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13. ABSTRACT (Maximum 200 words) Staphylococcal enterotoxin B is a superantigen produced by staphylococcus aureus. It is known to form complexes with the major histocompatibility complex class II and T-cell receptor. A recent study showed that the use of peptide antagonists failed to block the binding of SEB to MHC II and also failed to inhibit T-cell activation and cytokine production (Rajagopalan et al 2004). Since the immunopathology of SEB is T-cell dependent, inhibiting the binding to the MHC II-T-cell complexes would be a potential therapeutic strategy. We plan to develop in silico pharmacophore models to identify new potential SEB-MHC II inhibitors through compound database searches. The identified inhibitors will be tested in the appropriate in vitro assay. In silico pharmacophore modeling and identification of potential inhibitors for the interactions between SEB and MHC-II through compound database searches. In silico three dimensional pharmacophore model for inhibitors of the formation of SEB-MHC-II complex will be performed using the InsightII and CATALYST methodologies (Accelrys, Inc., San Diego, CA). 1- We will use Unix-based workstations to apply InsightII and CATALYST methodologies (Accelrys, Inc., San Diego, CA) to identify new potential inhibitors of the interactions between SEB and MHC II using a small molecule database. InsightII allows				
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Enclosure 1

Apoptosis Use Case: In silico evaluation of a library of small molecule pharmacophore models for blocking the formation of SEB-major histocompatibility class II complexes

Rasha Hammamieh

Introduction:

Staphylococcus aureus is a bacterium responsible for the production of a number of secreted toxins (named Staphylococcal enterotoxin (SE) A through H and Toxic Shock Syndrome Toxin 1). These toxins are closely related structurally (Marrak & Kapler, 1990) but categorize into 2 groups of greatest similarities SEA vs SEB. SEB is the most abundant toxin produced (in mg/ml quantities), while SEA has been somewhat more frequently identified as inducing classic symptoms of food poisoning, usually resulting from poorly stored meats and dairy products. Exposure to large quantities of SEB can also cause nonmenstrual toxic shock syndrome (TSS) and has been characterized as a triggering agent in many autoimmune disorders. SEB is an extremely stable toxin in aerosols, resistant to heat, and has very low lethal and incapacitating inhalation doses. Because of SEB's ability to cause wide spread systemic damage, multiorgan system failure, shock, and even death, and other factors, it is included as a Class B biological threat agent.

The purpose of discovering a method to prevent or treat illness induced by the SE toxins is of the utmost importance because of how common it is to encounter the potentially fatal bacterial products. There has been considerable research conducted to study biological actions of these toxins, and many reports have documented extensive food poisoning, but few of these cases have been severe enough to result in lethality. However, the unimaginable impact of accidental food poisoning was recently documented at a celebration of a Catholic priest's ordination ceremony in Brazil, by an incident involving approximately 8000 individuals who were exposed to food contaminated with SEA. One food handler was found to asymptotically carry toxin producing *S. aureus*, even on throat swabs. Within 4 hours of consumption of contaminated food, approximately 4000 people were incapacitated with intense nausea, emesis, diarrhea, severe abdominal pain, prostration, and dizziness. Approximately 2000 went to clinics or hospitals in small towns even miles away, 396 required subsequent admissions, and of these patients, 81 were admitted to intensive care units, and 16 died (Carmo, et al, Foodborne Illness. Dec 2004). The situation described above is uncommon due to the numbers of people affected in this single incident and although there are very many reports of SE-induced illness from food poisoning (Jett et al, 2001), the more serious complications arise from infections encountered in hospitals in which SE intoxication leads to highly lethal toxic shock (Jett et al, 2002). Of course other

hospital infections may be caused by Gram-negative organisms that produce lipopolysaccharide (LPS), a cell wall component that stimulates similarities with SE-induced lethality starting with flu-like symptoms, plunging blood pressure, and eventually leading to edema, vascular leakage, pooling of blood in the capillaries, hypoxia, hemorrhage into the tissues and to finally irreversible shock including multi-organ failure and vascular collapse (Karima et al 1999). Efforts to identify intervention strategies for septic (LPS) or toxic (SE, for example) shock have been studied for over a century, but “irreversible” shock is a formidable foe. As a result, development of a vaccine for prevention or an administered treatment is urgently needed. In my quest to discover a treatment to intervene in the toxicity induced by SEB, my project focused on the following:

Find a suitable treatment for SEB by linking the computer sciences world with the biological world by using computer-modeling technology to expedite the treatment search

Find potential SEB inhibiting compounds by using the three-dimensional *in silico* pharmacophore models

Demonstrate selected compounds’ peptide inhibition levels by fluorescence in a toxin-binding assay

Verify that the pharmacophore will not, itself, induce the same actions as would be initiated upon interactions with SEB.

I found that most of the published scientific studies about SEB either dealt with counteracting SEB by means of biological experimentation or three-dimensional analysis of SEB. I contemplated if it would be possible to combine the biological field of experimentation with mathematical computational analysis to find an appropriate treatment for SEB-induced toxic shock.

