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13. ABSTRACT (Maximum 200) Ricin is a potent cytotoxin which has been used by governments and terrorists as a poison. The three-dimensional structure of this toxic molecule was solved by X-ray crystallography, including an atomic description of its active site. The goal of this project was to use computer searches and other molecular modeling techniques to identify an inhibitor or ricin A chain (RTA). The program CHEM-X was used to predict that pteric acid (PTA) would bind to RTA. This was shown to be the case by kinetic assays, where PTA protected ribosomes against the action of RTA, and by X-ray crystallography. The affinity of PTA is weak, with a K_i estimated at 600 μ M. The pterin group of PTA was observed to make many polar interactions with RTA within the specificity site of the enzyme, and to bind more strongly than the natural substrate adenine. Further work will be required to increase the binding affinity of this class of inhibitors, and to improve their solubility if efficacious antidotes are to be designed from this lead.			
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FOREWORD

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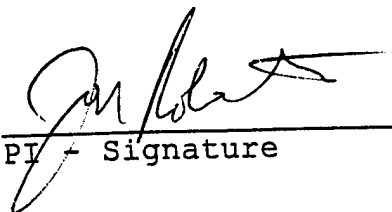

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TABLE OF CONTENTS

Front Cover

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Foreword

Table of Contents

Introduction Page 5

Goals of the Contract Page 6

Results and Discussion Page 7

Conclusion Page 12

References Page 12

Bibliography Page 15

Personnel Page 15

INTRODUCTION

The overall goal of this contract project was to use structural information to identify inhibitors of the cytotoxin called ricin. As will be developed below, this protein has been used as a poison and constitutes a potential threat to military and civilian personnel if used as a biological weapon. Our laboratory used X-ray diffraction to solve the three-dimensional structure of ricin and our mission was to use developing computer technology to identify potential ligands of the toxin which might serve as inhibitors. It was not our charge to synthesize novel inhibitors based on these leads, although we did in fact synthesize a few simple candidate inhibitors.

Ricin is a heterodimeric toxic protein which can be easily isolated from *Ricinus communis*--castor seeds (Olsnes and Pihl, 1982). It consists of a B chain lectin (RTB) and an enzyme A chain (RTA). RTB aids in cell surface binding and increases the efficiency of endocytotic uptake. RTA is able to cross endosomal, or other internal, membrane and enters the cytoplasm. It attacks ribosomes, removing a specific adenine base from the 28S rRNA (A_{4324} in rat) and thereby inhibits protein synthesis (Endo and Tsurugi, 1988). Ricin has an LD_{50} of ~ 1 mg/Kg body weight for mice, rats, and dogs and is ten times more potent against rabbits (Olsnes and Pihl, 1982). The toxic dose for humans is likely to be in the mg/Kg range and ranks it among the most toxic substances known.

Ricin gained notoriety when it was used in the famous "umbrella tip" assassination of Georgy Markov (Rich, 1992; Reuters, 1993) and was also used in an unsuccessful attempt to poison the famous Soviet dissident Alexander Solzhenitsyn (Remnick, 1992). Recently, ricin was prepared by a militant anti-tax group in Minnesota which planned to poison IRS personnel. Two men were arrested and convicted of the crime (deFiebre, 1995). The terrorists learned to prepare the toxin from an underground pamphlet circulated by a gun store; it referred to the poison as "the silent tool of justice". In a second incident, a man was arrested in Arkansas under antiterrorist statutes for possession of 130 grams of ricin. Two books describing the isolation of ricin were found in his home. The man had ties to survivalist groups and had been previously arrested for weapons trafficking; he committed suicide in his cell before he could be tried on the terrorist charges (Kifner, 1995).

Ricin can be dispensed as an aerosol and constitutes a major threat to military and civilian populations. There is strong evidence that Iraq has experimented with ricin disbursement as an element of its large biological warfare program. Ricin was loaded into 155-millimeter artillery shells and used against Iran in their recent war (Wright, 1995). The verified use of ricin as a poison by foreign governments and by terrorist groups emphasizes the importance of finding or developing inhibitors and antidotes and provides a strong rationale for undertaking this contract.

