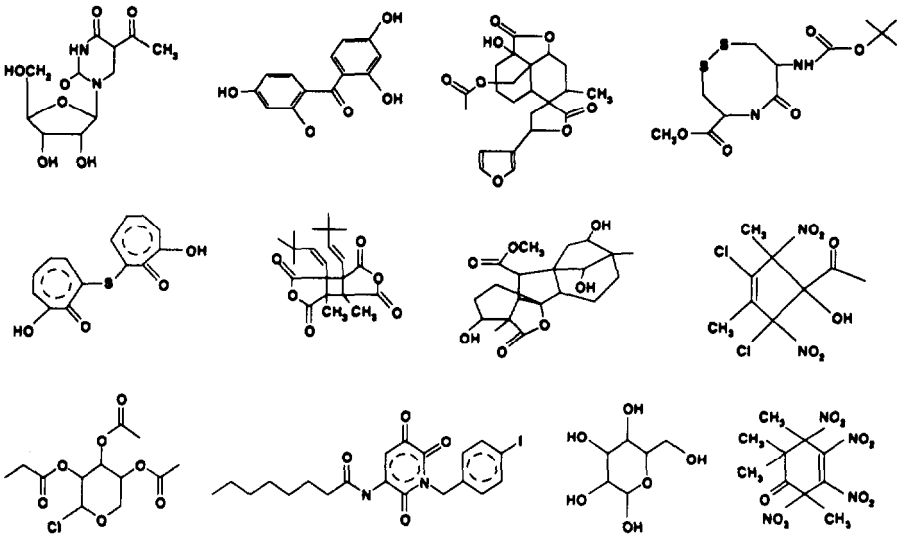


FIGURE 15. *Structures retrieved from a 3-D search*

those discussed here. It will involve searches, for example, for all molecules containing a ketone oxygen between 4 and 5 Å from an aliphatic chlorine *and* a hydroxyl separated from the ketone oxygen by 3.5 to 4.2 Å.

Databases that support such searches are becoming fairly common (Jakes et al. 1987; Sheridan et al. 1989; Moock et al. 1990), and the searches provide interesting lists of compounds that in principle all contain the pharmacophore that was defined in the search query. The lists can often be narrowed further with other criteria such as availability, solubility, toxicity, and so on, and it is often possible to identify and test a candidate compound very quickly. A search[1] for compounds containing a three-oxygen pharmacophore similar to that in phorbol, in which each of the three interatomic distances was expressed as a range, gave as hits a number of compounds; some of them are shown in figure 15. Many of these compounds are natural products, and biological activities are not known for many of them. Such retrieved structures offer many interesting possibilities with which to pursue the search for PKC inhibitors.

NOTE

1. This search was conducted at Pfizer Central Research in Sandwich, U.K.

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Purification and Cloning of Opioid Receptors

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INTRODUCTION

The opioid receptor was one of the first receptors discovered in the early 1970s (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). Nearly two decades after its discovery, opioid receptors have yet to be isolated and purified in sufficient amount for biochemical analysis. With the advent of gene-cloning techniques in the past decade, more than 50 genes of hormone and neurotransmitter receptor proteins have been cloned to date. Again, opioid receptor genes remain one of the elusive genes yet to be cloned. The slow progress in this area has been attributed to the lack of neuronal tissue sources rich in opioid receptors and the high lability of the protein. On the other hand, accumulated biochemical and pharmacological evidence suggests the existence of multiple types and subtypes of opioid receptors, including μ-, δ--,κ--, and ε–receptors and their subtypes (Chang 1984). Today, all clinically useful opiates are p-agonists. These drugs not only produce analgesic effects but also cause many side effects such as addiction, physical dependence, and respiratory depression. The undesirable side effects may be mediated by different subtypes of opioid receptors. To improve opiate analgesic medication, more selective and specific ligands for various opioid receptor subtypes must first be discovered. The specificity of current biochemical and pharmacological methods for drug screening is limited. One solution to the problem is to clone all possible types and subtypes of opioid receptors and use the recombinant receptors for drug discovery studies.

Formerly, a receptor protein was first purified by affinity chromatography to obtain information on the partial amino acid sequences of the receptor molecule (Noda et al. 1983; Kubo et al. 1986; Dixon et al. 1986; Kobilka et al. 1987; Schofield et al. 1987). The corresponding oligonucleotides were then synthesized to clone the full-length cDNA of the receptor's gene by the hybridization technique. The cloned cDNA was then expressed in appropriate cells. The identity of the expressed receptor was verified by either functional assay or receptor-binding assay with known agonists and/or

antagonists. Now, however, nearly all genes within a superfamily of receptors are cloned by molecular biology techniques without going through the traditional protein purification processes. The techniques include the low-stringency hybridization screening technique with known homologous oligonucleotides (Frielle et al. 1987; Emorine et al. 1989; Cotecchia et al. 1988; Peralta et al. 1987; Bunzow et al. 1988; Dearry et al. 1990; Sokoloff et al. 1990; Van Tol et al. 1991; Matsuda et al. 1990), the polymerase chain reaction (PCR) technique with degenerate oligonucleotide primers (Mahan et al. 1991; Zhou et al. 1990; Gantz et al. 1991; Buck and Axel 1991), the COS cell transient expression cloning system (Sakurai et al. 1990; Sasaki et al. 1991; Murphy et al. 1991; Ishihara et al. 1991; Juppner et al. 1991), the ligand affinity panning technique (Davis et al. 1991), and the *Xenopus* oocyte expression cloning method (Masu et al. 1987; Julius et al. 1988; McEachern et al. 1991; Arai et al. 1990; Masa et al. 1991).

Evidence indicates that opioid receptors belong to the superfamily of G protein-coupled receptors (Blanchard and Chang 1988). The signal-transduction system of this family of receptors consists of three protein components: the receptor, the G protein, and the effector (Gilman 1987; Birnbaumer et al. 1990). Many members of this family of receptors have been cloned. A comparison of the secondary and tertiary structures of those cloned receptors revealed a common topological feature, in that the receptors exhibit seven transmembrane domains. The corresponding oligonucleotides coding the seven transmembrane domains are also highly homologous among these receptors' genes. Because opioid receptors belong to this receptor family, it is reasonable to expect that opioid receptors also have a similar seven-transmembrane protein structure and should possess in the cDNA an oligonucleotide sequence homologous to other receptor genes.

The authors and researchers in many other laboratories have designed synthetic oligonucleotides homologous to many other receptor genes of this family to clone opioid receptors from cDNA libraries prepared from opioid receptor-rich cell lines and tissues. Both the low-stringency hybridization and the PCR techniques were used for cloning with these designed oligonucleotides and degenerate primers. So far, no one has succeeded in cloning opioid receptors, These two methods are extremely powerful and have been successful in cloning many subtypes of adrenergic (Lefkowitz et al. 1988), muscarinic (Peralta et al. 1987), and dopaminergic receptors (Bunzow et al. 1988; Dearry et al. 1990; Sokoloff et al. 1990; Van Tol et al. 1991; Sunahara et al. 1991), as well as in cloning adenosine (Mahan et al. 1991), histamine (Gantz et al. 1991), and odorant receptors (Buck and Axel 1991). The failure in cloning opioid receptors with these methods may be explained by the results of the recently cloned receptor for endothelin (Sakurai et al. 1990; Arai et al. 1990) and the metabotropic glutamate receptor (Masa et al. 1991). These receptors do not contain

sequences homologous to other G protein receptors, despite the presence of seven transmembrane domains and their coupling to G proteins, Opioid receptors may thus belong to a subfamily with a unique amino acid structure.

The *Xenopus* oocyte expression cloning method is an extremely powerful technique in cloning receptors that are coupled to phospholipase C to generate two second messengers, inositol-1,3,5-triphosphate and diacylglycerol (Masu et al. 1987; Julius et al. 1988; McEachern et al. 1991; Arai et al. 1990; Masa et al. 1991; Morel et al. 1992; Kimura et al. 1992). The increase of inositol-1,3,5-triphosphate triggers release of Ca^{++} from the intracellular pool. This results in increased cytoplasmic Ca^{++} concentration leading to activation of Cl' channel, which can be monitored by electrophysiologic techniques. However, there is no evidence to suggest that opioid receptors are functionally linked to the activation of phospholipase C and Cl^- channels, Thus, this method is unlikely to work for opioid receptor cloning.

The COS cell transient expression cloning method is suitable for receptors that can be detected by ^{125}I-labeled ligands. This method works for receptors whose high-affinity binding activity for the labeled ligand does not require the presence of affinity modifier such as G proteins. Currently available ^{125}I-labeled opioid ligands are all agonists that bind with a high affinity only to opioid receptors that are associated with G proteins. Thus, the expressed opioid receptors in COS cells must interact with appropriate G proteins to attain a high affinity for the detection by ^{125}I-labeled opioid agonists. This greatly reduces the chance of successful cloning. The lack of ^{125}I-labeled antagonists for binding assay also hampers the molecular cloning of opioid receptors by this technique.

There are only two other alternatives for cloning opioid receptors: (1) the traditional method of purifying opioid receptor protein with affinity chromatography to obtain partial amino acid sequences and (2) the ligand affinity panning method. The latter method is exemplified by the recent cloning of a receptor protein by Goldstein and colleagues (Xie et al. 1992). They reported expression cloning of cDNA encoding an opioid-binding protein from a κ· -rich human placenta cDNA library. By repeated panning of transfected COS-7 cells expressing κ--receptor binding activity, a cDNA encoding a 440-residue protein was cloned. Hydropathy plot of the protein revealed a seven-helix transmembrane domain typical of G protein-coupled receptors as expected for opioid receptors. The expressed receptor showed stereospecific binding for opioids and peptide as well as nonpeptide ligands, but showed noκ--selectivity. The affinity of the cloned receptor for agonist was about two orders of magnitude lower than that of native membrane receptors, and the affinity for antagonist was even lower. The lack of κ· -receptor selectivity and the lower affinity of the expressed receptor might be caused by, among other reasons, missing G proteins or other accessory

proteins in the COS-7 cells or by improper, or lack of, posttranslational modification of the expressed receptor for correct conformation. Interestingly, the cloned protein showed a high degree of homology with the human neuromedin K receptor at the transmembrane domain. The significance of this observation is not clear at this time.

Li and colleagues (1992) recently succeeded in purifying μ-opioid receptor to homogeneity. This chapter describes the methods used and discusses the results of the purification of opioid receptors from rat brain membranes.

SOLUBILIZATION OF OPIOID RECEPTOR FROM RAT BRAIN MEMBRANES

Rat brains (Sprague-Dawley) were homogenized in a buffer containing 50 mM Tris HCl, 1 mM ethylene glycol-bis (β·-aminoethyl ether) N,N,N',N' tetra acetic acid (EGTA), 10 mM $MgSO_4$, 1 mM benzamidine, 0.5 mM phenylmethyl sulphonylfluoride, 10 μg/mL leupeptin, and 10 μg/mL pepstatin in 0.34 M sucrose. The homogenate was centrifuged at 40,000 x g for 20 minutes and the pellet was resuspended in a hypotonic buffer containing 5 mM Tris HCl, 1 mM EGTA, and protease inhibitors and incubated at 4 °C for 25 minutes. The hypotonic treatment was repeated twice, and the final pellet was washed and resuspended in 50 mM Tris buffer containing ethylene glycol and protease inhibitors as described above. Opioid receptor was then solubilized from the above brain membrane preparations by incubating membranes in a mixture of CHAPS (3[(3-cholamindopropyl)-dimethylammonio]-1 -propane-sulfonate), digitonin, and 1 M NaCl in the 50mM Tris buffer. After centrifugation at 100,000 x g for 1 hour, the soluble fraction and pellet were assayed for opioid-binding activity, which was carried out at 24 °C for 1 hour with [^{3}H]diprenorphine (35 to 45Ci/mmol) in Tris buffer containing 1 M NaCl and 0.4 percent bovine serum albumin. Nonspecific binding was determined by the presence of 1 μM naloxone in the assay. When membrane material was used, the binding reaction was terminated by filtration through GF/C glass filter paper. When solubilized receptor was used, the reaction was terminated by precipitation with saturated ammonium sulfate solution in the presence of 0.1 percent r-globulin as protein carrier. The precipitate was filtered with GF/C glass filter paper.

Both CHAPS and water-soluble digitonin *were* reported to be successful in solubilizing opioid receptor from membranes (Simonds et al. 1980; Gioannini et al. 1985). The authors have investigated the efficiency of CHAPS and water-soluble digitonin in solubilizing opioid receptors from rat brain membrane preparations, It was found that either one alone could only partially solubilize receptor from membranes (figure 1). Optimal conditions appear to require both 7.5 mM CHAPS and 1 percent digitonin. It is also important to include 1 M NaCl in the buffer. Elimination of NaCl from the buffer caused irreversible

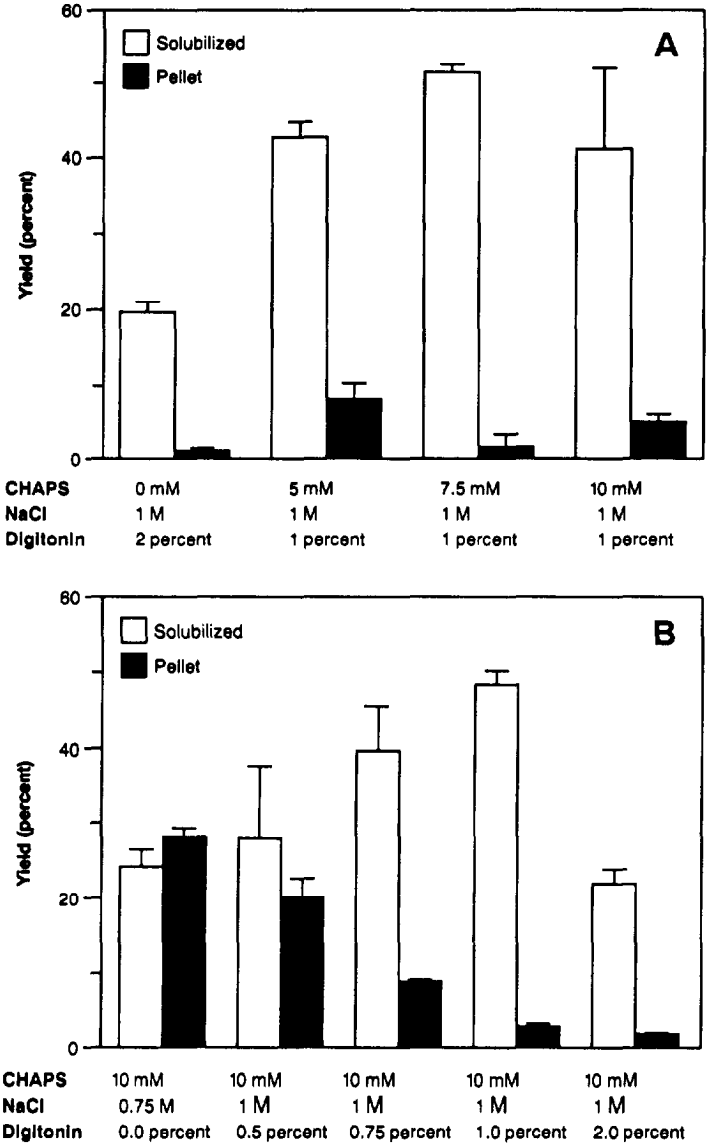


FIGURE 1. *Solubilization of opioid receptor from rat brain membranes. Membranes were incubated for 1 hour at room temperature with CHAPS (A) and digitonin (B) at concentrations indicated. After centrifugation, the resulting supernatant and pellet were assayed for specific binding of [^{3}H]diprenorphine, which is expressed as percentage yield of starting membrane material. Data were presented as means±SEM (n=3).*

aggregation of solubilized proteins and, thus, inactivation of opioid receptors. (It was also found later that the presence of 1M NaCl is essential for the binding of opioid antagonist to the purified receptors.) At least 30 minutes of incubation time is required to achieve effective solubilization (figure 2). Room temperature (24 °C) appears to be more effective than 4 °C, and the optimal protein concentration is about 7 mg/mL (figure 3). The final protocol for solubilization includes CHAPS (7.5 mM), water-soluble digitonin (1 percent), NaCl (1 M) in the solubilizing buffer, 60 minutes incubation at 24 °C, and about 6 to 10 mg/mL membrane protein.

Various protease inhibitors were systematically investigated for their abilities to protect opioid receptor from proteolytic degradation during solubilization and purification. A combination of EGTA, bezamidine, phenylmethyl sulphonylfluoride, leupeptin, and pepstatin at concentrations described above provides the best protection. The requirement of ethylene glycol was also investigated. It was found that 25 percent ethylene glycol improved

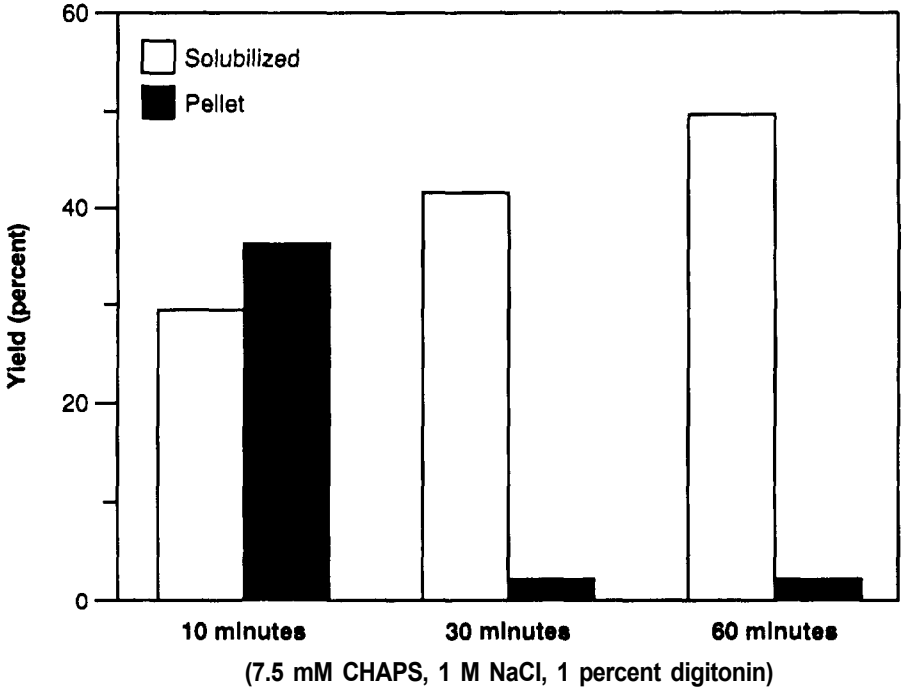


FIGURE 2. *The effect of incubation time on solubilization yield. Membranes were incubated up to* 60 *minutes at room temperature in the solubilization solution indicated. The membrane protein concentration was 4.0 mg/mL.*

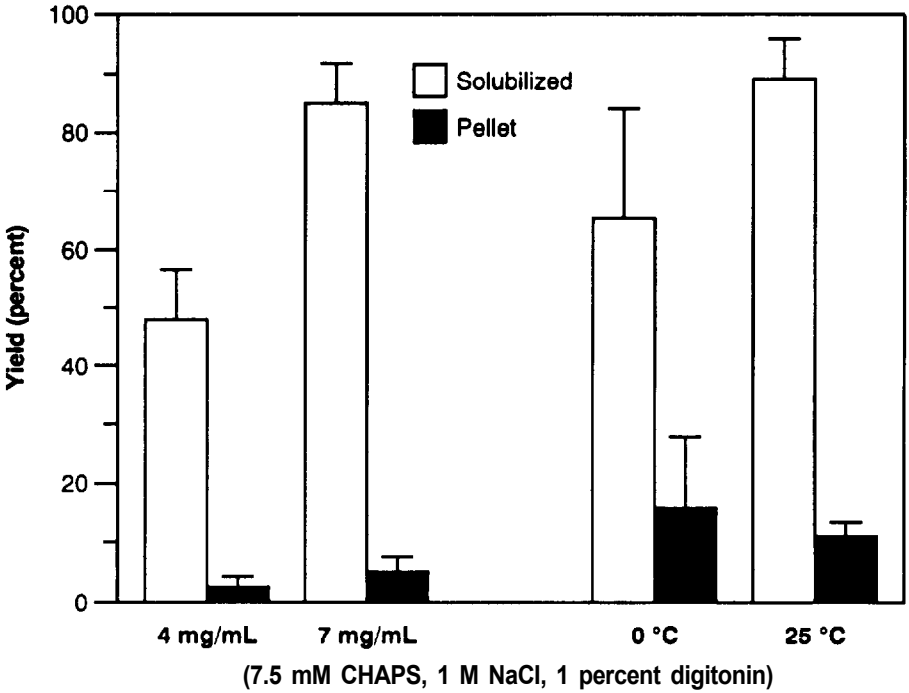


FIGURE 3. *The solubilization yield as function of temperature and protein concentration. Membranes were incubated for 60 minutes in the solubilization solution indicated. Data were presented* as *means±SEM (n=3).*

the stability of the solubilized receptors. Using this protocol, the yield of solubilization is routinely about 80 to 90 percent, and the solubilized receptors obtained can be stored at -70 °C for months without losing binding activity.

PURIFICATION TO HOMOGENEITY

Sequential opioid antagonist-affinity column and wheat-germ agglutinin (WGA) column were used to purify solubilized receptors (Li et al. 1992). The opioid antagonist used was N-cyclopropyl-methyl-7-amino-6,14-endoetheno-7,8-dihydromorphine (10 cd), which was covalently coupled to Affi-gel 202 by the addition of 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide HCl in 0.1 M HEPES buffer (pH 5.4). Four separate 2.5 mL size opioid antagonist-affinity columns were routinely set up in parallel. About 60 mL solubilized receptors (from eight rat brains) were applied to each column, which were preequilibrated with 0.1 percent digitonin in the buffer. After thorough washing, opioid receptors were

eluted with 100 μM naloxone and readsorbed onto a WGA-agarose column (1 mL) set up in series. The passed-through buffer was recycled back to opioid antagonist and WGA columns four times. After thorough washing, opioid receptors were then eluted from WGA column with N-acetyl-β-D-glucosamine (50 mM). Aliquots were tested for binding activity with [^{3}H]diprenorphine in the presence of 1 M NaCI. Active fractions were pooled and further purified by repeating the procedure described above but with smaller columns (0.5 mL size). The active fractions were then concentrated tenfold with microconcentrators, and the concentrated material was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (figure 4). A protein of diffused band with a medium molecular mass of 62 kD was observed to correspond to the peak binding activity of [^{3}H]diprenorphine. Two proteins of molecular mass of 67 and 58 kD were eluted in fractions 1 through 3 and were separated from the active fractions (fractions 4 through 6) in the last step of purification.

The kD value of purified receptor for [^{3}H]diprenorphine is 0.29 nM, a value very close to that observed in membrane preparation. The purified material binds the highly selective p-agonist PL017 ([N-Me-Phe^3, D-Pro^4]morphiceptin) with an affinity similar to that observed in membrane preparations determined Jnder identical conditions (Li et al. 1992). This material also binds selective u-antagonist (-)-naloxone with a Ki value of 4.3 nM, which is in the range of known values for μ-receptor. It shows a very low affinity for the biologic inactive isomer (+)-naloxone (Li et al. 1992). [^{3}H]diprenorphine binding is inhibited only 30 percent by (+)-naloxone at 100 μM. δ-Receptor antagonist naltrindole (Portoghese et al. 1987) and k-receptor antagonist nor-binaltorphimine (Portoghese et al. 1988) also show binding to the purified material, but the low affinities and the binding characteristics of these compounds strongly suggest that the purified material is predominantly μ-opioid receptor. It is estimated that 85 percent of the receptor is of the μ-type and 15 percent the δ-type. The specific binding activity of [^{3}H]diprenorphine was calculated to be 18.8 pmol/μg protein, which is near the theoretical value of homogeneity. The final product is about 65,000-fold purified over the initial solubilized material (Li et al. 1992).

CHARACTERIZATION AND RECONSTITUTION OF PURIFIED OPIOID RECEPTOR

As discussed above, opioid receptors belong to the G protein-coupled receptor superfamily, and the binding of opioid agonists is known to be regulated by guanine nucleotides such as guanosine triphosphate (GTP), guanosine diphosphate (GDP), or their stable analogs, GDP$_\beta$S Gpp(NH)p, and GTPS. The binding of μ-agonist PL017 to membranes and to solubilized opioid receptors was greatly reduced in the presence of GDP and GTPS (figure 5, panel A). This reduction of binding was even greater in the presence of Na^+ (Li et al. 1992). However, the binding affinity of PL017 was reduced for the partial as well as the purified opioid μ-receptors, and the regulatory effect of guanine nucleotides was

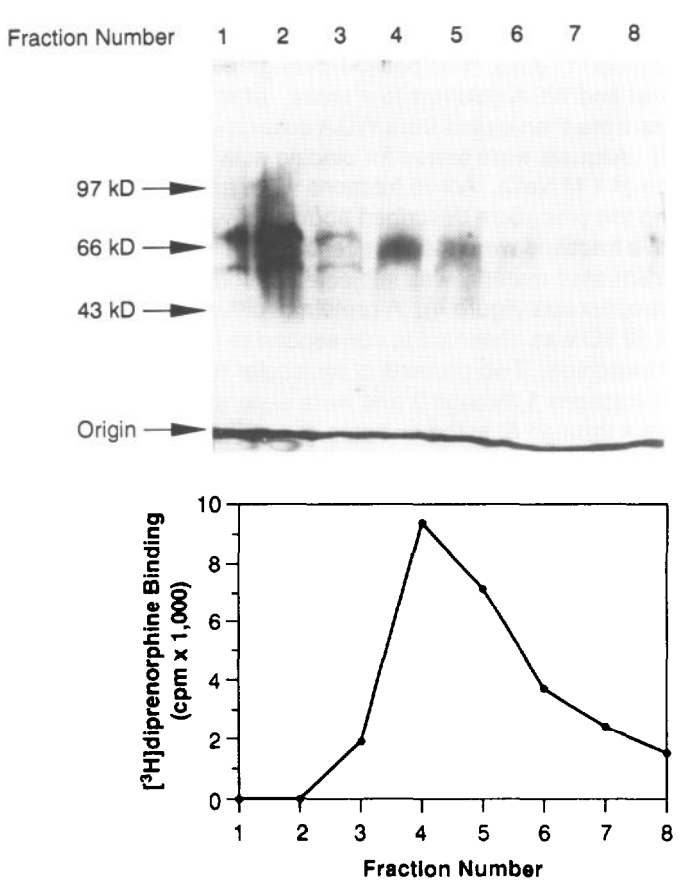


FIGURE 4. *The elution profile of receptor protein from the final WGA-agarose affinity chromatography and the sodium dodecyl-sulfate polyacrylamide gel eiectrophoresis result of the purified opioid receptor. Opioid receptor was purified by chromatography through repeated 10cd and WGA columns as described in the text. The specific binding activity of eluted fractions from the final WGA column was assayed, and aliquots of each fraction were subjected to electrophoresis (7.5 percent polyacrylamide gel). The top panel shows the silver staining pattern of the purified protein material. The positions of molecular weight standards are indicated by arrows. The bottom panel shows the corresponding binding activity of each fraction. Fractions 4 through 6 contain most of the binding activity and exhibit as diffused bands with a medium molecular mass of 62 kD. Fractions 1 through 3 contain two proteins of 58 kD and 67 kD with little or no binding activity.*

lost (figure 5, panel B). These data suggest that purified μ-opioid receptors are dissociated from G proteins and thus assume a low-affinity state for agonists. The high-affinity binding of PL017 and the regulatory effect of guanine nucleotides can be partially restored by reconstituting the purified receptor with the G proteins purified from bovine brains in phospholipid vesicles (figure 5, panel C).

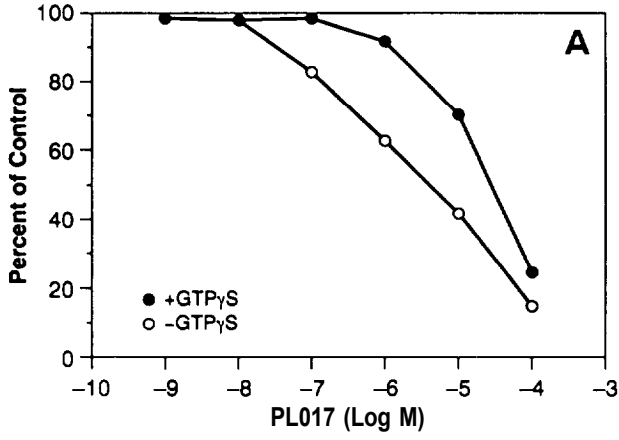
Another interesting observation is the requirement of Na^+ ions for the binding of opioid antagonist to the purified opioid receptors (Li et al. 1992). During the process of purification, it was found that the higher the purity of the receptor, the higher the concentrations of Na^+ ions required for the optimal binding of [^{3}H]diprenorphine. To reach half-maximal binding, the purified receptor requires about 250 mM of Na^+, a concentration 10 times greater than that required for membrane receptor. The reason for the increased Na^+requirement is unknown.

One can use this Na^+ dependency of opioid antagonist binding to design an affinity chromatography for the purification of opioid receptors (Li et al. 1992). This can be achieved by loading receptors onto an opioid antagonist column in the presence of 1 M NaCl and eluting receptors with a buffer without NaCl. The realization of the importance of sodium ions in the binding of opioid antagonist has eventually led the authors and colleagues to design an appropriate affinity chromatography and binding assay for the successful purification of μ-opioid receptor to homogeneity (Li et al. 1992).

The observation of the effect of sodium on opioid antagonist binding is not new. It was reported in the early 1970s at the time opioid receptors were discovered. The increase in opioid antagonist binding by sodium ions was originally proposed as a result of conversion of agonist conformation to antagonist conformation (Pert and Snyder 1974; Simon and Groth 1975; Simon et al. 1975; Pasternak et al. 1975). Nijssen and Childers (1987) reported that the effect of sodium ion on antagonist binding was to increase the number of opioid binding sites for antagonist. Wong and colleagues (1992) have recently confirmed this observation and further identified that the increased binding sites of labeled antagonist by sodium ions are low-affinity sites for an opioid agonist. This study on the purified receptor clearly suggests that opioid agonists and antagonists either do not bind or bind with low affinity to G protein-dissociated free opioid receptors in the absence of sodium ions. However, the free opioid receptors have a high affinity for antagonists but not agonists in the presence of sodium ions. This explains why sodium ions increase binding sites for labeled antagonist.

MOLECULAR MODEL OF OPIOID RECEPTOR

Based on the model proposed for a G protein-linked receptor superfamily by Gilman (1987) and Birnbaumer and colleagues (1990) and data presented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Percent of Control
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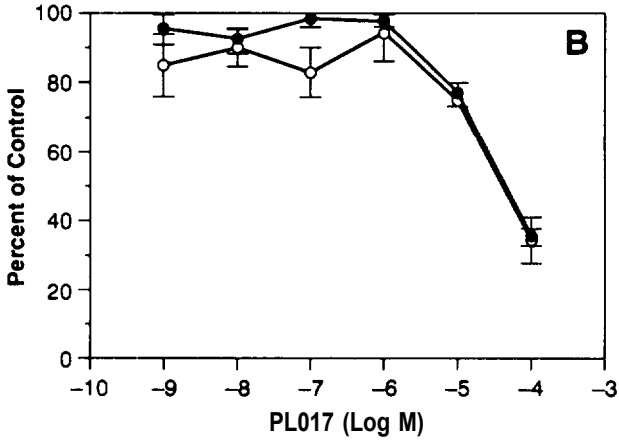
−10 −9 −8 −7 −6 −5 −4 −3
PL017 (Log M)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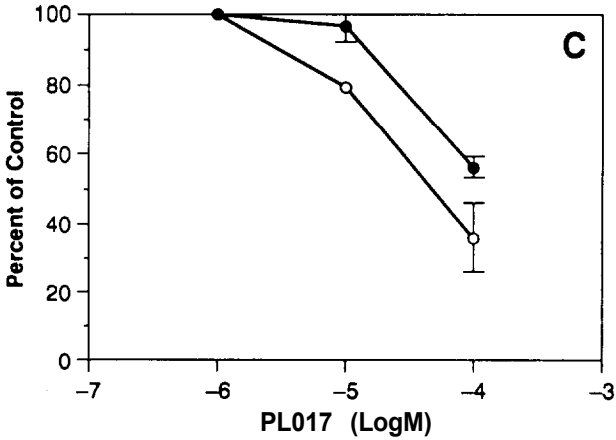
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FIGURE 5. *Effect of GTP$_{\gamma}$S on ligand competitive binding curves of crude membranes* and *solubilized, partially purified, and purified G protein reconstituted receptor preparations. Highly selective μ-agonist PL017* was *used in competition binding* against *[^{3}H]diprenorphine* with *rat brain membranes (A),* partially *purified receptor from WGA affinity column (B), and final purified opioid receptor reconstituted with G protein (C). Binding* assay was *carried out in the absence (O - O) and presence (• - •) of 0.1 mM GTP$_{\gamma}$S. The reconstitution experiment* was *carried out in phospholipid vesicles by a modification of* the *method described by Sternweis (1986). Briefly, Gi-proteins (purified from rat brain according to Sternweis [1986]) are incubated with* alcoholic solution *of phosphatidylcholine to form* a *vesicle suspension. The suspension* was *passed through a Sephadex G50 column to obtain* a *vesicle fraction containing G protein (30 pmol/mL estimated from ^{35}S-GTP$_{\gamma}$S binding* assay). *The vesicles (0.8 mL) were mixed with purified receptor solution (1.4 mL containing 2.5 pmol of opioid binding site) in the Tris buffer (same buffer described for homogenization in the text) containing 0.1 percent digitonin and 300 mM* NaCl. *[^{3}H]diprenorphine and PL017 were added,* and *the mixture was incubated overnight at 4 °C. The binding assay* was *terminated by filtration through GF/C paper. The results are expressed as percentage of control-specific binding* (mean ±*SEM) of at least three separate experiments.*

here and other data mentioned above, the authors propose a model of opioid receptor-G protein activation cycle as depicted in figure 6. Opioid receptors (R) exist in three forms: high-affinity ($G•R_H$) complex, low-affinity ($_{GDP}$-$G•R_L$) complex, and free (R_L) receptors. At resting physiological condition, the receptors are presumably in the low-affinity $_{GDP}$-$G•R_L•Na^+$ complex. When a binding assay is conducted in the presence of high concentrations of sodium ions and GDP, a condition that thermodynamically favors the formation of $_{GDP}$-$G•R_L•Na^+$ complex, all p-receptors detected are in the low-affinity state for agonists. Mg^{++}, together with opioid agonist Op, promote receptors into the high-affinity $G•R_H$•Op complex. In the absence of GTP, the reaction will not proceed. Thus, in membranes prepared in the absence of GTP and assayed with Mg^{++} and opioid agonist, all receptors stay in the high-affinity state. Wong and colleagues (1992) have recently described that pretreatment of brain membranes with sodium ions and GDP promoted the formation of a homogeneous high-affinity state of μ-opioid receptors for an opioid agonist when assayed in the presence of Mg^{++} ions.