Three-Dimensional Pharmacophore Model

Staphylococcal enterotoxin B is a bacterial superantigen i.e., proteins of bacteria that stimulate the release of T cells by cross-linking T cell antigen receptors (TCRs) and major histocompatibility complex class II (MHC II) molecules on monocyte/macrophages. This stimulation results in hyperactive responses causing T cell proliferation and deletion/sequestration of specific subsets of T-cells. A more inclusive definition of a superantigen is an antigen that interacts with the T cell receptor in a domain outside of the

antigen recognition site. This type of interaction induces the activation of larger numbers of T cells compared to antigens that are presented in the antigen recognition site.

Mutational analyses of proteins, such as superantigens, are most useful when combined with structural studies, such as X-ray crystallography. The abundant production of superantigens, like SEB (at ~1 mg/ml), along with soluble forms of the normally membrane bound MHC II and TCR molecules have made it possible to elucidate the interactions of these superantigen/MHCII/TCR 3-D structures. The combination of mutational information and 3-D X-ray crystallographic structures provide a wealth of information on the modus operandi of superantigens, and insight into how the immune system has evolved under the selective pressure from these toxins (detailed information can also be used for rational mutational design). This information is required for the production of superantigens as therapeutic agents.

MHC molecules, also known as human leukocyte antigens (HLA), are the products of a cluster of genes in the human DNA known as the MHC. These antigens are present on various human cells and enable T-lymphocytes to recognize epitopes and to identify self from non-self. When the SEB binds to MHC II, the T-lymphocytes are activated to release cytokines that cause the physical distress associated with the superantigen. The cells that were selected to be analyzed in the toxin-binding assay were the HLA-DR expressing cells.

Small molecule inhibition of protein (SEB) to protein (MHC) interaction would halt the devastating effects of the immune system in the human body. Small molecules possess numerous advantages to the drug discovery process as compared to peptides and natural products. The SEB protein consists of 239 amino acids and has the molecular weight of 28.4 kDa. The molecule consists of two tightly packed domains (as shown in figure 1).

Materials and Methods

A three-dimensional pharmacophore model for SEB binding was developed from the binding information of the SEB- MHC II complex (Jardesky, et al) using the CATALYST methodology [CATALYST Version 4.9 software, 2004, Accelrys Inc., San Diego, CA.]. The algorithms in CATALYST treat molecular structures as templates comprising of chemical functions localized in space that will bind effectively with complementary functions on the on the respective binding proteins.

It enables the use of X-ray structural data to generate geometric templates of pharmacophores, characterizing the binding interactions. The most relevant chemical features were extracted from the residues involved in the binding interactions to develop a complementary model for the inhibitors. Molecular flexibility was taken into account by considering each compound as an ensemble of conformers representing different accessible areas in a three dimensional space. The best searching procedure was applied to select representative conformers within 10 kcal/mol of the global minimum (Greenridge, Weiser. 2001). The geometric template was used to search existing 3-D multi-conformer databases to identify potential small molecule SEB inhibitors. *In silico* three dimensional pharmacophore model development for anti-MHC II activity was performed by identifying the binding interactions of the binding pocket, as observed in the crystallographic 3-D structure of the superantigen. The template for interactions such as hydrogen bond acceptors, hydrogen bond donors, and hydrophobic sites were obtained using the CATALYST methodologies (Accelrys, Inc., San Diego, CA). CATALYST allows generation of functional attributes of molecules to develop the pharmacophore for specific biological activity. The computations were carried out in unix-based workstations. The pharmacophore models and binding site analysis of the MHC II crystal structure allowed generation of a reliable template, which was used to search compound databases to identify new potential small molecule MHC II inhibitors. This procedure was pursued iteratively until optimal target specific inhibitors were obtained.

3-D Pharmacophore

The conformational model used for pharmacophore generation within CATALYST aims to identify the best three-dimensional arrangement of chemical functions; such as hydrophobic regions, hydrogen bond donor, hydrogen bond acceptor, and positively and/or negatively ionizable sites distributed over a three dimensional space explaining the activity variations among a training set. The hydrogen bonding features are vectors, whereas all other functions are points. The statistical relevance of the obtained pharmacophores were assessed on the basis of the cost relative to the null hypothesis and the correlation coefficient (Gunner, 2000). The pharmacophores can be used to estimate the activities of the training set. These activities are derived from the best conformation generation mode of the conformers displaying the smallest root-mean square (RMS) deviations when projected onto the pharmacophore.

The statistical significance of the reported pharmacophores should be within the recommended range [CATALYST Version 4.9 software, 2004, Accelrys Inc., San Diego, CA] of values of the CATALYST procedure. The difference between the fixed and the null cost should clearly indicate the robustness of the correlation. Moreover, as the cost difference between the first to the tenth hypothesis and the null hypothesis should vary between certain recommended limits so that it could be expected that for all the pharmacophores, there is 80-92% chance of representing a true correlation in the data. To further alleviate any problem of chance correlation, a Fischer randomization was performed (as implemented in the CatScramble module of the CATALYST [CATALYST Version 4.9 software, 2004, Accelrys Inc., San Diego, CA]) to generate several random spreadsheets from the training set and calculate the statistical significance of the models. The greater the number of randomized generated models that require a total cost value lower than the model under investigation would indicate greater possibility of confidence level of the generated pharmacophore model.