Structure-based Inhibitor Design

A number of stratagems are available to identify inhibitors for enzymes such as RTA. Historically, researchers have used trial and errors methods, testing the efficacy of

compounds which physically resemble the natural substrate. A given "lead compound" may then be improved upon by chemical synthesis, altering substituents in a random fashion.

Recently, academic and pharmaceutical laboratories have begun developing **structure-based drug design** paradigms. Knowledge of the molecular structure of the target protein allows identification or design compounds which are likely to be complementary in shape and charge to the active sites of enzymes; such compounds may act as inhibitors or drugs. Computer programs have been written to search the vast data banks of existing chemical compounds and to computationally fit them into the active site of the protein of interest.

The program DOCK searches small molecule structural data banks and computationally fits them into the active site of the protein of interest (Kuntz et al, 1982). It has been tested on known systems and shown to predict substrates and some known inhibitors for model cases (DesJarlais et al 1988) and also predicted that the known anti-psychotic drug haloperidol would bind to the HIV protease (DesJarlais et al, 1990). The program GRID can be used to map an enzyme active site with respect to the binding of various functional groups (Goodford, 1985). It was used in a classic study designing inhibitors for thymidylate synthase by the "iterative protein crystallographic algorithm" (Appelt et al, 1991). Similar crystallographic studies, together with computer aided search methods, were also used in the design of inhibitors of purine nucleoside phosphorylase (Ealick et al, 1991).

The Structure of ricin

As discussed above, knowledge of the molecular structure of RTA is vital to structure-based drug design. The X-ray structure of ricin was solved to 2.8 Å resolution (Montfort et al, 1987) and refined to 2.5 Å (Rutenber et al, 1991). The structure of the two individual chains has been described in detail (Katzin et al, 1991; Rutenber and Robertus, 1991). The crystal structures of two substrate analogs, formycin monophosphate (FMP) and the dinucleotide ApG, have also been solved and this allowed a molecular analysis of how RTA interacts with and binds its substrates (Monzingo and Robertus, 1992). It is important to note however, that although FMP bound into the active site of RTA, it is not an inhibitor in a protein synthesis assay. Its binding is too poor to compete with ribosomes.

The X-ray model allowed identification of a number of suspected active site residues for RTA. These include Glu 177, Arg 180, Tyr 80 and Tyr 123. Site directed mutagenesis of the cloned RTA gene has been used to examine the significance of all of these residues (Schlossman et al, 1989; Frankel et al, 1990; Ready et al, 1991). A putative mechanism of RTA action has been proposed which incorporates elements of the structural and kinetic analyses (Ready et al, 1991; Monzingo and Robertus, 1992).

GOALS OF THE CONTRACT

Results from this contract have been reported quarterly, and for the most part this document recapitulates those accomplishments, although in a more integrated fashion. The goals of the initial contract, as stated in the original program of work was described as:

During year one, to develop experience with the DOCK program. It is reasonable to expect that DOCK should predict the weak binding to RTA of formycin monophosphate (FMP) and the dinucleotide ApG in a fashion similar to that observed crystallographically. The DOCK parameters will be systematically altered, including the difficult choices of anchoring and sphere sets. By "tuning" the program for this protein we should be able to predict the known binding FMP. These results will effect the work over the next two years. It should tell us if the search needs to be run with several active site models, or if one large tetraloop binding site will work for all potential inhibitors.

In year two, to make a series of searches of the CSD to identify chemicals likely to bind to the RTA active site. These will be tested in kinetic assays and reasonable inhibitors analyzed crystallographically. The iterative method will be rigorously applied to search for a true RTA inhibitor. We hope to identify classes of compounds which appear to useful and therefore limit the search of the data bases. Crystal analysis of even fairly weak binding compounds will contribute greatly to our understanding of groups on the molecule which are most important to rational inhibitor design.

