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Article

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In Silico Discovery and Validation of Amide Based Small Molecule Targeting the Enzymatic Site of Shiga Toxin

Vinita Chauhan[a], Dilip Chaudhary[a], Uma Pathak[b], Nandita Saxena[c] and Ram Kumar Dhaked[a*]

Biotechnology Division[a], Synthetic Chemistry Division[b], Pharmacology and Toxicology Division[c], Defence Research and Development Establishment, Gwalior-474002, India

Abstract

Shiga toxin (Stx), a category B biothreat agent is ribosome inactivating protein and toxic to human and animals. Here, we designed and synthesized small molecules that block active site of Stx A subunit. Based on binding energy twenty molecules were selected for synthesis and evaluation. These molecules were primarily screened using fluorescence-based thermal shift assay and *in vitro* in Vero cells. Among 32 molecules (including 12 reported), six molecules offered protection with IC_{50} value of 2.60 to 23.90 μM. 4-Nitro-N-[2-(2-phenylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride is the most potent inhibitor with IC_{50} at 7.96 μM and selectivity index of 22.23 and is better than any known small molecule inhibitor of Stx. Preincubation with Stx offered full protection against Shiga toxin in mice. Surface plasmon resonance assay further confirmed that these molecules bind specifically to Stx A subunit. Further optimization is continued to identify potential candidate which will be *in vivo* effective.

Introduction

Shiga toxin (Stx) and Shiga-like toxins (Stx-1 and Stx-2) are structurally and functionally related toxins which are produced by *Shigella dysenteriae* type I and few strains of *Escherichia coli* respectively.[1,2] These toxins are listed as category B biological warfare agents by Centers for Disease Control and Prevention (CDC) and are responsible for life threatening bloody diarrhoea, hemorrhagic colitis, and fatal hemorrhagic uremic syndrome (HUS). So far, no effective therapy is available for Stx intoxication. Antimicrobial treatment is associated with increased risk of toxicity due to release of additional toxins after bacterial cell lysis.[3] Hence, treatment mainly relies on supportive therapy.

Stx belongs to the large family of ribosome inactivating proteins (RIPs) that irreversibly inactivate ribosome by removing specific adenine from the 28S rRNA resulting in inhibition of protein biosynthesis. Stx is constituted by non-covalent association of single A subunit with pentameric B subunit. A subunit is responsible for its N-glycosidase activity that blocks protein synthesis. While B subunit mediates retrograde trafficking of Stx which leads to productive infection.[4, 5]

A number of approaches are being used to develop treatment against Stx toxicity. Small molecules are of particular interest due to ease of access of a variety of desired compounds, convenient handling, reasonably stable and are suited for oral administration. Since retrograde transport is absolutely necessary for Stx toxicity, blocking of the toxin transport appears promising therapeutics. Recently small molecule inhibitors Retro 1 and 2 are reported which block retrograde trafficking of Stx from early endosome to trans-Golgi network thus protecting cells from the Stx cytotoxicity.[6] These compounds are also shown to provide protection in mice against lethal dose of Stx and ricin which is a phytotoxin, another member of RIP family.[6-8] Noel and co-workers reported further modifications of retro 2 compound by cyclization with improved protection efficacy against Stx toxicity.[9] Developing

small molecules that selectively target enzymatic activity of toxin can be safe and alternative option for the treatment.[8] N-Glycosidase activity of A subunit cleaves a highly conserved adenine nucleotide within the sarcin-ricin loop of eukaryotic 28S rRNA and leads to ribosome arrest and inhibit protein synthesis.[10] Since N-glycosidase activity removes adenines from 28S rRNA, addition of adenine protects ribosomes from inactivation by RIPs. Several adenine derivatives and analogues have been shown to inhibit toxicity by *in vitro* translation assay.[11]

In the present study we have identified and synthesized small molecules targeting N-glycosidase activity of Stx A subunit. In addition to these molecules we have also studied twelve previously reported purine derivatives and their analogues as an inhibitor of Stx activity of RNA and DNA depurination.[11] These small molecules were initially screened using fluorescent based thermal shift assay (FTS) and in *in vitro* assay using Vero cells. Compounds which were found to be promising in initial screening were further evaluated for their efficacy against Stx in Balb/c mice. Interaction of small molecule inhibitor with Stx is further validated by surface plasmon resonance (SPR). Interestingly, molecules which offered protection were all amide derivatives. Further optimization of these compounds may uncover drug lead against Stx toxin.

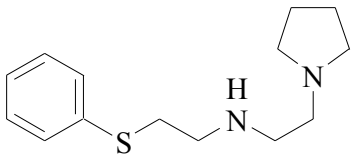
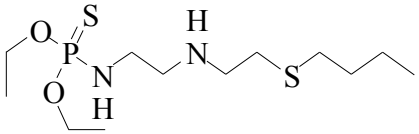
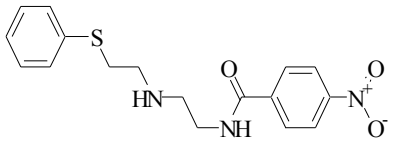
Results

In silico Screening of Small Molecule Inhibitors

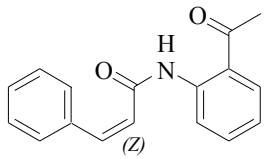
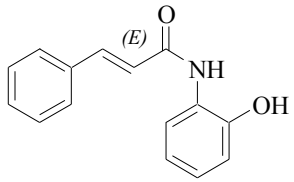
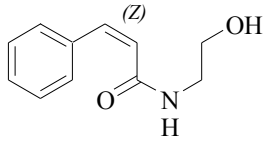
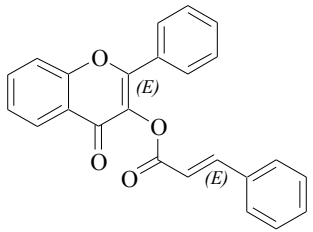
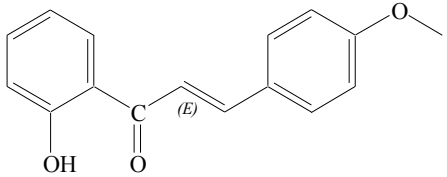
In an effort to identify potential inhibitors against Stx, 300 molecules were mined from NCBI, ChemBridge and ZINC databases. These molecules were subjected to *in silico* studies and docked on the active site of A chain of Stx using AutoDock 4.2. Scoring was done on the basis of minimum binding energy (kcal/mol) of protein-ligand complex formation. Of them most potent, twenty molecules were prioritized for *in vitro* screening. The binding energy varied from -10.06 to -4.59 kcal/mol and K_i values from 3.21 to 439.63 μM (Table1). Among

these; **1, 2, 3, 12, 16** and **18** are novel molecules and rest of the molecules are the reported, however to the best of our knowledge they have never been studied against Shiga toxin. These molecules were found to interact with the active site residues of the Stx A subunit which indicated their probability of efficient binding thus inhibition of the toxin. The docked models of top scoring compounds were visualized using UCSF Chimera[12] (http://www.cgl.ucsf.edu/chimera), to observe the interactions between the lead compounds and target protein (Figure S1). In order to perform the comparative analysis, previously reported twelve small molecule Stx inhibitors (R-1 to R-12) were also included in the study and the docking was done for these compounds using Autodock 4.2 (Table S1).