The WRAIR-CIS database has over 290,000 compounds and it was transformed into a multi-conformer database in CATALYST, using the catDB utility program as implemented in the software [CATALYST Version 4.9 software, 2004, Accelrys Inc., San Diego, CA]. The catDB format allows a molecule to be represented by a limited set of conformations, thereby permitting conformational flexibility to be included during the search of the database. The best fit mapping of the pharmacophore was utilized (in potent analogues) by using: a fast-fit algorithm, a principle component analysis, a partial least squares technique, a linear regression technique, or a non-linear regression technique. The pharmacophore was also converted into a 3-D shape based template containing all the chemical features necessary for potent SEB inhibition and used for WRAIR-CIS database searches. Shape-based template of the pharmacophore may be useful for generating the potent analogues to incorporate the steric factors associated with the small molecule inhibitors. The plan is to continue this procedure to obtain more potential inhibitors for the SEB MHC II.

Each compound in the WRAIR-CIS was converted into 3-D multi-conformations with an energy range of 0-20 kcal/mol, filtered by using the catDB algorithm of CATALYST and stored in a SGI Octane workstation. The procedure of compound identification and down selection will be furthered by generating several shape-based pharmacophore templates on a few potent analogues from the results of the inhibition study. This approach allowed the addition of the steric attributes

along with pharmacophoric features necessary for binding of the SEB target. Database searches based on this procedure have been reported in the identification of both similar and new compounds (Bhattacharjee et al., 2004 and Hicks et al., 2004).

These identified compounds were tested in the assay to validate the success of the model. Based on the activity data obtained from the experimental assay, a training set of SEB inhibitors was developed which may be iteratively refined with ongoing and further experimental biological activity data.

Toxin Binding Assay

The steps for culturing the cell lines CCL-86 and CRL-2769 were conducted as instructed by the manual. The cell lines were frozen in a liquid nitrogen vapor phase until the ready for use. The cells were taken out of the freezer, thawed rapidly and placed in culture fluid. Fetal Bovine Serum (FBS) at 20% was mixed into RPMI 1640 media. The cells were culture for 2 weeks to obtain stability prior to use. The medium was filtered and 10 mL were transferred into each of the culture flasks that already contained 5 mL of the cell suspension.

To begin the binding studies, the contents of one of the cell culture flasks was emptied into a 50 mL conical tube and centrifuged for 10 min at 400 x g. Then the media supernatant solution was decanted until a small amount of media and the pellet remained. The pellet was resuspended in the tube with the remaining media by carefully and smartly tapping the tube on a hard surface. I resuspended the cells in PBS/1% BSA/1% gelatin mixture and counted the cells using a hemacytometer.

Suspended cells (1×10^6) were placed in each tube for the binding assay and the tubes were labeled as follows: control, CCL-SEB-50 μ M, 10 μ M, and 1 μ M. The SEB-treated cells and control cells were incubated at 4°C for two hours. Then, the cells were washed once with a solution of 1% BSA/PBS. After that, the cells were incubated again with the addition of toxin-specific polyclonal rabbit antiserum diluted 1:100 (10 μ L/mL) in 1 mL of PBS/ 1%BSA/ 1% gelatin for one hour on ice. The cells were pelleted by centrifugation @400 x g for 10 min, resuspended and centrifuged again to wash away unbound SEB, then incubated on ice for 30 minutes with antisera specific for SEB (Toxin Technologies, Inc, Miami, FL). This is a sandwich antibody format that utilized specific rabbit antisera to SEB, then was followed by antisera raised to rabbit IgG. It was the second antisera that contained the fluoroisothiocyanate (FITC) molecule which provides the fluorescence. FITC-labeled goat anti-rabbit Ig-G diluted 1:100

(10 $\mu\text{L/mL}$) in 1 mL of PBS/BSA/gelatin solution, and the cells were washed twice, then resuspended in 1 mL of the same solution. Two hundred μL of each sample were transferred into a 96 well flat-bottom plate and scanned for fluorescence using FX scanner (BioRad) and image was analyzed using the QuantityOne program. So the amount of SEB bound to the washed cells is directly proportional to the bound fluorescent antibody. In order to test inhibition of SEB to MHCII, cells were first incubated with the test compound, the hypothesis being that if it occupies the MHCII binding site, it will prevent SEB from binding, depending on affinity constants.

The assay relies on the fact that SEB binds to the MHC II complexes on the cells and that may be with higher affinity than the small molecule. Fluorescence occurs when the antibody is tagged to the SEB that is bound to the complexes on the cells. The more SEB that binds to the cells, the higher fluorescence that is expressed. The tested compounds selected are those that interfered with the SEB's binding to the cells. The attachment of compounds inhibits these molecules, resulting in a decrease of fluorescence.