Year three will continue the iterative analytical program of year two. Design efforts will intensify within a limited class of molecules to optimize them as inhibitors. The program GRID will be brought to bear to assist in the rational analysis of inhibitors. The main goal is to identify compounds which may not have scored high in the computer search but which should be tested. Information from this process will be fed back into the DOCK parameters to improve its performance. Also this analysis, using GRID to complement chemical intuition, may predict the structures of inhibitors which are not available in the data bases and which may be the goal of chemical synthesis by others interested in this program. By the end of year three we should have identified a reasonable RTA inhibitor or suggested the design for one.

RESULTS AND DISCUSSION

The first program used to search for RTA inhibitors was DOCK 3.0, a program which requires that a topological "map" of the active site be created. Candidate molecules from the CSD were then systematically inserted and oriented based on shape complementary. As a test we initially tried to fit FMP to the active site because the X-ray structure had been solved and we knew how this compound bound to the RTA active site (Monzingo and Robertus, 1992). The goodness of fit is estimated by a "contact score" which was 220 for FMP. A comparison between the crystallographically observed binding of FMP and that predicted by DOCK 3.0 showed that DOCK makes a reasonable prediction of correct binding; this is illustrated in Figure 1. The results suggested it would be profitable to carry out a search of other compounds to identify inhibitors.

A number of anthraquinones scored well in the DOCK searches. Daunomycin had a contact score of 250, but was predicted to bind on the other side of the Tyr 80 ring from FMP. A second anthraquinone of interest was Cibacron blue. This dye has been used in RTA affinity columns and had been described as an RTA inhibitor (Watanabe and Funatsu, 1987). However, protein synthesis assays in our laboratory showed it was not an effective RTA inhibitor. Soaking the dye into the tetragonal crystals stained them a deep blue. This color could not be removed by back soaking, suggesting fairly tight binding. Difference Fourier analysis showed that no specific binding occurred, however.

Several thousand compounds from the CSD have been screened using DOCK 3.0 and the results have been disappointing. The program is excellent at fitting shapes; visual inspection of high scoring compounds revealed an excellent degree of shape complementarity. However, the snugly fitting compounds did not necessarily exhibit any chemical discrimination. The DOCK fitting does not attempt to match hydrogen bond donors and acceptors and many high scoring fits between RTA and candidate compounds were topologically satisfying but chemically meaningless. Furthermore, the scanning rate on the Silicon Graphics CRIMSON computer was slow, on the order of 50 to 100 compounds per hour. Our conclusion is that, despite apparent success in the literature, DOCK is NOT an effective tool for structure-based drug design. This is part of the reason we are applying for an extension of this contract. As described below we have developed a more successful paradigm for identifying inhibitors and we hope to exploit it find truly efficacious inhibitors of ricin.

Query based searches and energy calculations

A number of commercial firms have developed "query" based drug searching software. The basic idea is to list the chemical and/or geometric properties the investigator wishes to search for. These are coded to rapidly search an appropriate chemical data base to test if each listed compound has the desired properties. We initiated query based searches with the help of Dr. Robert Pearlman in the Pharmacy School at Texas. We used the program UNITY to search both the NCI data base and the available chemical data base. This brief collaboration produced a number of interesting inhibitor candidates, which proved upon kinetic testing, not to be inhibitors. It was also clear that Dr. Pearlman

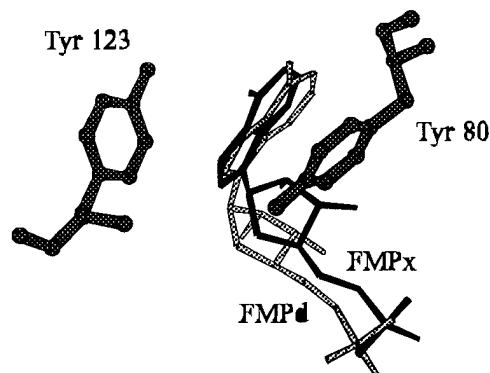


Figure 1: The predicted and observed binding of FMP to RTA. DOCK 3.0 predicted that FMP would bind between the rings of tyrosines 80 and 123, as shown in light bonds. The actual binding, as seen by X-ray diffraction, is shown in dark bonds.

did not have the personnel to continue to execute searches for our project, and it was also clear that the UNITY software was far too expensive for us to obtain.