GTP binds to the a-subunit of the high-affinity G protein-receptor complex. The GTP-bound G protein promptly undergoes subunit dissociation and the formation of the low-affinity free receptor (R_L). The GTP-bound a-subunit of

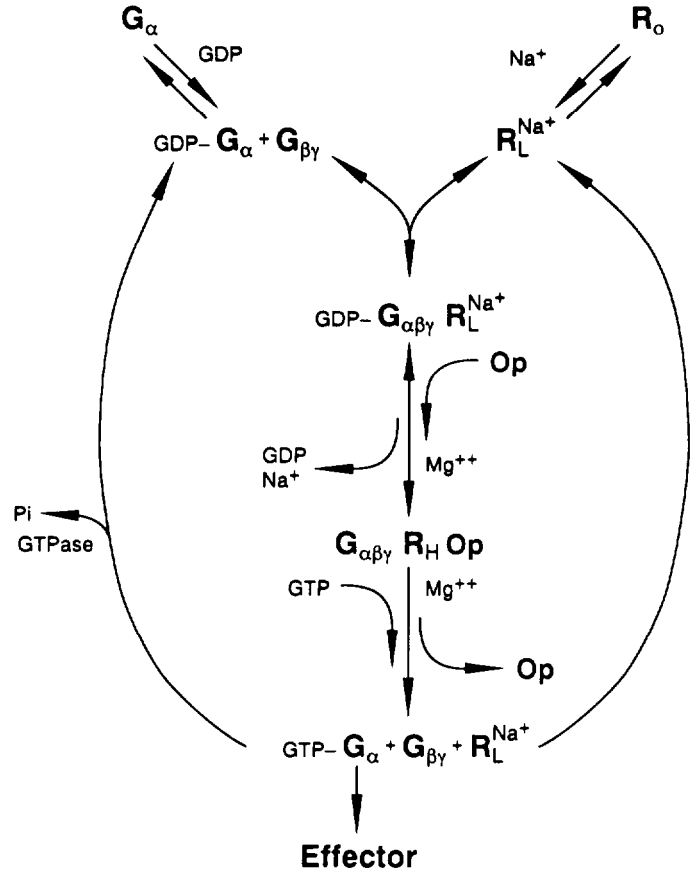

FIGURE 6. *Mechanism of opioid receptor-G protein activation cycle. In membranes, opioid receptor exists in the G protein-complexed low-affinity ($G{\bullet}R_L$) or high-affinity ($G{\bullet}R_H$) forms, or the low-affinity free receptor form (R_L). A nascent receptor form (R_o) is also proposed, which expresses itself in the membranes only in the presence of high sodium concentrations. Op is an opioid agonist that together with Mg^{++} facilitates the GDP/GTP exchange reaction. G_α is the α-subunit of G protein; $G_{\beta\gamma}$ is the $\beta\gamma$-subunit of G protein; GTP-G_α is the active GTP-bound G_α-subunit. The GDP-bound G_α-subunit (GDP-G_α) is an inactive form that reassociates with $\beta\gamma$-subunit. Guanine nucleotides, sodium, and magnesium ions regulate receptor affinity and receptor-G protein coupling. The rate-limiting step of effector activation is proposed to be the formation of high-affinity $G{\bullet}R_H{\bullet}Op$ complex. See text for details.*

G protein induces effector activation. The hydrolysis of GTP to GDP, catalyzed by an intrinsic GTPase on the α·-subunit, terminates the activation process and returns the receptor to resting state. However, if GTP is replaced by the nonhydrolyzable analogs of GTP such as Gpp(NH)p, the GTPase reaction will not proceed and the effector will be permanently activated because of the occupancy of the a-subunit by Gpp(NH)p. The subunits G_{α} and $G_{\beta\gamma}$ remain dissociated and unable to reform the G protein-receptor complex. Thus, all p-receptors will be in the low-affinity state of free R_L.

Sodium ions favor the low-affinity conformation and also facilitate effector activation by promoting the breakdown of the high-affinity receptor-G protein complexes. This is supported by the observation that all p-receptors are in the low-affinity state for agonists in the presence of Na^+, Mg^{++}, and Gpp(NH)p (Wong et al. 1992). When membranes are prepared with a buffer without sodium ions and guanine nucleotides, part of opioid receptors are converted to nascent receptors (R_o), which do not bind opioid agonists or antagonists. These nascent receptors can be recruited back to the low-affinity receptor pool by sodium ions. These sodium-associated receptors can further interact with GDP-bound G proteins to form the $_{GDP}$-G•R_L•Na^+ complexes (Wong et al. 1992).

Under normal physiological conditions, the rate-limiting step for this cycle of activation process is the interaction of opioid agonist with $_{GDP}$-Ga&•R_L complex in the presence of Mg^{++} to induce the dissociation of GDP and the formation of the high-affinity $G_{\alpha\beta\gamma}$·•R_H•Op complex. This is based on the fact that Na^+, Mg^{++}, and GTP exist in high concentrations under physiological conditions such that opioid agonist triggers the breakdown of the high-affinity $G_{\alpha\beta\gamma}$•R_H•Op complex immediately. Wong and coworkers (1992) have recently demonstrated that this step of the formation of the high-affinity $G_{\alpha\beta\gamma}$•R_H•Op state is attenuated in spinal membrane μ-receptors prepared from opioid-tolerant animals. This reduced conversion to the high-affinity state correlates the degree of opioid tolerance.

CLONING OF OPIOID RECEPTOR cDNA

As discussed above, many attempts to clone opioid receptor gene with modern molecular biology techniques have not been successful for various reasons. The authors have recently succeeded in purifying μ-opioid receptor to homogeneity with a reasonable yield. Currently, we can obtain about 5 to 10 pmol of purified receptor in a week. It is estimated that to obtain 100 to 200 pmol quantity of receptor for partial amino acid sequence analysis, it will take about 6 months to 1 year to accumulate enough quantity of purified receptor. We are actively attempting to obtain this accumulation.

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Computer-Assisted Design of Dopamine Transporter Ligands

Gilda H. Loew, Hugo O. Villar, Patricia A. Meguire, and M. Frances Davies

INTRODUCTION

Cocaine is a major drug of abuse that has potent effects in the central and peripheral nervous systems (Washton and Gold 1987; Spitz and Rosecan 1987). These effects are elicited by cocaine's interaction with multiple sites, including the sigma (Sharkey et al. 1988*a*), muscarinic (Sharkey et al. 1988*b*), and serotonergic (Ritz and Kuhar 1989) receptors. In addition, cocaine inhibits the reuptake of the monoamines from the synaptic cleft (Javitch et al. 1984). Among the monoamines there is growing evidence implicating the ability of cocaine to block the dopamine transporter and inhibit its reuptake as one of the major causes of its addiction liability (Dackis and Gold 1985).

For example, a specific link between the inhibition of dopamine reuptake and the potential for addiction has been provided by the observation that the reinforcing properties of several substances in self-administration studies in primates varied directly with their affinity for the site labeled by mazindol on the dopamine transporter (Ritz et al. 1987; Kuhar et al. 1991). The relationship between the reinforcing properties of cocaine and its interaction with the dopamine transporter has led to an increased interest in the molecular mechanisms of monoamine transport at the cellular and molecular levels.

For many years, researchers at the authors' laboratory have been involved in multidisciplinary studies of molecular mechanisms of central nervous system actions of abused drugs, which have recently been expanded to include cocaine. The insights gained from these studies provide a strong foundation for the rational design of other compounds that interact with a particularly targeted subset of sites that are recognized by the abused drugs. There are three important reasons for the design of drugs that interact with all or part of the same sites that interact with drugs of abuse: (1) to obtain novel compounds that permit probing the site of action at which drugs of abuse act; (2) to design drugs that can be used in the pharmacotherapeutic treatment of the conditions induced by drug abuse, such as the abstinence syndrome; or (3) to search for

therapeutic agents deprived of abuse potential that may substitute for commonly abused drugs in clinical practice. All three goals are relevant to the design of compounds that act at the same sites that cocaine does and, in particular, to those that bind to the dopamine transporter. Experimental efforts aimed at characterizing detailed aspects of the uptake mechanisms would profit from more extensive and varied probes. Moreover, because the uptake mechanism and the nature of the transporter protein are not fully understood, it is reasonable to search for a compound that affects cocaine binding to the transporter without altering dopamine uptake. Finally, there is a need in clinical practice for compounds with some of the proper-ties of cocaine. Cocaine was used for several years in nasal and ocular surgery because of its anesthetic properties and was abandoned only because of its powerful addicting properties.

Of equal importance as the hypothesis-based generation of novel compounds is the pharmacological evaluation of these novel drugs as a means of testing the soundness of current hypotheses (i.e., the candidate molecular properties identified as modulators of recognition and activation at the sites where abused drugs act). If the new drugs evaluated fail to have the predicted pharmacological profile, then the molecular hypothesis on which the design was based should be refined. In this iterative process, valuable insights are gained about the nature of the site recognized by the drug and useful new drugs ultimately are designed.

The use of the tools of computational chemistry has permitted the development of detailed hypotheses for the mechanisms of drug recognition and activation that have subsequently been applied to the design of therapeutic agents. The methods and approach used are different, depending on whether the three-dimensional (3-D) structure of the macromolecular target of the ligands is known or unknown. In the first instance, ligand-macromolecular interaction can be modeled explicitly, but with several important factors still to be resolved. Modifications in the ligands aimed at improving the complementarity between the properties of the ligand and those of the binding site can then be suggested. In contrast, commonly abused drugs, and the drugs used in the pharmacotherapeutic treatment of their effects, belong to the second category. They act at membrane-bound receptors and, although many of these receptors have been cloned and sequenced, none has a known 3-D structure. For example, the dopamine transporter with which cocaine interacts has been recently cloned and sequenced (Kilty et al. 1991; Shimada et al. 1991), but the information is of limited use because it is not possible to deduce a 3-D structure for the protein from its sequence alone.

If no structural information on the site of action is available, then the design of additional probes must be made by comparing the properties of known ligands to the properties of chemically related compounds that fail to bind to and/or activate the receptor. The most complete use of this approach involves calculation and examination of three types of properties—steric, electronic,

and environmental—as candidate molecular determinants of recognition and activation because all three types of properties relate to the ability of the candidate compound to participate in intermolecular interactions with the macromolecule. However, insights can be obtained at intermediate steps, short of the final goal.

In the work reported in this chapter, the authors describe the current status of our studies to identify and characterize the molecular determinants of recognition by cocaine congeners and other ligands of the dopamine transporter. A structural study of cocaine and its diastereoisomers has been made to characterize its bioactive form (Villar and Loew 1990). In addition, study has begun on the mechanistic significance of the three substructural elements common to all ligands that bind to the cocaine-binding site of the dopamine transporter: (1) an amine nitrogen, (2) a strongly lipophilic region, and (3) an aromatic moiety. Finally, the authors illustrate how using these substructural domains as the basis for even a two-dimensional (2-D) search of a chemical database for new families of ligands can facilitate the drug design process.

CHARACTERIZATION OF THE BIOACTIVE FORM OF COCAINE

The first step in the characterization of the bioactive form of any substance is a thorough study of its conformational and dynamic properties, If the compound has only one energetically accessible structure (i.e., is a rigid ligand), this unique structure would be the form recognized by the receptor and further studies are not required. However, for more flexible ligands, determining which of many accessible conformers is recognized by the binding site is a more complex process and cannot be done by consideration of only one ligand. Several ligands must be included, some of which do and some of which do not bind with high affinity to the binding site being studied. Conformational profiles of each must be obtained and properties calculated and compared for all accessible conformations. In this case, the form of the ligands that maximizes the similarities in the molecular properties of the high-affinity ligands should be regarded as the bioactive form. As an additional requirement, this form should not be energetically accessible in compounds that display low affinity. The consequence of such an approach is that the molecular properties common to all ligands with high affinity that are not present in the low-affinity analogs are selected as the determinants of recognition at the binding site.

To determine the bioactive form of cocaine, a disubstituted tropane bicycle, the authors carried out a systematic study of its conformational characteristics, using the AM1 semiempirical, quantum mechanical technique (Dewar et al. 1985). Systematic rotations of the side chains were performed in each of the four possible conformations for the tropane nucleus. The minimum energy structure of the free base was found to correspond to a chair conformation with the N-methyl substituent in an equatorial position. This preferred conformation

is shown in figure 1. The geometrical parameters characterized—bond distances, angles, and torsional angles—are similar to those found in the x-ray determination of crystal structure (Gabe and Barnes 1963). The only geometric difference between the x-ray structure and the lowest energy minima characterized with AM1 is in the torsion angle around the ester function in position 2. This difference could be attributed to the fact that our studies were made for the free base, whereas the x-ray determination was for the chlorohydrate form in which the amine nitrogen is protonated. Indeed, the structure of the lowest energy form for N-protonated cocaine (figure 2, panel A) is identical to the x-ray determined structure.

As discussed above, the relevance of the lowest energy form of a compound to the biological processes it initiates depends on how rigid it is at physiologically relevant temperatures. Cocaine has only one low-energy conformer. One method of assessing its flexibility is to perform a molecular dynamics calculation on it. Such computations for cocaine were performed at 310 K for more than 200 psec. The study revealed that the only portion of the molecule that freely rotates is the phenyl ring of the substituent in the 3 position. Despite the presence of other rotatable bonds, the rest of the molecule is surprisingly rigid. Therefore, the lowest energy structure of cocaine is likely to be the bioactive conformation.

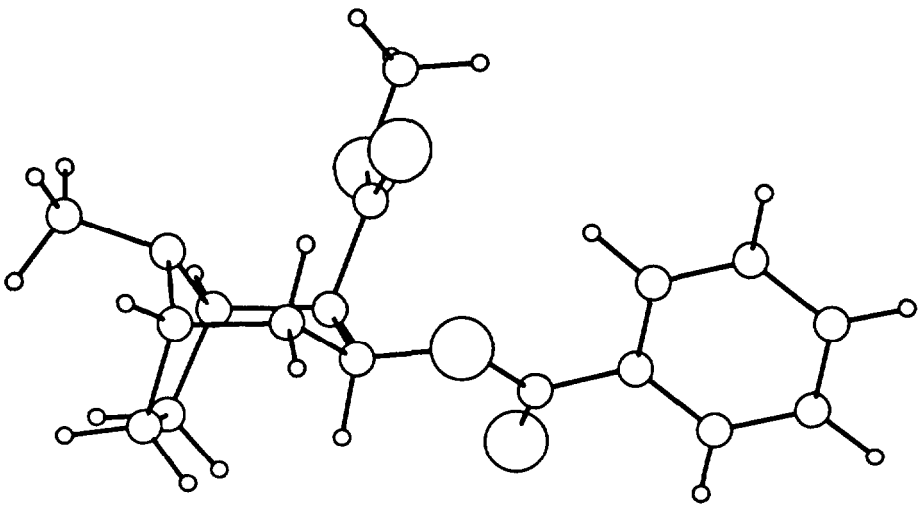


FIGURE 1. *Lowest energy structure characterized for cocaine in its neutral form*

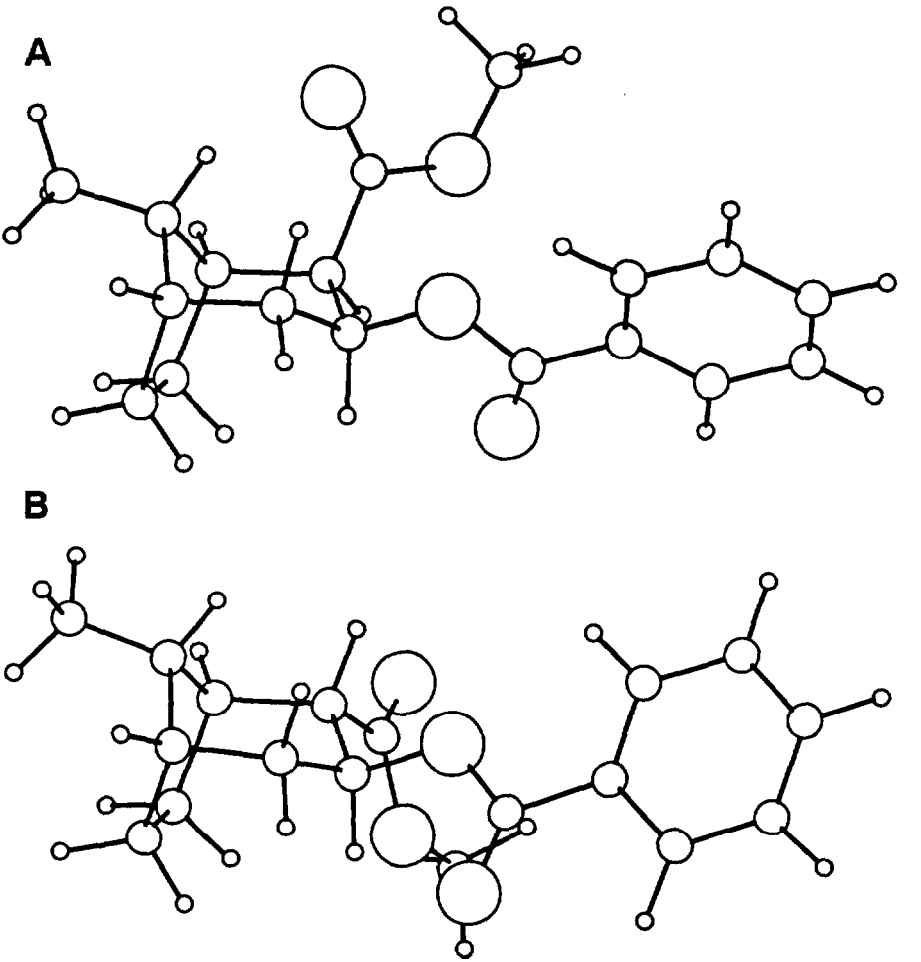


FIGURE 2. *Structure of (A) cocaine and (B) pseudococaine in their protonated forms. Note that cocaine has an internal hydrogen bond that stabilizes ifs protonated form. The hydrogen bond is not possible for pseudococaine because of its different stereoisomery.*

In addition to conformation, the state of protonation of the amine nitrogen in cocaine in its bioactive form was not completely resolved. To resolve this question, cocaine was compared with pseudococaine. These two compounds differ significantly in their affinity for the site labeled by [^{3}H] mazindol on the dopamine transporter, The inhibition constant in monkey striatum for /-cocaine is 0.64 μM; for *d*-pseudococaine it is 116 μM (Ritz et al. 1987). However, the

only structural difference between the two compounds is in the chirality of carbon 2, as shown in figure 3.

The authors have computed the heat of protonation of both analogs using the AM1 semiempirical method. For cocaine, the heat of protonation in vacuo is 139.4 kcal/mol, whereas for pseudococaine it is 148.3 kcal/mol, a much lower proton affinity. The affinity for the receptor then varies directly with the proton-accepting capabilities of the free bases. Therefore, because the rest of the structure remains unaltered, the ligands should be acting as proton acceptors (i.e., in their unprotonated forms). The difference in heat of protonation can be easily understood at the qualitative level. Cocaine can form an internal hydrogen bond, between the protonated amine nitrogen and the carbonyl oxygen, as shown in figure 2, panel A, whereas pseudococaine cannot form such an internal hydrogen bond (figure 2, panel B) and, therefore, is less able to stabilize its protonated form.

There is additional evidence to support the conclusion that the unprotonated form of cocaine is responsible for its biological activity. The pKa of cocaine is 5.5, significantly lower than the physiological pH of 7.4. Hence, at physiological pH, the ratio of protonated to unprotonated cocaine is 1:100. Moreover, in pH dependence studies (e.g., Calligaro and Eldefrawi 1987), cocaine has been shown to lose affinity for the site at pHs lower than 6.0.

Hence, theoretical and experimental evidence strongly suggests that the bioactive form of cocaine is its free base in a chair conformation with the N-methyl substituent in an equatorial orientation.

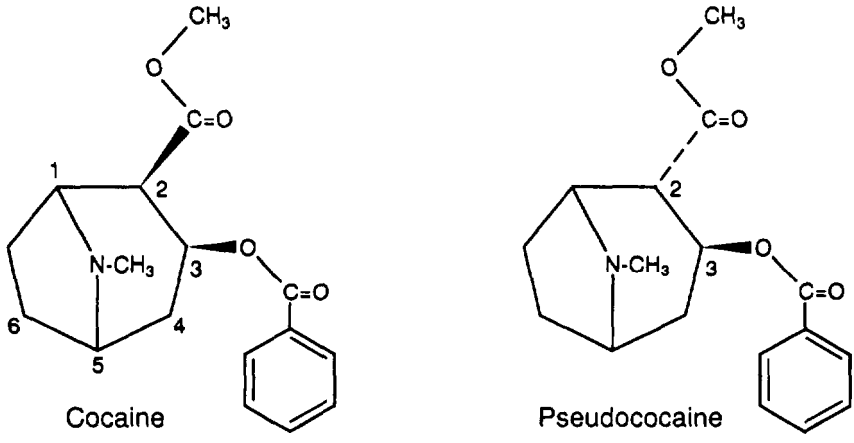


FIGURE 3. *Structure of cocaine and its diastereoisomer pseudococaine*

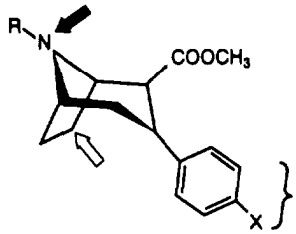
PHARMACOPHORE FOR THE DOPAMINE TRANSPORTER

Simple inspection of the ligands that displace [^{3}H] mazindol in striatum membranes reveals that all these compounds have three substructural elements in common (Villar and Loew 1990). Examples of those commonalities can be seen in figure 4.

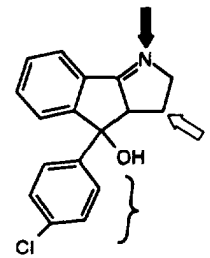
First, there is the existence of an amine nitrogen, similar to the one in cocaine. Second, there is a lipophilic region, an aliphatic lipophilic environment in which the amine nitrogen is embedded. Third, there is at least one aromatic ring. These are the three elements of an elementary pharmacophore for the site.

As discussed, the first element required for recognition, the amine nitrogen, interacts with a proton-donating residue in the binding cavity. The second element appears to be required for interaction with a lipophilic pocket, because it is a single aliphatic chain that embeds the amine nitrogen. The role of the third element, the aromatic rings, is more complex, and there are at least three possibilities: It could be interacting with a second lipophilic pocket; it could form a charge transfer complex with the transporter; or it could form a π-π stacked complex.

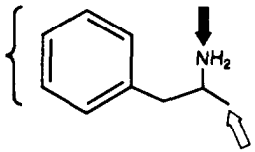
To study the mechanistic function of the aromatic moiety, the authors have evaluated molecular properties that relate to each of its three plausible roles using the quantum mechanical semiempirical program AM1. To this end, we chose a series of four closely related analogs of one of the ligands with the highest affinity for the site, 2ß-carbomethoxy-3ß+(4-fluorophenyl)-tropane (CFT) (WIN 35,428), shown in figure 5. The four compounds differ only in the nature of the substituent in position 4 of the aromatic ring. Hence, the differences in in vitro affinities can be directly related to changes in this ring. The three properties computed for the four ligands were the logarithm of the octanol water partition coefficient (logP), the nature and energy of the frontier orbitals and the product of the average molecular polarizability times the ionization potential. The logP, computed using a technique recently developed in our laboratory (Kantola et al. 1991), should indicate whether changes in the hydrophobic nature of the substituent affect the affinity of the ligand. The energies of the frontier orbitals relate to the ability of the ligand to form a charge transfer complex with the receptor. The energy of the lowest empty molecular orbital is a measure of the electron affinity, or electron-accepting ability, of the compound, whereas the energy associated with the highest occupied molecular orbital is a measure of its ionization potential, or electron-donating ability. Finally, the product of the polarizability times the ionization potential derives from the London dispersion term and is related to the ability of the ligand to form a π-π stacking complex. The calculated properties as well as the affinity data (Boja et al. 1990), using [^{3}H] CFT and rat striatum membranes, are shown in table 1.



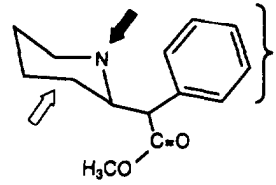
Winthrop Compounds



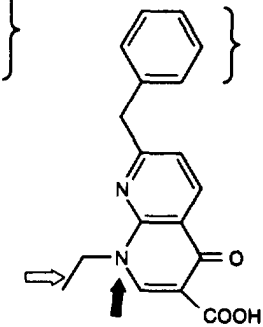
Mazindol



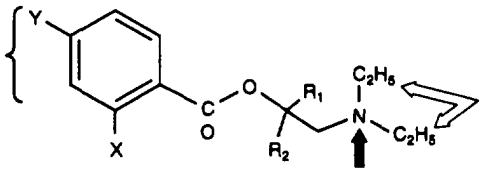
Amphetamine



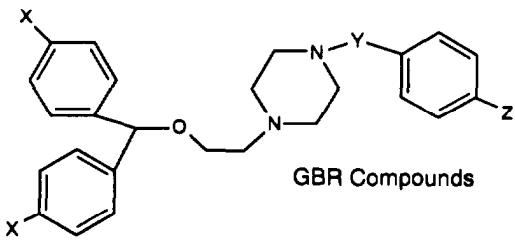
Methylphenidate



Amphonelic Acid

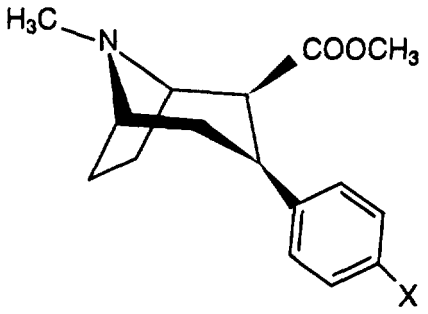


Dimethocaine	X=H; Y=NH_2; R_1=R_2=CH_3
Procaine	X=H; Y=NH_2; R_1=R_2=H
Chloroprocaine	X=Cl; Y=NH_2; R_1=R_2=H



GBR Compounds

FIGURE 4. *Structure of compounds reported to interact with the dopamine transporter. The brackets indicate the aromatic region, the solid arrow indicates a lipophilic center, and the empty arrow shows a proton-accepting atom.*



Compound	X
CFT or WIN 35,428	F
RTI-31	CH_3
RTI-32	Cl
WIN 35,065-2	H

FIGURE 5. *Analogs of CFT used in the* analysis *of the mechanistic role of the aromatic ring in recognition*

Comparing the affinities with the four properties calculated, no relationship is observed between the variation in electron-donating or electron-accepting ability of the ligands and the variation in affinities. However, the other two computed properties do appear to vary with the affinity. A decrease in the computed logP for the ligand, as a consequence of the different substitutions on the aromatic ring, parallels a decrease in the affinity. A similar observation can be made for the decrease in the ability of the ligand to form a π-π stacking complex. Thus, both lipophilicity and the ability of the aromatic ring to form a π-π stacking complex are potential determinants of recognition. Further studies are necessary, using a larger congeneric series that differs only in the nature of the substituents on the aromatic ring, to continue to probe the relative importance of these two properties. For such a study, additional in vitro pharmacological data, determined under similar experimental conditions and not currently available, will be necessary.

TABLE 1. *Properties computed for the compounds shown in figure 5*

Compound	LogP	IP(eV)	EA(V)	αIIP(Å^3.eV)	IC_{50} (mM)*
RTI-31	2.50	9.31	0.15	206.7	1.2
RTI-32	2.37	9.10	0.48	204.8	1.7
CFT	2.09	9.29	0.16	197.9	15.7
WIN 35,065-2	2.07	9.38	0.51	196.0	23.0

KEYS: Octanol water partition coefficient (logP); energy of the highest occupied molecular orbital (IP=ionization potential); energy of the lowest unoccupied molecular orbital (EA=electron affinity): polarizability times the ionization potential (αIlp); and IC_{50} from the literature data

* Data from Boja et al. 1990

DESIGN OF NOVEL FAMILIES OF DOPAMIN TRANSPORTER LIGANDS

As mentioned in the introduction, the identification of novel ligands for the dopamine transporter is essential for continued characterization of the pharmacological endpoints that can be elicited by modification of the normal functioning of this protein, Chief among this identification process is the possibility of finding compounds that may affect cocaine binding to the transporter without altering the uptake process itself. Compounds with these characteristics have not yet been identified but are worth searching for because the benefits of such a substance would be invaluable for efficient pharmacotherapy of the cocaine abstinence syndrome.

The requirements for recognition developed at different stages of investigation, using the tools of computational chemistry, can be useful not only in developing mechanisms of ligand-binding site recognition but also for searching chemical databases for novel compounds that satisfy these criteria. Searches using a chemical database can be done at two different levels. The first involves the use of 2-D, topological connectivity criteria to retrieve all compounds with the stipulated characteristics. The second, a more desirable and stringent level, is a search using 3-D structural information stored in the database. Only those structures that fulfill the 3-D arrangements of structural elements proposed in the pharmacophore are retrieved. Because this search does not depend on connectivity, the nature of the compounds that can be retrieved is more diverse than those found using the 2-D searching.

Because of the preliminary nature of our pharmacophore, we have not yet developed a full understanding of the 3-D relationship among its elements. Therefore, it is not possible to carry out a search of a 3-D database. However, 2-D searches can provide some useful initial ideas for the design of novel probes. The purpose of such searches is to identify novel skeletons that might display some affinity for the dopamine transporter. These basic structures can then be used to develop structure-activity relationships (SARs) that may lead to the discovery of a new family of ligands.

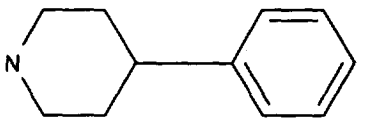
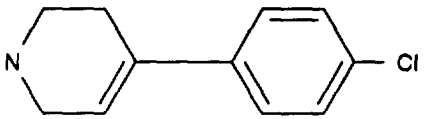
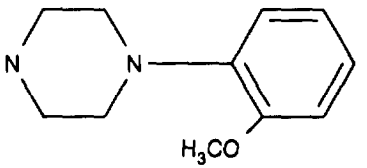
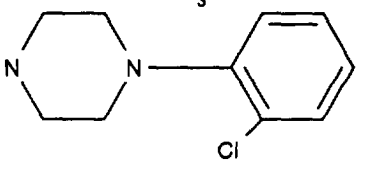
As a basis for the two-dimensional search, we used the presence of a secondary or tertiary amine, bound to aliphatic carbon atoms and separated by three atoms from an aromatic ring. A search in the Cambridge Crystallographic Database (Allen et al. 1963) permitted the characterization of more than 150 compounds, some of them closely related to 1 -methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the neurotoxin known to interact with the dopamine transporter. From the set of compounds retrieved, we selected four compounds for evaluation of their affinity for the dopamine transporter. Three of them are novel and one, a 4-phenylpiperidine, with known affinity for the dopamine transporter, was included as a control. The three new compounds belong to two chemical families: tetrahydropyridines and piperazines. They were tested for their ability to displace

[^{3}H] CFT at 0 °C from rat striatum membranes. The results shown in table 2 indicate that all the compounds have affinity in the low μM range similar to cocaine and therefore can be regarded as appropriate starting points for a more extensive SAR study.

CONCLUSIONS

The authors have developed a preliminary model for recognition of ligands by the dopamine transporter. Three elements are essential for recognition: a proton acceptor; a lipophilic region in which the proton acceptor is embedded; and, finally, an aromatic region that could function as another lipophilic region or in π-π stacking with aromatic residues at the binding site. More complete characterization of the molecular determinants of recognition of cocaine congeners and other families of ligands by the dopamine transporter requires

TABLE 2. *Inhibition constants (IC_{50}) for four compounds retrieved from the search in the chemical database using the elementary pharmacophore for the dopamine transporter*

Compound	IC_{50} (μM)
	1.9
	1.6
	20.9
	2.0

the generation of an augmented database of ligands that have been pharmacologically evaluated under the same experimental conditions.

However, using the elementary pharmacophore developed so far, we have illustrated the power of chemical databases in compound design, even in the initial stages of pharmacophore development. The results of a 2-D search permitted the identification of two new chemical families that could potentially be ligands for the site: tetrahydropyridines and piperazines. These analogs now can form the basis of an extensive SAR study, which should permit the generation of experimental data and the corresponding computations necessary to continue the development of a 3-D pharmacophore for the site.

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The Pharmacophore for Opioid Activity

Mark Froimowitz

INTRODUCTION

Although the phenyl ring in prototypical morphine is constrained to be axial on the piperidine ring, it is clear that phenyl-equatorial compounds are also capable of opioid activity. A good example of the efficacy of both classes of opioids are α- and β-azabicyclanes in which the piperidine and cyclohexane rings are interchanged to produce compounds, with the phenyl ring constrained to be either axial or equatorial (figure 1, panels A and B) (Froimowitz et al. 1984). Despite this major structural difference, the potency of both classes of opioids is enhanced by the addition of a phenyl *meta* hydroxyl (Froimowitz et al. 1984). To deal with these data, an opioid ligand model has been proposed that is consistent with the general structure-activity relationships (SARs) of opioids (Tecle and Hite 1976; Fries and Portoghese 1976; Froimowitz et al. 1984). To account for the potency-enhancing effects of a phenyl *meta* hydroxyl in both phenyl-axial and phenyl-equatorial opioids, the model suggests that the phenyl ring of both classes binds to the same region of the receptor. Superimposing the phenyl rings of opioids such as α- and β-azabicyclane (figure 1, panel C), one observes that, although the ammonium nitrogens are placed quite differently, the ammonium hydrogens are oriented toward the same point and could, therefore, interact with the same negatively charged region of the receptor.

The opioid ligand model is also consistent with general SARs concerning the N-alkyl group. An N-allyl or related group consistently produces opioid antagonists in phenyl-axial opioids (Archer and Harris 1965) but never in phenyl-equatorial opioids such as the phenylmorphans, meperidine, or the prodines (Ong et al. 1974; Casy et al. 1968). Similarly, an N-phenethyl group greatly enhances and is optimal for the potency of phenyl-axial opioids, but this is less true for phenyl-equatorial compounds (Portoghese 1978; Froimowitz and Matthysse 1986). This can easily be understood with the ligand model because the N-alkyl group is positioned differently in phenyl-axial and phenyl-equatorial opioids (figure 1) and, therefore, would interact with different portions of the receptor.

