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# Protein crystallography, computer graphics and drug design

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Abstract

Protein crystallography enables us to elucidate the three-dimensional structures of small and large proteins with great accuracy, if suitable crystals can be prepared. Such three-dimensional structures form a corner stone in a number of modern high-chem applications, such as design of new catalysts, vaccines, industrial enzymes, agro-chemicals and drugs. The way in which protein crystallography, computer graphics and molecular modelling helps, via a "rational drug design" in the development of future drugs is outlined in this contribution.

## 1. INTRODUCTION

The development of a new drug, or medicine, is a challenge of tremendous complexity and of truly multi-disciplinary character. It is currently also of a dual nature. Typical trial-and-error processes are carried out simultaneously with the most advanced biochemical, synthetic and computational procedures. In recent years two new techniques have received great interest from medicinal chemists: protein crystallography and computer graphics. The first allows the determination, at the atomic level, of three-dimensional structures of potential target proteins (as well as of other biomacromolecules such as DNA and RNA). The second is indispensable for detailed investigations of the experimentally obtained structures and provides a convenient interface with a variety of theoretical calculations and molecular modelling techniques.

## 2. A NEW STRATEGY FOR THE DESIGN OF DRUGS

Until recently design or discovery of new drugs has largely been based on the following strategies:

(i) in-depth investigations of active components in herbs and plants considered of medical importance by traditional medicine (e.g. quinine);
(ii) follow-up of accidental effects seen during modern physiological and biochemical investigations (e.g. penicillin);
(iii) massive screening programs (e.g. the anti-malarial mefloquine);
(iv) exploitation of known key metabolic pathways, or other biochemical activities, in man and pathogen (e.g. the anti-malarial pyremethamine).

Despite the ingenuity and sophistication that these four approaches have reached at the present time they all suffer from the fact that the target to be hit is invisible. A new strategy initiated in various laboratories starts from targets known at the atomic level.

The cornerstone of this new approach (1,2) is accurate knowledge of the three-dimensional structure of potential target proteins and other biomacromolecules. For this one needs *X-ray crystallography*, which is the only method available to obtain accurate three-dimensional information of biomacromolecules ranging in molecular weight from a few thousand to more than a million. One also needs *computer graphics* techniques because this is the only way to be able to analyse the complex structures elucidated by the crystallographers. It also provides a convenient interface with data bases of known structures and with hypothetical structures generated by *molecular mechanics* and *molecular dynamics* calculations as well as by other molecular modelling techniques.

Although each project differs in detail, a typical example of a modern drug design project is the following. Biochemists succeed in isolating small amounts of a medically important protein. A gas phase sequenator run establishes the amino acid sequence of the first twenty to eighty residues. This is followed by *oligonucleotide synthesis* of a series of DNA fragments that may hybridize with the gene encoding for this particular protein. Once the gene has been identified it can be sequenced rapidly and the complete amino acid sequence of the protein is established. Transfer of the gene into another organism where it