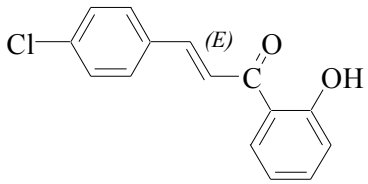
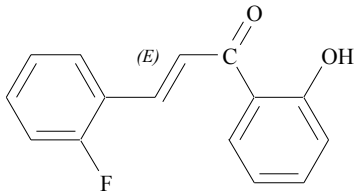
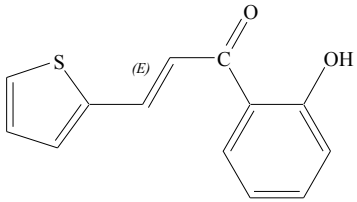
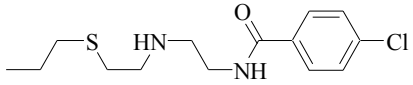
Table 1: Chemical structure of the synthesized compounds tested as inhibitors of the RNA-N-glycosidase activity of Stx. Binding energy of the molecules were (compounds 1-20) considered for in silico screening using AutoDock 4.2. Table is showing name, structure, binding energy (BE) and inhibitory constant (Ki) of the compounds.

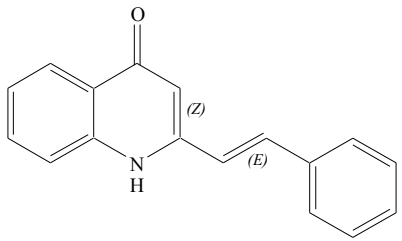
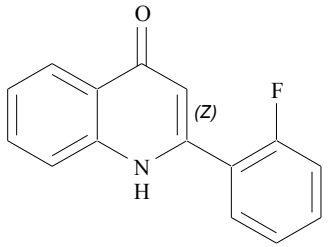
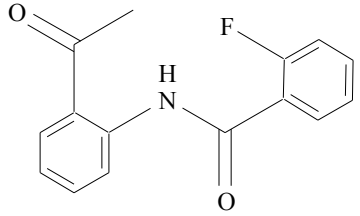
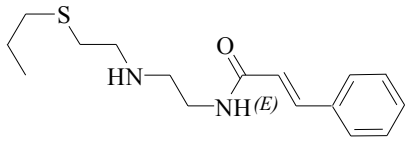
Compound Number	Structure	Name	Binding Energy (kcal/mole)	Ki (µM)
1		*N*-(2-(phenylthio)ethyl)-2-(pyrrolidin-1-yl) ethanamine hydrochloride	-5.64	73.78
2		*O,O*-diethyl(2-{[2-(butylsulfanyl)ethyl]amino}ethyl)phosphoramido-thioate hydrochloride	-5.04	53.78
3		4-Nitro-N-[2-(2-phenylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride	-5.94	42.46

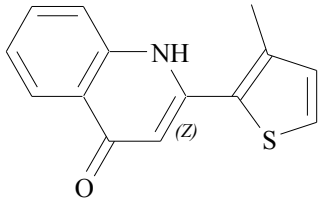
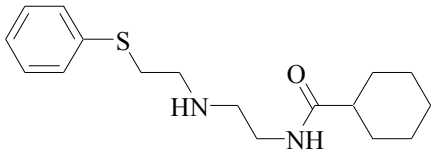
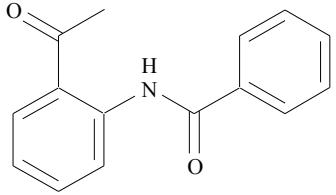
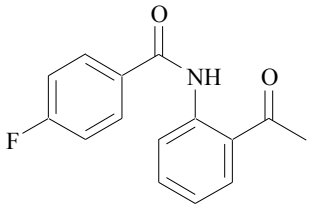
5

4		N-(2-Acetyl-phenyl)-3-phenyl-acrylamide	-7.06	6.69
5		N-(2-Hydroxy-phenyl)-3-phenyl-acrylamide	-6.45	18.68
6		N-(2-Hydroxy-ethyl)-3-phenyl-acrylamide	-6.05	37.05
7		3-Phenyl-acrylic acid 4-oxo-2-phenyl-4 h-chromen-3-yl ester	-6.64	8.78
8		(E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one	-6.83	9.93

6

9		3-(4-Chloro-phenyl)-1-(2-hydroxy-phenyl)-propenone	-6.77	10.88
10		3-(2-Fluoro-phenyl)-1-(2-hydroxy-phenyl)-propenone	-7.35	4.10
11		1-(2-Hydroxy-phenyl)-3-thiophen-2-yl-propenone	-7.16	5.96
12		4-Chloro-N-[2-(2-propylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride	-5.49	93.86

13		2-Styryl-1H-quinolin-4-one	-10.06	41.99
14		2-(2-Fluoro-phenyl)-1H-quinolin-4-one	-7.49	3.21
15		N-(2-Acetyl-phenyl)-2-fluoro-benzamide	-4.59	439.63
16		N-(1-Methylene-3-phenyl-allyl)-N'-(2-propylsulfanyl-ethyl)-ethane-1,2-diamine	-5.08	189.36

17		2-(3-Methyl-thiophen-2-yl)-1H-quinolin-4-one	-6.66	13.33
18		*N*-(2-{[2-(phenylsulfanyl)ethyl] amino}ethyl) cyclohexane carboxamide hydrochloride	- 6.09	34.50
19		N-(2-Acetyl-phenyl)-4-nitro-benzamide	- 7.23	5.00
20		N-(2-Acetyl-phenyl)-4-fluoro-benzamide	-6.16	3.36

Synthesis of Small Molecules

Compound 1 was prepared by treating phenyl-*S*-ethylamine hydrochloride with 1-(2-chloroethyl) pyrrolidine in the presence of triethylamine as a base. Compound 2 was prepared by selective thio-phosphorylation of N^1-(2-butylsulfanylethyl)ethane-1,2-diamine with diethylchlorothiophosphate. Selective amidation of N^1-(2-phenylsulfanylethyl)ethan-1,2-diamine with corresponding acyl halide resulted in formation of Compounds 3 and 18. Amides 4, 15, 19 and 20 were prepared by direct acylation of appropriate amine with acyl halide. Similarly, selective amidation of N^1-(2-propylsulfanyl-ethyl) ethan-1,2-diamine with 4-chlorobenzoyl chloride and cinnamoyl chloride yielded 12 and 16, respectively. Acylation of 2-aminophenol and 2-aminoethanol with cinnamoyl chloride in water utilizing sodium bi carbonate as base resulted in the formation of compounds 5 and 6, respectively. Compound 7 was prepared by esterifying 3-Hydroxyflavone with cinnamoyl chloride. Quinolones compounds 13, 14 and 17 were resulted by cyclisation of the amides obtained from 2-aminoacetophenone and corresponding acyl halides. Chalcones ie compounds 8, 9, 10 and 11 were prepared by the method used in reported literature.[13, 14]

Primary Screening using Fluorescence-Based Thermal Shift Assay (FTS)

In present study, FTS assay was used for initial screening of synthesized molecules for their interaction with recombinant Stx A subunit (rStx A). Thermal stability shift analysis is a fast and powerful platform for screening of molecules that affects thermal stability of proteins after binding interactions.[15] This assay operates on the principle that each protein unfolds at specific temperature and binding of ligands alters the thermal stability of the protein resulting in shift in unfolding temperature. A probe (sypro orange) provides fluorescence due to binding to hydrophobic surface resulted from unfolding of the protein. All 32 compounds were evaluated for their interaction with rStx A subunit. Seven molecules are found to bind with greater affinity and altered the stability of protein, resulting >2 ^{o}C change in transition

temperature (Δ*Tm*). Among these seven compounds, maximum shift in Δ*Tm* was recorded with compound 15 (7.25°C) followed by 4 (4.61 °C), R-7 (4.45 °C), 3 (3.87 °C), R-8 (3.75 °C), 16 (2.65 °C) and R-2 (2.32 °C) (Figure 1 and Table S1).