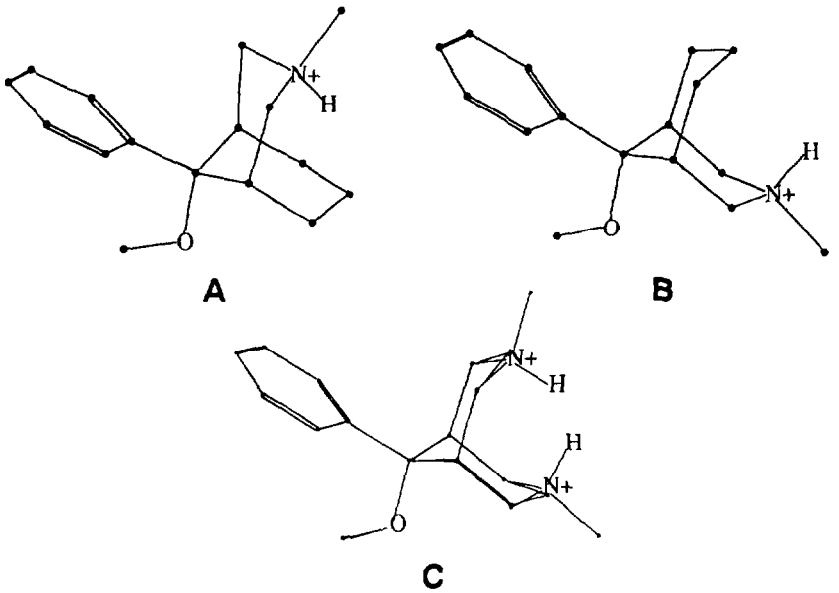

FIGURE 1. *Azabicyclane opioids in which the phenyl ring is constrained to be (A) axial (α-azabicyclanes) or (B) equatorial (β-azabicyclanes) on the piperidine ring, A superimposition of the phenyl rings for the two compounds is shown in panel C.*

Given the opioid ligand model, a more important structural factor may be the orientation of the phenyl ring. Although the phenyl ring is conformationally constrained in morphine, this is not the case for most phenyl-equatorial opioids. It has been noted that 4-phenylpiperidines with a symmetrically substituted piperidine ring, such as ketobemidone, 3-demethylprodine, meperidine, and azabicyclanes, contain two mirror-image conformers that are equally preferred (figure 2) (Froimowitz 1982; Froimowitz and Kollman 1984; Froimowitz et al. 1984), and it is unclear which one is responsible for the opioid activity. Both mirror images have been observed in the crystal structures of the azabicyclanes, ketobemidone, and meperidine (Froimowitz et al. 1984; Froimowitz and Cody, submitted for publication). In 4-phenylpiperidines with an asymmetrically substituted piperidine ring such as the prodines and phenylmorphans, one conformer becomes preferred. In prodines with an axial 3-methyl group (or 5-methyl group), the energy difference between the two conformers is a substantial 3 to 4 kcal/mol, and the concentration of one conformer will be considerably higher than that of the other due to the Boltzmann factor (Froimowitz and Kollman 1984). Interestingly, in a series of resolved prodine analogs, the same conformer is consistently observed in the crystal structure

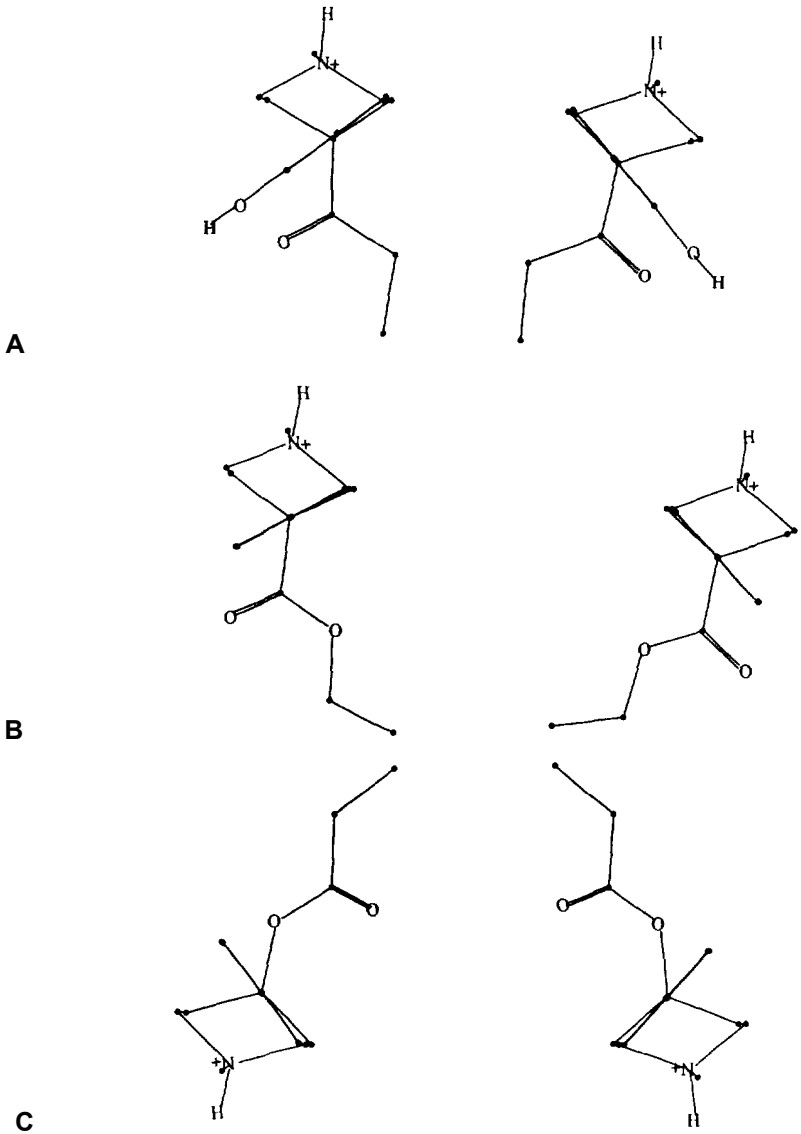

FIGURE 2. *The two mirror-image conformers that are preferred in (A) ketobemidone, (B) meperidine, and (C) 3-demethylprodine. The phenyl rings, which are shown edge on, eclipse either one or the other edge of the piperidine ring.*

of the more active enantiomer (Pottoghese 1978). However, the phenyl orientation of the preferred conformer of the more active prodine enantiomers is the opposite of that in morphine with the above ligand model (Portoghese 1978; Froimowitz and Kollman 1984).

A second series of 4-phenylpiperidine opioids with an asymmetrically substituted piperidine ring are the phenylmorphans. The (+)-enantiomer of phenylmorphan has three to five times the potency of morphine, and the compound is morphinelike in that it completely substitutes for morphine in dependent rhesus monkeys and rats (May and Takeda 1970; Ong et al. 1974; Awaya et al. 1984*a*). The (-)-enantiomer, with about the same potency as morphine for antinociception, appears to be an atypical opioid in that it does not substitute for morphine in dependent monkeys and rats but, conversely, will precipitate withdrawal symptoms in nonwithdrawn animals (May and Takeda 1970; Ong et al. 1974; Awaya et al. 1984*a*). Using the above ligand model, the phenyl orientation for the preferred conformer of (+)-phenylmorphan is a good match to that of morphine (Cochran 1974; Froimowitz 1984). Conversely, the preferred phenyl orientation of the atypical (-)-phenylmorphan is an essentially perfect fit to that of the more active prodine enantiomers. Interestingly, β-prodine also appears to be an atypical opioid agonist in that a phenyl *meta* hydroxyl, which generally enhances potency in opioids, converts the compound into a pure antagonist devoid of agonist properties (Zimmerman et al. 1978). In general, the addition of a *meta* hydroxyl to potent agonists in the prodine series abolishes or greatly weakens their activities (Portoghese et al. 1981; Casy and Ogungbamila 1985).

Thus, of the two mirror-image conformers in symmetrically substituted 4-phenylpiperidines, there appears to be a dichotomy between compounds in which the preferred conformer has a phenyl orientation that is morphinelike and those in which it has the mirror-image orientation. The morphinelike orientation appears to be associated with morphinelike activity in compounds such as morphine and (+)-phenylmorphan. Opioids with atypical properties or SARs such as (-)-phenylmorphan and the prodines appear to be associated with the nonmorphinelike phenyl orientation.

PHENYLMORPHANS

One question that must be addressed is whether phenyl-axial and phenyl-equatorial opioids do bind to the same receptors. Antinociceptive activity alone does not prove that a particular receptor is involved because opioids that are selective for u-,κ·-, and δ··receptors are all capable of producing antinociception and analgesia (Heyman et al. 1988; Millan 1990). Therefore, Froimowitz and colleagues (1992*a*) have examined the binding of the phenylmorphan enantiomers and some of their analogs to opioid receptor subtypes. It was of particular interest to determine whether the atypical

pharmacological profiles of some of the compounds, most notably the mixed agonist-antagonist (-)-phenylmorphan, were due to a change in affinity for receptor subtypes. However, the affinities of all the compounds, except one, were found to be greatest for μ-receptors, somewhat lower for κ-receptors, and low for δ-receptors. The exception was the (+)-9α-methyl analog, which suffered a large decrease in μ-receptor affinity, causing it to have a slightly higher affinity for κ-receptors. It was found that the in vivo antinociception potency difference in favor of (+)-phenylmorphan relative to (-)-phenylmorphan can be explained entirely by its proportionately increased affinity for μ-receptors.

In addition to determining the affinities of the enantiomers of phenylmorphan for opioid receptor subtypes, some in vivo pharmacological properties also were examined (Froimowitz et al. 1992*a*). Although the affinities of both enantiomers are clearly greatest for μ-receptors, it is possible that another opioid receptor subtype is responsible for the antinociceptive activity. To test this, mice were pretreated with the selective opioid antagonists naloxonazine (μ_1), β-FNA (μ_1 and μ_2), nor-BNI (κ), or naltrindole (δ) prior to testing in the tail-flick assay. Only the y-receptor antagonists were found to significantly attenuate the antinociceptive activity of (+)- and (-)-phenylmorphan. The antagonism by the μ_1-selective naloxonazine suggests that both compounds are μ, agonists, whereas intrathecal administration suggests that both are also μ_2 agonists (Pick et al. 1991). In contrast, nor-BNI and naltrindole did not significantly affect the antinociceptive activity at doses that attenuated the activity of the x-selective agonist U50488 and the δ-selective agonist DPDPE, respectively. Thus, both the morphinelike (+)-phenylmorphan and the atypical (-)-phenylmorphan appear to be producing their antinociceptive effects through μ-receptors.

As indicated above, the affinity of the (+)-9α-methyl analog is slightly higher for x-receptors than for μ-receptors. This is due to a large decrease in affinity for p-receptors relative to the two possible parent phenylmorphans. In terms of in vivo activity, the compound is devoid of agonist activity in three mouse assays of antinociception but is an opioid antagonist with a nalorphinelike potency (Awaya et al. 1984*a*). In contrast, the (-)-9α-methyl analog is a weak agonist relative to the parent compounds with atypical properties because the compound does not substitute for morphine in morphine-dependent animals (Awaya et al. 1984*a*). Because the pharmacological profiles of both 9α-methyl analogs are modified by the introduction of a single methyl group to the parent compounds, it was of interest to determine how this molecular change causes these dramatically different pharmacological profiles.

The first necessity was to determine the absolute configuration of the 9α-methyl analogs. This was accomplished through an x-ray crystallographic study of the chloride salt of the (+)-9α-methyl analog (Froimowitz et al. 1992*b*). The absolute configuration of this compound was found to be (1R,5S,9R); it therefore

corresponds to (-)-(1R,5S)-phenylmorphan. This implies that the (-)-9α-methyl analog corresponds to (+)-(1S,5R)-phenylmorphan. Thus, the addition of the 9α-methyl group to (+)-phenylmorphan converts the potent morphinelike agonist into a weaker agonist that no longer substitutes for morphine. The same change to the atypical agonist (-)-phenylmorphan converts it into a pure antagonist with no apparent agonist activity and is also associated with a tenfold drop in the μ-receptor affinity.

The phenylmorphans and their analogs provide a series of compounds with widely differing profiles of pharmacological activity (May and Takeda 1970; Ong et al. 1974; Awaya et al. 1984*a,* 1984*b*). The morphinelike opioids are (+)-phenylmorphan and the (±)-9α-hydroxy analog, which substitute for morphine in dependent animals. (-)-Phenylmorphan, although maintaining good in vivo antinociceptive activity and receptor-binding affinity, does not substitute for morphine and appears to have the antagonist property of precipitating withdrawal in morphine-dependent animals. The (-)-9α-methyl analog has even weaker agonist activity and also does not substitute for morphine. The (+9β-hydroxy analog is a weak agonist at best. Finally, the (+)-9α-methyl analog is devoid of agonist activity but does appear to be an antagonist with nalorphinelike potency.

As indicated above, the orientation of the phenyl ring relative to the piperidine ring may be an important factor for the pharmacological profile of an opioid. This has been examined in figure 3, which shows those regions of the conformation space between 0° and 180° that are within 1 kcal/mol of the global minimum for the various phenylmorphans. The relatively rigid phenyl-axial morphine (84°) (Gylbert 1973) and phenyl-equatorial racemic benzofuro [2,3-c]-pyridin-6-ols(92°) (Hutchinson et al. 1989) are included for reference purposes (Froimowitz et al. 1992*a*). The two compounds with the most morphinelike profiles—(+)-phenylmorphan and the (±)-9α-hydroxy analog—appear to most closely correspond to the rigid analogs, whereas the other compounds deviate. The only possible exception is the (-)-9α-methyl analog. However, for this compound the conformational energy of the region past 100" rises rapidly (Froimowitz et al. 1992*a,* 1992*b*). Interestingly, the receptor affinity of this compound is essentially the same as for (-)-phenylmorphan, although the former appears to be considerably weaker in in vivo assays of antinociception. The compounds that have the most difficulty in attaining this region of conformation space due to unfavorable energies are the (+9β-hydroxy and (+)-9α-methyl analogs. Additional compounds will allow further exploration of this aspect of molecular structure.

U50488

The opioid U50488 (figure 4) is the prototype for selective k-receptor agonists (Lahti et al. 1982; Piercey et al. 1982; Szmuszkovicz and VonVoigtlander 1982; VonVoigtlander et al. 1983). Unlike previously described opioids that interact

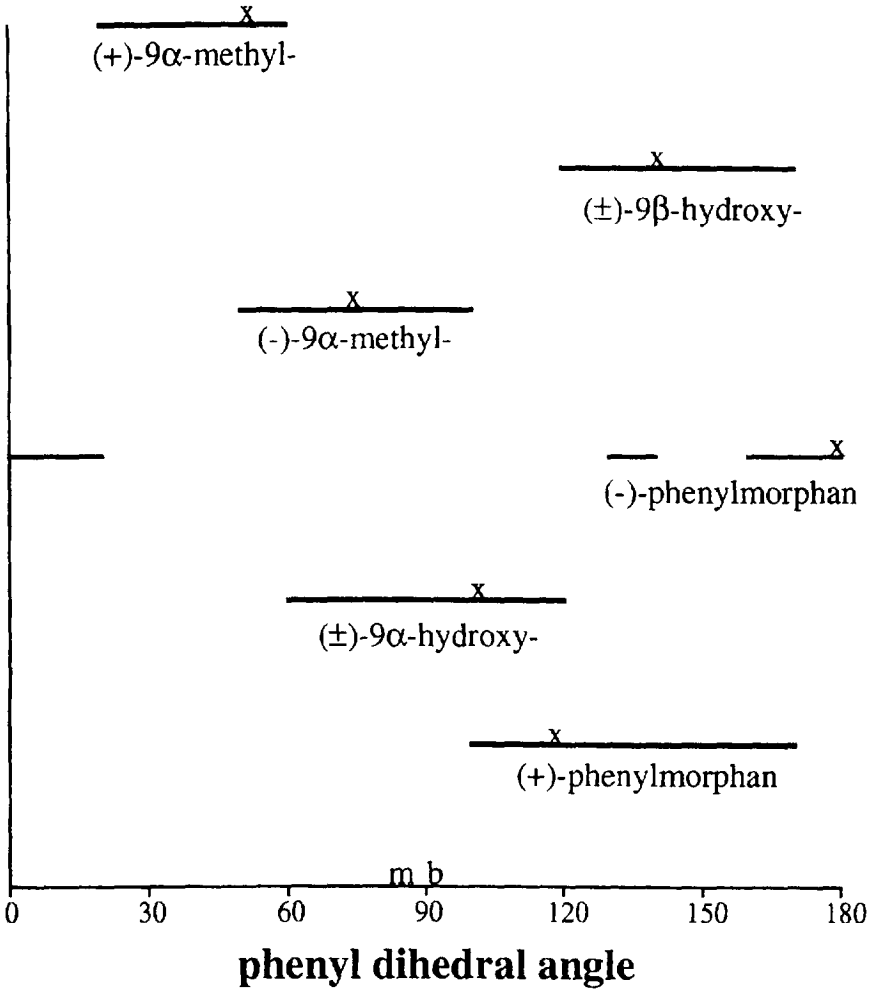

FIGURE 3. *The regions of the phenyl conformation space that are within 1 kcal/mol of the global minima for the various phenylmorphans. The global minima are indicated by the "x's." The equivalent value for the constrained phenyl-axial morphine and the phenyl-equatorial benzofuro[2,3-c]-pyridin-6-ols are indicated by the "m" and "b, " respectively.*

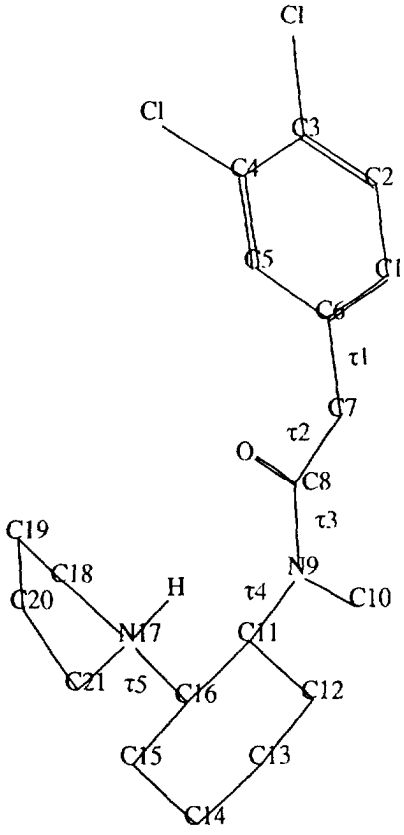

FIGURE 4. *The energy-minimized trans (with respect to τ2) conformer of U50488 that is most similar to the three x-ray crystal structures*

with κ-receptors, U50488 has little affinity for μ-receptors, and there is little cross tolerance between it and μ-agonists such as morphine. It is important to emphasize that the selectivity of U50488 for κ-receptors is primarily due to its loss of affinity for μ-receptors rather than enhanced affinity for κ-receptors. The conformational properties of U50488 have been studied using the molecular mechanics computer program MM2-87 for the isolated molecule and high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy for aqueous solutions of the compound (Froimowitz et al. 1992*c*). In addition, two crystal structures of U50488 with different counterions and the crystal structure of a closely related analog have been examined as well (Clark et al. 1988; Doi et al. 1990; Costello et al. 1991).

There are five dihedral angles that are important for the three-dimensional structure of U50488 (figure 4). These are τ1(C8-C7-C8-C5), τ2(N9-C8-C7-C6), τ3(C11 -N9-C8-C7), τ4(C12-C11 -N9-C8), and τ5(C18-N17-C16-C15). Preliminary MM2-87 calculations and the conformational preferences of simpler molecules were used to determine that there are two energy minima for τ1 (±90°), three for τ2 (two gauche and one *trans*), two for τ3 (0° and 180°), two for τ4 (110° and -70°), and three for τ5 (two *gauche* and one *trans*). Permuting the different combinations, there are 72 distinct conformational possibilities. All these were generated and energy minimized with the MM2-87 program. Because there is little energy difference between conformers that differ only with respect to τ1, these can be thought of as 36 pairs of minima. One of the pair of global minima-aside from a pair of artifactual conformers (Froimowitz et al. 1992*c*)– is shown in figure 5. This conformer was found to be especially stabilized by attractive van der Waals' interactions due to its compact structure.

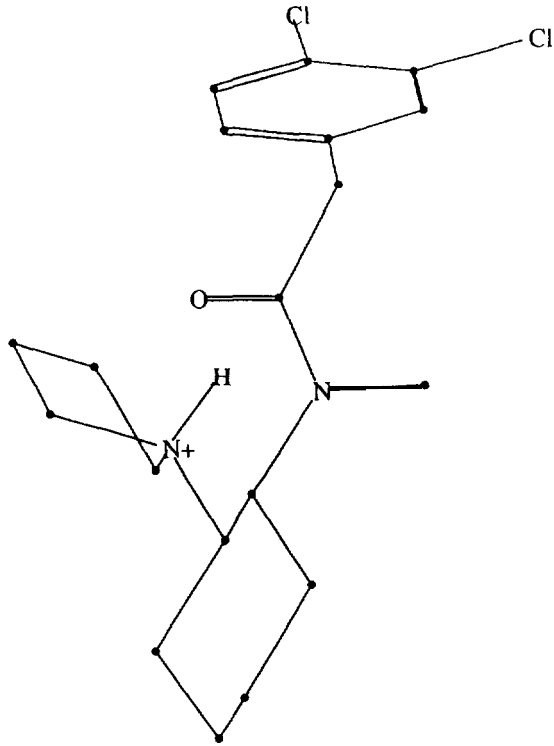

FIGURE 5. *The energy-minimized gauche (with respect to 52) conformer that appears to be the biologically active form for U50488. This conformer is especially stabilized by attractive van der Waals' interactions due to its compact structure.*

The NMR studies of U50488 in aqueous solution included one- and two-dimensional high-resolution ^1H NMR techniques with the interpretation of ^1H-^1H vicinal coupling constants, one- and two-dimensional nuclear Overhauser effect (NOE) experiments, and two-dimensional correlated spectroscopy experiments. Especially important were the NOE data because they provided important information regarding the conformation of the compound in solution. In particular, conformationally important NOEs were observed between the N-methyl protons and *both ortho* phenyl protons, the methylene protons H7, and the cyclohexyl proton H16 (see below).

There was generally good agreement between the MM2-87 calculations, the NMR studies, and the three crystal structures, although there were some notable differences as well. For τ_1, the MM2-87 calculations suggested that there was little energy difference (tenths of a kcal/mol) associated with flipping the phenyl ring over. This is consistent with the NMR data, which showed NOEs between the N-methyl protons and *both ortho* phenyl protons, which indicated that both conformers are populated in solution, However, all three crystal structures had only one orientation of the phenyl ring. With respect to τ_2, the MM2-87 calculations found that $\tau_2 \sim -60°$ was required to produce the compact and preferred structures that are stabilized by the attractive van der Waals' interactions. This is consistent with the NMR data because NOEs between the N-methyl protons and the phenyl protons are possible only in a gauche conformer. In contrast, all three crystal structures had a *trans* value for τ_2. For τ_3, the MM2-87 calculations suggested a slight consistent preference for the conformer in which the carbonyl group is *trans* to the N-methyl group. This is consistent with both the NMR data, which showed NOEs between the N-methyl protons and the methylene protons H7, and the three crystal structures, which all had a similar conformation. With respect to τ_4, the MM2-87 calculations consistently preferred a value of τ_4 4-110". This is consistent with both the NMR data, which showed NOEs between the N-methyl protons and the cyclohexyl proton H16, and the three crystal structures, which all had values of $\tau_4 \sim 110°$. For τ_5, the MM2-87 calculations suggest a small consistent preference for $\tau_5 \sim -60°$. All three crystal structures had values in this vicinity, whereas the NMR data did not provide any information regarding this dihedral angle.

Thus, there is good agreement between the MM2-87 calculations and either one or both experimental methods for each of the five important dihedral angles. With respect to τ_3 and τ_4, all three methods agree closely. For τ_1 and τ_2, there is good agreement between the MM2-87 calculations and the NMR data, which differ in reasonable ways from the three crystal structures. With respect to τ_5, there is good agreement with the three crystal structures, whereas the NMR data do not provide any information.

After the completion of the conformational analysis of U50488, the author became aware of some recent work on novel analogs of U50488 in which the amide bond

has been incorporated into a lactam ring resulting in two diastereomers with additional conformational rigidity (Cheng et al. 1990). The diastereomer that maintains good affinity and selectivity for x-receptors is shown in figure 6. The major conformational effect of the lactam ring is to fix τ3 -180° in agreement with the results outlined above. For the more potent and κ-selective diastereomer, the lactam ring also limits τ2 to negative values, whereas that for the less active diastereomer is limited to positive values. Thus, for the *two gauche* conformers of U50488 with respect to τ2, the one proposed for the biologically active form is the only one that is consistent with the more active and selective lactam-constrained diastereomer.

The conformational analysis of U50488 and the conformationally restricted lactam analog suggest that the energetically preferred conformer will be similar to that shown in figures 5 and 6. There are several rigid multicyclic κ-agonists available,

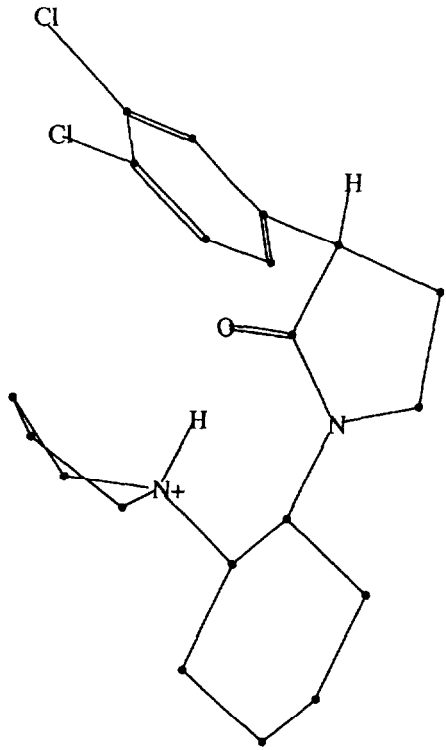

FIGURE 6. *The lactam-constrained analog of U50488 that retains good κ-receptor affinity and selectivity. The diastereomer with the phenyl ring and hydrogen atom interchanged is much less active and selective.*

although these are relatively nonselective. This includes ketazocine (figure 7), with which U50488 shares a carbonyl group. One would like to determine the common molecular features of κ-agonists and the aspect of the molecular structure of U50488 that makes it considerably more selective than compounds such as ketazocine.

Comparing the U50488 conformer in figure 5 with the crystal structure of ketazocine (Verlinde and De Ranter 1983), the overall geometries appear to be similar (Froimowitz et al. 1992*c*). The distance between the center of the phenyl ring and the ammonium nitrogen is 4.5 Å for both the U50488 conformer and ketazocine. Superimposing the two, it is possible to approximately align the phenyl ring, the carbonyl oxygen, and the ammonium nitrogen. Initially, however, it appeared that the orientation of the ammonium hydrogen in the two structures varied considerably. To resolve this, the pyrrolidine ring in U50488 was rotated to better match the orientation of the ammonium hydrogen of ketazocine, although this increased the conformational energy by 2.4 kcal/mol. However, it now appears that this is not necessary

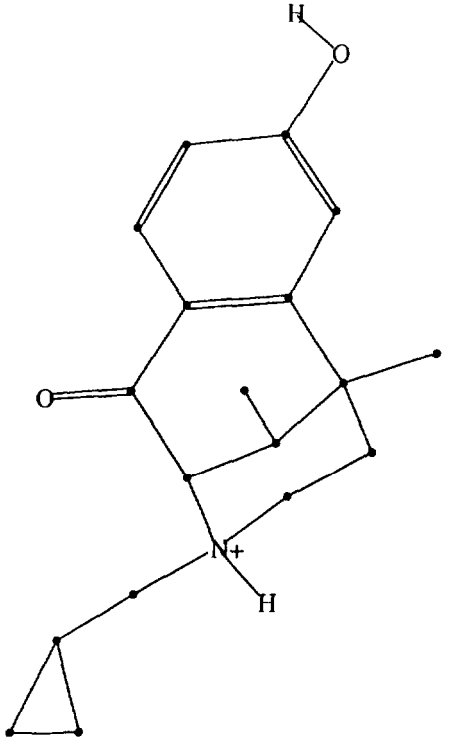

FIGURE 7. *The x-ray crystal structure of the κ--agonist ketazocine*

because the structures of phenyl-equatorial opioids such as the (+)-9α-methyl analog of phenylmorphan (figure 8) are a better match to the U50488 conformer without the rotation of the pyrrolidine ring. Thus, the proposed biologically active form of U50488 appears to be a better match to phenyl-equatorial opioids than to the phenyl-axial ketazocine.

Superimposing the U50488 conformer in figure 5 with ketazocine suggests that the κ-selectivity of the former is due to the presence of the cyclohexane ring, which occupies space that is not occupied in nonselective κ-agonists such as ketazocine. Thus, this added bulk appears to sterically hinder attachment to μ-receptors, whereas the κ-receptor appears to tolerate bulk in this part of the molecule. Additional κ-selective analogs of U50488 have been synthesized with considerable steric bulk, including naphthalene rings, in this portion of the molecule, and this does not adversely affect the affinity for κ-receptors (Lahti et al. 1985; Halfpenny et al. 1989, 1990, 1991; Freeman et al. 1991).

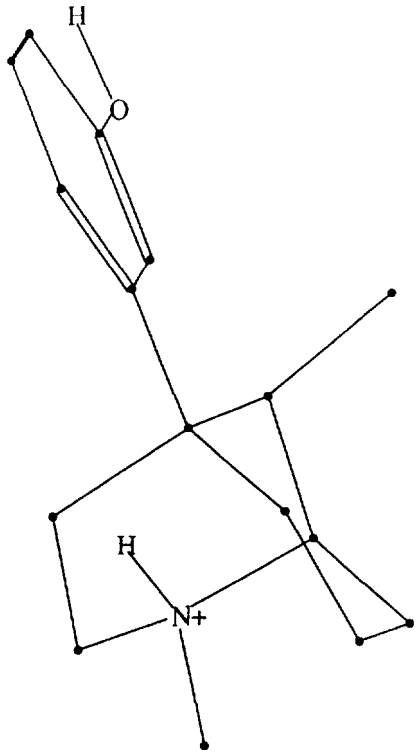

FIGURE 8. *The (+)-9α-methyl analog of phenylmorphan, which is a pure opioid antagonist*

CONCLUSIONS

A ligand model has been developed that is consistent with the diverse molecular structures that elicit opioid activity. Constrained phenyl-equatorial opioids like the phenylmorphans have been shown to produce their pharmacological effects through the same μ-receptors as more traditional phenyl-axial compounds such as morphine, and the opioid ligand model is consistent with these structures. Additional evidence has been presented regarding the importance of the phenyl orientation for morphinelike and atypical opioid activity. Currently, 4-phenylpiperidines are being synthesized, which will further explore the role of the phenyl ring orientation on opioid activity. Finally, a conformational analysis of the prototypical selective κ-agonist U50488 and its superimposition with less selective κ-agonists suggests that its cyclohexane ring is responsible for sterically interfering with its attachment to μ-receptors. This should make it possible to design additional κ-selective opioids through the introduction of steric bulk in the appropriate regions of opioid molecules.

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The Molecular Basis of Opioid Potency and Selectivity: Morphiceptins, Dermorphins, Deltorphins, and Enkephalins

Murray Goodman, Seonggu Ro, George Ösapay, Toshimasa Yamazaki, and Alexander Polinsky

INTRODUCTION

Naturally occurring peptide opioids have been isolated from the central nervous system (Pasternak et al. 1975; Numa 1984), frog skin (Montecucchi et al. 1981; Mor et al. 1989), and the enzymatic digestions of proteins (Chang et al. 1981). All natural peptide opioids contain an amino terminal tyrosine, a phenylalanine, and at least one spacer residue between them. Systematic modifications of these residues have shown that the free amine and phenolic groups of tyrosine at the first position and the aryl ring of the third or fourth residue represent the pharmacophoric array (Hruby and Gehrig 1989; Morley 1980). It is well known that each opioid peptide can bind to more than one receptor (μ, δ, κ . . .) and their subtypes. Such poor selectivities create difficulties in clinical applications and physiological studies of these molecules. Therefore, it is essential to design and synthesize potent opioid ligands that bind to only one receptor.

As with other peptide hormones, opioids assume specific three-dimensional structures when they interact with receptors. The flexibility of natural opioids allows them to adopt many different conformations that may account for their ability to bind to different receptors. To reduce such flexibility and to allow specific analogs to interact favorably with only one receptor, various constrained opioids have been devised and their conformations studied.

The authors' laboratories have employed an integrated approach that includes design, synthesis, bioassays and conformational analysis using nuclear magnetic resonance (NMR) spectroscopy and computer simulations. Many of the analogs synthesized in our laboratories have shown high activity, selectivity, and metabolic stability. In addition, the conformational studies of these analogs have suggested that the arrangements of the tyrosine and phenylalanine aromatic groups are important to determine the potency and

selectivity of the opioids. The authors recently proposed topochemical models to explain the bioactivities of morphiceptins, dermorphins, deltorphins, and a new class of cyclic opioids based on lanthionine. This work extends their prior studies on the structure-bioactivity relationships of enkephalin and related molecules.

STRUCTURAL CHARACTERISTICS OF MORPHICEPTIN AND DERMORPHIN

The biologically important Tyr^1 and Phe^3 are joined by a single amino acid in the highly μ-receptor selective morphiceptin (Tyr^1-Pro^2-Phe^3-Pro^4-NH_2) (Chang et al. 1981) and dermorphin (Tyr^1-D-Ala^2-Phe^3-Gly^4-Tyr^5-Pro^6-Ser^7-NH_2) (Montecucchi et al. 1981). As a result, the conformation and configuration of the second residue must play a significant role in orienting the pharmacophores. Interestingly, these two classes of opioids exhibit different structural features as residue. Morphiceptin requires an L-chirality for Pro^2, whereas dermorphin requires an amino acid with D-chirality. Dermorphin exhibits only the *trans* amide linkage between residues 1 and 2, whereas morphiceptin exhibits *cis* and *trans* isomers about the Tyr^1-Pro^2 amide bond in a ratio of 30:70 (Goodman and Mierke 1989). The *cis/trans* configuration about the Tyr^1-Pro^2 amide bond is particularly significant for the relative orientation of the biologically important Tyr^1 and Phe^3 residues.

In studies of morphiceptin analogs containing 2-aminocyclopentanecarboxylic acid (2-Ac^5c) as the second residue in place of proline Tyr-2-AC^5c-Phe-X^4-NH_2 [X^4=Pro and (L and D)-Val], the amide bond between residues 1 and 2 adopts a completely *trans* structure (Mierke et al. 1990; Yamazaki et al. 1991*a*). Among the four stereoisomers, only the analogs containing (1S,2R)-2-AC^5c in position 2 show opioid bioactivity. Although the 2-Ac^5c analogs can adopt only a *trans* amide configuration about Tyr-2-Ac^5c, the bioactive analogs Tyr-(1S,2R)-2-Ac^5c-Phe-X^4-NH_2 are topochemically equivalent to morphiceptin with the Tyr-Pro amide bond in a *cis* configuration (figure 1, structure A). However, the inactive analogs Tyr-(1R,2S)-2-Ac^5c-Phe-X^4-NH_2 share similar preferred conformations with morphiceptin adopting a *trans* configuration at the Tyr-Pro amide bond (figure 1, structure B). We therefore proposed that a *cis* configuration about the Tyr^1-Pro^2 amide bond is required for bioactivity of the morphiceptin-related analogs containing a proline as the second residue (Yamazaki et al. 1991*a*) .