As a consequence a similar program, CHEM-X for windows, was purchased from Chemical Design Ltd (Oxon, England). Unlike DOCK, CHEM-X cannot carry out precise shape fits, but it is vastly superior at finding chemically sensible candidates. Its speed is very impressive; for example, in a simple two dimensional search for chemical groups, CHEM-X can scan a 120,000 NCI data base on a time scale of a half hour. A more complex search, involving three dimensional constraints may require days run. The candidates listed by CHEM-X must be examined by eye to see if a given compound is worth pursuing with kinetic and crystallographic tests. To aid in this decision we have used the program SYBYL.

SYBYL (Tripos Inc., St. Louis) can calculate the energy (enthalpy) of interaction between the RTA active site and a ligand. Interaction energies are calculated using the Tripos force field with charges calculated by the method of Pullman (Berthod et al, 1967). SYBYL can also adjust structures for both the protein and the inhibitor model to optimize their fit. A full refinement of such a complex requires about 12 hours on the CRIMSON, and so must be used sparingly. As a standard, we found the interaction energy with adenine = - 37 Kcal/mole and with the formycin ring = -43 Kcal/mole.

Pterin based RTA inhibitors

X-ray models of FMP and ApG binding had shown us specific interactions between adenine and the RTA active site (Monzingo and Robertus, 1992). The nature and position of adenine chemical moieties formed the basis for a query based search which predicted that pterin compounds might bind to RTA. SYBYL predicted an interaction energy with pterin of -49 Kcal/mol. Among the pterins, pteric acid (PTA) looked intriguing because energy calculations predicted the benzoic acid moiety would bind on the opposite side of Tyr 80 from the pterin ring, forming a sort of clamp. This predicted binding is shown in Figure 2.

Pteric acid was purchased from Sigma Chemicals (St. Louis) and tested in the ribosome assay. The results are shown in Figure 3. The inset panel shows an apparent K_i for pteric acid of 600 μ M. This is relatively poor in absolute terms but it constitutes the first known inhibitor of ricin. Most importantly, it serves as a lead compound for design of better inhibitors using the iterative method.

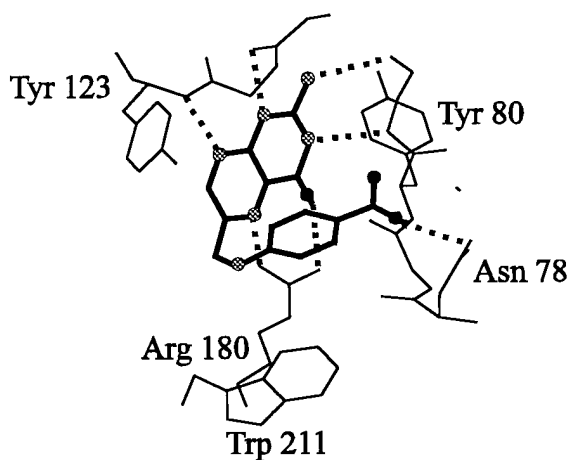


Figure 2: The predicted binding of pteric acid to RTA. RTA atoms are shown as thin bonds, and pteric acid as thick bonds. Nitrogens are shown as light and oxygen as dark dots. Hydrogen bonds are dashed lines.

Pteric acid was soaked into RTA crystals. It failed to bind to the tetragonal form at pH 5, but bound well to the monoclinic form at pH 9. The crystals suffered from some lack of isomorphism, but this was readily overcome by phasing with molecular replacement from the native protein using the program XPLOR (Brunger, 1988). The positioning of the inhibitor was straight forward. The geometric details of the binding are shown in Figure 4. Note that this is very close to the binding predicted from SYBYL and increases our confidence that the energy calculations will be a valuable tool in our inhibitor design protocol. The 2.6 Å distance between the O of Gly 121 and N1 of the pterin, presumably a hydrogen bond, tells us the tautomeric form of the inhibitor.