Results

Creation of Pharmacophore Model

To determine the distances between atoms in the hydrophobic pocket, *RasMol*® software was utilized to measure the distances between the vital amino acid sequences to create the elementary pharmacophore model. The following sequences were chosen based on a prior experiment (Jardetzky et al., 1994) that studied the structural crystallography of SEB (Figure 1), and they include the amino acids in the MHC II binding site region: phenylalanine 44, leucine 45, phenylalanine 47, tyrosine 13, methionine 36, alanine 37, leucine 60, isoleucine 63, alanine 64, and glutamine 18. The distances between the atoms were measured in Angstrom units ($\text{\AA}$). Distances of no more than 6 $\text{\AA}$ between atoms were used to narrow down the search of analyzing what compounds can readily fit into the pocket to inhibit the binding that took place (see figure 2). The final list is shown in table 1.

TABLE 1				
First Amino Acid Sequence	Atom	Second Amino Acid Sequence	Atom	Distance ($\text{\AA}$)
MET36	carbon	LEU45	carbon	4.917 $\text{\AA}$

TYR13	hydroxide	PHE44	oxygen	3.047 Å
PHE44	carbon	LEU60	oxygen	3.982 Å
PHE44	oxygen	ALA64	carbon	8.474 Å
LEU45	carbon	ALA64	carbon	8.322 Å
LEU45	carbon	TYR13	hydroxide	6.253 Å
PHE47	carbon	ALA37	oxygen	6.311 Å
MET36	oxygen	PHE47	carbon	4.642 Å
PHE47	carbon	LEU60	carbon	7.873 Å
ALA64	carbon	PHE44	carbon	3.923 Å
PHE44	carbon	ALA64	carbon	4.103 Å

Potential SEB Inhibiting Compounds

The pharmacophore developed from the analysis of the binding pocket of the 3-D crystal structure of the SEB MHC II protein is shown in Figure 3. The elementary pharmacophore generated following the above analysis contains two hydrogen bond donors, two hydrogen bond acceptors, one aromatic hydrophobic and one aliphatic hydrophobic site.

The pharmacophore allowed for the in-house Walter Reed Army Institute of Research (WRAIR)-Chemical Information System (WRAIR-CIS) database [The Chemical Information System, Division of Experimental Therapeutics, WRAIR, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500, U.S.A.] to search for potential SEB inhibitor sets' complementarity requirements. The search resulted in identifying 300 compounds from this database. The down selection of the identified compounds was carried out by evaluating the *in silico* ADME (absorption, distribution, metabolism and excretion) /Toxicity properties, and choosing only those compounds that have favorable properties. ADME/Toxicity evaluations were carried out by using Cerius² [Cerius² 4.9, 2003, Accelrys Inc., San Diego, CA] and TOPKAT [TOPKAT 6.1, 2003, Accelrys Inc., San Diego, CA] methodology as implemented in the software. The compounds were narrowed down to eighty-one. Fifteen compounds were initially tested in the cell binding assay. This part of the study had two goals: 1- to develop *in silico* pharmacophore models from the crystallographic information of the superantigen, and 2- identify new potential MHC II-cell inhibitors through compound database searches. These goals led to the testing of the identified inhibitors in the appropriate binding assay.

Two cell lines were initially evaluated since they both have high expression levels of MHCII, however, one of the cell lines, Raji lymphoid cells showed much better binding capability than did the other cell line (Figure 4). An example of one inhibition experiment, Figure 5, illustrates the results obtained upon testing these selected compounds to determine their ability to inhibit binding of SEB to MHC II on Raji cells. In this scan of fluorescence, inhibition of the binding of SEB to Raji cells was examined. Results are shown with compound WR93716 carried out in 4 replicates (rows) at concentrations (50, 10, 1 µg/ml, respectively, from left to right). Of the 81 highly relevant compounds, 15 were obtained and we proceeded to test their ability to inhibit SEB binding to Raji cells. Table 2 shows a compilation of the fluorescence intensities and identifies 3 of the compounds as showing good blocking of SEB interaction with the Raji cells. Those 3 compounds are described as follows:

WR093716: 2-Guanyl hydrazo-butyro phenone acetate (discrete – cannot be shown without clearance) (Fig. 6)

WR107322: 2-Chloro aceto amido-3-phenyl propionanilide (discrete – cannot be shown without clearance) (Fig. 7)

WR058190: 5,5'-dibromo 2,2'-dihydroxy benzyl (open compound, can be shown) (Fig. 8).

Discussion

The three-dimensional structure of the bacterial superantigen, *Staphylococcus aureus* enterotoxin B (SEB), bound to a human MHC II molecule (HLA-DR1) used X-ray crystallography with the resolution of 2.7 Å (Jardetzky et al., 1994). The topological view of the binding interface shows a distinct division of complementary surfaces of the DR1 and SEB molecule. Each binding surface is divided into two regions, one predominantly hydrophobic and one predominantly polar. The hydrophobic region of the interface consists of a ridge of non-polar residues (F44, L45 and F47), whereas the polar region of the binding interface is more apparent on the SEB interaction surface, which is left of the hydrophobic ridge. The polar pocket on the SEB surface is complemented by a protrusion from loop 3 of the DR1 molecule formed by lysine 39. This lysine forms a salt bridge with glutamic acid at position 67 of SEB, surrounded by hydrogen bonds from SEB Y89 and Y115. The mutation of lysine 39 in the DR1 molecule reduces SEB binding. Thus, the above wealth of information provides sufficient background to develop a

pharmacophore model for SEB binding and utilize the model to identify small molecule inhibitors through design or search of existing databases. The 3 compounds found to most successfully inhibit SEB binding to Raji cells clearly incorporate all of these properties described above. I am continuing to study other aspects of the inhibition seen with these three pharmacophores. This current study shows their capability to block SEB binding, but would they displace SEB that is already bound?