This suggestion is inconsistent with those proposed from the previous theoretical studies, in which the requirement of *trans* configuration of Tyr-Pro amide bond is implied for the bioactivity of morphiceptin (Momany and Chuman 1986; Lowe et al. 1986). Thus, systematic conformational studies concentrated on the second residue were undertaken to establish the configurational requirements of the Tyr^1-X^2 amide bond (where X^2 represents residue) of morphiceptin. Such studies can also provide insight into the “bioactive conformation” of dermorphin as well as morphiceptin because, as noted above, the array of pharmacophoric groups is determined primarily by the conformation of their second residues. Therefore, we

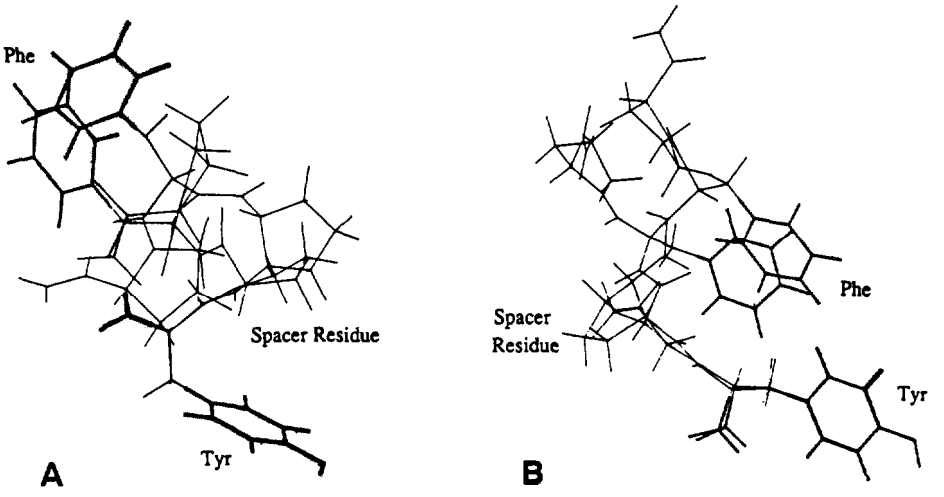

FIGURE 1. *Superposition of preferred conformations for (A) Tyr-cis-(1S,2R)-2-Ac^{5}c-Phe-Pro-NH$_2$ (active) and Tyr-Pro-Phe-Pro-NH$_2$ with cis Tyr-Pro amide bond and (B) Tyr-cis-2-Ac^{5}c-Phe-Pro-NH$_2$ (inactive) and Tyr-Pro-Phe-Pro-NH$_2$ with trans Tyr- Pro amide bond*

have designed and synthesized a series of analogs: Tyr^1-X^2-Phe^3-D-Pro^4-NH$_2$ (where X^2=Ala, D-Ala, Pro, D-Pro, L-[NMe]Ala, and D-[NMe]Ala) (Yamazaki et al. 1991 *b*). The in vitro bioactivities of these tetrapeptide analogs determined from the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are summarized in table 1.

Because these analogs differ only at the second residue, the differences in bioactivities can be explained in terms of the topochemistry resulting from the effects of changes at residue 2. To assess accessible $(\phi,,\psi)$ space for the second residues of the above-described analogs, energy calculations were carried out for six N-acetylamino acid-N'-methylamide derivatives (Ac-X^2-NHMe; X^2=Ala, D-Ala, [NMe]Ala, D-[NMe]Ala, Pro, or D-Pro). The derivatives Ac-X^2-NHMe display the greatest accessible space because they do not have neighboring residues that could restrict geometries for the X^2 residues in a peptide sequence (Zimmerman et al. 1977).

All the space accessible to the *trans* isomers of Ac-Pro-NHMe and Ac-(NMe)Ala-NHMe are also allowed for Ac-Ala-NHMe. The Tyr-Ala-Phe-D-Pro-NH$_2$ is inactive, whereas Tyr-Pro-Phe-D-Pro-NH, and Tyr-(NMe)Ala-Phe-D-Pro-NH$_2$ are active (table 1). Thus, the Tyr-X^2 amide bond of these two analogs cannot be *trans.* We therefore examined the conformational (ϕ,ψ) space accessible for the *cis* isomers of Ac-(NMe)Ala-NHMe and Ac-Pro-NHMe and

TABLE 1. *GPI and MVD assays of rerrapeprides related to morphiceptin and dermorphin*

Analog	GPI IC_{50}/nM	MVD IC_{50}/nM	MVD/GPI IC_{50}-ratio
Tyr-Ala-Phe-D-Pro-NH_2	28,100±7,500* 26,900±7,200†	Inactive* >100,000†	— —
Tyr-(NMe)Ala-Phe-D-Pro-NH_2	32.4±8.1* 30.6±6.1†	1,390± 1,430* 202±28†	42.9* 6.60†
Tyr-Pro-Phe-D-Pro-NH_2	28.7±2.4†	1,508±180†	52.5†
Tyr-D-Ala-Phe-D-Pro-NH_2‡	74±13	1,490±180	20.1
Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2	73.4± 15.9* 85.4±5.9†	2,046±391* 603±147†	27.9* 7.06†
Tyr-D-Pro-Phe-D-Pro-NH_2§	Inactive	Inactive	—

* Measured by the NIDA Opiate Compound Testing Program
† Measured by Dr. P.W. Schiller in the Clinical Research Institute of Montreal
‡ Measured by Matthies et al. 1984
§ Measured by Chang et al. 1981

noted that the two compounds share significant accessible space. Thus, we conclude that the L-chirality for the second residue and the *cis* configuration at the Tyr-X^2 amide bond are required for morphiceptin bioactivity (Yamazaki et al. 1991 *b*). In the 500 MHz ^{1}H-NMR studies, both Pro^2 and $(NMe)Ala^2$ analogs exhibit about 30 percent of *cis* amide structure for Tyr-X^2 bond, whereas Tyr-Ala-Phe-D-Pro-NH, is 100 percent *rrans* about the Tyr-Ala amide bond (Yamazaki et al. 1991 *b*).

The p-receptor selective Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2 also exhibits *cis/trans* isomerism at the Tyr-D-(NMe)Ala bond as does the inactive Tyr-D-Pro-Phe-D-Pro-NH, at the Tyr-D-Pro bond. The Tyr-D-Ala bond of dermorphin can adopt only a *trans* configuration. We therefore proposed that the bioactivity of D-$(NMe)Ala^2$ analog must be caused by the form of the tetrapeptide in which the Tyr-D-(NMe)Ala bond *is trans.* The opioid Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2 can be classified as a member of the dermophin family of opioids that requires both D-chirality and *trans* amide configuration at the second residue for activity at the μ-receptor. The (ϕ,,ψ}) conformation of the second residue required for dermorphin bioactivity could be estimated from accessible space for Ac-D-Ala-

NHMe and Ac-D-(NMe)Ala-NHMe but prohibited for Ac-D-Pro-NHMe (Yamazaki et al. 1991 *b*).

The ^{1}H-NMR and molecular modeling studies employing the above results provide the preferred topologies for Tyr-(NMe)Ala-Phe-D-Pro-NH_2 and Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2 and their torsional angles (figure 2 and table 2).[1] The Tyr-(NMe)Ala amide bond (figure 2, structure A) assumes a *cis* configuration, whereas the Tyr-D-(NMe)Ala amide bond (figure 2, structure B) adopts a *trans* configuration. As expected, most torsional angles, except for those that belong to the second residues, are extremely similar (table 2). Interestingly, in spite of the configurational and conformational differences of the second residue, the relative spatial arrangements of the pharmacophoric groups, that is, the amine and phenolic groups of Tyr[1] and the aromatic ring of Phe[3], are almost the same in both topologies. The chirality change of the (NMe)Ala[2] residue may be compensated by a 180° rotation about the amide bond between

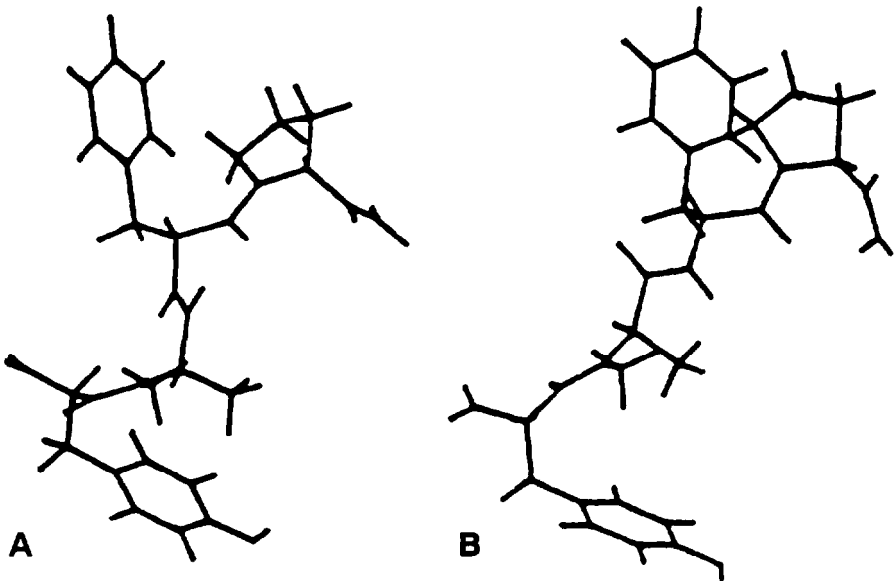

FIGURE 2. *The topochemical array for p-receptor bioactivity of (A) Tyr-(NMe)Ala-Phe-D-Pro-$NH_2$and(B)Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2. In structure A, the Tyr-(NMe)Ala amide bond assumes a cis configuration, whereas the Tyr-D-(NMe)Ala amide bond assumes a trans configuration in Stucture B.*

SOURCE: Yamazaki et al. 1991*b*. Copyright 1991 by Academic Press (Orlando, FL).

TABLE 2. *Conformations of Tyr[1]-X[2]-Phe[3]-D-Pro[4]-NH$_2$[X=(NMe)Ala, D-(NMe)Ala]*

	Tyr[1]			X[2]			Phe[3]			D-Pro[4]		
X[2]	ψ^1	χ^1	ω^1	ϕ^2	ψ^2	ω^2	ϕ^3	ψ^3	χ^3	ω^3	ϕ^4	ψ^4
(NMe)Ala	130	-159	14	-103	54	159	-105	93	-171	-177	76	-89
D-(NMe)Ala	129	-168	180	129	91	180	-144	130	-168	175	81	-82

residues 1 and 2. Because these analogs show high μ-receptor selectivity, these topologies are considered to be closely related to conformations necessary for the μ-receptor recognition.

CONFORMATIONAL STUDIES OF CYCLIC DERMORPHIN-DELTORPHIN ANALOGS

Dermorphin and deltorphins-deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH,), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2)—are isolated from the same source, and the possess common generalized N-terminal tripeptide sequences Tyr[1]-D-X[2]-Phe[3] (where D-X is either D-Ala or D-Met). Although dermorphin is p-receptor selective, deltorphins bind only to the δ-receptor (Erspamer et al. 1989). To elucidate the topochemical requirements for the bioactivities of these two opioid classes, conformationally constrained cyclic dermorphin-deltorphin analogs have been studied.

Most of the reported cyclic dermorphin-deltorphin analogs have been formed by side chain-to-side chain coupling with two different types of cyclization methods: amide bond formation and disulfide bridge formation (Schiller et al. 1987). A representative analog of the former is Tyr-c[D-Orn-Phe-Asp]-NH_2. The bioactivity of this compound is similar to those of corresponding linear analogs, but its selectivity for the p-receptor is one of the highest among the cyclic and linear dermorphin analogs. Among a series of disulfide-bridged analogs, Tyr-c[D-Cys-Phe-Cys]-NH_2 shows μ-selectivity with high activity at the μ-receptor and low activity at the δ-receptor. Introduction of a D-chirality at position 4 and a free C-terminal carboxylic group into this analog resulted in Tyr-c[D-Cys-Phe-D-Cys]-OH that is highly active at both μ- and δ-receptors and thus is nonselective.[2] Incorporation of penicillamine at the fourth position of this nonselective analog drastically decreases μ-receptor activity without significantly affecting δ·-receptor activity. The analog Tyr-c[D-Cys-Phe-D-Pen]-OH shows higher selectivity for the δ- over the μ-receptor (Mosberg et al. 1988). This result strongly suggests that the spatial arrays of the three pharmacophoric

groups within the N-terminal tripeptide sequence define the bioactivity and selectivity of dermorphin and deltorphin.

Calculations of a series of molecules based on the structure Tyr-c[D-Orn-Phe-Asp]-NH, indicated that a tilted stacking orientation of the two aromatic side chains at residues 1 and 3 is a structural requirement for high μ-receptor affinity (Wilkes and Schiller 1990). However, another theoretical study that employed various cyclic dermorphin-deltorphln analogs, including analogs from the Wilkes and Schiller study, suggested different topologies for μ-receptor recognition (Shenderovich et al. 1991). It should be noted that the bioactive conformation of Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2proposed in the previous section is not in agreement with any of the above topologies.

To avoid such ambiguities and to establish a conformational model for dermorphin bioactivity, we synthesized and evaluated analogs with a new cyclic structure formed by the cyclization between a side chain and the backbone.[3] Among them, the 12-membered cyclic analog Tyr-c[D-Orn-Phe-Gly], which contains the amino terminal tetrapeptide sequence of dermorphin, shows interesting features. Its bioactivity profile is similar to that of dermorphin (table 3). Although it is highly p-receptor selective, the analog shows bioactivity at the a-receptor unlike the Tyr-c[D-Orn-Phe-Asp]-NH_2 that is active only at the μ-receptor. The analog Tyr-c[D-Orn-Phe-Gly] is more constrained than Tyr-c[D-Orn-Phe-Asp]-NH_2 because the cyclization achieved by a side chain-backbone coupling produces a smaller ring (13 vs. 12 membered).

A conformational analysis was carried out employing NMR spectroscopy and computer simulations. Among the resulting preferred conformations of both analogs are conformations (figure 3, structures A, B, and C) that are topologically similar to that responsible for the μ-receptor activity of the Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2 (figure 2, structure B). These topologies contain a

TABLE 3. *Biological activities of cyclic dermorphin-deltorphin analogs*

Analog	GPI* IC_{50}[nM]	MVD* IC_{50}[nM]	MVD/GPI IC_{50}-ratio	In Vivot ED_{50}[μg]
Tyr-c[D-Orn-Phe-Gly]	8.8±0.8	145±15	18.9	0.1
Tyr-c[D-Orn-Phe-Asp]-NH_2	36.2±3.7	3,880±840	107	—
Tyr-D-Ala-Phe-Gly-NH_2‡	45.2±3.2	510±31.8	11.3	—
Morphine	58.8	844	11	5.8

*The GPI and MVD activities are measured in Dr. P.W. Schiller's laboratories.
† Values are measured in Dr. T. Yaksh's laboratories.
‡ Measured by Salvadori et al. 1982.

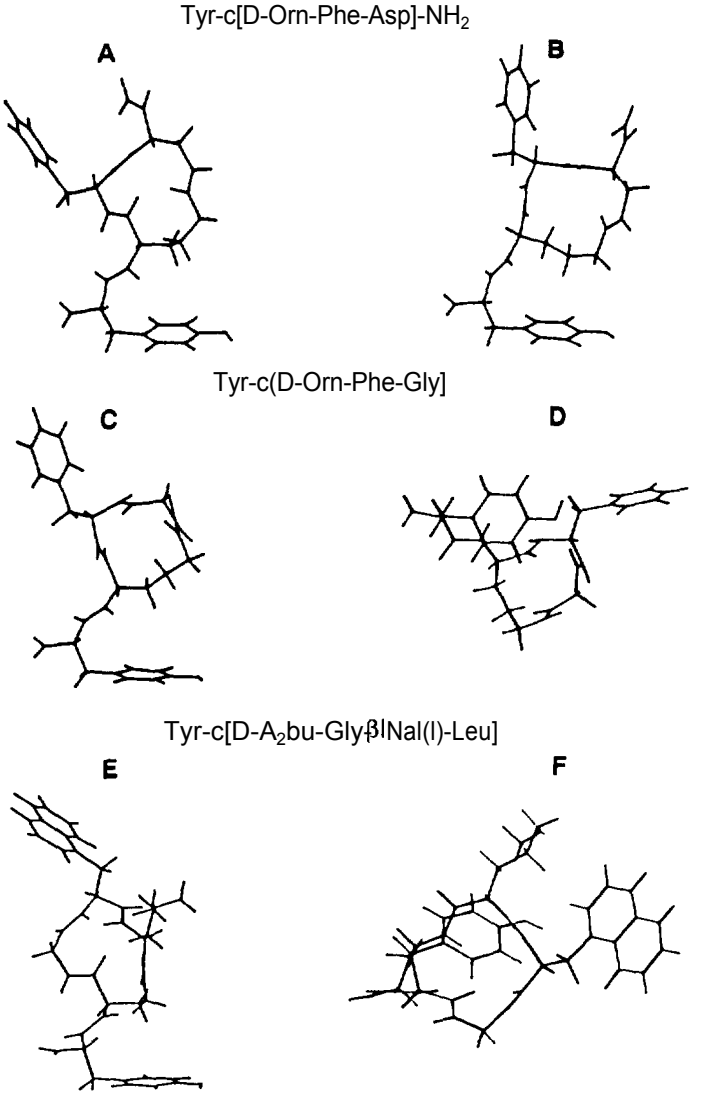

FIGURE 3. *Preferred conformations of (A and 8) and (C and D). Tyr-c[D-Orn-Phe-Asp]-NH_2 is biologically active at the μ-receptor. Tyr-c[D-Orn-Phe-Gly] is μ-receptor selective but also shows bioactivity at the δ·-receptor. Structures E and F represent bioactive conformations estimated for 14-membered cyclic enkephalin analogs containing β·-naphrhylalanine(1)as the fourth residue at the μ- and δ-receptors, respectively.*

common structure in which the aromatic side chains of the Tyr^1 and Phe^3 project away from each other. We therefore propose that dermorphin must adopt a topochemical structure with this topology for μ-receptor recognition. It is worthwhile to mention that μ-receptor active conformations of 14-membered cyclic enkephalin analogs (figure 3, structure E) (figure was estimated from a study described by Yamazaki and colleagues [1991*c*]) and morphiceptin (figure 2, structure A) also adopt similar topologies for the same receptor. Topochemical similarity of the bioactive conformations of enkephalin, morphiceptin, and dermorphin indicates that these three classes of opioids are likely to interact with the same μ-receptor subtype.

Among the preferred conformations of the Tyr-c[D-Orn-Phe-Gly] analog, a structure that places the two aromatic side chains in close proximity was observed (figure 3, structure D). This structure is topochemically similar to the conformation responsible for δ-receptor recognition of 14-membered cyclic enkephalin analogs (figure 3, structure F) (Yamazaki et al. 1991*c*). This type of molecular array was not observed among the preferred conformations of δ-receptor inactive Tyr-c[D-Orn-Phe-Asp]-NH_2. Therefore, the bioactivity of the 12-membered cyclic analog at the μ- and b-receptors can be explained by the presence of extended and folded forms among the preferred structures. Extended forms are defined as structures in which the phenol group of the tyrosine at position 1 and the aromatic group of phenylalanine at position 3 project away from each other. Folded forms are defined as structures in which the same aromatic groups are in close proximity to each other.

DESIGN, SYNTHESIS, AND CONFORMATIONAL STUDIES OF LANTHIONINE ENKEPHALIN

A new class of peptide opioid structures was designed and synthesized based on the incorporation of a lanthionine residue in the sequence. Lanthionine structures have been observed in naturally occurring “lantibiotics” such as nisin, subtilin, and epidermin (Shiba et al. 1986). In figure 4, the first lanthionine-bridged enkephalin analog, H-Tyr-c[D-$Ala_L$$Gly_s$-Phe-Ala,]-$NH_2$, is depicted where Ala_L denotes each of the lanthionine amino acid ends linked by a monosulfide bridge. The ring size in this new analog is reduced to 13 atoms as compared with 14 in the Tyr-c[D-Cys-Gly-Phe-Cys]-NH_2 analog (DCLCE) resulting in a more conformationally constrained molecule (Schiller et al. 1981).

For the preparation of lanthionine opioid analog, the method that most closely simulates the biosynthesis of lanthionine peptides was selected: The sulfide bridge was formed by the addition of the Cys^5 SH group to the double bond of dehydroalanine-2 in the solid phase. The peptide synthesis was carried out on a methylbenzhydrylamine resin using Boc chemistry with the symmetrical anhydride peptide coupling method. Serine was incorporated at position 2, then converted to dehydroalanine using disuccinimidocarbonate. The S-protecting

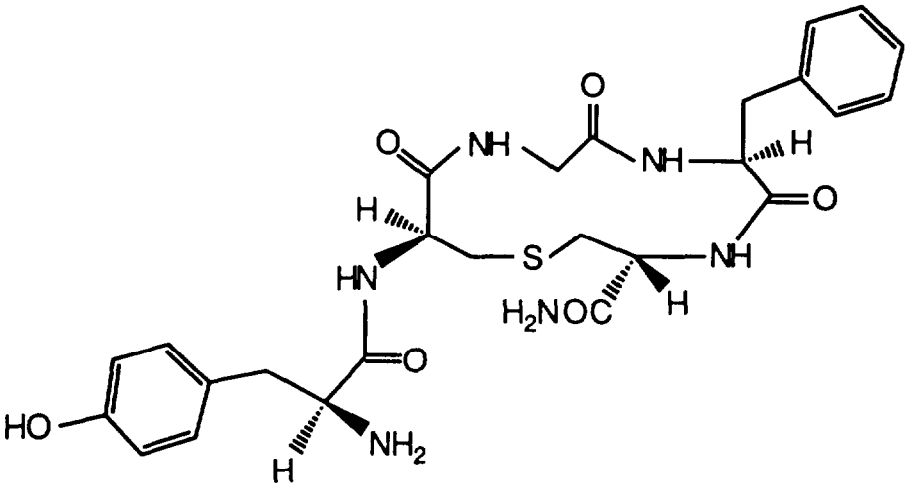

FIGURE 4. *The structure of Tyr-c[D-Ala$_L$-Gly-Phe-Ala$_L$]-NH$_2$*

fluorenyl methyl group was selectively removed with piperidine. A slightly basic milieu (5 percent piperidine/DMF) promoted the Michael addition of the SH group to the double bond. "Low-high HF cleavage" was used to cleave the peptide from the resin and to remove the benzyl and benzyloxycarbonyl protecting groups. Purification of the resulting crude product was achieved by preparative reverse phase high-performance liquid chromatography.

In vivo bioactivity of the compound was determined from the rat hot-plate test after intrathecal injection (Yaksh et al. 1991). The analog Tyr-c[D-Ala$_L$-Gly-Phe-Ala$_L$-NH$_2$ shows 37 times higher bioactivity than morphine (table 4).In vitro assays with GPI and MVD preparations, this analog exhibits 400 times greater bioactivity at the p-receptor and 20 times higher at the δ·-receptor than (Leu^5]-enkephalin. These values are higher than those of its disulfide-bridged counterpart, DCLCE (Schiller et al. 1981). The lanthionine analog shows preference to neither the μ- nor the δ·-receptors with an IC_{50} ratio (MVD/GPI) of 0.88.

To determine the conformational features of this molecule that are responsible for such behavior, we carried out a rigorous conformational analysis employing both NMR spectroscopy and molecular modeling. The torsional angles $\chi^1(Ala_L^2)$, $\psi(Ala_L^2)$, ϕ_{Gly}, ψ_{Gly}, and ϕ_{Phe} remain quite stable during molecular dynamics (figure 5). Although no methyl substituents are present on the β·-carbon of the D-Ala$_L^2$ to provide steric hindrance that would restrict rotations about the C_α-C_β and C_β·-S bonds, this part of the ring is nonetheless

TABLE 4. *Biological activities of the lanthionine-bridged enkephalin analog*

Analog	GPI* IC_{50}[nM]	MVD* IC_{50}[nM]	MVD/GPI IC_{50}-ratio	In Vivo† ED_{50}[μg]
Tyr-c[D-Ala_L-Gly-Phe-Ala_L]NH_2	0.6	0.5	0.88	0.15
Tyr-c[D-Cys-Gly-Phe-Cys]-NH_2	1.5	0.8	0.50	—
Leu^5-enkephalin	246	11.4	0.05	>100‡
Morphine	58.6§	644§	11	5.6

* The GPI and MVD activities are measured in Dr. P.W. Schiller's laboratorles.
† Values are measured in Dr. T. Yaksh's laboratorles.
‡ It only shows 50 percent of maximum effect at 100 μg dosage.
§ Data taken from Hruby and Gehrlg 1989.

constrained. Dynamics reveals that some local flexibility exists about residues 4 and 5 (Phe and Ala_L^5), enabling pairs of torsional angles in this region to undergo concerted transitions. This small degree of flexibility does not influence the mutual orientations of the two aromatic rings, because the D-Ala_L^2-Gly region is highly constrained. Although the flexibility of the main ring has been decreased, the distance between the aromatic rings is still highly variable and therefore no increase in selectivity has been achieved compared to parent analog DCLCE

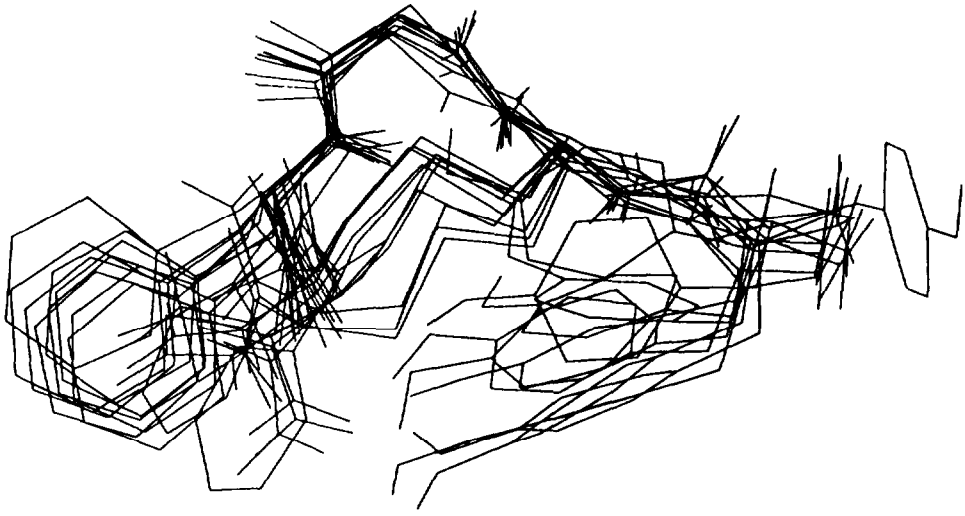

FIGURE 5. *Superposition of structures of Tyr-c[D-Ala_L-Gly-Phe-Ala_L]-NH_2 resulting after every 10 psec of dynamics simulation*

(Schiller et al. 1981). The bioactivity profile of the new 13-membered lanthionine analog is consistent with conformational models for opioid activities of the (14-membered) cyclic enkephalins (Yamazaki et al. 1991 *a*).

CONCLUSIONS

From the above studies, the authors obtained several analogs with novel structures and of reduced ring size such as lanthionine enkephalin. Because they are highly potent and exhibit interesting conformational features, they can be key analogs for opioid drug discovery.

These studies also enabled us to classify peptide opioids into three families by their structural characteristics as well as their origin. The morphiceptin family requires an L-chiral residue between Tyr^1 and Phe^3 that adopts a *cis* configuration at the Tyr-X^2-(spacer) amide bond. The dermorphin-deltorphin family contains a D-chiral X^2 residue that assumes a *trans* amide configuration. The enkephalin family can have a D-chiral or an achiral residue at position 2 and an additional achiral residue at position 3. According to such classifications, the authors are now synthesizing cyclic morphiceptin analogs with N-alkylated-L-amino acids at position 2.

For each family of opioid peptides, topochemical models have been developed to explain bioactivity and receptor selectivity based on the accessible space of conformationally constrained analogs. The analogs incorporate various peptidomimetics and cyclic structures. All the topochemical structures proposed as "bioactive conformations" of the three families for each μ- and δ-receptor recognition are quite similar. Therefore, we propose that in general the folded structure with the tyrosyl aromatic ring in close proximity to another aryl ring is required for δ-receptor activity. For bioactivity at the μ-receptor, the structure in which the two aromatic side chains project away from each other with a large separation (>11Å) is required.

NOTES

1. The details of our conformational studies will be discussed in a subsequent paper.

2. Unpublished data of Dr. M. Goodman's laboratories.

3. Similar work also was carried out by Darlak and colleagues (1991).

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Molecular Determinants of Cannabinoid Activity: Toward the Design of Cannabinoid Analgesics With Reduced Psychoactive Liability

Patricia H. Reggio

INTRODUCTION

Cannabinoids are the group of C_{21} compounds typical of and present in the Indian hemp, *Cannabis sativa* L. Both hashish and marijuana are derived from cannabis and have been used for centuries for their medicinal effects as well as for their psychotomimetic effects (Mechoulam and Gaoni 1967). Current scientific interest in the cannabinoids followed Mechoulam and Gaoni's (1967) and Gaoni and Mechoulam's (1971) report that (-)- *trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) (figure 1, panel A) is the major psychoactive component of cannabis. Subsequent to this report, more than 300 cannabinoids have been synthesized and tested (Razdan 1986). Some cannabinoids have been shown to have possible therapeutic value as analgesics, bronchodilators, anticonvulsants, antiemetics, and antiglaucoma agents (Mechoulam 1986). For example, interest in the development of a cannabinoid analgesic has been spurred by the demonstration that some classical and nonclassical cannabinoids possess analgesic activity comparable to that of the strong opiates. However, these cannabinoid analgesics do not act at opiate receptors and have neither the physical dependence liability nor the respiratory depression properties of the strong opiates, yet they possess oral activity with long duration (Razdan 1986).

11 -Hydroxylation has been shown to be a primary metabolic event for molecules such as Δ^9-THC (Nilsson et al. 1970). Although studies have shown that Δ^9-THC can exert its psychotomimetic effects without metabolic activation (Martin et al. 1975, 1977; Carney et al. 1979), it has been proposed that 11-hydroxylation in the tetrahydrocannabinols (THCs) *is* a prerequisite for the production of analgesic activity (Wilson and May 1975). During the course of their work on a metabolic activation requirement for cannabinoid analgesia, Wilson and coworkers (1976) synthesized (-)-9-nor-9-alpha-hexahydrocannabinol [(-)-9-nor-9α-OH-HHC]and(-)-9-nor-9-beta-hexahydrocannabinol (-)-9-nor-9β-OH-HHC]. The 9α compound (in which the 9 hydroxyl group is axial) (figure 1, panel B)

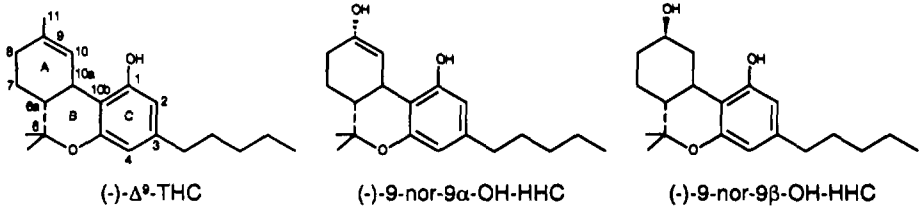

FIGURE 1. *(A) One numbering system commonly employed for (-)-Δ⁹-THC. (B) A drawing of the structure of (-)-9-nor-9α-OH-HHC. (C) A drawing of the structure of (-)-9-nor-9β-OH-HHC.*

was found to possess low psychoactivity and no analgesic activity, whereas its 9β epimer (in which the 9 hydroxyl group is equatorial) (figure 1, panel C) was found to possess both significant analgesic and psychotomimetic activity. Later, Johnson and Melvin (1986) worked with (-)-9-nor-9β-OH-HHC, making modifications to this structure that improved analgesia. Unfortunately, these modifications also produced a parallel increase in psychoactivity, rendering the new cannabinoid compounds less desirable as therapeutic agents.

Results such as those for cannabinoid analgesics discussed above underscore a major problem in the cannabinoid field: the inability to separate the psychotomimetic effects of the cannabinoids from their therapeutic effects. When the author began work on the cannabinoids, the initial research goal was to use the methods of theoretical chemistry o shed some light on the molecular basis for the actions of the cannabinoids. As work on the molecular determinants for cannabinoid activity progressed, I became interested in this problem of separation of activities. One of the major goals of this work is to accomplish such a separation, that is, to design cannabinoid analgesics with reduced psychoactive liability. This chapter reviews the progress made toward this goal. For a more detailed discussion of the work presented here, the reader is referred to Reggio and Mazurek (1987), Reggio and colleagues (1989, 1990*a,* 1990*b,* 1991), and a recent review by Reggio (1992).

Historically, the well-known high lipophilicity of the cannabinoids led many investigators to hypothesize that the cannabinoids exerted their effects by perturbing biological membranes (Roth and Williams 1979). However, evidence of stereospecificity of action in the cannabinoids led other investigators to hypothesize the existence of a cannabiniod receptor (Binder and Franke 1982). This second group was recently rewarded by the discovery of the first cannabinoid receptor. The potent radiolabeled nonclassical cannabinoid ligand [^{3}H]CP-55,940 was used by Devane and coworkers (1988) to develop membrane homogenate and tissue section binding assays for the characterization and

localization of a cannabinoid receptor in rat brain. The [^{3}H]CP-55,940 binding site has been reported to exhibit characteristics expected for a neuromodulator receptor associated with a G protein. Autoradiographic techniques using [^{3}H]CP-55,940 have revealed a heterogeneous distribution of this receptor throughout the brain, with greatest abundance of sites in the basal ganglia, hippocampus, and cerebellum (Herkenham et al. 1990). The cloning and expression of a complementary DNA that encodes a cannabinoid receptor both in rat (Matsuda et al. 1990) and in humans (Gerard et al. 1991) also has been reported recently. To date, no cannabinoid antagonist has been discovered.

The site or sites of the analgesic action of the cannabinoids in the central nervous system (CNS) are not yet established. The concentration of low levels of [^{3}H]CP-55,940-labeled receptors in the spinal cord gray matter in the substantia gelatinosa of the dorsal horn suggests an action at the spinal level. However, this finding does not rule out action at higher levels in the brain (Howlett et al. 1990).

Agonist binding data for the [^{3}H]CP-55,940 binding site are rather limited (Devane et al. 1988; Herkenham et al. 1990). Over the years, a wealth of pharmacological data for the cannabinoids has been published. The majority of these data has come from whole animal assays such as the rhesus monkey behavioral assay (Razdan 1986), the rat drug discrimination assay (Weissman 1978; Browne and Weissman 1981) and a battery of tests in mice that have been shown to be highly predictive of cannabinoid effects (Razdan 1986; Martin et al. 1984). In the work presented here, results have been correlated with those of the selected whole animal assays mentioned above.

Because one of the major goals of the work presented here is the design of cannabinoid analgesics with reduced psychoactive liability, efforts have been directed toward the elucidation of the molecular determinants for cannabinoid psychopharmacological activity and for cannabinoid analgesic activity. To this end, the methods of theoretical chemistry have been used. Several assumptions are implicit in this work. (1) It is assumed that the interactions between drugs and their biological targets are dependent on the same molecular parameters that determine chemical interactions and reactions. A molecular reactivity characteristic (MRC), such as the molecular electrostatic potential (MEP), is a molecular property that determines specific chemical reactivity. In this work the calculation of the MEP has been emphasized as an MRC. (2) Because studies have shown that differences in the penetration and distribution of cannabinoids in the CNS do not correlate with activity (Ohlsson et al. 1980; Binder et al. 1984), it has been assumed that changes in activity caused by molecular modification can be attributed to changes in the ability of the molecule to interact at its receptor or site of action. (3) Because the compounds studied are clearly structurally related, it has been assumed that they may be unambiguously superimposed on one another and that all compounds interact in analogous

orientations with their receptor or site of action, However, the recent report (Pacheco et al. 1991) that a new class of compounds, the aminoalkylindoles, interact with a cannabinoid receptor may soon produce the first test of these assumptions of superimposability and analogous orientations of all compounds.