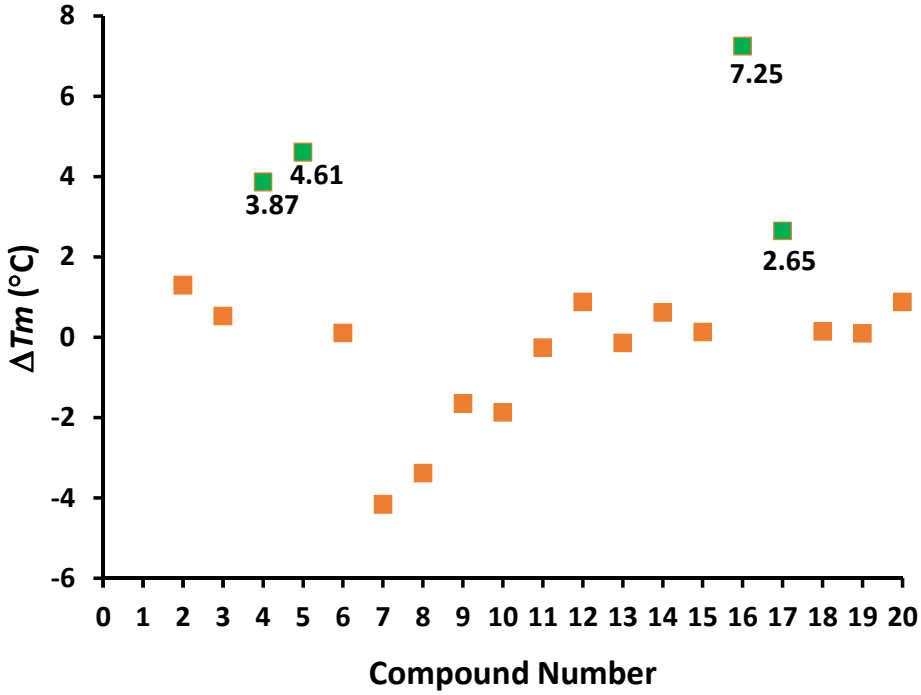


Figure 1: Fluorescence based thermal shift assay result for interaction between small molecules and Stx. The *ΔTm* depicts the difference between in the Tm of Stx mixed with ligand and the Tm of Stx only reactions Values are average of three replicates. Small molecules showing various degrees of thermal shift ranging from -4.16 °C to 7.25 °C.

Stx Induced Cytotoxicity in a Dose and Time Dependent Manner in Vero Cells

To examine the cytotoxic effect and 50% cytotoxic concentration (CC_{50}) of Stx in cells, the Vero cells were treated with different concentration of Stx ranging from 0 to 120 ng/ml for 24 h, and the viability was determined by3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and neutral red (NR) dye uptake assay. From 20 ng/ml, Stx caused a

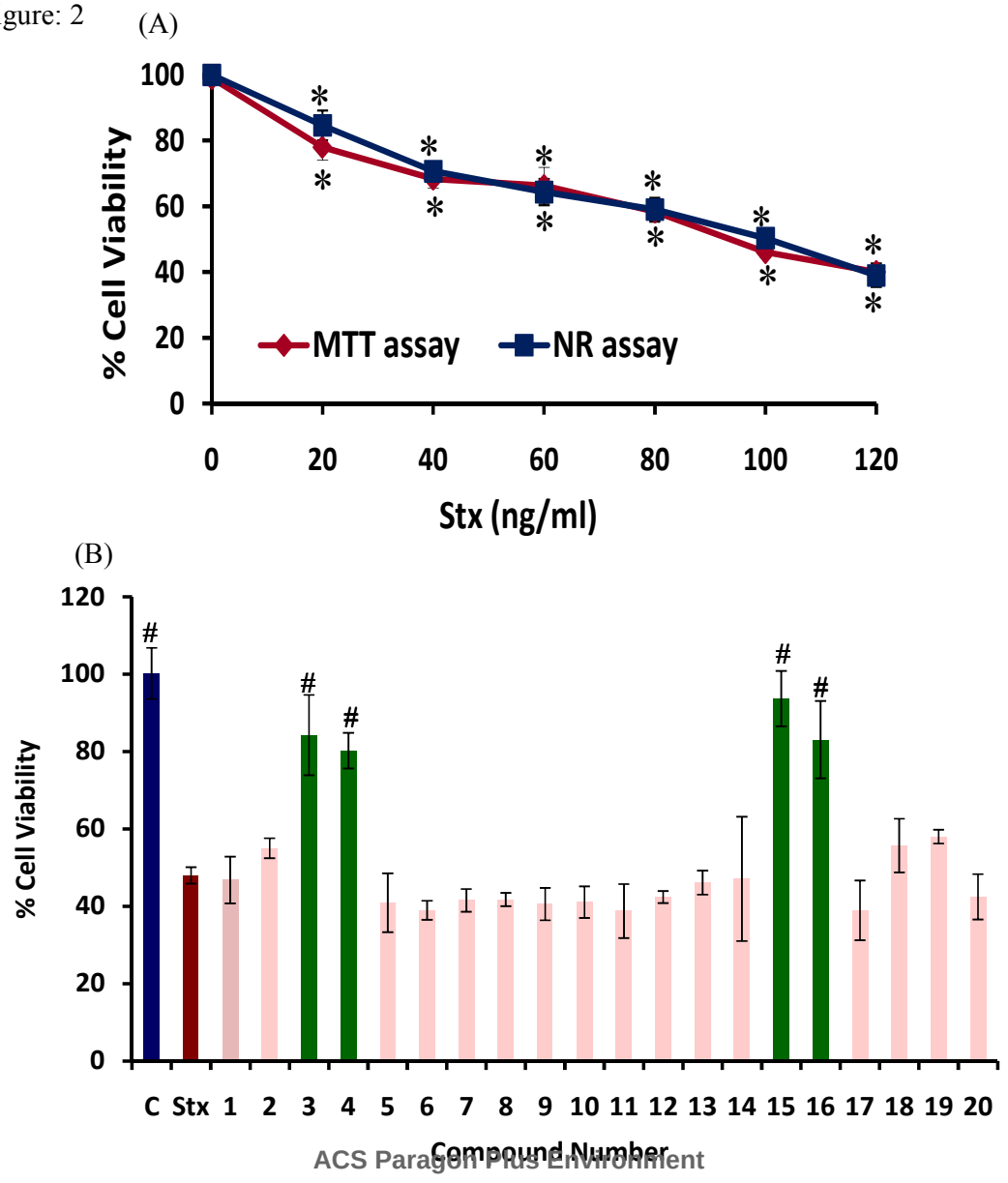
significant dose dependent decrease in viability with increase in concentration at 24h. Concentration that is 50% cytotoxic (CC_{50}) was found to be 100 ng/ml at 24 h (Figure 2A). This CC_{50} value of 100 ng/ml was used in further experiments.