On the basis of conformational energy level, absorption level, polar surface area, atom-based LogP (AlogP98), blood brain barrier penetration level (BBB), solubility level, and rule of 5 violation compounds were eliminated from the CIS database list for determination of the selection of compounds for inhibiting the binding site. Polar surface area gives a measure of lipophilicity or hydrophobicity of a compound. This property is important for blood-brain barrier (BBB) penetration. Favorable ADME is taken into account because ADME provides a measure of bioavailability of certain compounds due to oral absorption. AlogP98 is the numerical measure of lipophilicity or hydrophobicity of a compound based on its atomic composition. According to the Rule of 5 Violation, poor absorption of a compound is likely when several of the following occur: there are more than 5 hydrogen bond donors, there are more than 10 hydrogen bond acceptors, the molecular weight is over 500, or the log P is over 5. In vitro ADME models cut down on animal tests by acting as a pre-screen. The models are based around real tissue samples, which have similar properties to those in the body. The compounds examined were short-listed to 237. These results were used to perform the in vitro toxin-binding assay to determine if the potential inhibitors truly do inhibit the SEB protein from binding to the MHC protein. If so, a drug could be created from the observed results, and the devastating effects of SEB on the human body would no longer be a threat to those exposed to this toxin because there would be a known antidote. The *in silico* theoretical inhibition with a small molecule is a tremendous step forward, in not only the link between computational analysis and experimentation, but also for drug researchers everywhere.

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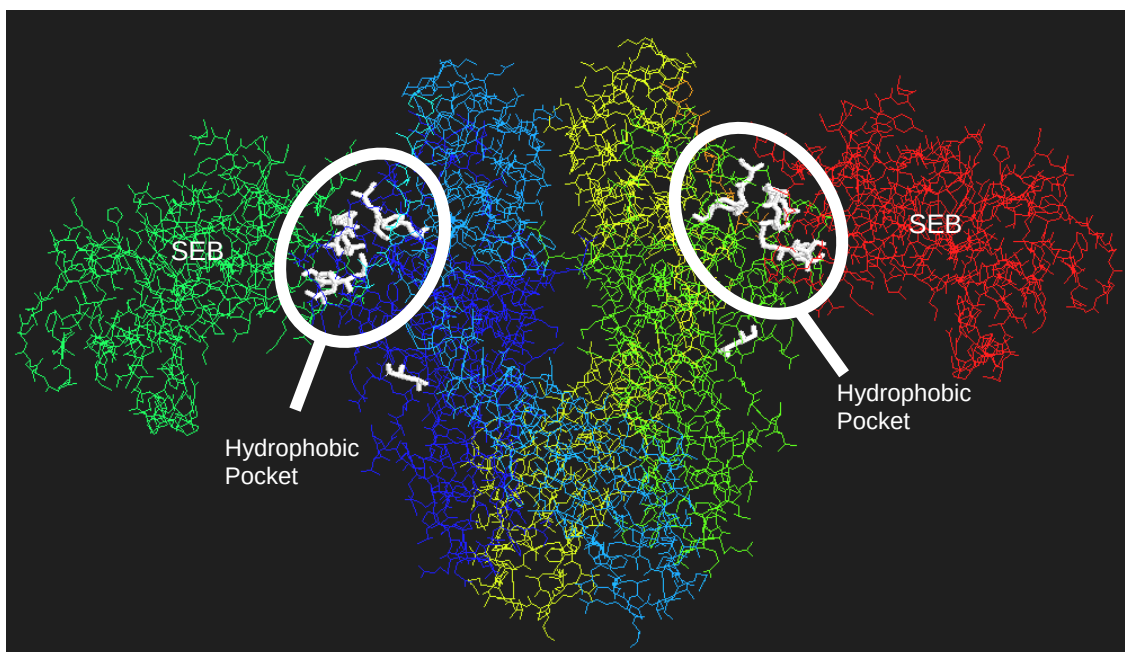


Figure 1. The highlighted amino acid sequences comprise the binding site.