TEMPLATE SELECTIONS AND HYPOTHESES

This work of determining the MRCs necessary for cannabinoid psychopharmacological activity and for analgesic activity began by identifying template molecules for each activity. These template molecules are compounds with demonstrated activities whose MRCs can be used as a basis for comparisons with those of other cannabinoids. For psychopharmacological activity, Δ^9-THC (figure 1, panel A) was chosen as the template molecule because this molecule has been reported to be the major psychoactive component of cannabis (Mechoulam and Gaoni 1967; Gaoni and Mechoulam 1971) and because studies indicate that it can exert behavioral effects without metabolic activation (Martin et al. 1975, 1977; Carney et al. 1979). The first working hypothesis concerning cannabinoid psychoactivity employed in the work reported here was that there are two components of the structure of Δ^9-THC that confer on the molecule reactivity characteristics crucial to activity. These components are (1) the orientation of the carbocyclic ring, ring A, and its C9 substituent relative to the phenyl group hydroxyl and (2) the orientation of the lone pairs of electrons of the phenyl group hydroxyl oxygen,

For cannabinoid analgesic activity, (-)-9-nor-9β-OH-HHC was chosen as the template molecule (figure 1, panel C) because 11- or 9β-hydroxylation has been reported to be a prerequisite for the production of cannabinoid analgesia (Wilson and May 1975; Wilson et al. 1976) and because this molecule seems to embody the minimum requirements for activity as evidenced by the fact that the simple change of the 9-OH group from equatorial to axial abolishes analgesic activity (Wilson et al. 1976). The author's working hypothesis concerning cannabinoid analgesia was that the analgesic activity of 9-nor-9β-OH-HHC is caused by the presence and relative location of two regions of negative potential in the top half of the molecule. These negative potential regions are generated by the equatorial alcohol (i.e., the 9β-OH group) and by the phenyl group hydroxyl at C-1 (Reggio et al. 1990*a*).

MRC COMPARISONS

Having identified a template molecule for each activity, the strategy was to characterize each template and then to compare the MRCs calculated for each template molecule with those of other active and inactive cannabinoids to refine the hypothesis for each activity. The initial focus was on the MRCs for psychopharmacological activity.

Carbocyclic Ring Orientation

Molecular mechanics calculations [MMP2(85)] (Molecular Design, Ltd., San Leandro, CA) revealed that Δ^{9}-THC (figure 1, panel A) is a nonplanar molecule. Because the carbocyclic ring (ring A) is in a half-chair conformation, the top of this ring lifts up off the page and toward the viewer, protruding into what is called the top or β face of the molecule. The spatial relationship of the carbocyclic ring with the rest of the molecule can be better visualized by turning the molecule sideways so that the side chain comes forward and the carbocyclic ring (ring A) goes to the back (i.e., viewing the molecule parallel to the direction of the vector from C2 to C10b) (figure 2, panel A). From this perspective the "top" (β') face will be to the left of the molecule, and the "bottom" α) face will be to the right. In figure 2, panel A, it is clear that the top portion of ring A clears the bottom (α') face of the molecule and protrudes instead into the top (β') face. The shape of the template molecule, Δ^{9}-THC, was assumed to be characteristic of active molecules, with the idea that only molecules that possess no portion of their carbocyclic ring protruding into the bottom (α') face will be active.

Because the first part of the working hypothesis for cannabinoid psychopharmacological activity concerned the orientation of the carbocyclic ring relative to the phenyl group hydroxyl oxygen, work was begun by considering active, minimally active, and inactive cannabinoids that all possess identical side chains and free phenyl group hydroxyls but that differ in the extent and position of unsaturation in the carbocyclic ring. The compounds chosen for study were the active cannabinoids Δ^{8}-THC and 11β-HHC, the minimally active cannabinoid 11α-HHC, and the inactive cannabinoids Δ^{7}-THC and $\Delta^{9,11}$-THC (Reggio et al. 1989). The activity of each of these compounds had been assayed in rhesus monkey behavioral tests (Razdan 1986).

Molecular mechanics calculations revealed that only 11α-HHC possessed more than one accessible minimum energy fused ring conformation. A 3 kcal/mol cutoff for accessibility was used in this analysis. For the active molecules in this set, molecular mechanics calculations revealed that, just as in Δ^{9}-THC, the top part of the carbocyclic ring and its C9 substituent clears the bottom (α') face and protrudes instead into the top (β') face of each molecule. For the inactive cannabinoids in this set, molecular mechanics calculations revealed an obvious shape difference. In inactive cannabinoids, the C9 substituent at the top of the carbocyclic ring protrudes instead into the bottom (α) face of the molecule (see Δ^{7}-THC in figure 2, panel B).

To quantitate the orientation of the carbocyclic ring and its C9 substituent in each molecule, several nonbonded torsion angles were measured. It was found that the value of one of these angles, τ (C11-C9—Cl-O), correlates well

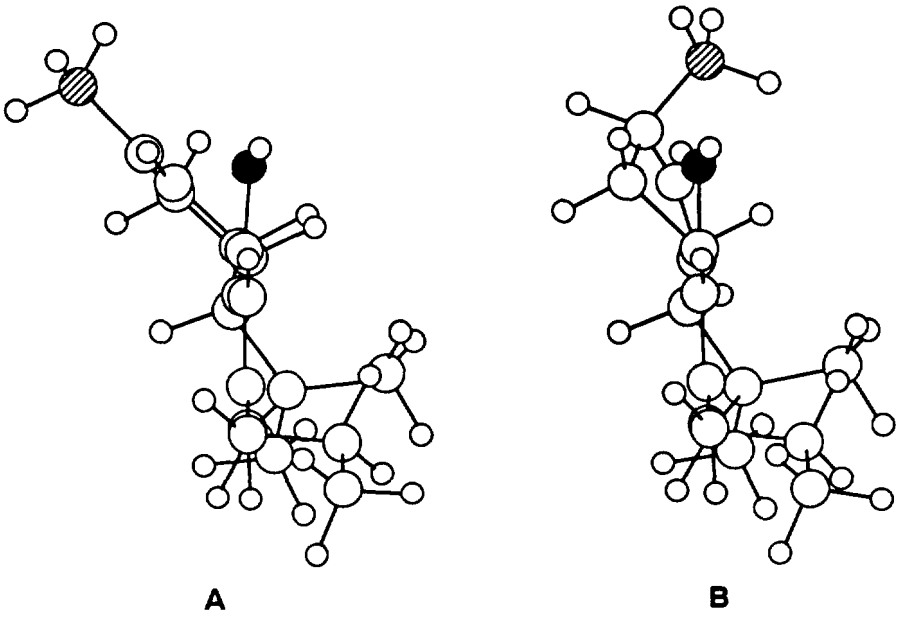

FIGURE 2. *(A) A side view of the conformation of the global minimum energy conformation of (-)-Δ^9-THC (with propyl side chain) as determined by MMP2(85). The perspective is along the vector from C2 to C10b. The phenyl group hydroxyl oxygen is shown here as a blackened circle. The carbon of the C9 substituent (i.e., C11) is shown as a hatched circle. (B) A side view of the conformation of the global minimum energy conformation of (-)-Δ^7-THC (propyl side chain) as determined by MMP2(85). In this compound the C11 methyl group is axial. See panel A for other details.*

with activity. The τ-angle measures the inclination of the C9 substituent relative to the phenyl group hydroxyl oxygen. All active cannabinoids (including Δ^9-THC) were found to possess a negative value for this angle. A negative r-value means that the C9 substituent points into the β-face of the molecule or to the left relative to the phenyl group hydroxyl oxygen in the side view. All inactive molecules in the set were found to possess positive values of τ. A positive τ-value means that the C9 substituent points into the α-face of the molecule or to the right of the phenyl group hydroxyl oxygen in the side view.

This correlation between shape (as quantitated by τ) and activity was taken to be an indication that there may be a region of steric interference at the site of action of the cannabinoids. In relation to the structure of the cannabinoids,

this region must be located near the top of the carbocyclic ring in the bottom face of the molecule. Any molecule whose conformation would cause it to protrude into this region of steric interference may not be shaped properly to fit at its site of action and thus may be rendered inactive (Reggio et al. 1989). A recent comparative molecular field analysis study of several cannabinoids has supplied additional evidence for a region of steric interference near this portion of the molecule (Thomas et al. 1991).

The idea of a region of steric interference was then extended to suggest why compounds such as 11-α-HHC are minimally active (i.e., only active at higher doses). Molecular mechanics studies showed that the carbocyclic ring of 11-α-HHC can exist in two conformations (a CHAIR form and a TWIST form, figure 3). It was found that for the CHAIR form of 11-α-HHC (which will be the predominant form in solution), τ is positive (figure 3, panel A). Thus, in its lowest energy form, 11-α-HHC resembles the inactive molecules previously studied. However, the higher energy (ΔFSE=1.72 kcal/mol) TWIST form of 11-α-HHC was found to possess a negative r-value (figure 3, panel B). Thus, the higher energy form of 11-α-HHC resembles the active cannabinoids previously studied. In the TWIST form the C9 substituent protrudes into the top (β') face. Therefore, the diminishment in activity of 11-α-HHC was attributed to the fact that only the higher energy TWIST form of 11-α-HHC is shaped properly to fit at the site of action (Reggio et al. 1989).

These results for the orientation of the C9 substituent suggested one way to reduce psychoactivity in the cannabinoids, that is, create a molecule in which the C9 substituent protrudes into the bottom (α) face of the molecule (τ: >0). This idea was incorporated into the design of new cannabinoid analgesics. Unfortunately, preliminary work has indicated that both psychoactivity and analgesic activity share a common shape requirement for activity. Thus, it appears that protrusion of the C9 substituent into the bottom (α') face of the molecule is associated with diminishment of analgesic activity as well as with diminishment of psychoactivity (Reggio et al. 1991).

Lone-Pair Orientation

The second part of the author's working hypothesis for cannabinoid psychopharmacological activity concerned the orientation of the lone pairs of electrons of the phenyl group hydroxyl oxygen. In this part of the work, studies were conducted on Δ^{9}-THC and other cannabinoids that possess similar shapes but which differ at C-1 (the phenyl group hydroxyl position in Δ^{9}-THC, figure 1, panel A). Both molecular mechanical and ab initio studies of the rotational behavior of the phenyl group hydroxyl of Δ^{9}-THC revealed that, although the phenyl group hydroxyl is capable of existing in any orientation by expenditure of a modest amount of energy, it possesses two minimum energy positions (Reggio and Mazurek 1987; Reggio et al. 1989). In the global

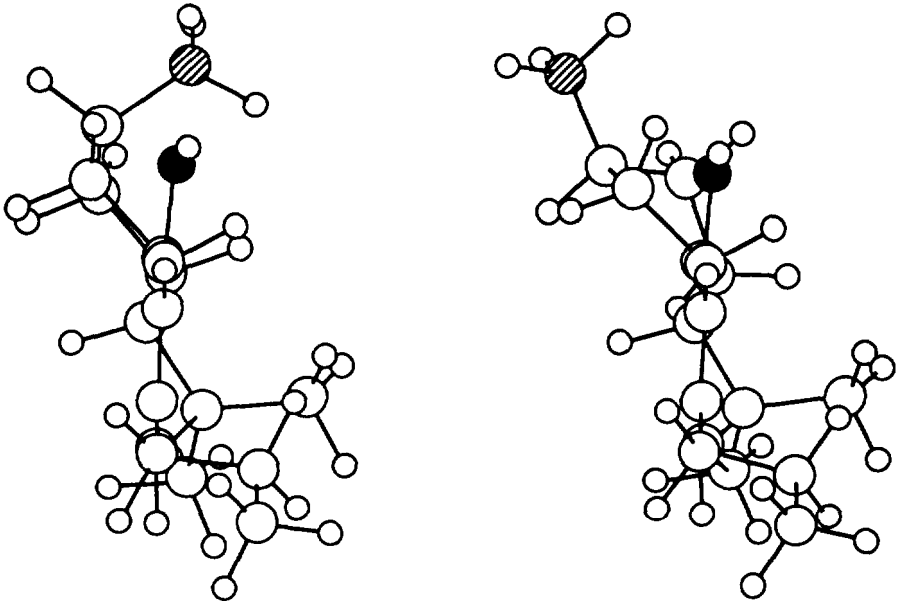

FIGURE 3. *(A) A side view of the global minimum energy conformation of 11α-HHC. The carbocyclic ring is in a CHAIR conformation. (B) A side* view *of the second minimum energy accessible conformation of 11α-HHC. The carbocyclic ring is in* a *TWIST conformation. See figure 2, panel A, for other* details.

SOURCE: Reggio, P.H. A search for properties which produce discrimination between cannabinoid psychoactivity and analgesic activity. *Int J Quantum Chem* 44:165-179, 1992. Copyright 1992 by Wiley-Liss, a division of John Wiley & Sons, Inc.

minimum energy conformer, the phenyl group hydroxyl hydrogen points away from the carbocyclic ring. In this position the lone pairs of electrons of the phenyl group hydroxyl oxygen point toward this ring. The C2-C1-O-H torsion angle is 7° (figure 4, panel A). In the second minimum energy conformer, the phenyl group hydroxyl hydrogen points essentially in the opposite direction, toward the carbocyclic ring, and the lone pairs point away from the carbocyclic ring. The C2-C1-O-H torsion angle is 167° (figure 4, panel B). A comparison of the MEPs generated by each of these forms revealed that these two different phenyl group hydroxyl positions generate MEPs that are distinguishable from one another (Reggio and Mazurek 1987).

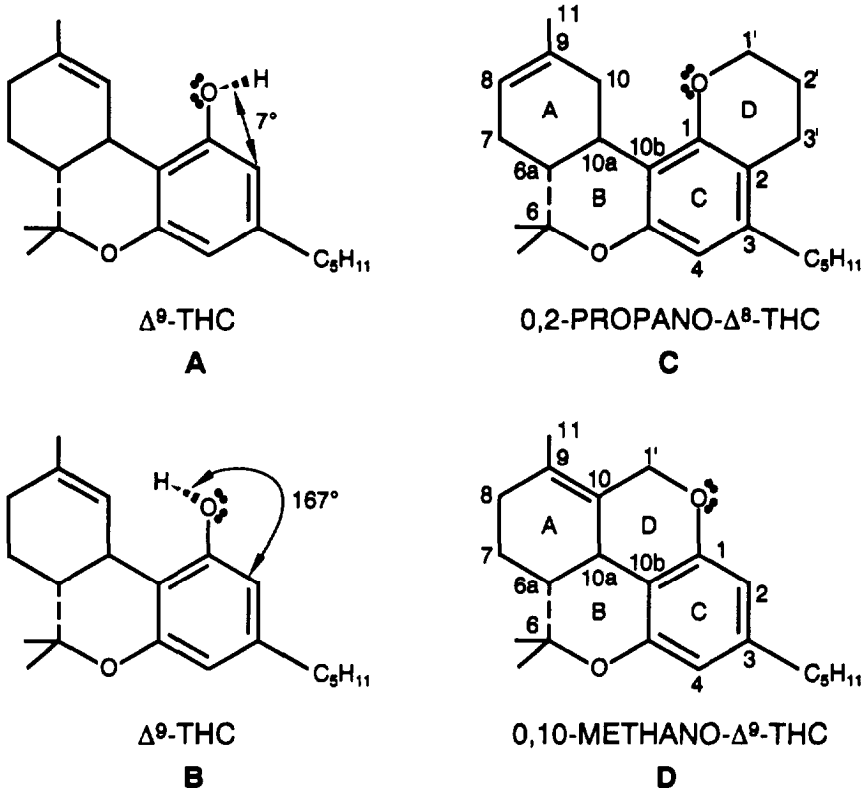

FIGURE 4. *(A) The phenyl group hydroxyl position in the global minimum energy conformation of (-)-Δ^9-THC. The C2-C1-O-H torsion angle, which has a value of 7°—as determined by MMP2(85)—is indicated by the double-headed arrow. (B) The second minimum energy phenyl group hydroxyl position in (-)-Δ^9-THC. The C2-C1-O-H torsion angle, which has a value of 167°—as determined by MMP2(85)—is indicated by the double-headed arrow. (C) A drawing of the structure of O,2-propano-Δ^8-THC. (D) A drawing of the structure of O, 10-methano-Δ^9-THC. In each of these drawings, the two pairs of dots have been drawn (according to the Lewis Dot formalism) for each phenyl group hydroxyl oxygen to symbolize (in two dimensions) the lone pairs of electrons of this oxygen.*

SOURCE: Reggio, P.H. A search for properties which produce discrimination between cannabinoid psychoactivity and analgesic activity. *Int J Quantum Chem* 44:165-179, 1992. Copyright 1992 by Wiley-Liss, a division of John Wiley & Sons, Inc. (New York).

New Compound Design

When the work presented here was begun, one of the traditional conclusions drawn regarding the structure-activity relationship of the cannabinoids was that the position of and the environment around the phenyl group hydroxyl were critical for activity (Razdan 1986). A free phenyl group hydroxyl was believed to be necessary, presumably for the hydrogen of the phenyl group hydroxyl to participate as a donor in a hydrogen-bonding interaction at the site of action. However, it should be noted that the phenyl group hydroxyl possesses not only a phenolic hydrogen that can participate in a hydrogen-bonding interaction as a donor but also possesses lone pairs of electrons (on the oxygen) that can participate as acceptors in a hydrogen-bonding interaction. To study the importance to cannabinoid psychoactivity of the lone pairs and their orientation, two new etherified or oxygen-bridged analogs were designed. In each analog, the phenyl group hydroxyl oxygen was incorporated into a new fourth ring (ring D) (Reggio et al. 1990*b*). In these new compounds, the orientation of the lone pairs was restricted to one of two general directions. In O,2-propano-Δ^{8}-THC (figure 4, panel C), the lone pairs point toward the carbocyclic ring, mimicking the lone-pair orientation in the global minimum energy phenyl group hydroxyl position of Δ^{9}-THC. In O,10-methano-Δ^{9}-THC (figure 4, panel D), the lone pairs point away from the carbocyclic ring, mimicking the lone-pair orientation in the second minimum energy phenyl group hydroxyl position of Δ^{9}-THC.

For O,2-propano-Δ^{8}-THC, the compound designed to mimic the first phenyl group hydroxyl position in Δ^{9}-THC, molecular mechanics calculations revealed that there are two accessible minimum energy fused ring conformers. Conformer 2 is 0.81 kcal/mol higher in steric energy than conformer 1 (figure 5). In both, the carbocyclic rings exist in a half-chair conformation. The major difference between the two conformers is in the conformation of the new fourth ring (ring D). In figure 5, it is clear that in both conformers of O,2-propano-Δ^{8}-THC, the C9 substituent of the carbocyclic ring protrudes into the top (β) face of the molecule (or to the left of the phenyl group hydroxyl oxygen in the side view), as do the C9 substituents of active cannabinoids discussed previously.

MEP maps of each conformer were then calculated. The spatial distribution and relative strength of potential regions in the MEP map form a characteristic pattern, or "fingerprint," for a molecule in a given conformation. In the work reported here, the idea has been employed that molecules that interact at the same site generate MEP patterns that are similar. Thus, at least in the recognition stage of the interaction of the drug with its receptor, the receptor recognizes the drug because each generates an MEP that is complementary to the other. Therefore, the MEP may be thought of as a recognition pattern for the site of action. If the MEP of a new analog is similar to that of a known active compound both in shape and in the position of minimum potential

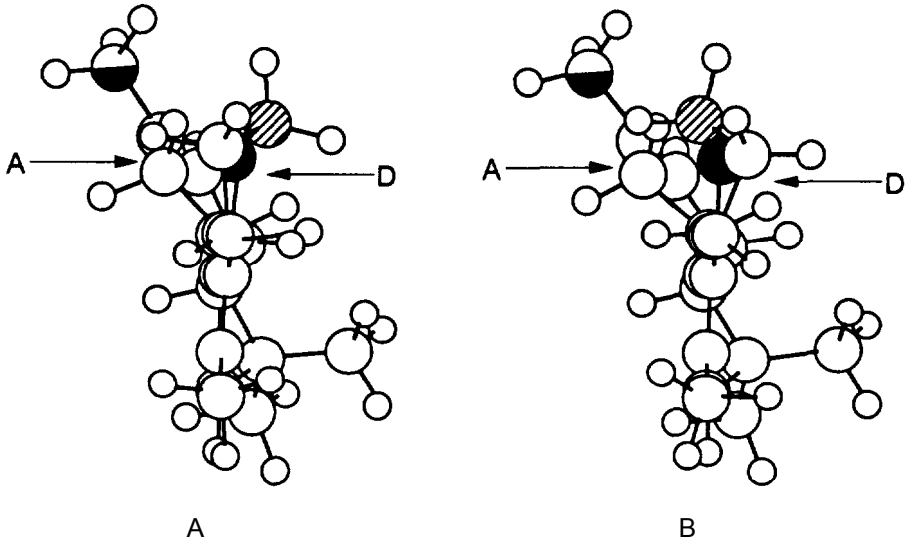

FIGURE 5. *Side views of conformer 1 (A) and conformer 2(B) of O,2-propano-Δ^{8}-THC (with methyl side chain). The perspective of ring A is viewed in the direction from C2 to C10b. Therefore, the side chain is forward and the carbocyclic ring (ring A) is in the back. The ring D oxygen is shown as a blackened circle, the C11 carbon (C9 substituent) is shown as a half-blackened circle, and the C1' carbon of ring D is shown as a hatched circle.*

values, then the new compound should be recognized at any site that recognizes the known active compound. When the MEPs generated by the conformers of O,2-propano-Δ^{8}-THC were compared with that generated by Δ^{9}-THC in its global minimum energy phenyl group hydroxyl position, the MEPs were found to be similar. Figure 6 shows a comparison of the MEP map generated by conformer 1 of O,2-propane-Δ^{8}-THC on the left, with the MEP map generated by Δ^{9}-THC in its global minimum energy phenyl group hydroxyl position on the right. These MEPs were calculated in a plane parallel to the aromatic ring using the Gaussian system of programs and the LP-3G basis set at a distance of 1.5 Å from the aromatic ring in the bottom face of the molecule (Reggio and Mazurek 1987; Reggio et al. 1990*b).*

In figure 6, it is clear that the overall shapes of the MEPs generated by conformer 1 of O,2-propano-Δ^{8}-THC and by Δ^{9}-THC in its global minimum energy phenyl group hydroxyl position are similar. In addition, it is clear that the position and values of analogous minima (labeled here by stars) in the two MEP maps are also similar. Conformer 2 of O,2-propano-Δ^{8}-THC also generated a

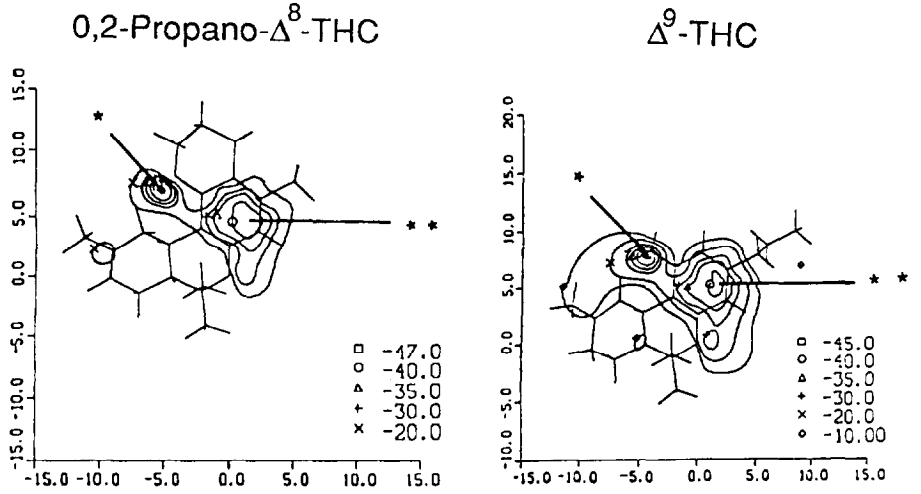

FIGURE 6. *A comparison of the MEPs in a plane parallel to the plane of the aromatic ring, at a distance of 1.5Å below the aromatic plane, in (A) conformer 1 of O,2-propano -Δ⁸-THC and (B) the global minimum energy phenyl group hydroxyl position of Δ⁹- THC. *, the minimum associated with the ring D oxygen in (A) and with the phenyl group hydroxyl in (B); **, the minimum associated with the aromatic ring in each. The potential values are in kcal/mol.*

similar MEP map (Reggio et al. 1990*b*). It was concluded from this comparison that O,2-propano-Δ⁸-THC should be recognized by any site that recognizes Δ⁹-THC in its global minimum energy phenyl group hydroxyl position,

For O,10-methano-Δ⁹-THC, the compound designed to mimic the second minimum energy phenyl group hydroxyl position of Δ⁹-THC, molecular mechanics calculations revealed that there are three accessible minimum energy fused ring conformers (figure 7). Conformer 2 is 0.40 kcal/mol higher in steric energy than the global minimum conformer (conformer 1). The third conformer is 1.99 kcal/mol higher in steric energy than conformer 1 (Reggio et al. 1990*b*). It is clear in figure 7 that the C9 substituent in each conformer protrudes into the top (3) face (or moves to the left of the phenyl group hydroxyl oxygen in the side view) as do the C9 substituents of active cannabinoids discussed previously.

Figure 8 shows a comparison of the MEP map generated by conformer 1 of O,10-methano-Δ⁹-THC on the left and the MEP map generated by Δ⁹-THC in its second minimum energy phenyl group hydroxyl position on the right (Reggio and Mazurek 1987; Reggio et al. 1990*b*). It is clear in figure 8 that the MEP generated by conformer 1 of O,10-methano-Δ⁹-THC is similar to

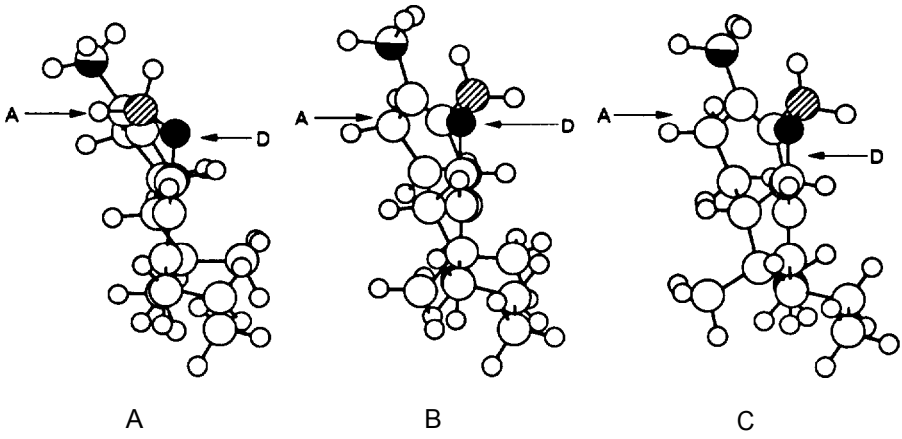

FIGURE 7. *Side views of conformer 1 (A), conformer 2 (B), and conformer 3 (C) of O, 10-methano-Δ^9--THC (with propyl side chain). See figure 5 for other details.*

that of Δ^9-THC. Again the positions of analogous minima are labeled with stars. It was concluded from this comparison that O,10-methano-Δ^9--THC should be recognized by any site that recognizes Δ^9-THC in its second minimum energy phenyl group hydroxyl position.

Synthesis and Pharmacological Evaluation of New Compounds

0,2-propano-Δ^8-THC and O,10-methano-Δ^9-THC were synthesized (Seltzman et al. 1991) and pharmacologically evaluated in a series of tests in mice and rats, which have been shown to be predictive of cannabinoid effects (Reggio et al. 1990*b).* The pharmacological results for both compounds are shown in comparison to the pharmacological results for active cannabinoids Δ^9-THC and Δ^8-THC in table 1.

Pharmacological evaluation of O,10-methano-Δ^9--THC revealed that this compound was inactive in all mouse tests as well as in rat drug discrimination. Several possible explanations were explored for the inactivity of O,10-methano-Δ^9-THC, including possible steric effects. Ultimately, it was concluded that the inactivity of this compound is caused by the inappropriate directionality of the lone pairs of electrons of the phenyl group hydroxyl oxygen (Reggio et al. 1990*b*).

Pharmacological evaluation of O,2-propano-Δ^8--THC revealed that this compound possesses a unique activity profile. This compound is similar to Δ^8-THC in that it depresses rectal temperature and produces antinociception

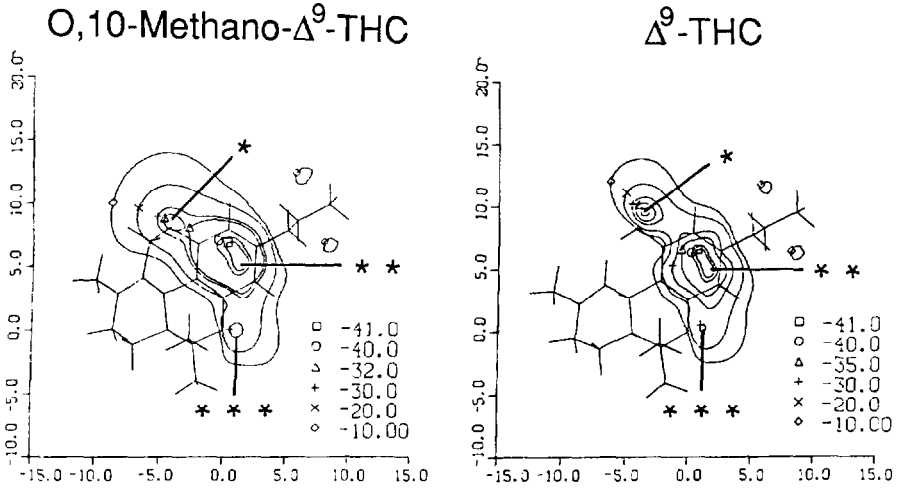

FIGURE 8. *A comparison of the MEPs in a plane parallel to the plane of the aromatic ring, at a distance of 1.5Å below the aromatic plane, in conformer 1 of O, 10-methano-Δ9-THC (A), and the second minimum energy phenyl group hydroxyl position of Δ9-THC (B). *, the minimum associated with the ring D oxygen in (A) and with the phenyl group hydroxyl oxygen in (B); **, the minimum associated with the aromatic ring in each; ***, the minimum associated with the pyran oxygen in each. The potential values are in kcal/mol.*

(analgesia) and ring-immobility (catalepsy) in mice. However, it differs from Δ9-THC and Δ8-THC in that it only weakly depresses locomotor activity, and it fails to substitute for Δ9-THC in the rat drug discrimination paradigm. Drug discrimination In animals has been reported to be reflective of or highly correlated with the subjective "high" in humans (Järbe et al. 1981). Thus, 0,2-propano-Δ8-THC appears to produce a separation of cannablnoid activities, with psychoactivity diminished or absent but with other activities such as antinociception (analgesia) remaining.

The methyl ether of Δ9-THC(O-methyl- Δ9-THC) has been reported to be inactive in the rhesus monkey behavioral assay (Razdan 1986). Because of etherification, this compound lacks a free phenyl group hydroxyl hydrogen. A molecular mechanics conformational study of this molecule revealed that there is only one minimum energy position for the methoxy group in O-methyl-Δ9-THC (Reggio 1987). This minimum energy position is analogous to the position of the phenyl group hydroxyl in the global minimum energy conformer of Δ9-THC. Thus, the lone-pair orientation in O-methyl- Δ9-THC is analogous

TABLE1. *Pharmacologica/ activity of THC and O-bridged analogs*

Pharmacological activity of THC and O-bridged analogs on mouse locomotion (5 to 15 minutes), tail flick antinociception (20 minutes), rectal temperature (60 minutes), and ring-immobility (90 to 95 minutes), following tail-vein injection, as well as activity in the rat discriminative stimulus paradigm following intraperitoneal injection. ED, values are presented (mg/kg) with their 95-percent confidence limits. The maximum effect produced in each mouse evaluation is indicated in parentheses below the ED_{50} (% inhibition vs. control, °C decrease, or %).

Analog	**Motor Activity** (% Inhibitor)	**Hypothermia** (°C)	% Immobility	**% Maximum** Possible Effect	**Discriminative** Stimulus
Δ^9-THC	1.0 (0.5-1.4) (78%)	1.4 (1.2-3.8) (4.2)	1.5 (0.4-2.7) (49%)	1.4 (0.5-3.4) (100%)	0.8 (0.4-1.5)
Δ^8- -THC	1.9 (1.2-2.2) (79%)	15.5 (8.1-17) (-5.9)	5.2 (3.1-8.9) (58%)	1.5 (1.0-2.3) (100%)	0.9 (05-1.8)
O,2-propano- Δ^8- -IHC	5.4 (4.1-7.0) (54%)	16.5 (11-26) (-4.9)	2.6 (0.3-23) (44%)	3.5 (1.6-7.2) (100%)	>30
O,10-methano-Δ^9-THC	>30	>30	>30	>30	>10

SOURCE: Reggio, P.H.; Seltzrman, H.H.; Compton, D.R.; Prescott, W.R., Jr.; and Martin, B.R. An investigation of the rote of the phenolic hydroxyl in cannabinoid activity. *Mol Phamacol* 38:854-862,1990*b*. Copyright 1990 by American Society for Pharmacology and Experimental Therapeutics (Bethesda, MD).

to that in O,2-propano-Δ^8-THC. Recently, Burstein and coworkers (1988) repotted that O-methyl-Δ^9-THC exhibits activity in the mouse tail flick test for antinociception (analgesia) comparable to the activity of Δ^9-THC. Therefore, these pharmacological results for O-methyl-Δ^9-THC support the results for O,2-propano-Δ^8-THC and reinforce the idea that, although a free phenyl group hydroxyl may be necessary for the production of psychopharmacological activity in the cannabinoids, lone-pair orientation may be an important molecular determinant for the production of cannabinoid analgesia.

FUTURE DIRECTIONS

The results for O,2-propano-Δ^8-THC hold promise in the search for a way to separate cannabinoid analgesia from cannabinoid psychoactivity. Before pursuing a new line of compounds based on O,2-propano-Δ^8-THC, however, prudence dictates that two crucial questions be answered. First, can the analgesic effects produced by O,2-propano-Δ^8-THC be mediated by some mechanism other than a unique cannabinoid mechanism? Twitch response tests of the mouse vas deferens and of the guinea pig ileum have revealed that the effects of O,2-propano-Δ^8-THC are not blocked by naloxone (R.G. Pertwee, unpublished data). These results imply no involvement of opiate receptors in the activity of O,2-propano-Δ^8-THC, Currently, other possible mechanisms for the production of antinociception (analgesia) by O,2-propano-Δ^8-THC are being pursued. Second, what are the metabolic products of O,2-propano-Δ^8-THC? Previous work has indicated that 11-hydroxylation is a prerequisite for the production of analgesia in compounds such as Δ^9-THC (Wilson and May 1975). Currently, the metabolic products of O,2-propano-Δ^8-THC are being studied to determine whether 11-hydroxylation is a major metabolic route for this compound. Analogs of O,2-propano-Δ^8-THC that cannot be metabolized at this position are also being studied to see whether these analogs are still capable of producing analgesia. In addition, the metabolic studies are probing whether an opening of ring D occurs during the metabolism of any of the analogs. If the results of these two lines of inquiry reveal O,2-propano-Δ^8-THC (or one of its metabolites) to be an analgesic that acts by a unique cannabinoid mechanism, then the author intends to design new analogs of this compound that possess enhanced analgesia.