Effect of Compound on Cells and Stx Induced Toxicity

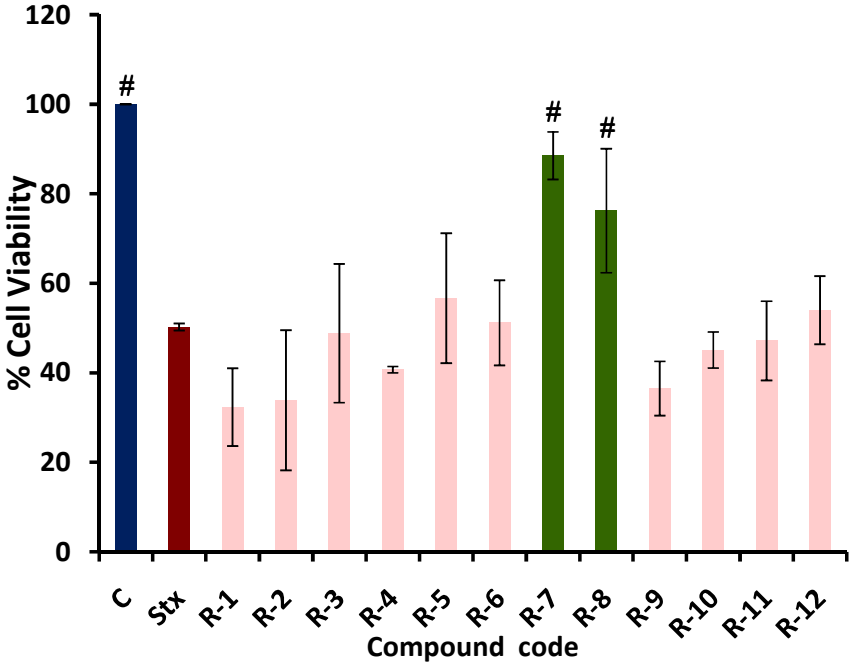
All 32 small molecules were evaluated for maximum tolerated dose (MTD) in Vero cells. For determination of MTD, cells were treated with varying concentration of compounds for 24 h and then cell viability was measured by NR assay. MTD values of synthesized compounds varied from 5.00±1.23 μM to 123.00±2.45 μM and for reported compounds 9.30±1.53 μM to 250.00±1.75 μM. (Table 2A and 2B). Further, to test the efficacy of the small molecules as an inhibitor of Stx by NR assay, cells were pre-treated with compounds for 1h followed by Stx exposure. Among the 32 compounds evaluated, four synthesized compounds (3, 4, 15 and 16) and two reported compounds (R-7 and R-8) showed 75- 95% inhibition of Stx induced toxicity (Figure 2 B and C). Efficacy of these six compounds was further validated by MTT and lactate dehydrogenase (LDH) release assay. As shown in figure 2D these small molecules were found to protect cells from Stx toxicity by MTT assay. Similarly, these compounds also showed significant inhibition of Stx induced LDH release and protected cell membrane integrity (Figure 2E). Other compounds showed no significant reversal of Stx induced toxicity in Vero cells. Those compounds which offered protection was also tested for their dose depended potential to inhibit Stx toxicity. In Figure S2 (supporting information) compound 3 has been shown to inhibit Stx toxicity in dose dependent manner with highest inhibition at approximately 12 μM. The doses of compounds (3, 4, 15, 16, R-7 and R-8) inhibiting 50% toxicity of Stx (IC_{50}) were determined in cells treated with different concentrations of compounds by NR assay (Table 2). These six compounds were also tested for their 50% cytotoxic concentration (CC_{50}) using NR assay and results are mentioned in table 2. Based on the CC_{50} and IC_{50} values, the selectivity index ($SI=CC_{50}/IC_{50}$) of each

compound was determined which reflects the quantity of compound that is active against the toxin but is not toxic towards the host cell. SI of compounds which offered protection were 22.23, 11.29, 15.50 and 15.00 for compounds 3, 4, 15 and 6, respectively. Although, compounds 3, 4, 15 and 16 significantly inhibited the Stx toxin induced cell death, but compound 3 was found to be most potent with SI value of 22.23 (Table 2A). Among the purine derivatives included in the study, guanine (R-7) showed maximum 88% inhibition and IC_{50} value of 11.50 µM and 76% inhibition was observed with allopurinol (R-8) with an IC_{50} value of 23.90 µM. The SI values were found to be 11.80 and 17.90 for R-7 and R-8, respectively (Table 2B).

Figure: 2



(C)


120
100
80
60
40
20
0
% Cell Viability
#
#
#
C
Stx
R-1
R-2
R-3
R-4
R-5
R-6
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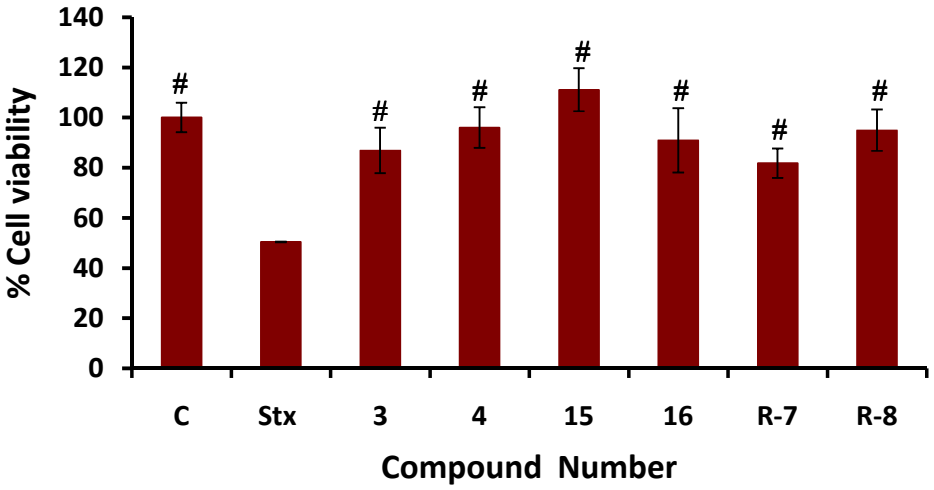
R-8
R-9
R-10
R-11
R-12
Compound code

(D)


140
120
100
80
60
40
20
0
% Cell viability
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C
Stx
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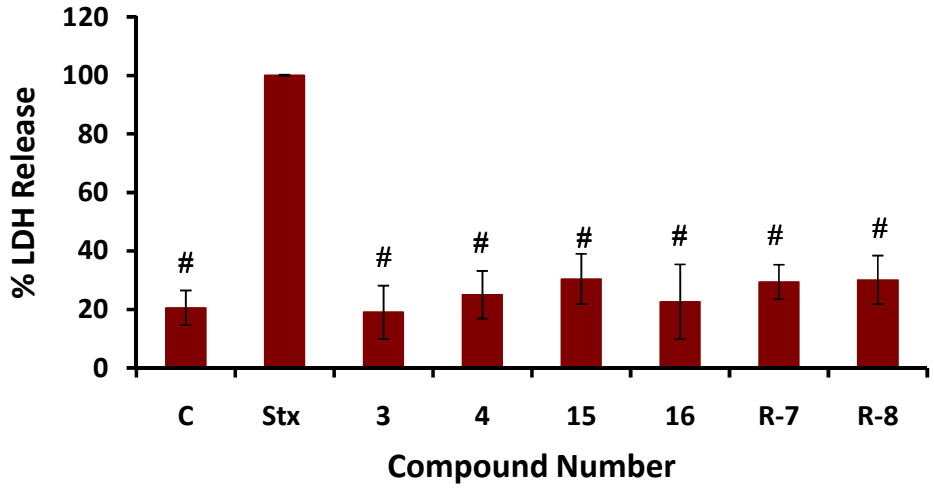
R-7
R-8
Compound Number