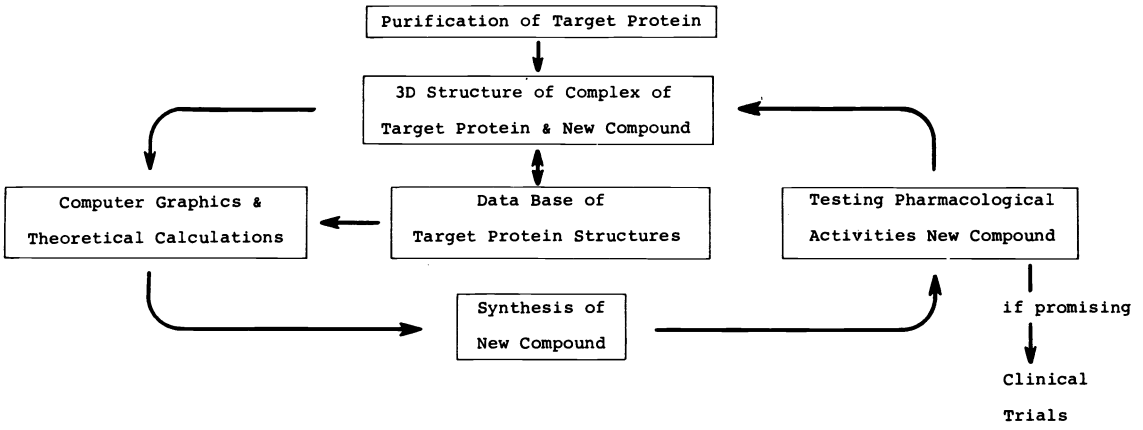


Fig.1. A rational drug design cycle

is brought to expression allows production of large quantities of the desired protein. After purification, which can sometimes be astonishingly simple if suitable *monoclonal antibodies* can be used, gram amounts of the protein are available. This is then a starting point for a full biochemical and kinetic characterization as well as for the growth of suitable crystals for an X-ray investigation. If crystals are obtained, protein crystallography yields, in a number of sophisticated steps, the three-dimensional structure of the target molecule with great accuracy.

Subsequently, computer graphics, quantum mechanics, molecular mechanics, molecular dynamics and a range of molecular modelling techniques are used and result in numerous suggestions as to which kinds of molecules might be useful drugs. These molecules have to be synthesized and tested for their effectiveness. This is little different from ongoing approaches in drug design projects. However, knowledge of the three-dimensional structure of target proteins allows an important feedback mechanism: structures of target proteins complexed with potential drugs can often be determined rapidly by X-ray crystallography once a first structure of the protein molecule is known.

Such three-dimensional structures of a protein complexed with various small molecules provide a powerful starting point for the design of new molecules. After all, the small molecules may bind differently than expected because the predictive procedures are still far from perfect. Also, structures of complexes may reveal major or minor conformational changes of the protein which might be exploited when designing new compounds. One may speak of a "rational drug design cycle" (Fig. 1) where each structure of a protein complexed with a new compound expands the data base from which firmer ideas for pharmaceutically more active compounds can be generated. The goal is, of course, to leave the cycle as soon as possible by obtaining a successful new drug.

## 3. THREE CASES

Three different cases for the development of new drugs on the basis of target protein structures can be considered:

(i) a human protein is not functioning properly and, hence, only that protein has to be considered - no proteins from infective agents need to be studied;
(ii) the potential target is a protein unique for the infectious organism and, consequently, no analogous human protein needs to be taken into account;
(iii) the target is a protein from a harmful organism which has to be inhibited in such a manner that the human isozyme is not impaired.

## 4. DRUGS WHICH AFFECT HUMAN PROTEINS

### 4.1. Sickle Cell Anemia

Hemoglobin was one of the first protein structures known, and it was also one of the first structures employed for designing new drugs. For instance, an attempt to design compounds which alleviate the clinical symptoms caused by sickle haemoglobin, HbS, has been reported (3). Substituted benzaldehydes were designed to bind preferentially to the oxy conformation of human haemoglobin at a site between the amino terminal residues of the α-subunits. The compounds produced indeed the desired effect: left-shifting of the oxygen saturation curve of dilute haemoglobin solutions and of whole blood. Most interestingly, one of the predicted compounds is also a potent inhibitor of the sickling of erythrocytes of sickle cell disease patients. It may prove to be a clinically useful anti-sickling agent (3).

Several other groups are using the three-dimensional structure of the hemoglobin molecule for designing anti-sickling agents, e.g. Walder et al. (4) and Abraham et al. (5). The latter collaboration is revealing the structures of a series of compounds complexed with hemoglobin, several of which appear to be bound in a rather unexpected manner (Perutz, personal communication).

It appears that at present hemoglobin is one of the very few human proteins of great interest for drug design for which an accurate three-dimensional structure is known. However, knowledge of the three-dimensional structure of an analogous protein from another organism may provide a starting point for obtaining a structure of the human protein - although one always has to bear in mind that more, and less, subtle changes in conformation may arise from amino acid substitutions. A few examples of such potentially useful structures are discussed below.