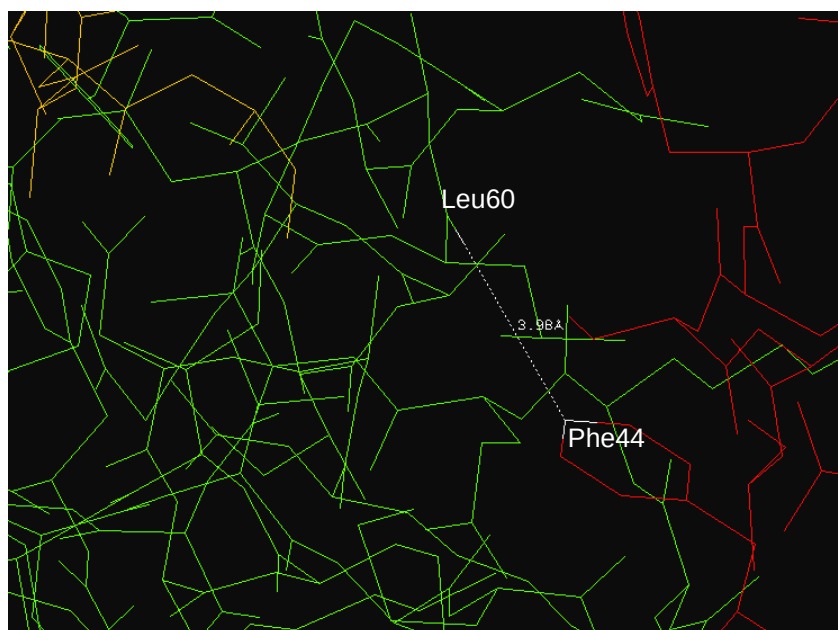


Figure 2. The distance of leucine 60 (carbon)-phenylalanine 45 (carbon) measured in angstroms.

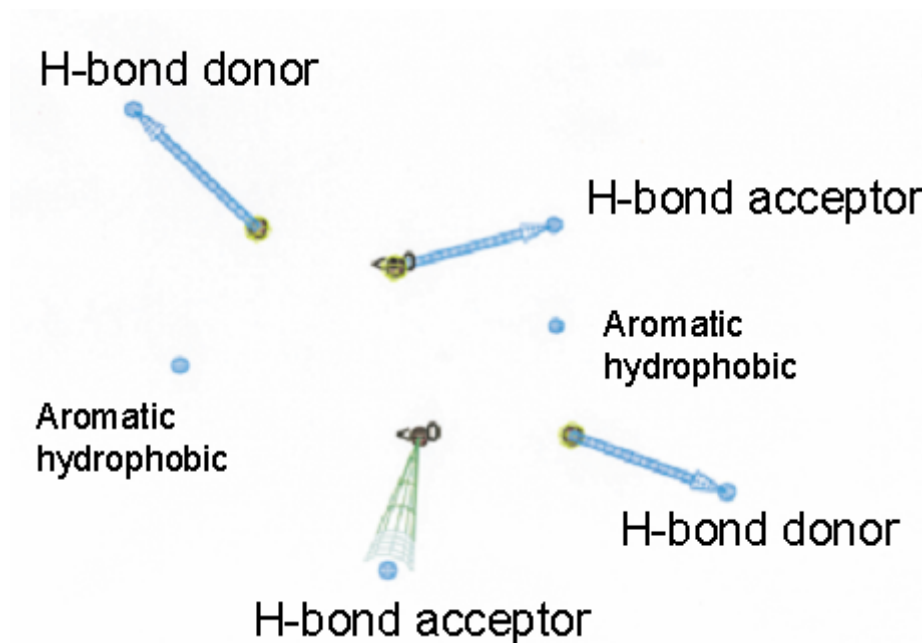


Figure 3. The elementary pharmacophore model must have a hydrogen bond donor connected to every hydrogen bond acceptor.