In addition to pteric acid we have tested several related compounds both kinetically and crystallographically. These include folic acid, neopterin, and 6-carboxy pterin. To date, pteric acid is the best inhibitor. Folic acid is not an inhibitor; presumably the large and negatively charged groups on the R substituents are repulsed by a net negative charge within the second pocket. That pocket is lined by, or contains residues such as aspartates 75, 96, 97, and 100. It seems likely that compounds should be polar, but neutral or slightly positively charged to have optimum binding in the second pocket. Neopterin binds to the crystals and acts as an inhibitor, although it is poorer than PTA. There is only one apparent interactions between RTA and the R group of neopterin, a hydrogen bond donated by the substituent alcohol to the side chain of Glu 177. This suggests that most of the binding forces arise from the pterin ring itself. In contrast to neopterin, the 6-carboxy pterin does not inhibit RTA or bind to the crystal. Two possible explanations are that the 6 carboxyl group may experience charge repulsion by Glu 177, or that it is too insoluble to be useful. The carboxyl derivative could only be made about 0.1 mM under assay or crystallization conditions and this may simply be inadequate to bind the compound significantly. Neither neopterin nor the 6- carboxyl pterin are able to bind into the second pocket and so both lack the favorable interactions seen there for PTA.

Based on the success with PTA, efforts were made to identify related compounds which would bind more strongly to RTA. Computer aided data base searches failed to

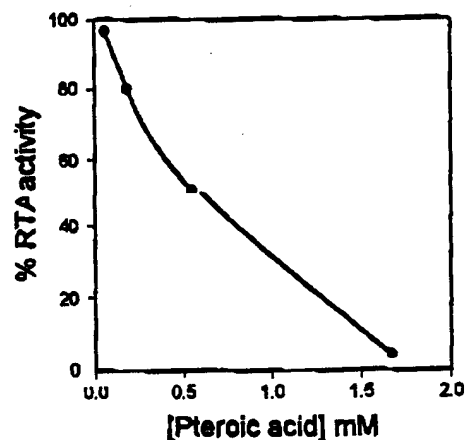


Figure 3. Inhibition of RTA by pteric acid. The methods employed in the assay are described in Ready et al., 1983.

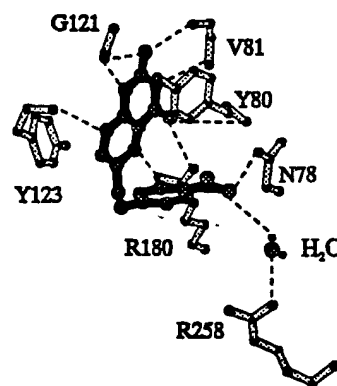


Figure 4. The observed crystallographic model of pteric acid (PTA) binding in the active site of RTA

identify any similar pterin-based compounds whose structure appeared likely to make stronger interactions with RTA than does PTA.

We then searched for novel compounds which could be easily synthesized and which might have better binding. It was possible to synthesize a pterin derivative, which we call TH-1, by the route shown in Figure 5. The predicted interaction energy for this compound is close to that for PTA, and encouraged us to examine it as an inhibitor. TH-1 was purified using silica filtration to at greater than 99% homogeneity as judged by mass spectrometry and tested as an inhibitor of RTA. Unfortunately, the novel compound was less potent than PTA.

The analysis of PTA called attention to the notion that slightly larger pendent containing derivatives might reach into a "second cleft" pocket on the ricin surface. Late in the contract period we explored this idea more fully by creating a model inhibitor which could be synthesized by the same chemistry shown in Figure 5, but used to couple PTA to commercially available compounds such as benzyl amine or a dihydroxy derivative of benzyl amine. We used SYBYL to dock and orient the latter derivative into RTA, and found that it was predicted to have an interaction energy substantially more favorable than simple PTA. The theoretical mode of binding is shown in Figure 6. It shows the same pterin interaction observed for PTA (Figure 4) and shows that the pendent benzyl group makes many additional hydrogen bonds within the second pocket. As the project ended we carried out a successful trial synthesis of the compound and are preparing to scale it up for future work.