(E)


120
100
80
60
40
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0
% LDH Release
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#
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C
Stx
3
4
15
16
R-7
R-8
Compound Number

Figure 2: Stx-induced cytotoxicity and evaluation of small molecule inhibitors for *in vitro* protection in Vero cells. (**A),** Stx-induced toxicity: Cytotoxic effect of Stx in Vero cells was determined by MTT and NR assays. For determination of 50% cytotoxic concentration (CC_{50}), the cells were seeded on to a 96 well plate and treated with varying concentration of Stx for 24 h. The CC_{50} was calculated as 100 ng/ml. Data are the mean of three independent experiments each in triplicate with standard error *$p<0.05$ compared with untreated cells. **(B),** Pre-treatment of compounds followed by Stx (100 ng/ml) exposure for 24 h. Cells were analyzed by NR assay. Compounds 3, 4, 15 and 16, significantly reversed Stx induced cytotoxicity. Data are the mean of three independent experiments each in triplicates with standard error. #$p<0.05$ compare to Stx treated cell, **(C),** Pre-treatment of previously reported compounds followed by Stx (100 ng/ml) exposure for 24 h. Cells were analyzed by NR assay. Compounds R-7 and R-8 were found to be promising; Data are the mean of three independent experiments each in triplicates with standard error. #$p<0.05$ compare to Stx treated cell, **(D),** Pre-treatment of compounds 3, 4, 15, 16, R-7 and R-8 followed by Stx (100 ng/ml) exposure for 24 h. Cells were analyzed by MTT assay. All six small molecule inhibitor offered protection against Stx induced toxicity. Data are the mean of three independent experiments each in triplicates with standard error. #$p<0.05$ compare to Stx treated cell, **(E),** Pre-treatment of compounds 3, 4, 15, 16, R-7 and R-8 followed by Stx (100 ng/ml) exposure for 24 h. Cells were analyzed by lactate dehydrogenase (LDH) release assay. All six compounds could significantly block Stx induced LDH release. Data are the mean of three independent experiments each in triplicates with standard error. #$p<0.05$ compare to Stx treated cell. Untreated cells (C), only toxin treated (Stx), Pretreatment of compounds for 1 h (Compounds #1-20 and R-1 to R-12) followed by Stx.

Table 2: Determination of maximum tolerated dose (MTD), 50% cytotoxic concentration (CC_{50}), inhibitory concentration (IC_{50}) and selectivity index (SI) of the small molecules in Vero cells; (A) synthesized compounds; (B) reported compounds.

Table 2A

Compound Code	**MTD (µM)**	**CC_{50} (µM)**	**IC_{50} (µM)**	**SI**
1	63.30±1.53	-	-	-
2	32.30±1.72	-	-	-
3	15.00±1.01	177.00±7.10	7.96±1.90	22.23
4	6.96±1.00	29.71±5.40	02.63±0.53	11.29
5	32.00±2.65	-	-	-
6	31.60±1.53	-	-	-
7	64.00±2.00	-	-	-
8	7.35±0.58	-	-	-
9	6.00±2.05	-	-	-
10	5.67±1.64	-	-	-
11	17.00±.3.06	-	-	-
12	123.60±2.45	-	-	-
13	9.33±1.53	-	-	-
14	5.00±1.23	-	-	-
15	37.00± 1.00	120.00±3.24	7.70±1.10	15.50
16	38.00±2.36	123.00±4.23	8.20±0.50	15.00
17	5.33±1.00	-	-	-
18	38.00±1.45	-	-	-
19	17.33±1.53	-	-	-
20	10.00± 1.23	-	-	-

Table 2B

Compound code	MTD (μM)	CC_{50} (μM)	IC_{50} (μM)	SI
R-1	9.30±2.30	-	-	-
R-2	125.00±3.24	-	-	-
R-3	18.70±5.60	-	-	-
R-4	125.00±1.50	-	-	
R-5	9.30 ±2.80	-	-	-
R-6	9.30±1.53	-	-	-
R-7	125.00±1.87	136.50±2.50	11.50±0.90	11.80
R-8	250.00 ±1.75	428.00±5.60	23.90±2.00	17.90
R-9	250.00±3.60	-	-	-
R-10	18.70±4.20	-	-	-
R-11	125.00±3.20	-	-	-
R-12	75.00±3.45	-	-	-

Binding Studies of Small Molecule Inhibitors with Stx A

To quantitatively assess the binding affinity of compounds 3, 4, 15 and 16 with Stx we performed SPR experiments using immobilized rStxA on GLH sensor chips at ~6000 RU (resonance units) as described in experimental section. As shown in figure 3, compound 3 binds to rStxA in a concentration dependent manner with high affinity and yielding the equilibrium dissociation constant (KD) of $7.16X10^{-7}$ M with a fast on rate (k_a=$9.46X10^3$ 1/Ms) and fast off rate (k_d=$6.77X10^{-2}$1/s). Other compounds 4, 15 and 16 also interacted to StxA but comparatively with lower affinities yielding KDs of $3.09X10^{-6}$, $9.68X10^{-2}$ and $8.67X10^{-2}$ M, respectively (figure S3).