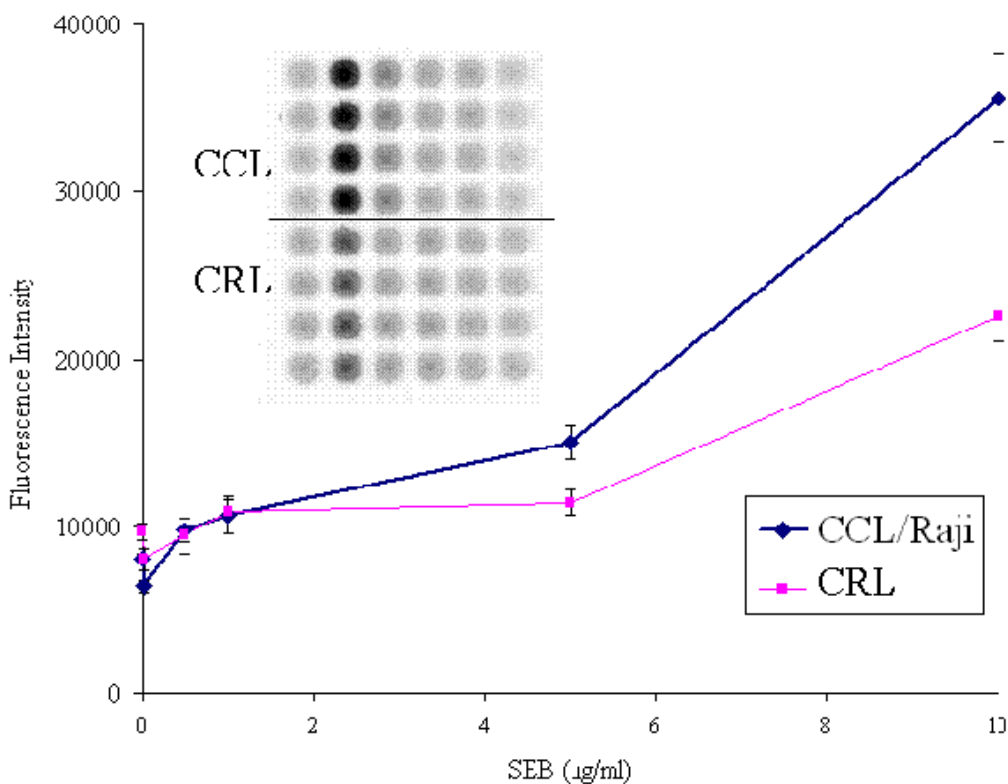


Figure 4: Binding of SEB to 2 MHC II bearing lymphoid cell lines.

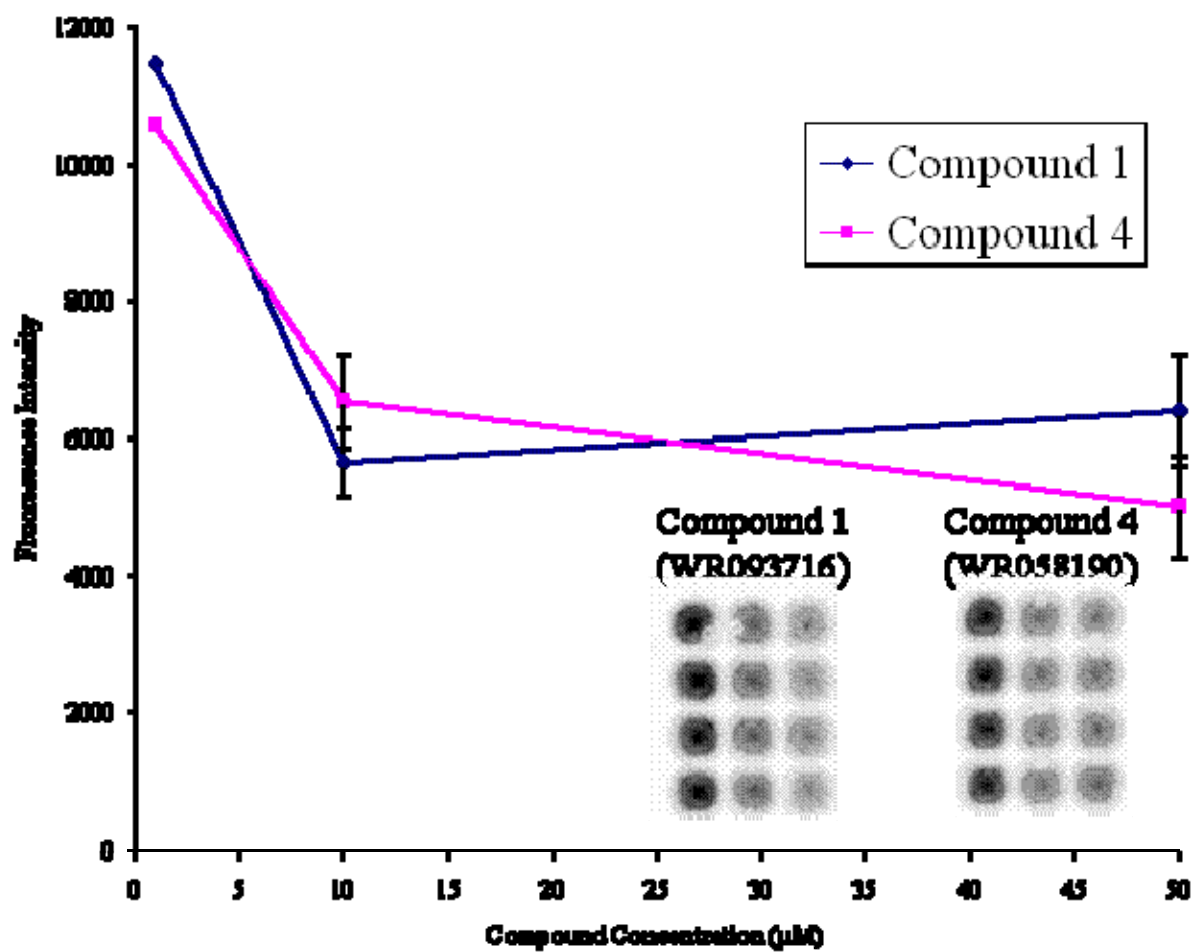


Figure 5: Compounds 1 and 4 percentage of inhibition of SEB binding to Raji lymphoid cells.