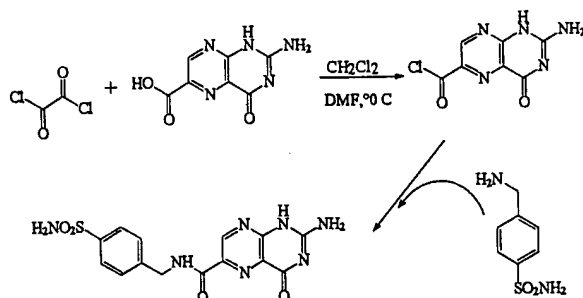


Figure 5. The synthesis of a potentially improved RTA inhibitor, called TH-1. All materials are commercially available. The one step reaction is carried out under argon in dichloromethane. Dimethyl formamide, DMF, acts as a catalyst.

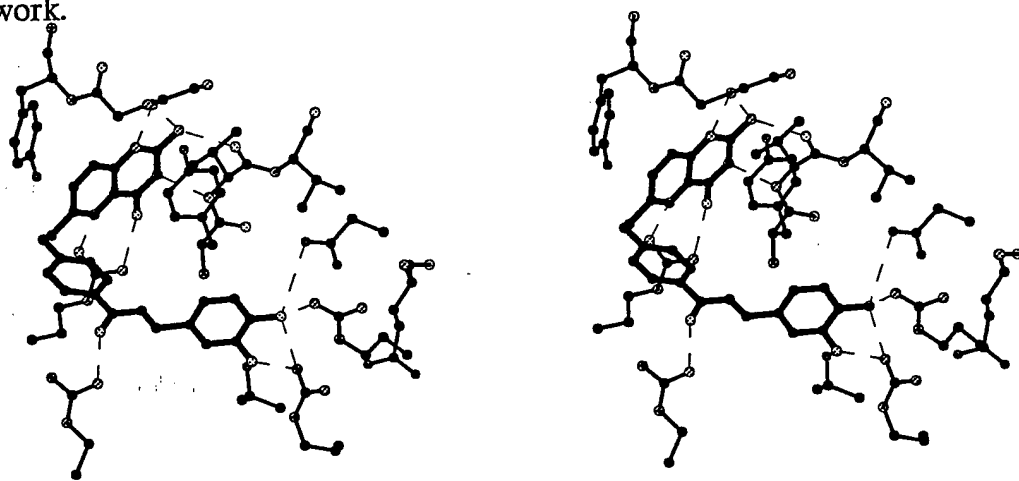


Figure 6: A stereo picture of the binding of a PTA based inhibitor, with a dihydroxybenzylamine pendant. The inhibitor is shown in heavybonds and the RTA atoms as lighter bonds. Hydrogen bonds are dashed lines.

CONCLUSION

Several computer programs were tested as aids to structure-based inhibitor design. In our hands the most useful was CHEM-X, a query based search engine. It is our opinion that experimenter observation and model building will be essential to the inhibitor search protocol. Computer programs are superb at repetitive searches but have limited imagination to widen their searches. Energy calculations may also be of help in predicting inhibitor interactions, but only if used cautiously. The programs available today are very crude; none deals with solvent effects or ligand conformational entropy in a realistic way. Calculations may be helpful if made within a class of related compounds for which solvation and entropic effects are comparable.

Our search has revealed pterioic acid (PTA) to be an inhibitor of RTA, but it can be considered only a starting point for further research. PTA is poorly soluble at neutral pH and even at pH 9 has an apparent K_i of only 600 μM . The pterin ring of PTA makes a large number of interactions with the RTA active site "specificity" pocket and nearly saturates the polar groups in that site. Pterin makes more interactions than the natural adenine substrate. It may be possible to define related moieties with improved interactions and solubility, but this is not obviously the case at this time. If pterin is close to an optimal specificity site ligand, then it may be that specifically-binding pendant groups will be the key to the design of efficacious RTA inhibitors, which might eventually serve as toxin antidotes.

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