### 4.2. Hypertension

Hypertension is a major health problem in industrial societies contributing to cardiovascular disease, stroke and renal failure. Although its mode of action is not fully understood the hormone angiotensin II is an important factor in regulating blood pressure, with higher concentrations of the hormone leading to higher blood pressures. The hormone is produced in two steps. First, the enzyme *renin* generates angiotensin I from the inactive prohormone angiotensinogen. Second, the *angiotensin converting enzyme* (ACE) removes the carboxyl terminal dipeptide from angiotensin I, releasing angiotensin II. Inhibition of either renin or ACE, or both, would lead to a smaller angiotensin II concentration and contribute to lowering the blood pressure. No three-dimensional structure of ACE is known as yet but it is a zinc-peptidase and the structures of two such proteases are known currently with great accuracy: carboxypeptidase A and thermolysin (6,7).

The active site analogy of ACE and carboxypeptidase A has been utilized by Cushman et al. (8) to guide the design of specific ACE inhibitors. The first carboxyalkanoyl amino acid synthesized, succinyl-L-proline, had an $I_{50}$ value of $3.3 \times 10^{-4}$ M for inhibition of rabbit lung ACE. Starting from here, many modified compounds were synthesized, constantly keeping the general architecture of the carboxypeptidase A active site in mind. The most potent inhibitor found was D-3-mercapto-2-methyl-propanoyl-L-proline, with an $I_{50}$ of $2.3 \times 10^{-8}$ M (8). This inhibitor is now on the market as captopril and may be considered as the first generally available drug designed, albeit partly, on the basis of a known crystal structure of a protein molecule.

The aspartyl protease renin is considered a promising alternative candidate for inhibiting the angiotensinogen to angiotensin II pathway. This is based on the great specificity of this enzyme. Due to the availability of high resolution X-ray structures of several bacterial aspartyl proteases (9-11) and to the knowledge of the human renin amino acid sequence (12,13), computer graphics methods could be used to derive models of the human enzyme, which form the basis for making predictions of substrate binding (14,15). Detailed crystallographic data on the structure of an aspartyl protease complexed with an inhibitor showed that the predictions made were essentially correct (T.L. Blundell, personal communication). Clearly, considerable activity is going on in this area, further illustrated by the report on successful crystallization of mouse renin and renin-inhibitor complexes by an American drug company (16).

### 4.3. Inflammation and asthma

Prostaglandins, thromboxanes and leukotrienes are mediators of hypersensitivity reactions and inflammation (e.g. 17-19). They are formed as a result of complex metabolic pathways, in response to various stimuli, with the formation of arachidonic acid as a central step. This step is catalysed by the enzyme phospholipase $A_2$, more precisely the intracellular $Ca^{2+}$-dependent phospholipase $A_2$. In spite of its great importance, relatively little is known about this enzyme. However, Okomato et al. (20) showed recently that these intracellular phospholipases $A_2$ are immunologically related to the secretory phospholipases $A_2$, which have been characterized in great detail. Several three-dimensional structures of pancreatic phospholipases $A_2$ are known (21-24), while the structures of the snake venom and pancreatic enzymes appear to be very similar indeed (25,26). Clearly, this structural information can be a starting point for designing inhibitors of intracellular phospholipase $A_2$, because the immunological relationship (20) almost certainly implies a considerable similarity in the active site region. It would have been of great help if the structure of a phospholipase $A_2$ complexed with a phospholipid analogue were known but this has turned out to be difficult to achieve so far. The surprising conformational sensitivity of the pancreatic enzyme for even minute alterations (24) may have been one of the causes of the problems encountered. Nevertheless, the structure of the covalent inhibitor p-Br penacyl bromide bound to the active site histidine is currently known at 3.0 Å resolution - despite the fact that only a single suitable crystal was available (R. Renetseder, B.W. Dijkstra & J. Drenth, personal communication). This inhibitor binding mode may form a first experimental starting point for designing specific inhibitors.