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Cocaine Receptor: A Structure-Activity Relationship Study

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Abuse of (-)-cocaine (1) an alkaloid obtained from the leaves of E. coca, is a major U.S. health problem. While (-)-cocaine has many physiological effects, the most relevant, from the point of view of drug abuse, include its ability to produce euphoria and its reinforcing properties. The latter are readily demonstrated in animal models, and the euphorigenic effects of cocaine are amply documented in human subjects (Johanson and Fischman 1989; Griffiths et al. 1980). In addition to being a powerful reinforcer, cocaine has properties common to other drugs of abuse. For example, tolerance develops to some of its effects, and its psychological withdrawal syndrome takes place over a long period, which includes periods of craving during which relapse to drug use may often occur (Johanson and Fischman 1989; Gawin and Kleber 1986).

To understand the biochemical mechanisms of the above processes, it is necessary to identify the molecular site(s) where the drug interacts to produce its initial physiological effects. Cocaine has several sites of action in the central nervous system; however, it is the site associated with the dopamine transporter that has been implicated in the reinforcing properties of cocaine (Kuhar et al. 1991; Ritz et al. 1987). In fact, the so-called “dopamine hypothesis” (Kuhar et al. 1991; Ritz et al. 1987) (see figure 1 for a pictorial representation of the hypothesis) stipulates that the reinforcing effects of cocaine are brought about by blockage of dopamine reuptake, thereby potentiating dopaminergic transmission. Thus, the cocaine “receptor” (i.e., the dopamine transporter) is ultimately responsible for cocaine’s reinforcing properties. A goal of the research conducted at the Research Triangle Institute in collaboration with the Addiction Research Center of the National Institute on Drug Abuse (NIDA) is to gain information about the structural features of the cocaine receptor.

To obtain information relating to the structural features of (-)-cocaine that lead to potent and selective binding at the cocaine site on the dopamine transporter, the authors and colleagues at the Research Triangle Institute and the Addiction

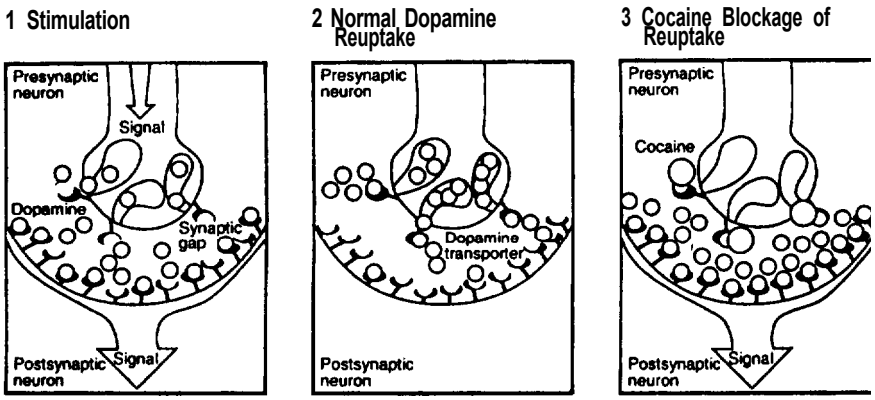
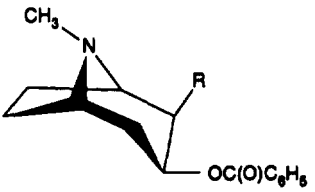


FIGURE 1. *A hypothesis of cocaine's mechanism of* action

Research Center have examined the effects of systematic variations in the structure of cocaine on binding affinity at the dopamine transporter. Studies from our laboratories as well as from other laboratories have shown that, in addition to the overall molecular configuration, the carbomethoxy, the aromatic ring, and the nitrogen moieties of cocaine are important in receptor interactions (Carroll et al. 1991 *a,* 1991 *b*; Reith et al. 1986; Ritz et al. 1990; Abraham et al. 1991; Lewin et al. 1991).

Prior to the work of Carroll and colleagues (1991 a) and Lewin and colleagues (1991), it had been pointed out that the presence of a β-substituent (Reith et al. 1986; Ritz et al. 1990), preferably a carbomethoxy group (Ritz et al. 1990), at C-2 contributed substantially to binding affinity. The results of Carroll and colleagues (1991 *a)* and Lewin and colleagues (1991) (table 1) confirm these observations and suggest a role of the 2β-substituent. The substantial reduction in binding potency observed on replacement of the carbomethoxy group in cocaine by a hydrogen atom (**1a**, no hydrogen acceptor capability) or by a methylenehydroxy group (**1d**, single hydrogen bond acceptor) and the partial restoration of binding potency with a methyleneacetoxy group (1e, with potential for accepting two hydrogen bonds) are consistent with a model in which the 2β-substituent enhances affinity to the receptor by presenting to it one and possibly two hydrogen bond acceptor sites (Lewin et al. 1991). Replacement of the carbomethoxy methyl group in cocaine by bulky and lipophilic groups (table 1, **1f** through **1q**) had only minor effects on binding affinity (Lewin et al. 1991). This tolerance to substitution suggested that the C-2 site may be amenable to the introduction of chemically or photochemically active residues, leading to irreversible ligands; thus, analogs **1r** through **1t** were

TABLE 1. *Potencies of cocaine and 2-substituted analogs in inhibition binding of 0.5 nM [^{3}H]WIN 35,428**



Compound	R	IC_{50}(nM)
Cocaine	CO_2CH_3	102
1a	H	5,180
1b	CO_2H	19,500
1c	$CONHCH_3$	3,180
1d	CH_2OH	561
1e	CH_2OCOCH_3	215
1f	$CO_2C_2H_5$	130
1q	$CO_2(CH_2)_2CH_3$	191
1h	$CO_2CH(CH_3)_2$	211
1i	$CO_2C_6H_5$	112
1j	$CO_2CH_2C_6H_5$	257
1k	$CO_2(CH_2)_2C_6H_5$	248
1l	$CO_2(CH_2)_3C_6H_5$	139
1m	$CO_2CH_2CH = CHC_6H_5$	371
1n	$CO_2(CH_2)_2C_6H_4NO_2$	601
1o	$CO_2(CH_2)_2C_6H_4Cl$	271
1p	$CO_2(CH_2)_2C_6H_4NH_2$	71
1q	$CO_2(CH_2)_2C_6H_4NHCO(CH_2)_2CO_2Et$	86
1r	$CO_2(CH_2)_2C_6H_4$4-N_3	227
1s	$CO_2(CH_2)_2C_6H_4$4-NCS	196
1t	$CO_2(CH_2)_2C_6H_4$ 4-$NHCOCH_2Br$	61

*All values are the mean of four to five experiments performed in triplicate.

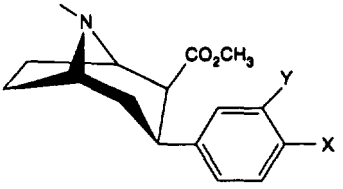
prepared (Lewin et al. 1991). These could be attached to resins and used in affinity chromatography; they also could be prepared with radioisotopic labels and utilized as chemical probes.

The most potent compounds in binding and behavioral studies reported before the authors' studies began were 3β-phenyltropan-2β-carboxylic acid methyl ester (**2a**, WIN 35,065-2) and 3β-(p-fluorophenyl)-tropan-2β-carboxylic acid methyl ester (**2b**, WIN 35,428), the so-called WIN compounds, reported by Clarke and coworkers (1973). Correlation of the IC_{50} values of a series of 3β-(p-substituted phenyl)-tropan-2β-carboxylic acid methyl esters (2) for inhibition of [^{3}H]WIN 35,428 binding to the dopamine transporter (Carroll et al. 1991 *b)* (table 2) with the structural features was carried out by conducting a quantitative structure-activity relationship (QSAR) study using the Comparative Molecular Field Analysis (CoMFA) program (Carroll et al. 1991 *b)*. The substituents (H, F, Cl, CH_3, CF_3, NO_2, NH_2, C_2H_5OCONH, CH_3CONH, C_2H_5CONH, Br, and I) were selected to represent systematic variations. The final model, constructed without cross-validation, had good statistical parameters and a high predictive value. Extension of these studies has revealed that whereas m-substitution of WIN 35,065-2 had virtually no effect on binding potency (table 2, **2p** through **2r**), m-substitution of **2b**, **2c**, and **2i** enhanced binding potency by factors ranging from 5 to 18 (table 2, **2t** through **2w**). These effects are under investigation (Carroll et al. 1992).

The high potency of **2c** prompted investigation of analogs with chemically and photochemically active residues at C-2 (table 3). Highly potent compounds were obtained; however, no correlation of the effect of the C-2 substituent on the binding potencies of the cocaine analogs and of the analogs of **2c** was observed (Carroll et al., in press). Thus, whereas a p-bromoacetamidophenethyl-carboxylate slightly enhanced the potency to displace [^{3}H]WIN 35,428 binding in the cocaine series (**1t**), a fivefold decrease in potency was observed for the **2c** analog **3e** (Carroll et al. 1992). Similar observations were made for other C-2 substituents (see tables 1 and 3), suggesting that additional studies are needed to determine whether cocaine and its WIN analogs bind to the same site.

The effects of substitution at the nitrogen atom (Abraham et al. 1991) of cocaine suggest that decreased electron density is detrimental to binding potency (table 4). An extreme example is the 111 -fold decrease in potency observed for cocaine methiodide (**4d**, full positive charge on nitrogen). Similarly, the acetamide (**4c** partial positive charge on nitrogen) is 33 times less active than cocaine. On the other hand, binding potency is only slightly affected by steric bulk. High binding potency may not require the nitrogen atom to be at the 8 position of the bicyclic skeleton; similar binding potencies are exhibited by **6a** and **5b** (table 4), which are 8-aza and 6-aza analogs of cocaine, respectively.

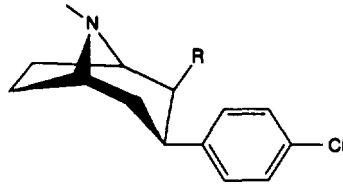
TABLE 2. *Potencies of cocaine and related compounds in inhibiting binding of 0.5 nM [^{3}H]WIN 35,428**



Compound	X	Y	IC_{50}(nM)
(-)-Cocaine	—	—	102
2a	H	H	23.0
2b	F	H	15.7
2c	Cl	H	1.17
2d	CH_3	H	1.71
2e	CF_3	H	13.1
2f	CH_3O	H	8.14
2g†	H†	H	257
2h	NO_2	H	10.1
2i	NH_2	H	24.8
2j	C_2H_5OCONH	H	316
2k	CH_3CONH	H	64.2
2l	C_2H_5CONH	H	68.0
2m	Br	H	1.81
2n	I	H	1.26
2o	N_3	H	2.12
2p	H	Cl	10.6
2q	H	F	23
2r	H	I	26.1
2s	NH_2	I	1.35
2t	F	CH_3	2.95
2u	NH_2	Br	3.91
2v	Cl	Cl	0.79
2w	Cl	CH_3	0.81

*All values are the mean of four to five experiments performed in triplicate.
†3β-(2-Thiophenyl)-tropan-2 β-carboxylic acid methyl ester.

TABLE 3. *Potencies of cocaine end analogs in inhibiting binding of 0.5 nM WIN 35,428**

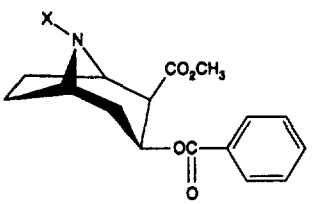


Compound	R_2	IC_{50}(nM)
Cocaine	CO_2CH_3	102
2c	CO_2CH_3	1.17
3a	$CO_2(CH_2)_2C_6H_4$4-NO_2	2.71
3b	$CO_2(CH_2)_2C_6H_4$4-NH_2	2.16
3c	$CO_2(CH_2)_2C_6H_4$4-N_3	6.17
3d	$CO_2(CH_2)_2C_6H_4$ 4-NCS	5.3
3e	$CO_2(CH_2)_2C_6H_4$ 4-$NHCOCH_2Br$	1.73
3f	$CO_2(CH_2)_2C_6H_3$ 3-I, 4-NH_2	2.51
3g	$CO_2(CH_2)_2C_6H_3$ 3-I ,4-N_3	14.5
3h	CO_2H	2,070

*All values are the mean of four to five experiments performed in triplicate.

The progress made in the development of highly potent ligands for the cocaine receptor at the dopamine transporter has enabled the identification of a neuroaminidase-sensitive polypeptide of approximately 80 kDa by photoaffinity labeling with [^{125}I]3g (Patel et al. 1992). Apparently, the same polypeptide was labeled by [^{125}I]1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl] piperazine (DEEP) (Grigoriadis et al. 1989), providing strong evidence that WIN analogs and GBR analogs may bind to the same protein in rat striatal membranes (Patel et al. 1992). Furthermore, [^{123}I]RTI-55 (compound 2n, table 2) has been used to image the dopamine and serotonin transporters in vivo (Carroll et al. 1991*c*).

TABLE 4. *Potencies of cocaine and* analogs *in inhibiting binding of [^{3}H]WIN 35,428**



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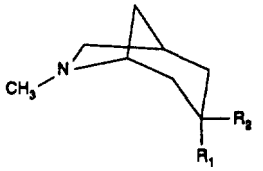

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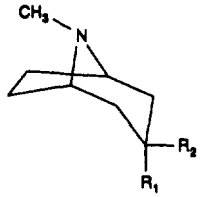

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Compound	X	R_1	R_2	IC_{50} (μM)
Cocaine	CH_3			0.102
4a	H			0.303
4b	$C_6H_5CH_2$			0.676
4c	CH_3CO			3.37
4d	$(CH_3)_2^+$			10.70
5a		$C_6H_5CO_2$	H	19.8
5b		H	$C_6H_5CO_2$	4.95
(+)-5b		H	$C_6H_5CO_2$	2.94
(-)-5b		H	$C_6H_5CO_2$	2.95
6a		H	$C_6H_5CO_2$	5.18
6b		$C_6H_5CO_2$	H	21.2

*All values are the mean of four to five experiments performed in triplicate.

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The Use of Conformational Restriction and Molecular Modeling Techniques in the Development of Receptor-Specific Opioid Peptide Agonists and Antagonists

Peter W. Schiller, Thi M.-D. Nguyen, Grazyna Weltrowska, Nga N. Chung, Carole Lemieux, Brian J. Marsden, and Brian C. Wilkes

INTRODUCTION

Conformational restriction is now generally recognized as an important principle in the design of analogs of peptide hormones and neurotransmitters with certain desired biological activity profiles. Many of the linear, bioactive peptides are very flexible molecules that exist in a conformational equilibrium in solution and that are not very selective in their interactions with different receptor types because conformational adaptation to various receptor topographies is possible. Obviously, the incorporation of conformational constraints into such peptides enhances their conformational integrity and, in case the resulting analogs retain biological activity, conformational studies aimed at elucidating bioactive conformations become possible. The results of such conformational studies by either theoretical or experimental techniques may suggest further structural modifications or rigidifications that ultimately will lead to the development of peptidomimetics (for a review, see Schiller, in press). Conformational restriction also may affect both receptor affinity and the intrinsic activity ("efficacy") of a peptide. In a situation of receptor heterogeneity, conformationally restricted peptide analogs often show improved receptor selectivity because the introduced conformational constraints may be compatible with the conformational requirements of one preferred receptor class only and no longer permit significant interaction with other receptor types, as first demonstrated with conformationally restricted enkephalin analogs (Schiller and DiMaio 1982). Conformational restriction of a peptide hormone or neurotransmitter may also decrease its intrinsic activity, resulting in either antagonists or partial agonists. This was unambiguously demonstrated for the first time with the development of the δ--opioid receptor antagonist H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2) (Schiller

et al., in press) (see below). Conformational restriction of peptides can be achieved either locally at a particular amino acid residue through incorporation of backbone or side-chain conformational constraints or more globally through peptide cyclizations.

Whereas the existence of at least three major opioid receptor classes (μ, δ, κ) is now well established, the development of potent opioid agonists and antagonists with high specificity for each receptor type and of ligands with receptor-specific agonist/antagonist properties continues to be an important goal in opioid pharmacology. Progress made in the development of receptor-specific opioid peptide analogs with agonist or antagonist properties has been described in recent review articles (Hruby and Gehrig 1989; Renugopalakrishnan et al. 1990; Schiller 1991). Receptor-selective peptide ligands have been developed by using the classical amino acid substitution/ deletion approach, the bivalent ligand strategy, or the concept of conformational restriction. The use of these three design principles produced highly selective μ- and δ-agonists and antagonists, whereas the development of opioid peptide-derived agonists and antagonists with selectivity for κ-OpiOid receptors has been less successful to date. Conformationally restricted peptide agonists and antagonists with high preference for either μ- or δ-opioid receptors include both cyclic analogs and analogs in which local conformational constraints were imposed at a particular amino acid residue.

In this chapter, the authors describe various opioid peptide analogs in which conformationally restricted analogs of Phe and/or Tyr (figure 1) were substituted. In the first part of the chapter, we show that replacement of the Phe^3 residue in the relatively nonselective cyclic dermorphin analog H-Tyr-D-Orn-Phe-Glu-NH_2 with 2-aminoindan-2-carboxylic acid (Aic) or 2-aminotetralin-2-carboxylic acid (Atc) (figure 1) resulted in highly μ-selective compounds. We then discuss how substitution of the exocyclic Tyr residue in the Aic^3-analog with 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) permitted further structural rigidification without producing a significant change in the opioid activity profile. Finally, we describe a new class of peptide analogs containing the N-terminal sequence H-Tyr-Tic-Phe- that are potent and highly selective δ-antagonists (Tic=tetrahydroisoquinoline-3-carboxylic acid).

CYCLIC DERMORPHIN ANALOGS CONTAINING CONFORMATIONALLY RESTRICTED PHENYLALANINE AND TYROSINE RESIDUES

The cyclic dermorphin tetrapeptide analog H-Tyr-D-Orn-Phe-GIu-NH_2 (1) displayed very high affinity for p-receptors but also bound quite strongly to δ-receptors and, therefore, was only slightly μ-selective (table 1) (Schiller et al. 1991). Configurational inversion at the 3-position residue (analog 2) resulted in a more than 1,000-fold drop in affinity for both μ- and δ-receptors. This finding confirms the strict requirement for L-configuration at the 3-position

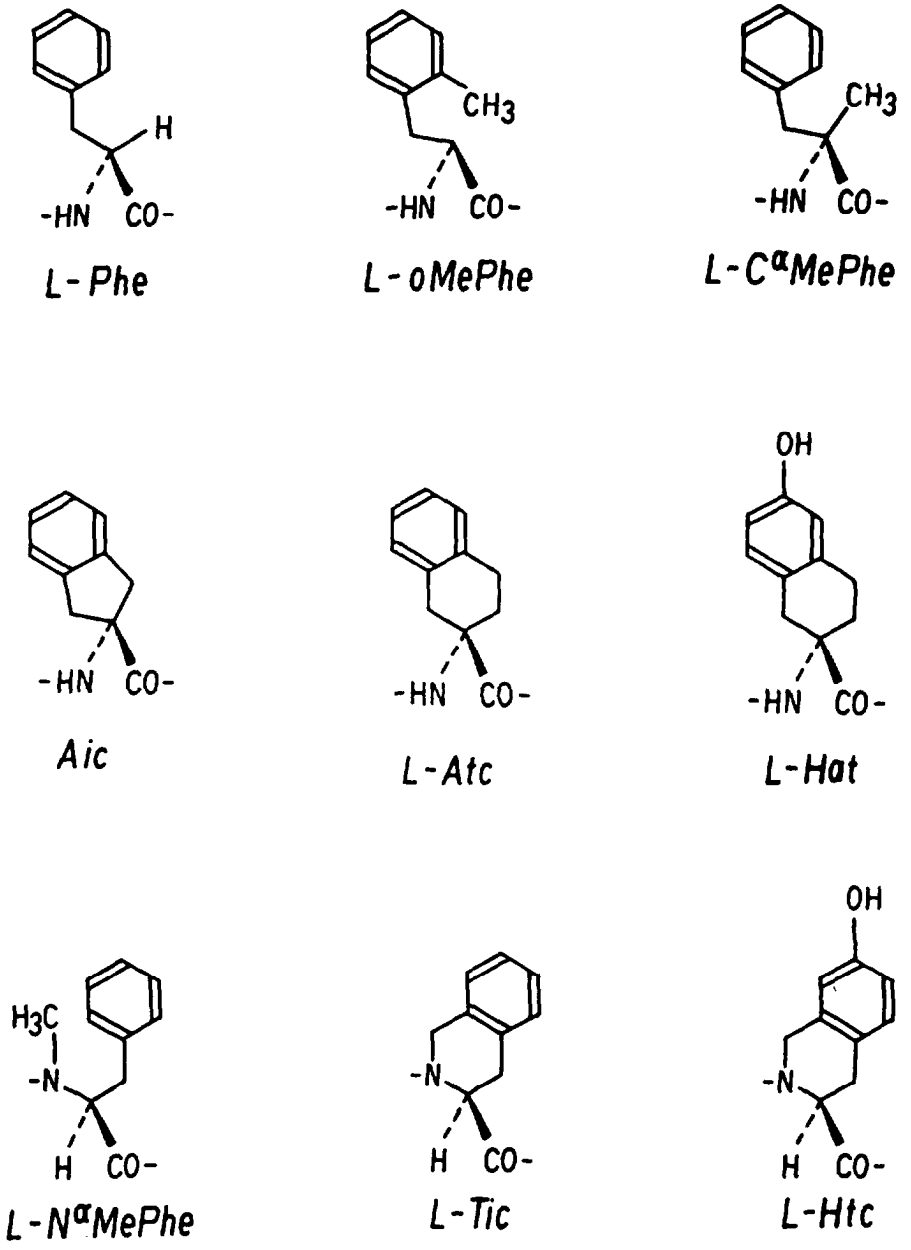

FIGURE 1. *Structural formulas of conformationally restricted phenylalanine residues*

of both linear and cyclic opioid peptide analogs that are structurally related to the dermorphin family (Schiller et al. 1987). Methylation of the Phe^3 α-nitrogen of parent peptide **1** also resulted in a weakly active analog (**3**). The more than 100-fold decrease in μ-affinity observed with this compound could be due to the introduced local conformational restriction of the peptide backbone (φ-angle), the unavailability of the amide proton in the 3-position for the formation of an important hydrogen bond, or the bulk of the N-methyl group that might not be tolerated at the receptor. In view of the low affinity of analog **3**, it was not surprising that the Tic^3-analog (compound **4**) was also very weakly active. Introduction of a methyl group in the ortho position of the Phe^3 aromatlc ring (analog **5**) had little effect on μ-receptor affinity and selectivity. Methylation of the α-carbon of the Phe^3 residue (analog **6**) produced a sevenfold decrease in μ-affinity and a similar decrease in δ-affinity, resulting in a compound with still only modest preference for μ-receptors over δ-receptors. C^{α}-methylation of an amino acid residue in a peptide drastically reduces the conformational space available to the peptide backbone around that residue insofar as the torsion angles at the position of the methylated residue are limited to values around φ=-50°, ψ =-50° and φ=+50°, ψ=+50° (Marshall and Bosshard 1972). The

TABLE 1. *Receptor affinities and μ-selectivity of cyclic dermorphin analogs**

No.	Compound	K_i^{μ} [nM]	K_i^{δ} [nM]	K_i^{δ}/K_i^{μ}
1	H-Tyr-D-Orn-Phe-GluNH$_2$	0.981	3.21	3.27
2	H-Tyr-D-Orn-D-Phe-Glu-NH,	1,660	14,000	8.43
3	H-Tyr-D-Orn-N$^{\alpha}$MePhe-Glu-NH$_2$	1,000	12,800	12.8
4	H-Tyr-D-Orn-Tic-Glu-NH$_2$	2,410	50,100	20.8
5	H-Tyr-D-Orn-oMePhe-Glu-NH$_2$	1.99	9.22	4.80
6	H-Tyr-D-Orn-C$^{\alpha}$-MePhe-Glu-NH$_2$	7.17	54.8	7.82
7	H-Tyr-D-Orn-Aic-Glu-NH$_2$	4.21	209	49.8
8	H-Tyr-D-Orn-(D or L)-Atc-Glu-NH$_2$ (I)	8.28	1,570	190
9	H-Tyr-D-Orn-(D or L)-Ate-Glu-NH$_2$ (II)	26.3	3,510	133
10	H-(D or L)-Htc-D-Orn-Phe-Glu-NH$_2$ (I)	1,680	18,200	10.8
11	H-(D or L)-Htc-D-Orn-Phe-Glu-NH$_2$ (II)	235	7,050	30.0
12	H-(D or L)-Hat-D-Orn-Phe-Glu-NH$_2$ (I)	2.91	10.8	3.71
13	H-(D or L)-Hat-D-Orn-Phe-Glu-NH$_2$ (II)	54.2	74.7	1.38
14	H-(D,L)-Hat-D-Orn-Aic-Glu-NH$_2$	7.68	119	15.5
	[Leu^5]enkephalin	9.43	2.53	0.268

**Determinations based on displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane binding sites*

slight affinity drop observed with analog **6** could be due either to the backbone conformational constraints introduced at the 3-position or to steric interference by the C^{α}-methyl group.

More extensive conformational restriction at the 3-position was achieved through substitution of the conformationally restricted phenylalanine analog Aic(figure 1). In comparison with parent peptide **1**, the cyclic peptide analog H-Tyr-D-Orn-Phe-Glu-NH_2(**7**) showed only 4 times lower u-receptor affinity but 65 times lower affinity for &receptors and, consequently, greatly improved μ-selectivity (K_i^{δ}/K_i^{μ}=49.6). Because analogs **5** and **6** are distinguished from analog 7 merely by the opening of one or the other of two adjacent bonds in the five-membered ring structure of Aic^3 (see figure 1), comparison of the receptor binding activity profiles of these three analogs permits the unambiguous conclusion that the high p-receptor selectivity of the Aic^3-analog is *exclusively* the consequence of the imposed side-chain conformational restriction. Replacement of Phe^3 with Aic in the cyclic peptide structure produces conformational constraints both in the side chain and in the peptide backbone at the 3-position. Torsional angles of the 3-position side chain in the Aic^3 analog are limited to values of x_1=-80°, x_2=-20° and x_1=-160°, x_2=+20°, whereas the ϕ·, ψ·-backbone torsional angles around the 3-position are limited to the same values as in the case of the C^{α} $MePhe^3$ analog. These torsional angles were indeed observed in molecular dynamics simulations that were carried out at 600 K and lasted for 100 ps. The limited conformational space available to the Aic^3 side chain and its transitions between the two conformational states defined by the x_1-, x_2-angles indicated above are evident in figure 2, which shows superpositions of 20 snapshots of the Phe^3- and Aic^3-analogs taken at 5-ps intervals along the dynamics trajectory.

The two diastereoisomers of the Atc^3-analog (compounds **8** and **9**) show even higher μ-selectivity (K_i^{δ}/K_i^{μ} values of 190 and 133, respectively) due to very weak affinity for the b-receptor, while μ-receptor affinity comparable to that of [Leu^5]enkephalin is maintained (table 1). As in the case of the Aic^3 analog, it is the conformational restriction per se introduced at the 3-position that is directly responsible for the drastic selectivity enhancement. Again, the Atc residue essentially is able to adopt only two side-chain conformations that are characterized by the torsional angles x_1=180° (t), x_2=+25° and x_1=-60° (g^-), x_2=-25° for L-Atc, and x_1=180° (t), x_2=-25° and x_1=+60° (g^+), x_2=+25° for D-Atc. Evidently, these side-chain conformational constraints are even more detrimental to δ-receptor affinity than those present in the Aic^3 analog that has about 10 times higher δ·-affinity than the Atc^3 analogs. Interestingly, the computer simulations showed that in the cyclic opioid peptide analogs described here both the L- and the D-Atc side chains underwent fewer conformational transitions than the Aic side chain (Wilkes and Schiller, in press).

FIGURE 2. *Twenty snapshots of H-Tyr-D-Orn-Phe-Glu-NH_2 (left) and H-Tyr-D-Orn-Aic-Glu-NH_2 (right) taken at 5-ps intervals along the dynamics trajectory*

As indicated above, the μ-receptor affinity of the cyclic dermorphin analog H-Tyr-D-Orn-Phe-Glu-NH_2 is more than 1,000 times higher than that of the corresponding D-Phe[3]-analog, whereas the μ-receptor affinities of both diastereoisomers of H-Tyr-D-Orn-(D,L)-Atc-Glu-NH_2 are high and differ from one another by a factor of only about 3. This loss of stereospecificity as a consequence of side-chain conformational restriction may be due to the fact that the D-Atc[3]-analog binds to the receptor in a manner different from that of the D-Phe[3]-analog. In the case of the D-Phe[3]-analog, a stepwise process of binding according to the "zipper"-type model (Burgen et al. 1975) may occur such that the D-Phe[3] side chain never has a chance to bind to the hydrophobic receptor subsite with which the L-Phe[3] aromatic ring interacts. On the other hand, the process of binding of not only the L-Atc[3]- but also the D-Atc[3]-analog is such that the Atc aromatic ring is forced to interact with this binding site as a consequence of the conformational constraints existing at the 3-position of the peptide sequence (Schiller et al. 1991).

As the results of the computer simulations (figure 2) show, the exocyclic tyrosine residue in the cyclic analogs described above retains considerable orientational freedom and also needs to be conformationally restricted to obtain better insight into the μ-receptor-bound conformation of this type of cyclic analog. This was achieved by replacement of Tyr[1] with the conformationally restricted tyrosine analogs 7-hydroxytetrahydroisoquinoline-3-carboxylic acid (Htc) or Hat (see figure 1). Both diastereoisomers of H-(D or L)-Htc-D-Orn-Phe-Glu-NH_2 (**10**, **11**) had relatively low receptor affinity, indicating that the conformational constraints resulting from replacement of Tyr[1] with Htc

are not well tolerated. On the other hand, one of the diastereoisomers of H-(D or L)-Hat-D-Orn-Phe-Glu-NH_2 (**12**, **13**) showed an opioid receptor affinity profile similar to that of **1**. High p-receptor affinity was also displayed by H-(D,L)-Hat-D-Orn-Aic-Glu-NH_2 (**14**) that essentially contains only two freely rotatable bonds (figure 3) and, therefore, represents the most rigid opioid peptide analog reported to date. Considering the fact that [Leu^5]enkephalin contains 16 freely rotatable bonds, the structural rigidification achieved with analog 14 is indeed quite remarkable. A molecular mechanics study revealed that the lowest energy conformation of H-Hat-D-Orn-Aic-Glu-NH_2 is still characterized by a close proximity between the two aromatic rings (figure 4), a structural feature that may be important for high μ-receptor affinity within this class of cyclic opioid peptide analogs (Schiller and Wilkes 1988).

All the cyclic opioid peptide analogs described above behaved as agonists in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, and none of them showed significant affinity for κ-receptors.

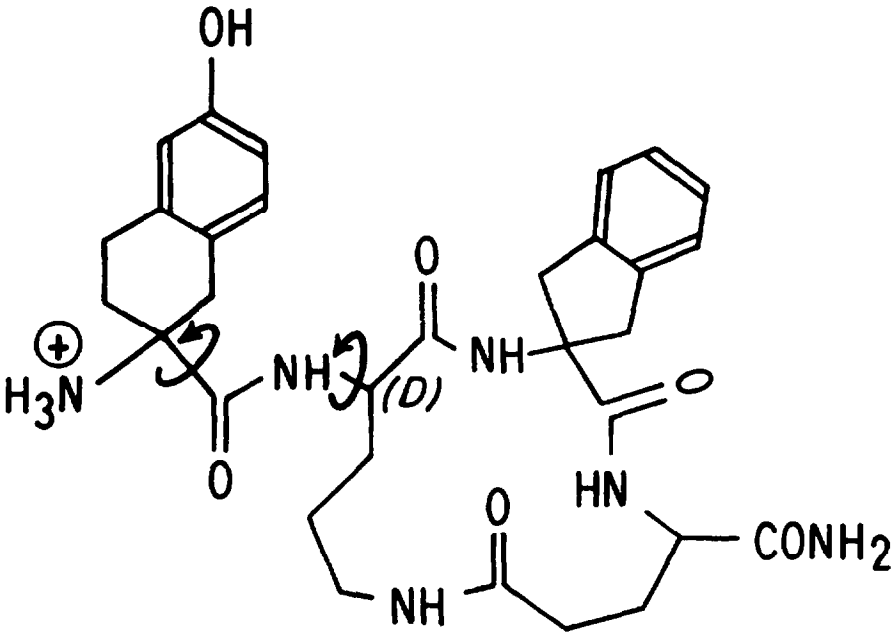

FIGURE 3. *Structural representation, of the rigidified cyclic opioid peptide analog H-(D,L)-Hat-D-Orn-Aic-Glu-NH_2*

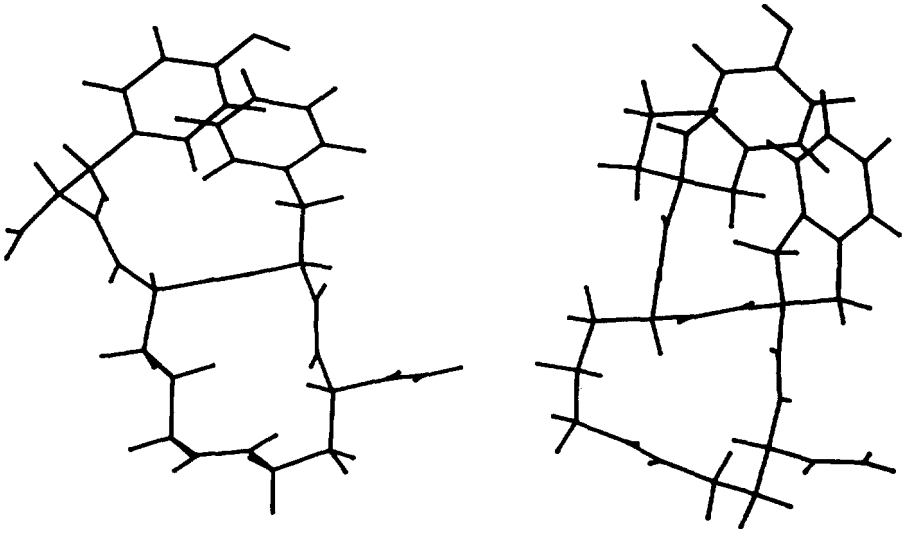

FIGURE 4. *Lowest energy conformers of H-Tyr-D-Orn-Phe-Glu-NH_2 (left) and H-Hat-D-Orn-Aic-Glu-NH_2 (right)*

THE TIPP PEPTIDE FAMILY: A NEW CLASS OF POTENT AND HIGHLY SELECTIVE δ-ANTAGONISTS

The a-position residue in opioid peptides can be replaced with a number of D-amino acid residues, including aromatic ones, and the resulting compounds generally not only are resistant to degradation by aminopeptidases but also often show high opioid activity. For example, the tetrapeptide H-Tyr-D-Phe-Phe-Phe-NH, is a potent, full agonist with preference for u-receptors over b-receptors (Schiller et al., in press). The latter peptide consists entirely of aromatic amino acid residues that can be conformationally restricted in a number of ways and that provide an opportunity to systematically study the effect of such conformational constraints on opioid receptor affinities and on the intrinsic activity ("efficacy"). Conformational restriction at the 2-position of the peptide sequence through substitution of a D- or L-Tic residue turned out to be particularly interesting (Schiller et al., in press). The D-Tic[2] tetrapeptide analog (**15a**) was a potent u-receptor ligand with considerable preference for μ-receptors over δ-receptors (table 2). On the other hand, the L-Tic[2]-analog (**15b**) showed relatively weak u-affinity and, very surprisingly, greatly enhanced δ-affinity. Thus, H-Tyr-D-Tic-Phe-Phe-NH_2(**15a**) turned out to be μ-selective, whereas its diastereoisomer, H-Tyr-Tic-Phe-Phe-NH_2(**15b**), showed considerable δ-selectivity (K_i^{μ}/K_i^{δ}=26.3). The opposite receptor selectivity profiles of analogs **15a** and **15b**, which contain stereoisomers of

TABLE 2. *Receptor affinities of opioid peptide analogs containing Tic at position 2**

No.	Compound	K_i^{μ} [nM]	K_i^{δ} [nM]	K_i^{μ}/K_i^{δ}
15a	H-Tyr-D-Tic-Phe-Phe-NH_2	7.30	519	0.0141
15b	H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2)	76.8	3.00	28.3
16	H-Tyr-Tic-Phe-Phe-OH (TIPP)	1,720	1.22	1410
17a	H-Tyr-D-Tic-Phe-NH_2	121	396	0.306
17b	H-Tyr-Tic-Phe-NH_2 (TIP-NH_2)	624	12.0	52.0
18	H-Tyr-Tic-Phe-OH (TIP)	1,280	9.07	141
	Naltrindole	12.2	0.687	17.8
	[Leu^5]enkephalin	9.43	2.53	3.73

*Affinities were determined as described in footnote to table 1.

a rigid amino acid at the 2-position, demonstrate that μ- and δ-receptors have different stereochemical architectures.