Figure: 3

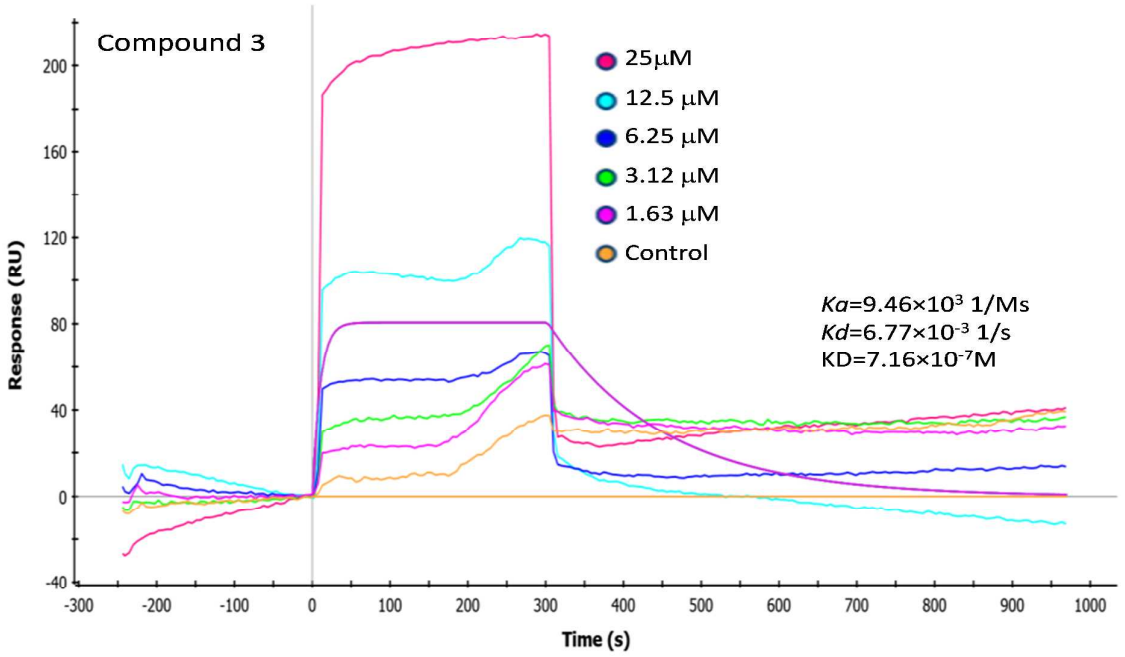

Figure 3: Surface plasmon resonance experiments to analyze the binding curves and fitted results of compound 3 to immobilized rStxA on GLH sensor chip. The concentrations of compound used were ranging from 25.00 to 1.625 μM. PBS with tween-20 was used as a control. The data were fitted to the simplest Langmuir 1:1 interaction model. The inset is showing the association rate (*k*a), dissociation rate (*k*d), and equilibrium dissociation constant binding (*K*D) constants.

***In vivo* Evaluation of Compound 3, 4, 15, 16 and R-7, R-8 in Mouse Model**

To assess whether our finding is restricted to cultured cell lines, we analyzed the efficacy of compounds in mice against supra lethal dose of Stx (5x LD_{50}). In Stx treated group, all mice died between 12 to 24 h with symptoms of shigellosis. The groups in which mice were exposed to Stx with pre or post treatment of compounds resulted in the extension of survival time where mice death was recorded in 24 to 48 h. Interestingly, in the group where compounds were pre-incubated with 5x LD_{50} of Stx and then administered, all the animals survived and no animal showed any symptomatic effect of toxin (figure 4). This finding

suggests neutralizing potential of these compounds against Shiga toxin toxicity. Surprisingly, R-7 and R-8 did not offer any protection even when they administered after pre-incubating with Stx. In vehicle control and treatment with compound alone groups did not show any sign of toxicity (data not shown).

Figure: 4

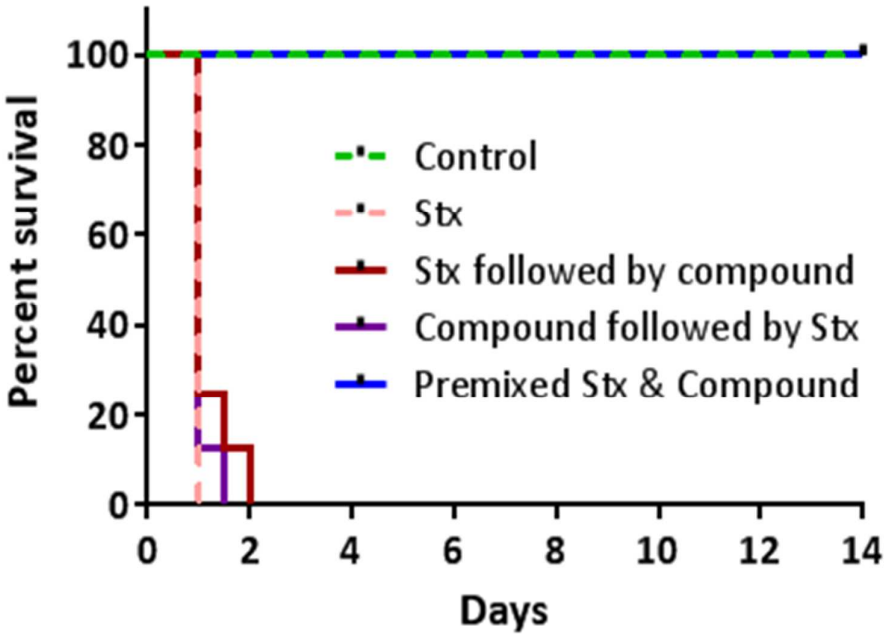

Figure 4: *In vivo* efficacy studies of small molecules. Balb/c mice were administered intraperitoneally with 5x LD_{50} of Stx. Compounds were given either 1h before or 1 h after treatment of Stx. In another group Stx and compounds were premixed and then administered to mice. For all treatment 2 mM compounds was used.

Discussion

Stx is a category B biothreat agent, therapeutic agents that inhibit Stx toxicity are urgently required to prevent the harmful consequences of Stx that are not addressed with current antibiotic-based treatments. Various novel approaches have been attempted to develop inhibitors of various stages of Stx intoxication including cell uptake, trafficking and release to

the cytoplasm.[6,7,9,16-20] The small molecules identified using high throughput screening that inhibit endogenous trafficking of toxin may affect other endogenous cargo proteins and can lead to other undesirable effects. Hence, only those molecules that directly interfere with active site and inhibits its N-glycosidase activity may be helpful.

Structure based small molecule inhibitors is an attractive approach for the treatment of Stx intoxication. In this study, we utilized *in silico* screening to identify Stx inhibitors selectively targeting N-Glycosidase activity of A subunit of Stx. Initial molecular screening of several small molecules *i.e.* amides, chalcones, esters and quinolones, led us to identify the best predicted inhibitors against shiga toxin. Finally, based on binding energy and K_i, we identified 20 molecules for synthesis and screening. Twelve reported purine derivatives were also included in the study.[11] These molecules act as an uncompetitive inhibitor of the RNA-N-glycosidase reaction and protect inactivation of ribosome by Stx.

Initial screening of these compounds was done using FTS assay and *in vitro* assay. FTS assay is an inexpensive method for screening of small molecule inhibitors that rely on change in thermal stability of the proteins after binding of the ligands.[15] Among 32 compounds evaluated, seven compounds were found to alter the stability of rStxA, where shift in transition temperature (ΔTm) was recorded >2 ^{o}C. These 32 compounds were also evaluated in cell based assay against Shiga toxin, out of those six compounds offered protection, four of them were new compounds (3, 4, 15 and 16) and two were reported inhibitors (R-7 and R-8). Interestingly, all the newly identified compounds were amides, suggesting amide derivatives may have potential to inhibit catalytic activity of Stx. We observed a good agreement between our initial screening methods, FTS and cell based assay. The compounds which were showing maximum thermal shift were consistent with the result that these small molecules owe the most potent *in vitro* inhibition activity. The only exception was observed with R-2 that was not able to protect cells after Stx challenge (table 2A & B).

Selectivity index (SI) value defines the compound's ability to preferentially produce a particular effect and is important parameter for analysing protective effect of compounds. Among all the compounds studied, compound 3 was found to be most promising in terms of protection against Stx with the highest SI value of 22.23. The magnitude of inhibition was also higher than those of earlier reported small molecule inhibitors including retro 1 and 2 where the IC_{50} was reported to be at 25.00 μM in cell culture.[19] Whereas in present study, IC_{50} of lead compounds was varied from 2.63±0.53 to 8.20±0.50 μM. Lower IC_{50} value in cell based protection assay reflects the higher efficacy of inhibitors, suggesting the greater therapeutic potential of present leads.