## 5.INHIBITING PROTEINS UNIQUE TO INFECTIOUS ORGANISMS

### 5.1. Inhibition of bacterial cell wall synthesis

Penicillin is undoubtedly the best known example of a compound that inhibits, in an infectious agent, a vital metabolic step that has no counterpart in humans. The penicillin family has been enlarged by cephalosporins and other chemically related compounds, so that a whole range of antibacterial compounds, interfering with cell wall synthesis, is now available. This has caused a revolution in the treatment of bacterial infections in the twentieth century (27). Nevertheless, there remains a constant threat of drug resistance, which is in several cases caused by enzymes such as β-lactamases which are able to degrade members of the penicillin family rendering them harmless to the pathogen.

The X-ray structure of a $Zn^{++}$-containing D-alanyl-D-alanyl-carboxypeptidase from *Streptomyces albus* has been solved at 2.5 Å resolution by Diderberg et al. (28). This enzyme is penicillin-resistant with only the β-iodipenicillanate binding irreversibly to its catalytic site (28). Once the structures of this enzyme and of complexes with its inhibitor are known in greater detail, they may prove to be of importance for suggesting further modifications of the penicillin molecule enabling it to block classes of resistant carboxypeptidases.

Another important structure is that of a penicillin-sensitive D-alanyl carboxypeptidase-transpeptidase solved by Kelly et al. (29). These investigators provided also the first results of crystallographic binding studies of three types of β-lactams and a desazacyclobutanone (30). Very recently, structures of β-lactamases have been reported (31,32). Protein crystallography is clearly providing a solid framework for new drug development.

### 5.2. Virus coats

High resolution structures of viruses and of viral coat proteins are obviously of tremendous importance for the development of new vaccines against several common human diseases. But also from the point of view of rational drug design, the crystallographic studies on rhino- and poliovirus (33,34), and also on the influenza virus coat proteins haemagglutinin (35) and neuraminidase (36), are of potential use. For instance, inspection of the intricate subunit-subunit interactions of the viral coat proteins may reveal binding sites for small molecules interfering with the assembly of the viral particles. Alternatively, there would seem to be ample opportunity for designing precisely tailored cross-linking reagents - following lines used for the development of bivalent anti-sickling compounds (3): extensive cross-linking may render viruses into harmless entities. A third possibility is the design of compounds which fit tightly to the receptor binding sites of the rhino and polio virus and in this way prevent cell entrance. Obviously, these most impressive achievements of protein crystallography are rich sources of inspiration for medicinal chemists designing new anti-viral agents.

## 6. SELECTIVE INHIBITION OF PROTEINS FROM INFECTIOUS ORGANISMS

There are only a few examples in the literature of crystallographic projects related to inhibition of an enzyme from a pathogen while trying to avoid inhibition of an analogous enzyme in the human host. One well-known case is formed by the bacterial dihydrofolate reductases. Another example is that of the glycolytic enzymes from *Trypanosoma brucei*, the causative agent of sleeping sickness.

### 6.1. Dihydrofolate reductase

Dihydrofolate reductase (DHFR) is a key enzyme in the metabolism of numerous organisms and it is the target protein of several widely employed drugs. The enzyme is vital for proliferating cells because it is required for maintaining adequate levels of fully reduced folate, which in its turn is necessary for the synthesis of thymidylate.

In a most interesting paper, the group of Matthews and Kraut in San Diego recently described X-ray structures of not less than ten different compounds complexed with their target dihydrofolate reductase (37). These structures, together with crystal structures of the anti-bacterial drug trimethoprim bound to chicken and *E. coli* DHFR (38), provided an explanation of the differential binding of the drug to bacterial and mammalian DHFR's at the atomic level; residues on opposite sites of the active site cleft in the *E. coli* enzyme are ~ 1.5 - 2.0 Å closer to each other than equivalent residues in the chicken enzyme.

Space does not permit to go here into any details regarding this series of DHFR structures which gives marvellously detailed insight into the mode of action of drugs and related compounds. It almost certainly will lead, by going through the rational drug design cycle (Fig. 1), to new antibacterial compounds in the next decades. Reports on several investigations along these lines have already appeared in the literature (e.g. refs. 39-41).