Analogs **15a** and **15b** were both full agonists in the μ-receptor-representative GPI assay with potencies that were in agreement with their relative p-receptor affinities determined in the binding assays. In the δ-receptor-representative MVD assay, the D-Tic^2-analog was a relatively weak but nearly full agonist, Thus, H-Tyr-D-Tic-Phe-Phe-NH_2 is a potent and selective p-agonist. Most surprisingly, the L-Tic^2-analog showed no agonist potency in the MVD assay at concentrations as high as 10 μM despite its demonstrated higH δ-receptor affinity, and it turned out to be a potent, selective δ-antagonist with K_e values ranging from 14 to 18 nM against various selective δ-agonists, including [D-Ala^2]deltorphin I and [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (table 3). The observation that configurational inversion at a single conformationally restricted amino acid residue can turn an agonist with selectivity for one receptor type

TABLE 3. *K, values determined for δ-antagonists against variousδ--agonists in the MVD assay*

	K_e [nm]		
Antagonist	[Leu^5]enkephalin	[D-Ala^2]deltorphin I	DPDPE
TIPP	5.88	2.98	4.80
TIPP-NH_2	15.7	14.4	18.0
TIP	11.7	12.6	16.1
TIP-NH_2	43.9	58.9	96.8
Naltrindole	0.850	0.632	0.636

into an antagonist with preference for another receptor class is unique and to the best of our knowledge has not been made previously in the peptide field.

It was interesting to note that the δ--antagonist H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2) was a full agonist in the GPI assay with about 15 percent the potency of [Leu^5]enkephalin. The effect of this compound on the ileum was very sensitive to naloxone inhibition (K_e=1.74±0.45 nM), indicating that It was mediated by μ-receptors. It thus turns out that TIPP-NH_2 is the first known opioid compound with μ-agonist/ δ--antagonist properties.

Recently, it has been demonstrated that pretreatment of mice with the nonpeptlde bantagonist naltrindole (Portoghese et al. 1988) prevented the development of morphlne tolerance and dependence (Abdelhamld et al. 1991). This important observation led to the suggestion that compounds with μ-agonist and δ--antagonist properties might have potential as analgesics that do not produce tolerance and physical dependence.

Because it has been clearly established that enkephalin analogs with a free C-terminal carboxylate function are in general more δ-selective than corresponding peptlde amides (Paterson et al. 1984), a Tic^2-tetrapeptide analog (16) with a free C-terminal COOH group was synthesized in an attempt to obtain a more selective δ-antagonist. This compound, H-Tyr-Tic-Phe-Phe-OH (TIPP), showed further improved δ-receptor affinity (K_i^{δ}=1.22 nM) and drastically diminished affinity for μ-receptors (table 2). In fact TIPP displayed extraordinary δ-receptor selectivity (K_i^{μ}/K_i^{δ}=1,410) and in direct comparisons turned out to be more selective than any other δ-agonist or antagonist described to date. In the MVD assay, TIPP was a potent antagonist (K_e=3 to 6 nM) against various δ-agonists (table 3) and had no agonist effects at concentrations as high as 10 μM. As expected on the basis of the receptor binding data, TIPP was a very weak agonist in the GPI assay (IC_{50}>10 μM)

and, most important, produced no antagonist effects at concentrations up to 10 μM in this μ-receptor-representative bioassay system.

In comparison with the nonpeptide antagonist naltrindole, TIPP showed about twofold lower δ-receptor affinity in the binding assays (table 2) and fivefold to sevenfold higher K_e values against δ-agonists in the MVD assay (table 3). Therefore, TIPP appears to be a somewhat less potent antagonist than naltrindole but, on the other hand, is about 80 times more δ-selective than the nonpeptide δ-antagonist (table 2). Naltrindole has u-receptor affinity in the nanomolar range and is a μ-antagonist with a reported K_e value of 29 nM against morphine in the GPI assay (Portoghese 1991). TIPP is also at least 10 times more potent and 10 times more δ-selective than the enkephalin-derived δ-antagonist N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI174864) (Cotton et al. 1984; Corbett et al. 1984). Because of its high potency, unprecedented δ-selectivity, and complete lack of μ-antagonist properties, TIPP offers several advantages as a δ-antagonist for use in opioid research.

The role of the Phe^4 residue in defining the opioid activity profiles of compounds **15a**, **15b**, and **16** was assessed by preparing and characterizing the corresponding des-Phe^4-tripeptide analogs (**17a**, **17b**, **18**). Like the D-Tic^2-tetrapeptide analog(**15a**),H-Tyr-D-Tic-Phe-NH_2(**17a**) was a μ-selective agonist, but its p-receptor affinity was lower, and it was less μ-selective (table 2). Its diastereoisomer H-Tyr-Tic-Phe-NH_2 (TIP-NH_2) (**17b**) was about twice as δ-selective as its tetrapeptide parent (TIPP-NH_2), but it had about four times weaker δ-antagonist potency. As in the case of the tetrapeptides, the corresponding tripeptide with a free C-terminal carboxyl group, H-Tyr-Tic-Phe-OH (TIP) (**18**), showed higher δ-affinity, was more δ-selective, and was a more potent δ-antagonist against various δ-agonists in the MVD assay with K_e values ranging from 12 to 16 nM. In comparison with its tetrapeptide parent (TIPP), TIP was 10 times less δ-selective and had about 4 to 7 times lower δ-antagonist potency. It can be concluded that deletion of the C-terminal Phe residue in compounds **15a, 15b,** and **16** produced tripeptide analogs with opioid activity profiles that were qualitatively similar to those of the corresponding parent tetrapeptides.

None of the D- or L-Tic analogs described above showed significant affinity for κ-OpiOid receptors, as determined by displacement of [^{3}H]U69,593 from guinea pig brain membranes. It is highly unlikely that the activity profiles determined with these analogs were distorted by enzymatic degradation, because incubations in the receptor binding experiments were performed at 0°C (DiMaio and Schiller 1980). Furthermore, nearly all the Tic^2-analogs described here should be quite enzyme-resistant even at higher temperatures because of their structural characteristics.

A molecular mechanics study based on a systematic energy minimization approach was performed with the tripeptides H-Tyr-D-Tic-Phe-NH_2 (**17a**) and H-Tyr-Tic-Phe-NH_2 (**17b**). The resulting lowest energy conformations of the two compounds were compared by superimposing their Tyr and Tic residues (figure 5). Most strikingly, this spatial superimposition revealed that the Phe^3 side chains of these diastereoisomeric peptides are located on opposite sides of the plane defined by the Tic residue as a consequence of the conformational constraints existing at the Tic^2 residue. This difference in the orientation of the Phe^3 residue may explain the different opioid activity profiles of the two diastereoisomers in structural terms.

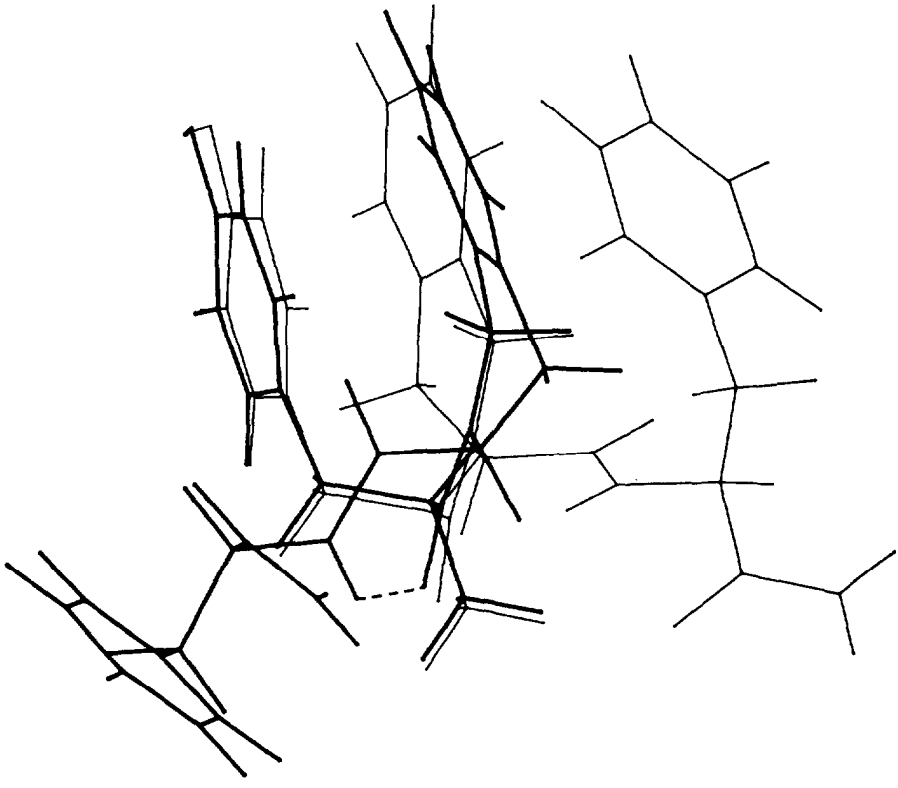

FIGURE 5. *Superimposition of lowest energy conformers of H-Tyr-Tic-Phe-NH, (heavy lines) and H-Tyr-D-Tic-Phe-NH_2 (light lines). An inverse c-turn stabilized by a Tyr^1-CO←HN-Phe^3 hydrogen bond is observed in the case of the L-Tic^2-analog.*

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Probes for the Cannabinoid Sites of Action

Alexandros Makriyannis

INTRODUCTION

Cannabinoids produce a complex pattern of pharmacological actions, some of which are believed to be related to their effects on cellular membranes (Martin 1986; Hillard et al. 1990; Leuschner et al. 1984); others are thought to be produced through an interaction with one or more cannabinoid receptors (Devane et al. 1989, 1988; Herkenham et al. 1990; Bidaut-Russel et al. 1990). Existing evidence indicates that the membrane effects involve cannabinoid interactions with noncatalytic amphipathic sites, resulting in perturbation of the membrane and a modification of the functions of membrane-associated proteins. There is also evidence that the cannabinoid:membrane interactions are governed by stereoelectronic requirements of a varied degree of specificity.

Regarding the receptor-mediated effects, cannabinoids are believed to first partition in the membrane where they assume a specific orientation and location and then laterally diffuse across the bilayer to reach their receptor site(s). Thus, for a productive interaction with its active site(s), a cannabinoid must fulfill two criteria. First, it must possess certain pharmacophores in a specific stereochemical arrangement (stereoelectronic requirements). Second, it must assume an appropriate orientation and location in the bilayer that would allow an optimal alignment of its pharmacophores with the corresponding binding components at the active site (drug:membrane interaction requirements).

CANNABINOID RECEPTORS

Although it had been generally recognized that at least some of the cannabinoid effects are due to interactions with specific receptor sites, direct evidence for a cannabinoid receptor has become available only recently. This evidence comes from experiments in which the tritiated cannabinoid-like ligand [^{3}H]CP-55940 was shown to bind stereospecifically to a brain membrane preparation (Devane et al. 1989). The relative abilities of cannabinoid analogs to inhibit [^{3}H]CP-55940 binding were found to parallel their potencies for producing behavioral effects in animals (Devane et al. 1988) and for regulating adenylate cyclase in vitro (Hewlett 1985). The same tritiated ligand was also used in autoradiographic

experiments for cannabinoid receptor distribution in brain sections from several mammalian species, including humans (Herkenham et al. 1990). The study revealed a unique and conserved distribution, with the highest "receptor" density in the basal ganglia, hippocampus, and cerebellum. More recently, the receptor was cloned. The cDNA was then injected in CHO-K_4 cells, and a cannabinoid-responsive G protein-coupled receptor was expressed (Matsuda et al. 1990).

CANNABINOID:LIPID INTERACTIONS

Interactions of drug molecules (including cannabinoids) with membrane lipids are generally discussed in terms of changes in the bulk properties of the lipid component, which are often described as changes in membrane fluidity. However, there is increasing evidence from a variety of sources for specific drug:membrane interactions. For example, evidence has been presented for the formation of Δ^9-trahydrocannabinol (Δ^9--THC):phospholipidcomplexes, the stoichiometry and stability of which varied depending on the nature of the phospholipids in the membrane (Bruggemann and Melchior 1983). More detailed evidence comes from the work of the author and colleagues showing that cannabinoids assume specific orientation in the phospholipid bilayers (Makriyannis et al. 1989).

Because of their high lipid solubility, cannabinoids partition preferentially in the lipid component of the membrane. The cannabinoid:lipid interactions can play two roles in determining drug activity: (1) Cannabinoids may affect the functions of membrane-associated proteins by perturbing their lipid environment, and (2) cannabinoids may diffuse across the membrane lipid bilayer (lateral diffusion) to reach a specific site of action on the cannabinoid receptor. It has been argued for other groups of lipophilic drugs (Herbette et al. 1986), and evidence was provided, that the location and orientation of the drug molecule in the membrane is critical in determining its ability to reach its site(s) of action in the proper orientation and/or conformation for a productive interaction. The author and coworkers have studied the location and orientation of several cannabinoids in model and biological membranes and have developed arguments correlating these properties with their abilities to interact with the receptor (Makriyannis et al. 1991; Mavromoustakos et al. 1990) .

DESIGNING PROBES FOR CANNABINOID RECEPTORS

To better understand the molecular basis of cannabinoid activity, the author and colleagues have sought to study in detail the interactions of some important cannabinoid analogs with their respective sites of action. Such studies have involved the combined use of a variety of biophysical and biochemical methods that could only be realized with the successful design and synthesis of

appropriate molecular probes. This chapter's goal is to describe some of these molecular probes, their design, and their applications.

The various methods used are aimed at answering the following questions:

- What are the conformational properties of the drug molecule in solution and in the membrane?
- What orientation does the drug molecule assume when it partitions in the membrane?
- What is the location of the drug molecule in the membrane bilayer?
- What is the distribution of cannabinoid receptors in mammalian brains? How can one follow the effects of a cannabinoid on the biochemistry of brain function?
- How can the cannabinoid receptor be covalently labeled? Can this approach be used for receptor isolation and characterization?

Cannabinoid Conformations

To explore the conformational requirements for interaction with their sites of action on the cannabinoid receptor, pairs of cannabinoid analogs are compared that differ only in one structural feature but exhibit widely different pharmacological potencies and affinities for the receptor. The conformational properties of the drug molecules in solution and in membrane-like environments can be studied using multinuclear one- and two-dimensional high-resolution nuclear magnetic resonance (NMR). For example, the results of comparing the two isomeric cannabinoids (Δ^{9}-THC and $\Delta^{9,11}$-THC) (Kriwacki and Makriyannis 1989) led to the suggestion that a slight deviation from planarity in the carbocyclic ring C of the cannabinoid, as is the case with the pharmacologically potent Δ^{9}-THC, is a requirement for activity (figure 1). Conversely, the much less potent $\Delta^{8,11}$-analog has all three rings coplanar. The same conclusions were reached by Reggio and colleagues (1989), who compared the theoretically obtained conformations of the above two analogs. Currently, the author's research group is synthesizing suitably designed cannabinoids to test the hypothesis regarding the role of ring C conformation in pharmacological activity.

Cannabinoid Orientation in the Membrane

As with many other membrane-active lipophilic molecules, cannabinoids preferentially partition in the membrane where they assume a thermodynamically favorable orientation and location. In this preferred orientation and location, the

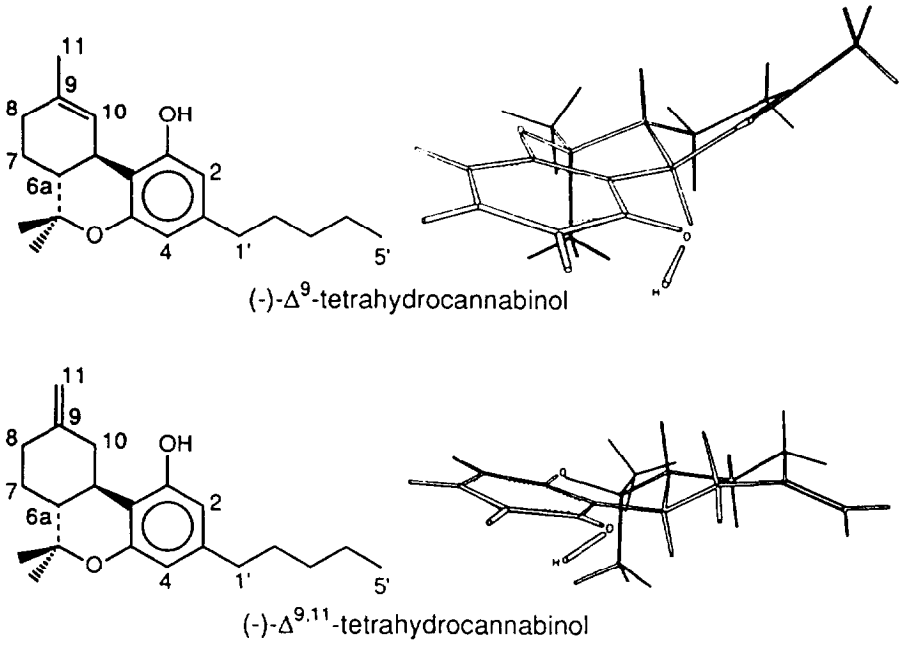


FIGURE 1. *Left: Structures of Δ9-tetrahydrocannabinol and Δ9,11-tetrahydrocannabinol. Right: Drawings showing the experimentally determined conformations of Δ9-THC and Δ9,11-THC.*

SOURCE: Kriwacki, R.W., and Makriyannis, A. The conformational analysis of Δ9- and Δ9,11-tetrahydrocannabinols in solution using high resolution nuclear magnetic resonance spectroscopy. *Molecular Pharmacology* 35:495-503, © American Society for Pharmacology and Experimental Therapy, 1989.

cannabinoid experiences fast lateral diffusion within the membrane bilayer. As mentioned earlier, these features of the drug:membrane interaction are believed to play an important role in determining the drug's ability to interact with its site of action on the receptor.

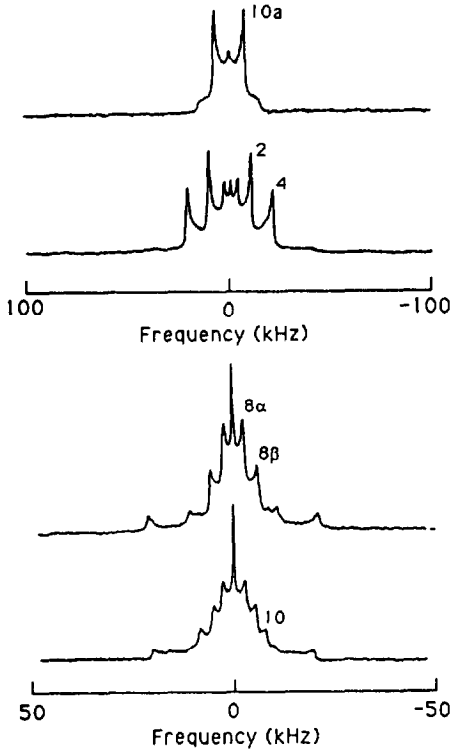
The orientation of the cannabinoid molecule in the membrane can be calculated from the solid-state [^{2}H]-NMR spectra of membrane preparations into which the appropriate [^{2}H]-labeled analog is introduced. The [^{2}H]-labels are introduced in strategic positions of the tricyclic ring system. Each experiment involves the singly [^{2}H]-labeled drug molecule. However, preparations in which the drug molecule is labeled in more than one position can be used if each

[^{2}H]-subspectrum in the composite [^{2}H]-spectrum can be positively assigned to an individual label, The approach was initially used to study the orientation of cholesterol in the membrane and subsequently expanded in the author's laboratory into a more general method (Makriyannis et al. 1989).

Studies with Δ^{9}-THC required the stereospecific introduction of six individual deuterium atoms in the tricyclic component of the molecule (figure 2). The author and coworkers found that the cannabinoid assumes an awkward orientation in the bilayer, with the long axis of its tricyclic system perpendicular to the bilayer chains (Makriyannis et al. 1989, 1991). This can be accounted for by assuming that this amphipathic drug molecule uses its phenolic hydroxyl group to anchor itself at the interface of the amphipathic membrane bilayer; small-angle x-ray results confirmed this interpretation. In addition, it was discovered that cannabinoids with two hydroxyl groups orient in a manner that allows both hydroxyls to be near the interface facing the polar surface of the membrane (Makriyannis and Yang 1989; Yang et al. 1991). However, if the phenolic hydrogen of THC is replaced with a methyl group, the cannabinoid orients with its natural long axis parallel to the chains (Yang et al. 1992).

Cannabinoid Topography in the Membrane

Information on the location of the drug in the bilayer can be obtained from small-angle x-ray diffraction experiments using carefully hydrated membrane samples into which the cannabinoid analog is introduced (Mavromoustakos et al. 1990). The well-layered membrane preparation is exposed to a small-angle x-ray beam, from which several diffraction orders are obtained. After transformation these orders can provide an electron density profile of the membrane bilayer (figure 3) in which the highest electron density corresponds to the phosphate headgroups and the lowest to the ends of the chains in the center of the bilayer. In such an experiment, three membrane preparations are compared. The first of these contains no drug molecule. The second preparation contains the cannabinoid molecule under study, and the third contains the same cannabinoid analog into which a high electron density substituent (e.g., Br, I) is introduced as a marker. The differences among the electron density profiles of the above three preparations reveal the positions of the cannabinoid tricyclic ring system and that of the heavy atom and thus provide direct information on the location of the drug in the membrane. If the high electron density atom is placed at the end of the cannabinoid chain, then the experiment also provides information on the conformation of this flexible portion of the drug molecule in the membrane. Experiments with Δ^{9}-THC, Δ^{8}-THC, and their inactive O-methyl analog provided data that complemented the [^{2}H]-NMR results. Such experiments required the synthesis of these cannabinoids as well as analogs having a halogen (I) substituent in the 5'-position of the cannabinoid side chain (figure 4).



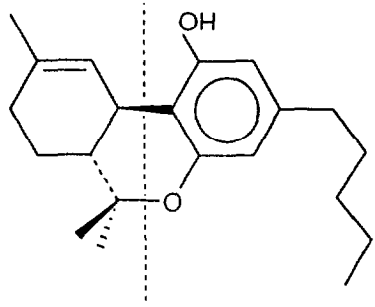


FIGURE 2. *Left: Solid-state [^{2}H]-NMR spectra of model membrane samples containing specifically [^{2}H]-labeled Δ^9-THC. Four deuterated Δ^9-THC analogs have [^{2}H]-labels in the 2, 4, 8α,,8β,,10, and 10a positions. Right: Orientation of Δ^9-THC in the membrane as determined from the solid-state [^{2}H]-NMR results. The dashed line represents the direction of the lipid acyl chains in the membrane.*

SOURCE: Reprinted from *Pharmacology, Biochemistry and Behavior,* Volume 40. Yang, D.P.; Banijamali, A.; Charalambous, A.; Marciniak, G.; and Makriyannis, A. Solid State ^{2}H-NMR as a method for determining the orientation of cannabinoid analogs in membranes, 553-557, Copyright (1991), with permission from Pergamon Press Ltd., Headington Hill Hall, Oxford, OX3 OBW, UK.

The study, which made use of membrane preparations from partially hydrated phosphatidylcholine, showed that the biologically active Δ^9-THC and Δ^8-THC analogs are located in the upper portion of the phospholipid bilayer, with the phenol hydroxyl anchored at the bilayer interface (Yang et al. 1992). However,

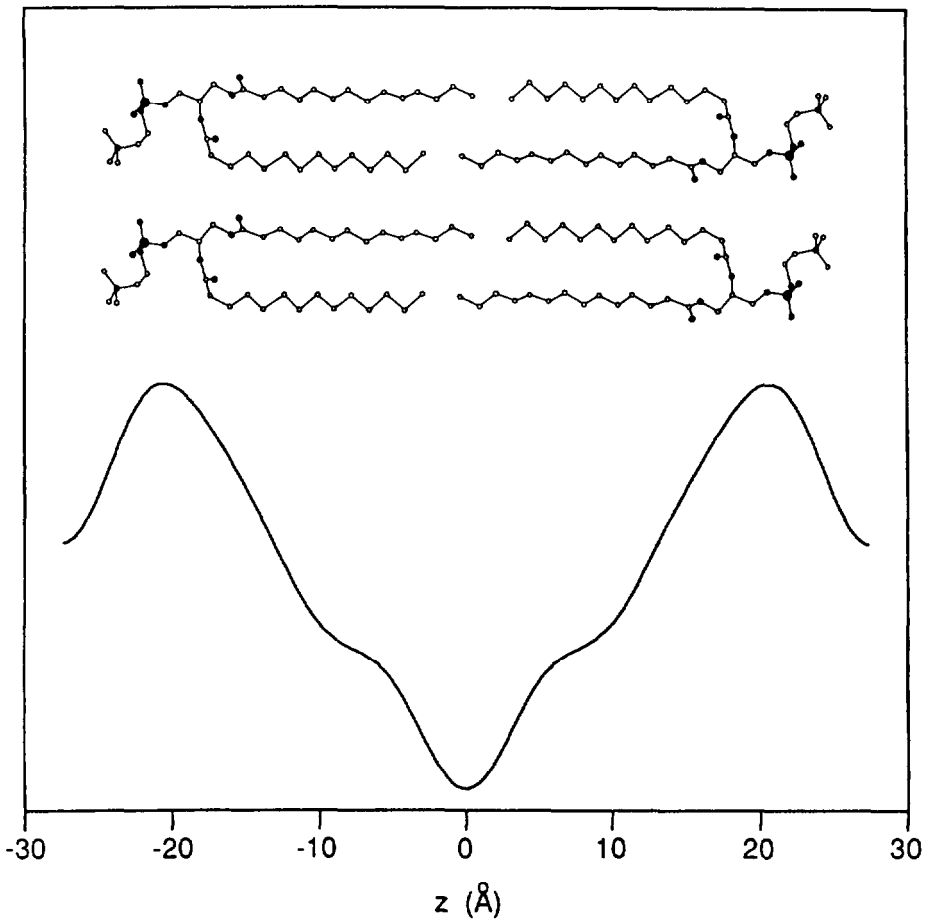
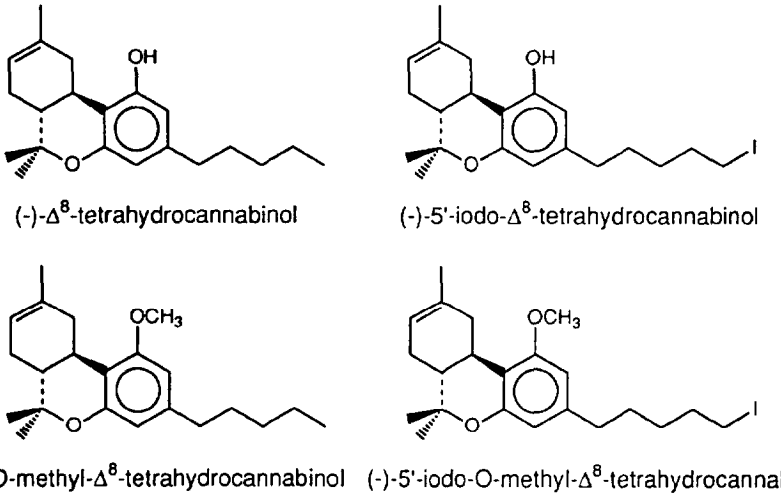


FIGURE 3. *Electron density profile obtained by Fourier transformation of small-angle diffraction intensities from a dimyrisroylphosphatdylcholine (DMPC) model membrane. A molecular graphic representation above the profile shows the space correlation in the dimension across the bilayer.*

SOURCE: Reprinted from *Pharmacology, Biochemistry and Behavior,* Volume 40. Mavromoustakos, T.; Yang, D.P.; Broderick, W.; Fournier, D.; and Makriyannis, A. Small angle x-ray diffraction studies on the topography of cannabinoids in synaptic plasma membranes, 547-552, Copyright (1991), with permission from Pergamon Press Ltd., Headington Hill Hall, Oxford, OX3 OBW, UK.



(-)-Δ^8-tetrahydrocannabinol (-)-5'-iodo-Δ^8-tetrahydrocannabinol

(-)-O-methyl-Δ^8-tetrahydrocannabinol (-)-5'-iodo-O-methyl-Δ^8-tetrahydrocannabinol

FIGURE 4. *Structures of (-)-Δ^8-tetrahydrocannabinol (Δ^8-THC) and (-)-O-methyl-Δ^8-tetrahydrocannabinol (Me-Δ^8-THC), along with the respective iodinated analogs 5'-I-Δ^8-THC and 5'-I-Me-Δ^8-THC.*

when the polar phenoiic hydroxyi is replaced with the more lipophilic methoxyl group, the cannabinoid sinks deeper into the biiayer. The study also revealed that the iodine atom's location in the biiayer requires that the cannabinoid side chain be parallel with the biiayer chains in a fully extended all-*trans* conformation. When studies were extended from model to biological membranes obtained from rat or calf brain synaptosomes, there was no significant change in the manner in which the cannabinoid analogs orient in the membrane (Mavromoustakos et al. 1991). However, the x-ray data revealed a small but significant variation in the location of the drug in the biiayer. It was found that Δ^8-THC and its haiogenated analog are located 2 to 3 Å deeper in the membrane preparations obtained from calf brain synaptosomes compared with the model membrane preparations. The data from the synaptosomai plasma membrane preparations also showed that the THC side chain assumes a more compact, *gauche-trans-gauche* conformation, unlike the fully extended conformation observed in the phosphatidylchoiine preparations. When molecular modeling was used to represent data from the biological membranes, the phenoiic hydroxyi in the cannabinoid could still interact with the biiayer interface through hydrogen bonding with either one of the phospholipid carbonyis or with a water molecule located slightly below the interface (figure 5).

A biophysical method that can be used in an analogous manner with the x-ray method described above is *neutron scattering.* in this method, the high electron density halogen marker used in the x-ray experiment is now replaced with

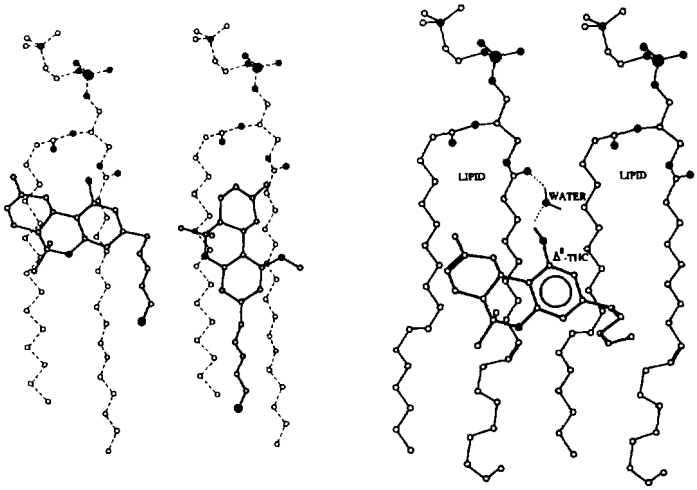


FIGURE 5. *Left: Graphical representation of the locations and orientations of 5'-I-Δ⁸-THC and 5'-I-Me-Δ⁸-THC In a DMPC model membrane based on results from a small-angle x-ray diffraction* and *solid-stare [²H]-NMR. Right: Graphical representation of a Δ⁸-THC molecule in a synaptosomal plasma membrane.*

SOURCE: Reprinted from *Pharmacology, Biochemistry and Behavior,* Volume 40. Mavromoustakos, T.; *Yang,* D.P.; Broderick, W.; Fournier, D.; and Makriyannis, A. Small angle x-ray diffraction studies on the topography of cannabinoids in synaptic plasma membranes, 547-552, Copyright (1991), with permission from Pergamon Press Ltd., Headington Hill Hall, Oxford, OX3 OBW, UK.

deuterium atoms. The two membrane preparations compared in this method contain, respectively, unlabeled and specifically [^{2}H]-labeled cannabinoids. The location of the [^{2}H]-label is then revealed by subtracting the data from the two preparations. One advantage of this method is that the two membrane samples being compared are almost identical. This avoids the potential pitfall encountered in the x-ray experiment where the samples being compared are not totally identical and may have somewhat different phase properties, thus requiring rigorous controls. Recent data using [^{2}H]-labeled Δ^8-THC in neutron diffraction experiments (P. Martel and A. Makriyannis, unpublished results) have confirmed, to a large extent, the earlier x-ray data described above.

The combined data from the solid-state [^{2}H]-NMR and small-angle x-ray diffraction experiments support the hypothesis that the ability of a cannabinoid to orient “properly” in the amphipathic membrane system can seriously influence its ability to bind to its active site(s) on the receptor. This “proper” orientation is

one in which all the cannabinoid's polar hydroxyl groups face the polar side of the membrane bilayer and, in turn, is determined by the position and relative stereochemistry of these hydroxyi groups in the cannabinoid molecule.