Evaluation of molecules in Vero cells produced interesting results. Compounds having amide functionality were found very effective, while chalcones, quinolones and ester of 3-hydroxyflavone did not provide any protection. Compounds 1, 3 and 18 were relatively similar except the terminal amide moiety. The modification of terminal amine functionality to pyrrolidine (compound 1) was not found effective. In 3 and 18 the terminal amine was acylated with 4-nitrobenzoylchloride (aromatic moiety) and cyclohexane carbonyl chloride (alicyclic moiety), respectively to generate the amide group. Amidation with aromatic moiety (compound 3) was found much more effective than alicyclic moiety (compound 18). Compound 3 contained two aromatic rings with one amine and one amide functionality was the most effective inhibitor in cell culture studies. Among the cinnamides, 4, 5, 6 and 16; the most effective compound was 16 followed by 4. Cyclisation of amides derived from 2-amino acetophenonequinolone, 14 and 17 were ineffective in cytoprotection and at the same time compound toxicity was also increased. Placing fluoro group at ortho position (compound 15), compared to para position in aromatic ring (compound 20) proved to be more useful, as compound 15 was impressively effective while other did not provide any protection.

Evaluation of reported compound in Vero cells also revealed interesting results. Guanine (R-7) and allopurinol (R-8) are reported the least efficient inhibitors of RNA-N glycosidase activity by *in vitro* protein synthesis assay but same showed highest efficacy in Vero cells against Stx in our study.[11] Probably cell based assay used in present study is closer to biological system than *in vitro* protein synthesis inhibition assay. It could also be possible that both compounds may exhibit some additional effect like interference of toxin binding to its receptor, trafficking or processing in the ER. 4-Aminopyrazolo[3,4-d]pyrimidine (4-APP), which was reported to be most efficient small molecule inhibitor could not be evaluated due to the non-availability of the compound with us.[11] Guanine was also showing good inhibition against enzymatic activity of ricin, substantiate the hope for finding common inhibitor working against all RIP.[17]

To investigate the possibility of non-specific or multi-targets binding of these leads, pan assay interference compounds (PAINS) filter was applied. None of the lead molecules (compounds 3, 4, 15 and 16) were recognized as potential PAINS. Moreover, screening system used in *in vitro* evaluation is based on Vero cell viability for the estimation of potential inhibitor unlike other HTS methods where above mentioned interference are major hurdle.[21] Compound 5 having the substructure included in PAINS was also evaluated in cell based assay and showed no inhibitory activity, this further strengthen that compounds 3, 4, 15 and 16 are true positive hits.

SPR is a highly sensitive label free technique to measure binding and the affinities of potential inhibitors to ligand. It provides information about kinetics of interaction and the half-life of the complex. These biosensors can be used for secondary screening to confirm whether the hit is real and can also provide information about their dynamic interaction in real-time. SPR experimentation showed that the amide based small molecules were interacting with rStxA in concentration dependent manner with high affinities and

equilibrium dissociation constants (KD) varied from $7.16X10^{-7}$ to $8.67X10^{-2}$ M. There was considerable correlation in SI values obtained from cytoprotection assay and the KDs of interactions between rStxA subunit and amide based compounds. On the basis of both values compound 3 was found to be most effective. The only exception was compound 4 which provided least SI value but interacted with higher KD than compound 15 and 16**.** As shown in figure 5, when compound 3 is docked in the active site pocket of Stx, the amide group of compound forms a hydrogen bond with Ser112 and is interacting with active site of toxin *i.e.* Tyr77, Val78, Tyr114, Glu167, Ala168, Trp203 and Arg170, thus contributes to the specificity and potency of this inhibitor. The amide of compound 4 formed a hydrogen bond with Arg170 that is reported to involve in substrate binding.[17] In docked conformations compound 15 was found to interact with Arg170 and Tyr114, whereas compound 16 was seen interacting with Ser112 and Val78, which are reported as active site residues of Stx.

Our findings from *in vivo* assay revealed that all 4 amide compounds are highly effective in neutralizing toxin by pre-incubating and protecting mice against Stx toxicity. There were no symptoms observed of Stx toxicity in animals. Protection obtained by pre-incubating indicates the efficacy of lead compound as Stx inhibitor and their potential to neutralize extracellular toxin. Significant extension in survival time was observed when the mice were challenged with Stx followed by pre- and post-treatment of inhibitors. However, no protection suggests the possibility of modification of compound or pharmacokinetics inside biological system. This may also be due to distribution, absorption or/and stability of the compound in body system. Both the earlier reported compounds (guanine and allopurinol) were not found effective in all three modes of treatment. Though the efficacy of leads identified in present study needs further optimization with respect to neutralization of toxin in pre- and post-challenge. All these leads are amide derivatives but compound 3 was found to be most promising and efficacious and require further study. Further these compounds are

non-optimized hits and modification in the chemistry of compounds may further improve *in vivo* efficacy.

Conclusion

Our findings indicate that targeting the catalytic site with small molecules represent a feasible therapeutic approach that may allow successful reversal of Shiga toxicity. Since our molecules are specific inhibitors of enzymatic activity of Shiga toxin hence they do not cause any undesirable effects unlike inhibitors of retrograde pathway which may affect trafficking of other vital endogenous cargo protein. Among all the compounds studied, 4-Nitro-N-[2-(2-phenylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride (compound 3) is found to be most promising in terms of protection against Stx with the highest SI value of 22.23 and the magnitude of inhibition is higher than those of earlier reported small molecules with the IC_{50} of 2.63±0.53 μM. Pre-incubation of Shiga toxin with these amides based small molecules also protected animals. Lower IC_{50} values in cell based assay reflects the higher efficacy of inhibitors, suggesting the greater therapeutic potential and their activities can be enhanced by further lead optimization efforts which are under progress.