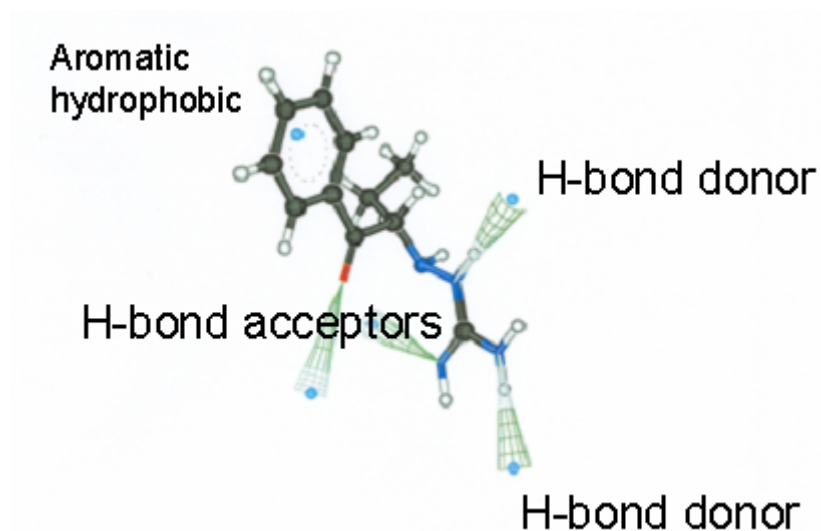


Figure 6. Using Accelrys, Inc. software, the elementary pharmacophore model was mapped onto the compound WR093716 from the CIS database list.

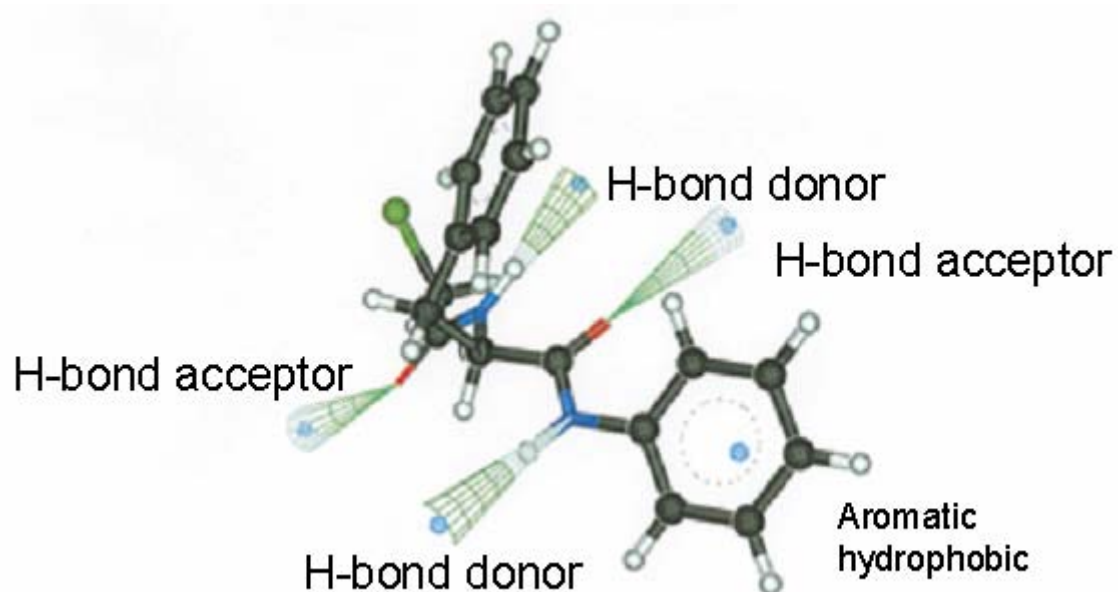


Figure 7. Using Accelrys, Inc. software, the elementary pharmacophore model was mapped onto the compound WR107322 from the CIS database list.

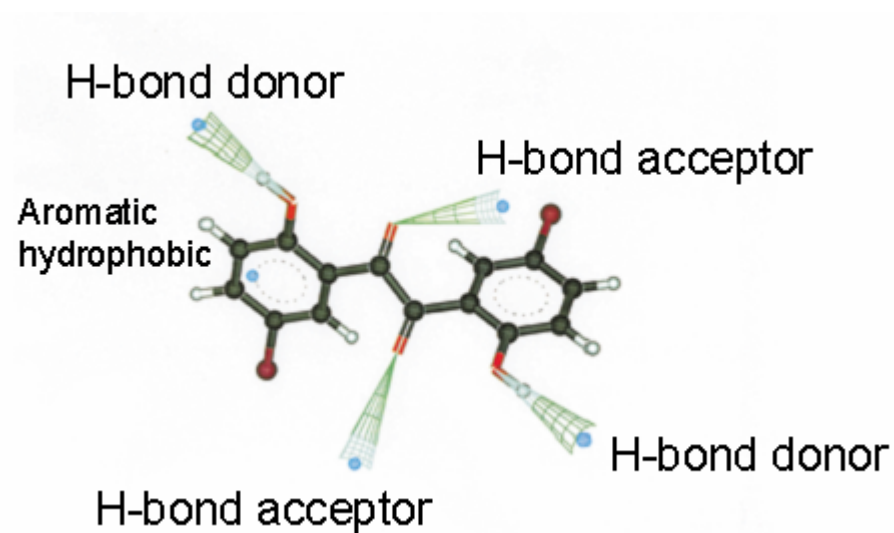


Figure 8. Using Accelrys, Inc. software, the elementary pharmacophore model was mapped onto the compound WR058190 from the CIS database list.