### 6.2. Glycolytic enzymes and sleeping sickness

Sleeping sickness, or human trypanosomiasis, in sub-Saharan Africa is considered by the World Health Organization as one of the six most important current tropical diseases (42). The disease is caused by the protozoan parasite *Trypanosoma brucei*. Only three drugs are presently available for treatment of the disease, each exhibiting serious side effects (43). Clearly, new and better drugs are urgently needed.

Three groups, in Amsterdam, Brussels and Groningen, collaborate in an attempt to design new drugs against sleeping sickness. These groups focus on a unique feature of *Trypanosomatidae*: the presence of glycosomes. This are peroxisome-like organelles that are vital for the energy supply of the bloodstream form of the trypanosome (44-46). The major glycosomal proteins are nine enzymes involved in glucose and glycerol metabolism. All these nine enzymes are now available in pure form (47), while for five glycolytic trypanosomal enzymes the amino acid sequence has been determined by sequencing the corresponding genes. Crystallization of trypanosomal enzymes is even more difficult than usual due to the small amounts of material available. Nevertheless, using ~ 1 mg of protein, high quality crystals of the enzyme triosephosphate isomerase (TIM) were obtained (48), which allowed the solution of its three-dimensional structure (R.K. Wierenga, personal communication). Fortunately, TIM is also one of the enzymes for which the *T. brucei* sequence is available (49). Moreover, the three-dimensional structure of the chicken enzyme is known (50), which gives a good idea of the structure of the human enzyme due to the high sequence homology. The *T. brucei* TIM structure still needs considerable refinement before it provides a solid basis for rational drug design but it is already obvious that the design of compounds which inhibit catalysis of the trypanosome enzyme while leaving the human TIM undisturbed will not be trivial. The reason is the great similarity of the active site regions in the human and parasite proteins. One of the differences closest to the active site resides in residues 101-103, which are Ala-Tyr-Tyr in *T. brucei* and His-Val-Phe in man (49). To exploit this difference for the design of selective inhibitors one has to bridge a distance of ~ 20 Å between the substrate binding site and residue 102. This can certainly not be achieved in a single shot. However, via the rational drug design cycle it might possible to increase gradually the size of the compounds synthesized, see how they bind and deduce new modifications which eventually may provide the necessary selectivity.

Selective enzyme inhibition is not the only way, however, by which the presence of glycosomes can be exploited for the rational design of drugs against sleeping sickness. Unlike their human counterparts, glycosomal enzymes have to enter the glycosome and this provides opportunities to interfere with the import mechanism. Analysis of the sequence information on glycosomal enzymes, together with the known three-dimensional structures of triose phosphate isomerase, phosphoglycerate kinase and glyceraldehydephosphate dehydrogenase has led to a proposal for glycosome import (51): two areas of highly positive charge, ~ 40 Å apart, are considered to be essential elements of the import signal. Interestingly, this suggests a possible mode of action of suramin, one of the few drugs effective against sleeping sickness (43). Suramin is a quite complex molecule that has two tri-sulphonated naphtalene ring systems, ~ 40 Å apart in its extended conformation. The drug, known to interfere with glycolysis, might exert its therapeutic effect by preventing import of enzymes into glycosomes. Further investigations are required, and underway, to prove these hypotheses. Whether or not these speculations are correct, the glycosomal import mechanism is an interesting target for rational drug design because it may have no close analogue in human beings.

Amidst all these exciting new possibilities for rational drug design it is good to remember the long list of obstacles to be overcome before a compound showing a desired activity in a test tube can be sold on the market as a useful drug. Drug delivery and safety, possibilities of metabolic alterations and economics of production all need to be given great attention and are time-consuming. Nevertheless, in view of the great advantage of this strategy over existing ones it is not hard to predict that a considerable percentage of new drugs coming on the market by the turn of the century will be based on approaches outlined in this paper.

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