Cannabinoid Binding Sites in Mammalian Brains

The distribution of a cannabinoid in the brains of live animals can be studied using positron emission tomography (PET). The method requires the labeling of the drug probe with a short-lived positron-emitting isotope. To carry out such an experiment, the author and coworkers synthesized an ^{18}F-containing analog of the parent Δ^{8}-THC, namely (-)-5'-[^{18}F]-Δ^{8}-THC. The probe was administered to live baboons, and the presence and distribution of the drug in the brain was followed using computerized tomography. Among the findings (Charaiambous et al. 1991) was that the drug reaches its maximum concentration in the brain (approximately .02 percent dose/cc) during the first 5 minutes and persists (.005 percent dose/cc) up to 2 hours afler injection (figure 6). High drug concentrations were found in the striatum, thaiamus, and cerebellum. A shortcoming in these early experiments was the high iipophilicity of the probe as well as its relatively low affinity for the receptor sites. Both factors resulted in relatively high levels of nonspecific binding by the ligand in the brain. Currently under way is the synthesis of novel cannabinoid PET probes possessing higher affinities for the receptor and more favorable partitioning properties,

Cannabinoid Photoaffinity Probes

The task of obtaining direct information on the molecular features of the cannabinoid receptor binding sites can be successfully pursued with the help of cannabinoid affinity labels so that, after interacting with the cannabinoid binding sites, they covalently attach themselves to the receptor. The affinity probes should be especially useful for the isolation and characterization of the cannabinoid receptor. The most successful of the first-generation cannabinoid probes was (-)-5'-azido-Δ^{8}-THC (figure 7), the first photoaffinity label for the cannabinoid receptor. This molecule was shown to specifically bind and inactivate the receptor after ultraviolet irradiation (Charaiambous et al. 1992). Furthermore its ^{125}I-radiodinated analog, when incubated with two different membrane preparations, was shown to covaientiy label the receptor (Burstein et al. 1991). The use of an aliphatic azide as a photaffinity label represents a departure from the generally used aromatic azides, which upon photoactivation are transformed into aromatic nitrenes as the reactive species and are known to be considerably more stable than their aiiphatic counterparts. in this photoaffinity probe, the aiiphatic azido group is expected to be transformed to an aiiphatic nitrene when subjected to ultraviolet irradiation. However, it is still unclear whether the originating highly unstable aliphatic nitrene directly reacts with the receptor or whether it first rearranges to a more stable intermediate

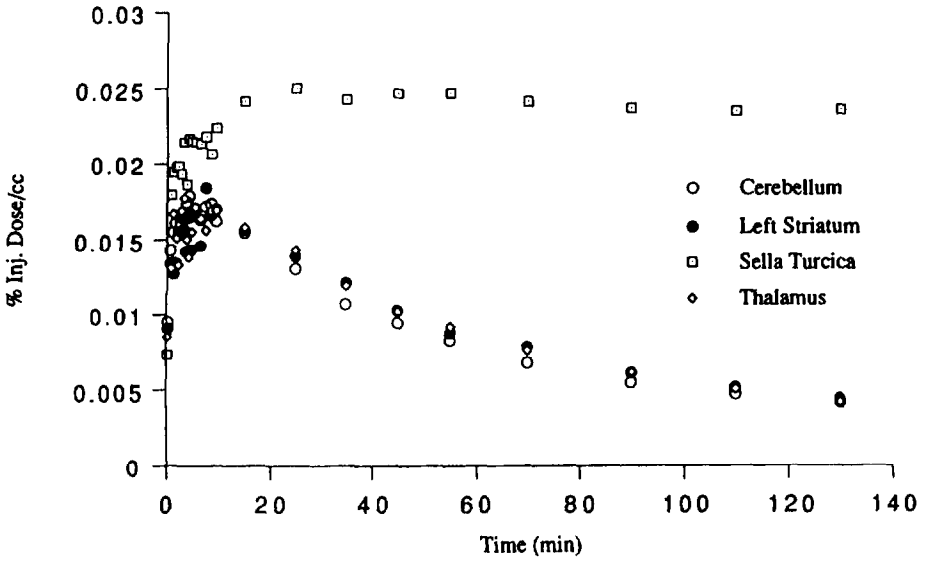
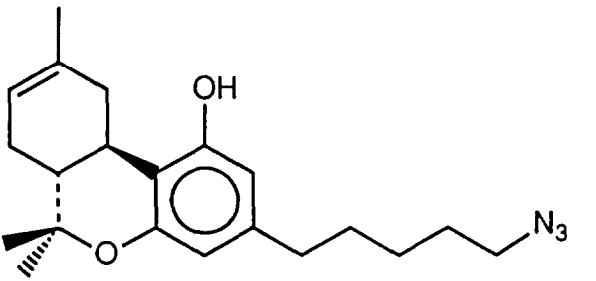


FIGURE 6. *Time courses for (-)-5'-[^{18}F]-Δ^{8}-THC distribution in baboon brain, showing the rates of accumulation and clearance of the radioactivity in the various areas of the brain.*

SOURCE: Reprinted from *Pharmacology, Biochemistry and Behavior,* Volume 40. Charalambous, A.; Marciniak, G.; Shiue, C.-Y.; Dewey, S.L.; Schlyer, D.J.; Wolf, A.P.; and Makriyannis, A, PET studies in the primate brain and biodistribution in mice using (-)-5'-^{18}F-Δ^{8}-THC, 503-507, Copyright (1991), with permission from Pergamon Press Ltd., Headington Hill Hall, Oxford, OX3 OBW, UK.

that, in turn, covalently attaches itself at or near the active sites on the receptor. Currently, the author's group is investigating the mechanism of this photoactivation reaction.

Recently, we have developed second-generation photoaffinity probes that possess considerably higher affinities for the cannabinoid receptor, as exemplified by the one (IC_{50}~0.25 nM) depicted in figure 8 (Charalambous et al., in press). These novel probes should be ideally suited for pursuing the important but difficult task of labeling and characterizing the cannabinoid receptor.



(-)-5'-azido-Δ^8-tetrahydrocannabinol

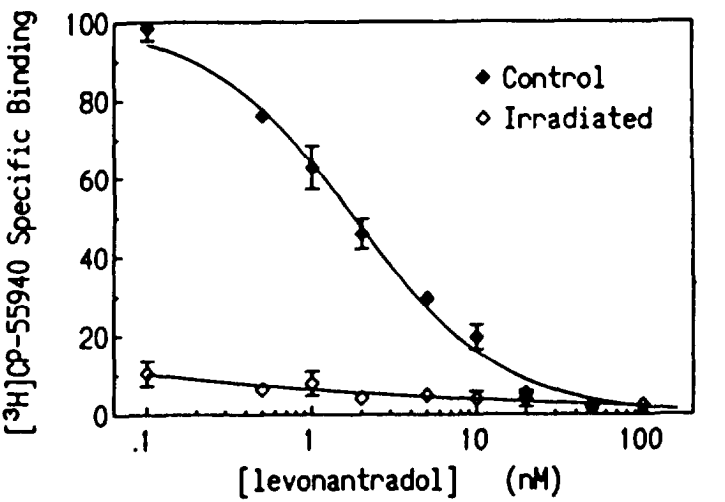
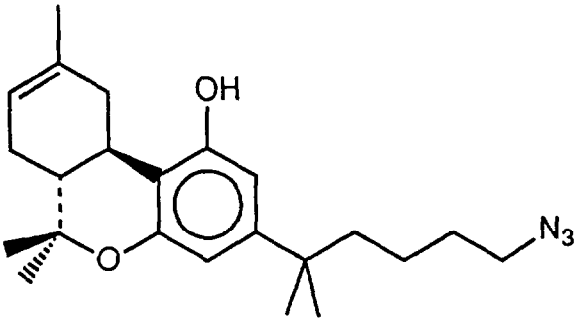


FIGURE 7. *Photoirradiation of (-)-5'-azido-Δ^8-THC with forebrain membranes inhibits specific binding of [^{3}H]CP-55940 to the cannabinoid receptor. Heterologous displacement by levonantradol is shown for membranes irradiated in the absence or after equilibration with 5'-azido-Δ^8-THC.*

SOURCE: Reprinted with permission from Charalambous, A.; Guo, Y.; Houston, D.B.; Hewlett, A.C.; Compton, D.R.; Martin, B.R.; and Makriyannis, A. (-)-5'-azido-Δ^8-THC; a novel photoaffinity label for the cannabinoid receptor. *J Med Chem* 35:3076-3079, 1992. Copyright (1992), American Chemical Society.



(-)-1',1'-dimethyl-5'-azido-Δ^8-tetrahydrocannabinol

FIGURE 8. *Structure of (-)-1',1'-dimethyl-5'-N_3-Δ^8-THC*

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A Model for the Structural Basis of δ_2-Opioid Receptor Selectivity

Henry I. Mosberg and Frank Porreca

Although opioid receptor heterogeneity has long been established, and the existence of distinct μ-, κ-, and δ-types of opioid receptors has been widely accepted since the late 1970s convincing evidence of subtypes of these major categories of opioid receptors is of more recent origin. In particular, the clear demonstration of δ-receptor subtypes in vivo has evolved only during the past 2 years. As is often the case, this demonstration of receptor subtypes followed from the observation of disparate effects mediated by compounds considered to be selective for the same receptor, in this case the δ-opioid receptor. Several of these observations have uncovered pharmacological differences between Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE) (Mosberg et al. 1983) where penicillamine (Pen) is β β-dimethyl cysteine, and deltorphin II (DELT II, Tyr-D-Ala-Phe-Glu-Val-Val-GlyNH^2) (Erspamer et al. 1989), a naturally occurring opioid peptide originally isolated from frog skin. The earliest such observation was that, although both DPDPE and DELT II displayed b-receptor-mediated antinociceptive activity in mice following intracerebroventricular (ICV) administration, pretreatment with two δ-selective, irreversible antagonists, Tyr-D-Ala-Gly-Phe-Leu-Cys (DALCE) (Bowen et al. 1987) and natrindole-5'-isothiocyanate (5'-NTII) (Portoghese et al. 1990), resulted in differential effects: the former antagonizing DPDPE but not DELT II, the latter antagonist blocking the antinociceptive effect of DELT II, but not DPDPE (Jiang et al. 1991). This result was interpreted as evidence that the antinociceptive actions of DPDPE and DELT II were affected by interaction with different subtypes of δ-receptor. This interpretation was supported by a later series of experiments that demonstrated a three-way lack of antinociceptive cross-tolerance between DPDPE, DELT II, and the μ-selective agonist Tyr-D-Ala-Gly-NMePhe-Gly-ol (DAMGO) (Mattia et al. 1991), indicative of mediation by three different receptors or receptor subtypes, Similar conclusions regarding δ-receptor subtypes in mouse brain have been reached by Takemori and coworkers from in vivo studies employing the δ-selective antagonists naltrindole and naltriben (Sofuoglu et al. 1991).

Although considerable evidence that DPDPE and DELT II act via distinct δ-receptor subtypes has been compiled, the structural basis for the disparate actions of these peptides has not yet been addressed. In what follows, the

authors propose ahypothesis implies insufficient evidence hypothesis for the structural basis of the subtype selectivities of DPDPE and DELT II and examine the structural features that endow DELT II with δ- vs. μ- and κ-selectivity. We then describe how this analysis has led us to reexamine a series of tetrapeptide opioids that we have developed over the past 5 years in the new context of δ-receptor subtype selectivity.

The structures of DPDPE and DELT II are shown in figure 1. Two significant structural differences between the peptides stand out. First, whereas DPDPE has a standard enkephalin-like pentapeptide structure, DELT II has an extended C-terminus culminating in a carboxamide function. As discussed below, this C-terminal extension is important for conferring b-receptor affinity to DELT II. The second obvious structural difference is in the location within the primary sequence of the important Phe residue, as residue 4 in DPDPE and as residue 3 in DELT II. Our working hypothesis, for which there is as yet, to our knowledge, no confirming or refuting evidence, is that this structural difference is a key element underlying the δ-subtype selectivity differences in DPDPE and DELT II. Results presented below, although not sufficient to prove this hypothesis, are at least consistent with it.

Structural features of DELT II that confer δ- vs. μ-selectivity (κ-affinities of DPDPE and the deltorphins are very low and are ignored here) can be deduced from the increasingly well-established structure-activity relationships for the deltorphins. Table 1 summarizes some of these data. As can be seen in table 1, deltorphin I (DELT I), which has been shown, like DELT II, to be selective for the δ_2-opioid receptor (Porreca, unpublished observations), and

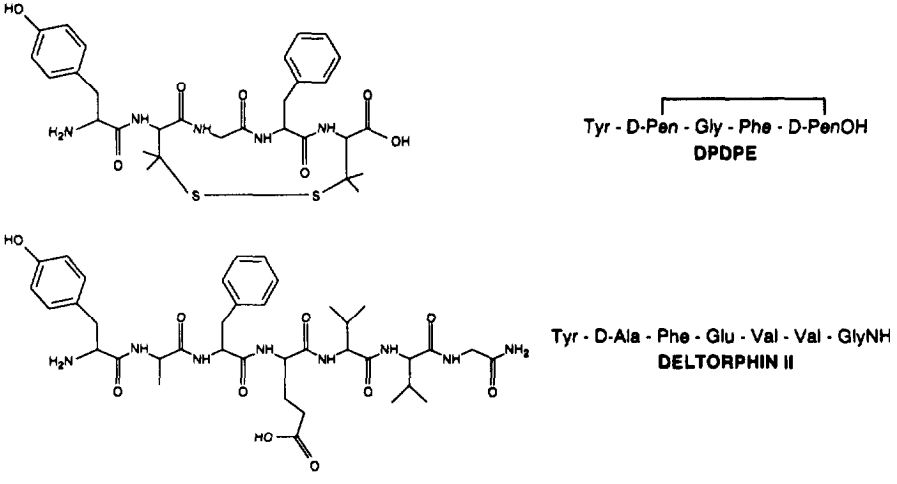


FIGURE 1. *Structures of DPDPE and DELT II*

TABLE 1. *Role of residue 4 side chain in DELTI and II; opioid receptor binding in membrane homogenates from guinea pig brain*

Peptide	K_i (nM) [^{3}H]DAMG0	[^{3}H]DPDPE	K_i (μ)/K_i (δ)
Tyr-D-Ala-Phe-Glu-Val-Val-GlyNH$_2$	1,310	2.69	487
Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$	677	1.73	391
Tyr-D-Ala-Phe-Asn-Val-Val-GlyNH$_2$	69.7	3.66	19
Tyr-D-Ala-Phe-Gly-Val-Val-GlyNH$_2$	4.7	2.60	1.81

DELT II differ only in residue 4, which in both cases has a negatively charged carboxylate side chain. The importance of this side chain is apparent by comparing the receptor-binding results obtained for these opioids with those for the corresponding amidated side chain in [Asn^4]deltorphin. As shown in table 1, this latter modification has only a minor effect on δ-receptor affinity but increases μ-affinity by approximately an order of magnitude, thus effecting a reduction in δ-selectivity. Removing all steric effects of the side chain by substitution with Gly in residue 4 again has no significant effect on δ-affinity but leads to a further order of magnitude increase in μ-affinity. These results strongly support the conclusion that the negatively charged side chain in residue 4 of DELT I and DELT II is critical for the δ-receptor selectivity displayed by these compounds but has no significant effect on δ-receptor-binding affinity. Rather, the δ-selectivity arises from adverse interactions at the μ-receptor attributable to both steric and charge effects of the residue 4 side chain. This same interpretation of similar results was proposed by Lazarus and coworkers (1991).

The role of the tripeptide C-terminal tail of DELT I has been examined independently by Melchiorri and colleagues (1991) and Salvadori and coworkers (1991). Both studies revealed that, whereas truncation to the hexapeptide, Tyr-D-Ala-Phe-Asp-Val-ValNH$_2$, resulted in only modest (threefold to fivefold) losses in b-binding affinity, further truncation led to increasingly pronounced reductions in δ-affinity. Affinity for μ-receptors was relatively unaffected, although a slight increase in affinity was observed for the shorter fragments. Consequently, δ-selectivity decreases with decreasing peptide length with the tetrapeptide, Tyr-D-Ala-Phe-AspNH$_2$, displaying slight μ-selectivity. The results clearly indicate the importance of the complete heptapeptide sequence for optimal δ-binding affinity and selectivity. A possible

explanation of the role of the tripeptide C-terminal tail may lie in the lipophilicity of this segment, in particular the valine residues. These lipophilic side chains may, for example, play a direct binding role with a complementary lipophilic subsite on the δ-receptor. This might account for the relatively high δ-affinity of the hexapeptide that retains both valine residues. Alternatively, the tripeptide tail may play a conformational role by energetically facilitating a relative orientation of other portions of the peptide that is required for δ-receptor recognition. Possible support for this latter explanation comes from conformational studies that suggest that the C-terminal tail of DELT I is folded on the N-terminal portion via a p-turn involving residues 3 through 6 (Balboni et al. 1990). In summary then, we can identify three structural features that may be critical for the $\delta_{1,2}$-receptor selectivity of the deltorphins: (1) a negative charge in the side chain of residue 4 that reduces p-receptor affinity; (2) the tripeptide C-terminal tail that enhances δ-affinity, perhaps via a conformational role; and (3) the presence of a Phe^3 residue rather than the enkephalin-like Phe^4 residue that may underlie the $\delta_{1,2}$-subtype selectivity of the deltorphins.

Support for the above structural basis for the selective opioid actions of deltorphin comes indirectly from attempts to determine the bioactive conformation of DPDPE, an area of interest in our laboratories as well as several others. Our approach for the elucidation of this bioactive conformation has combined experimental evidence of the solution conformation of DPDPE gained from extensive nuclear magnetic resonance (NMR) studies with computational methods (Mosberg et al. 1990). In particular, interproton distances calculated from nuclear Overhauser effect buildup rates were used as constraints for distance geometry calculations of conformers consistent with these constraints. Each of the conformers generated was then energy minimized, and those conformers of lowest energy were examined further for consistency with additional conformation-dependent NMR parameters. In this manner three low-energy conformers, I through III in table 2, were identified. Of these, conformer III was most consistent with all experimental evidence and was further modified to take into account calculated major conformations of the Tyr and Phe side chains. Further energy minimization led to a significantly lower energy conformer, conformer III', that was consistent with all experimental data, This conformer was then proposed as the active conformation of DPDPE (Mosberg et al. 1990). Many other conformational models, based primarily on computational studies, have also been proposed for DPDPE (e.g., Hruby et al. 1988; Froimowitz 1990; Chew et al. 1991; Nikiforovich et al. 1991; Wilkes and Schiller 1991). In general, however, agreement among these models is lacking. Among other interpretations, this lack of agreement raises the possibility that DPDPE may sample many low-energy conformations in solution; that is, it is more flexible than is usually assumed. Consequently, conformation-dependent NMR parameters interpreted in terms of a single conformation may reflect some significant degree of conformational averaging and thus may result in erroneous conclusions regarding "the" solution conformation. Naturally, extrapolations

TABLE 2. *Low-energy conformers of DPDPE*

Residue	Dihedral Angle	Model I	II	III	III'
Tyr[1]	ϕ	–	–	–	
	ψ	-36	150	155	163
	ω	178	-176	-173	-177
	χ^1	-62	-179	69	-173
	χ^2	96	-125	-69	-115
Pen[2]	ϕ	-79	135	142	149
	ψ	-129	-29	-153	-153
	ω	171	180	-173	-175
	χ^1	-88	-69	-82	-78
	χ^2	-175	-168	176	178
Gly[3]	ϕ	82	-80	96	78
	ψ	-66	49	-130	-111
	ω	-171	176	-168	-164
Phe[4]	ϕ	-105	-153	-79	-85
	ψ	-53	-81	27	38
	ω	-174	178	-178	172
	χ^1	-60	-54	63	-64
	χ^2	-73	-86	93	105
Pen[5]	ϕ	140	131	76	61
	ψ	–	–	–	–
	ω	–	–	–	–
	χ^1	-85	-69	-91	-87
	χ^2	62	72	61	60
	c-s-s-c	109	110	102	110
Energy (Kcal/mol)		-43.7	-43.8	-44.3	-51.5

SOURCE: Adapted with permission from Mosberg, H.I.; Sobczyk-Kojiro, K.; Subramanian, P.; Crippen, G.M.; Ramalingam, K.; and Woodard, R.W. Combined use of stereospecific deuteration, NMR, distance geometry and energy minimization for the conformational analysis of the delta opioid receptor selective peptide, [D-Pen[2], D-Pen[5]]enkephalin. *J Am Chem Soc* 112:822-829, 1990. Copyright 1990 by American Chemical Society, Washington, D.C.

to the receptor-bound, active conformation would in this case be unwarranted. Similarly, residual flexibility in DPDPE would devalue the significance of computationally based conformational models because the existence and calculation of many low-energy conformers does not allow the convincing identification of the pharmacologically relevant conformation.

Some degree of flexibility in DPDPE can be expected in the side chains, especially those of the Tyr and Phe residues. Such side-chain flexibility is ubiquitous in small peptides containing natural amino acids and can be addressed by replacement with conformationally restricted analogs of these residues. Correlation of biological activity with side-chain conformational features of the corresponding peptides allows deductions regarding side-chain geometry in the bioactive conformation of the parent peptide. The backbone variability In the conformational models for DPDPE suggests that residual flexibility also exists here. As depicted in figure 2, the likely source of such flexibility is the Gly^3 residue of DPDPE, which, lacking a side chain, has many low-energy conformations available. In fact, we had earlier proposed, based on the similar δ-activity but dissimilar Gly^3 NMR parameters of a series of DPDPE-like analogs with various combinations of Cys and Pen in residues 2 and 5, that the Gly residue acts as a "hinge" allowing the receptor-required relative orientation of key structural elements throughout the series (Mosberg 1987).

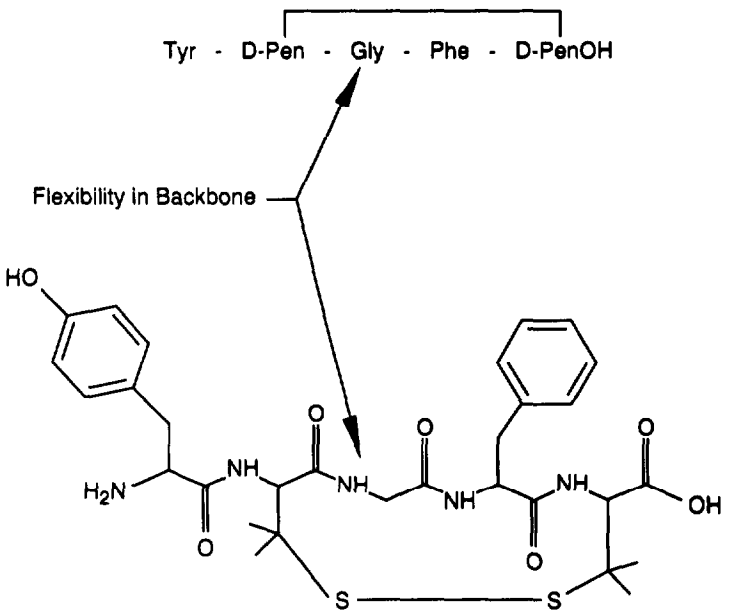


FIGURE 2. *Origin of backbone flexibility in DPDPE*

To examine the role of the Gly residue in conferring &selectivity in DPDPE and as an approach to increase the rigidity in this series, several years ago we began to Investigate the effect of eliminating the Gly residue in analogs otherwise similar to DPDPE. At that time, the deltorphins were still unknown; however, the p-selective frog skin heptapeptide dermorphin, Tyr-D-Ala-Phe-Gly Tyr-Pro-SerNH_2, had been described (Montecucchi et al. 1981) as had several cyclic, lactam-containing tetrapeptides of the general form, Tyr-c[D-Xxx-Phe-Yyy]-NH_2 (Schiller et al. 1985), that also displayed μ-selectivity. Our initial study (Mosberg et al. 1988), in addition to identifying several relatively nonselective tetrapeptide carboxamides, also reported the tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]-OH (JOM-13) (figure 3) that was shown to exhibit similar δ-binding affinity and selectivity as DPDPE. Further study of this series revealed that, unlike the DPDPE series in which δ-affinity is maintained with various combinations of D-Pen or D-Cys in residue 2 and D- or L-Pen, or D- or L-Cys as the C-terminal, any such variation in JOM-13 greatly reduces δ-affinity (Heyl et al. 1991). This is consistent with the view, expressed above, that the flexibility of the Gly residue in the DPDPE series allows all members to interact favorably with the δ-receptor, whereas the increased rigidity of the des-Gly^3 tetrapeptide series results in a much greater dependence on the nature of the residues involved in the cyclization.

Although the tetrapeptide series that resulted in JOM-13 was designed as a des-Gly^3 DPDPE set of compounds, the subsequent discovery of the deltorphins and the elucidation of the structural features underlying the δ-selectivity of the deltorphins lead to an alternate view—JOM-13 as a truncated, conformationally

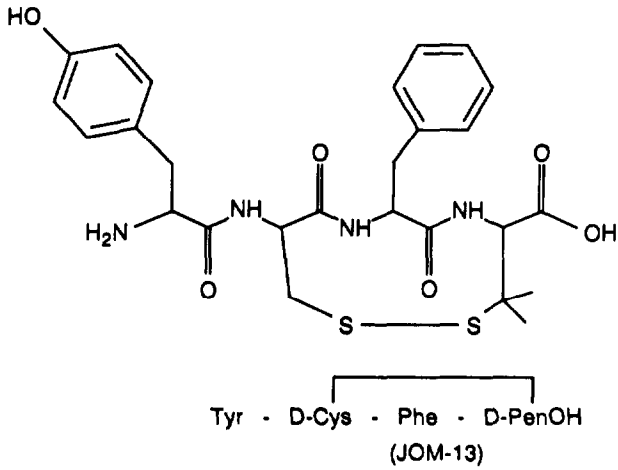
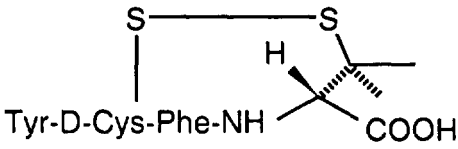


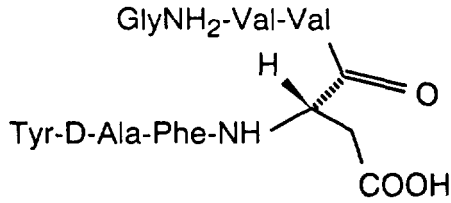
FIGURE 3. *Structure of JOM- 13*

restricted analog of DELT I and DELT II, which is represented in figure 4. As depicted in this figure, the side-chain to side-chain cyclization in JOM-13, which restricts this tetrapeptide to compact, folded conformations, serves the same structural function as the proposed turn-inducing C-terminal tail of DELT I and II, discussed above. In both cases then, relative orientations of key structural elements in the N-terminal tetrapeptide segment, which are required for δ-receptor recognition, are stabilized. Also, as shown in figure 4, the C-terminal carboxyl function of JOM-13 can be seen as corresponding to the residue 4 side-chain carboxyl group of DELT I (as shown) or DELT II. This correspondence between α--carboxyl in the former and side-chain carboxyl in the latter results only because of the chirality difference (D-stereochemistry for JOM-13, L-stereochemistry for the deltorphins) at residue 4 between the peptides.

In addition to the similarities pointed out above, JOM-13 shares with DELT I and II an additional structural feature, the presence of a Phe[3] (rather than an enkephalin-like Phe[4]) residue. Because we have proposed that this structural feature may be crucial for the $\delta_{1,2}$-subtype selectivity displayed by the deltorphins, a critical test of the view of JOM-13 as a deltorphin analog is the in vivo evaluation of its actions. This evaluation is presented in figures 5 and 6. Figure 5 displays the antinociceptive activity of JOM-13, following ICV injection, in the mouse tail-flick model under control conditions as well as following pretreatment with the μ-selective antagonist β-FNA or the δ-selective antagonist ICI 174,864. As can be seen from figure 5, the antinociceptive dose-response line of JOM-13 is unaffected by β-FNA but is shifted to the right by ICI 174,864, a behavior indicative of δ-receptor mediation of the antinociceptive action of JOM-13. The control antinociceptive potency of JOM-13 (A50=1.4 nmol) is comparable



JOM-13



DELT I

FIGURE 4. *JOM-13 viewed as a conformationally restricted deltorphin analog*

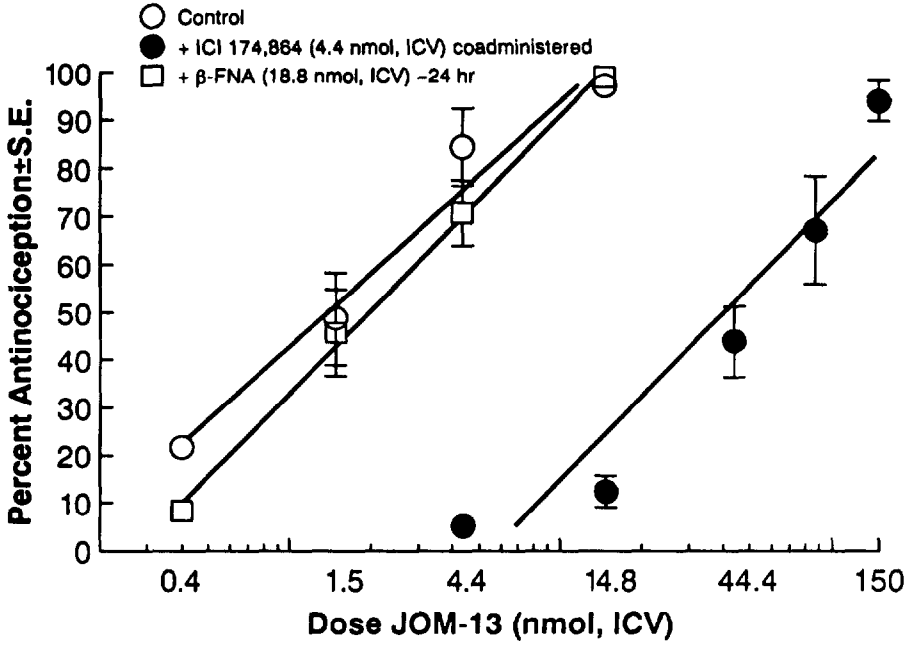


FIGURE 5. *Antinociceptive potency of JOM-13 alone (O), in the presence of coadministered ICI 174,864 (●), or after pretreatment with β-FNA (•),*

to that found for DELT II (Jiang et al. 1991). Figure 6 further examines the effect on this antinociceptive action of pretreatment with either the δ^1-selective antagonist, DALCE, or the δ_2-selective antagonist, 5'-NTII. As earlier demonstrated for DELT II (Jiang et al. 1991), the antinociceptive action of JOM-13 is blocked by 5'-NTII, but not by DALCE, a profile expected for a δ_2-selective agonist. Clearly then, JOM-13 can best be viewed as a truncated, conformationally restricted deltorphin analog. Furthermore, this demonstration of δ_2-selectivity for JOM-13 lends support for the structural and conformational model of deltorphin selectivity presented above.

Compared with the native deltorphins, JOM-13 presents several advantages. First, its smaller molecular weight and compact size may result in improved absorption or blood-brain barrier penetration via passive diffusion mechanisms. Second, both the conformationally constrained nature and the alternating D- and L-amino acid sequence should render JOM-13 highly resistant to metabolism by peptidases. Finally, as a tool for the elucidation of the bioactive conformation at the δ_2-receptor, JOM-13, which was initially designed as a more rigid analog of

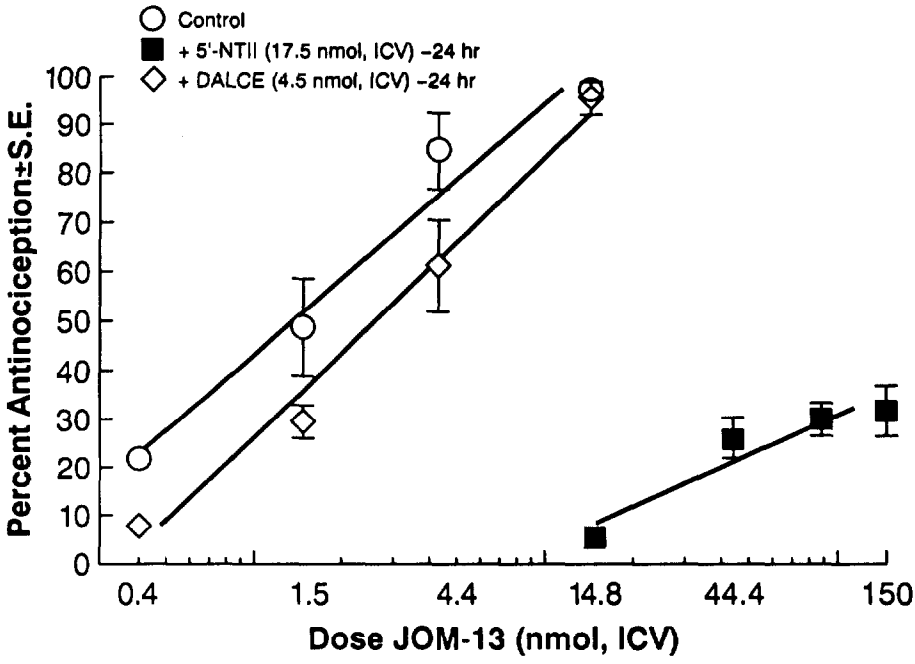


FIGURE 6. *Antinociceptive potency of JOM-13 alone (O), or after pretreatment with 5'-NTII (■), or DALCE (◇).*

DPDPE, is ideal because conformational averaging should not significantly influence experimental measurements.

Despite the potential advantages of JOM-13, a significant disadvantage is apparent from the comparison of μ- and δ-binding affinities shown in table 3. Although JOM-13 displays high δ-binding affinity, comparable to DELT I and DELT II, its δ-selectivity is severalfold lower. This reduced δ-selectivity, however, is not a limitation, because rather straightforward analogs of JOM-13 display highly improved selectivity. This, too, can be seen in table 3 where binding data for two examples, [2-Nal^3]JOM-13 (2-Nal=2-naphthylalanine) (Heyl and Mosberg 1992*a*) and [p-ClPhe^3]JOM-13 (Heyl and Mosberg 1992*b*), are presented. Both of these analogs display δ-selectivity comparable to that of DELT I and DELT II with [p-ClPhe^3]JOM-13 also showing significantly higher δ-affinity.

In summary, the δ- vs. μ-selectivity of JOM-13 supports the view that the δ- (vs.μ-) selectivity of DELT I and DELT II results from the negatively charged side chain in residue 4 (which lowers μ-affinity) and a conformational effect of

TABLE 3. *Binding affinities and selectivities of JOM- 13 analogs*

Peptide	K_i (nM)		
	[^{3}H]DAMG0	[^{3}H]DPDPE	$K_i(\mu)/K(\delta)$
DELT I	677	1.73	391
DELT II	1,310	2.69	487
DPDPE	810	3.98	204
JOM-13	107	1.79	60
[2-Nal^3]JOM-13	590	2.23	265
[p-ClPhe^3]JOM-13	327	0.98	334

the C-terminus, which stabilizes a reverse-turn structure favoring δ-receptor recognition. The δ_2-subtype selectivity displayed by JOM-13 is consistent with the proposal that the Phe^3 residue in DELT I and DELT II is important for such selectivity. The results presented clearly establish that JOM-13, originally developed prior to the discovery of the deltorphins as a more conformationally restricted analog of DPDPE, can more correctly be viewed as a conformationally restricted, truncated deltorphin analog. No other class of small, potent, and selective deltorphin analogs has been reported.

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