Figure: 5

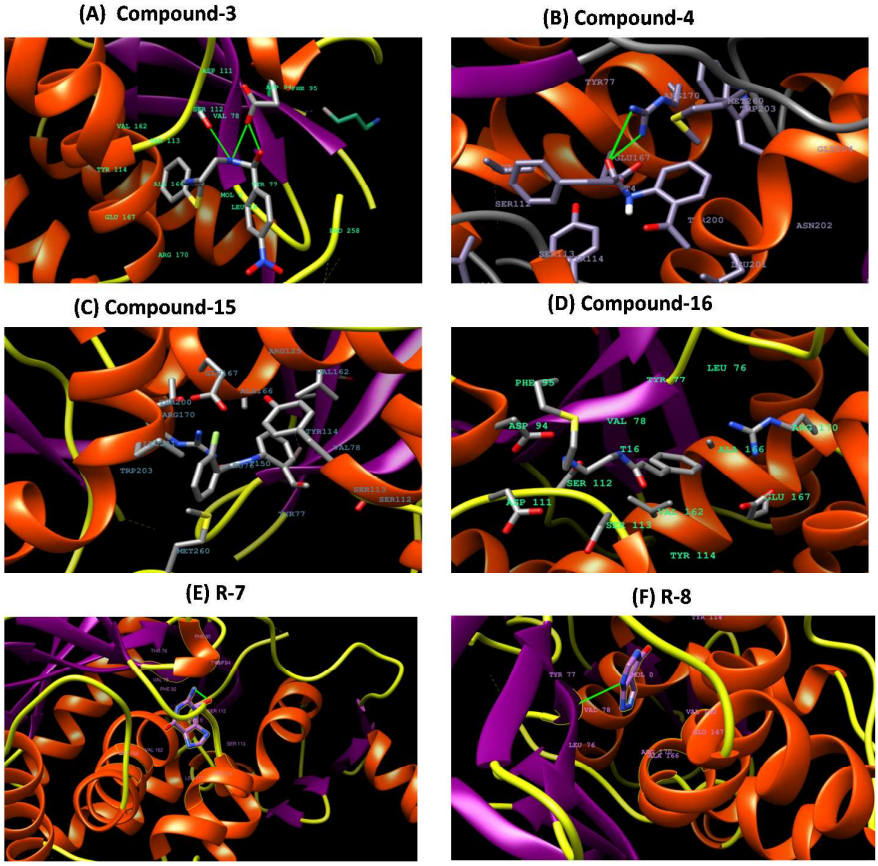

Figure 5: Close view of the compounds 3, 4, 15, 16, R-7 and R-8 that offered protection, to show the interaction and active site of Shiga toxin (PDB code 1DM0) with the docked ligands using UCSF chimera.

5(A): Hydrogen bond between amide group of compound 3 and Ser112.

5(B): The amide group of compound 4 forming hydrogen bond with Arg170.

5(C): Compound 15 is located in pocket surrounded by active site residues Arg170 and Tyr114.

5(D): Compound 16 is located in pocket surrounded by active site residues Ser112 and Val78.

5(E): R-7 is located in pocket surrounded by active site residues.

Experimental Section

1. Chemistry Methods

Reagents of analytical grade (>98%) were obtained from commercial suppliers and used without further purification. Column chromatography was performed on silica gel (60 - 120 mesh). ^{1}H, ^{31}P and ^{13}C NMR spectra were recorded on Bruker AVANCEII 400 MHz and Bruker AVIII 600 MHz. Chemical shifts were expressed in parts per millions (δ) downfield from the internal standard tetramethylsilane and were reported as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Melting points were measured by scientific-MP-DS apparatus. Mass spectra were obtained from Agilent-5975C GC-MS and elemental analysis was performed on Elementar vario MICRO-cube CHNS analyser. Purity was checked by elemental analysis and were >98% for all the compounds synthesized. The reported compounds (R-compounds) were purchased from Sigma-Aldrich (St Louis, MO) and were >99% pure except R-3, R-5 and R-9 where the purity was ~98%.

2. Synthesis of Compounds

***N*-(2-(phenylthio)ethyl)-2-(pyrrolidin-1-yl) ethanamine hydrochloride (1).** Phenyl-*S*-ethylamine hydrochloride 10 mmol (1.89 g) and 1-(2-Chloro-ethyl)-pyrrolidine 2.0 mmol (2.60 g) was dissolved in 10 ml of ethanol. The contents were heated to reflux. To this, triethylamine 5 mmol (7.50 ml) in 30 ml of ethanol was added drop-wise over a period of 3-4 h. After the addition is over reaction mixture was heated till the reaction was complete, which was monitored by GC-MS and TLC (mobile phase CH_3OH:$CHCl_3$:NH_3OH; 3:2:1). On evaporation of ethanol, solid residue was obtained, which was dissolved in chloroform and washed with sodium carbonate. Evaporation of chloroform resulted in an oily mass. The oil was dissolved in acetone and converted into its hydrochloride salt with dry HCl. The hydrochloride salt of *N*-alkyl derivative was obtained as solid precipitate which was purified by recrystallization from acetone-methanol mixture.

White solid; mp- 175-176 °C; ^{1}H NMR (400 MHz, CD_3OD): δ=7.42-7.40 (m, 2H), 7.33 (t, 2H, *J* = 8.0 Hz), 7.28 (d, 1H, *J* = 6.8 Hz), 3.51-3.47 (m, 2H), 3.40-3.36 (m, 3H), 3.22 (s, 5H), 1.99 (br s, 4H); ^{13}C NMR (150.9 MHz, CD_3OD) δ 135.02, 131.58, 130.66, 128.51, 55.70, 49.57, 44.45, 30.55, 24.15, 0.11.MS (EI): m/z 55, 70, 84, 98, 117, 155. Anal. calcd for $C_{14}H_{22}N_2S$: C, 67.15; H, 8.86; N, 11.19; S, 12.81. Found: C, 67.17; H, 8.84; N, 11.17; S, 12.85.

***O,O*-diethyl(2-{[2-(butylsulfanyl)ethyl]amino}ethyl)phosphoramido-thioate hydrochloride (2).** A two neck flask was charged with dry dichloromethane (DCM) and N^1-(2-(butylthio) ethyl) ethane-1, 2-diamine dihydrochloride[23], 10 mmol (2.12 g). The mixture was stirred and then triethylamine was added very slowly to reaction mass. Reaction mixture was stirred further for 5-10 min. This was followed by drop wise addition of diethyl chlorothiophosphate dissolved in dichloromethane at 20-25 ºC. On complete addition of diethyl chlorothiophosphate 12 mmol (2.2 ml), the reaction mixture was stirred for additional 1-2 h. The reaction was monitored by GC-MS. After completion of the reaction, organic layer of DCM was washed with $NaHCO_3$, and DCM layer was dried over anhydrous Na_2SO_4. The solvent (DCM) was evaporated and an oily residue was obtained. The residue was dissolved in acetone and acidified by dry HCl gas. The acidic acetone solution was evaporated to a half volume by purging nitrogen. Then diethyl ether was added. White precipitate was appeared which was filtered and washed with diethyl ether and dried under vacuum. Compound was recrystallized by acetone-ether solvent system.

White shiny crystal; mp- 99-101 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 5.70-5.63 (m, 4H), 5.36-5.31 (m, 2H), 5.02 (d, 4H, *J* = 5.2 Hz), 4.78 (d, 2H, *J* = 5.2 Hz), 3.99 (t, 2H, *J* = 7.2 Hz), 2.97-2.76 (m, 2H), 2.76-2.67 (m, 6H), 2.29 (t, 3H, J = 7.2 Hz). EIMS: m/z 117, 170, 196, 225, 226; ^{13}C NMR (150.9 MHz, CD_3OD) δ 64.57, 48.25, 39.03, 32.93, 32.63, 28.68, 23.17, 16.56, 14.25, 0.24; MS (EI): m/z 61, 73, 97, 117, 135, 153, 170, 196, 225, 251. Anal. calcd

for $C_{12}H_{29}N_2O_2PS_2$: C, 43.88; H, 8.90; N, 8.53; S, 19.52. Found: C, 43.85; H, 8.95; N, 8.51; S, 19.56.

4-Nitro-N-[2-(2-phenylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride (3). N^1-(2-Phenylsulfanyl-ethyl)-ethane-1,2 diamine dihydrochloride 1 mmol (0.269 g) was taken round bottom flask and to this 10 ml dry DCM was added. To this mixture triethylamine 2 mmol was added very slowly. The content was stirred till the clear solution was formed. After that 1.1 mmol (0.204 g) 4- nitro -benzoyl chloride in 10 ml of DCM was added slowly (in 2 h) at 20-25 °C. Reaction was monitored by GC-MS and TLC (mobile phase CH_3OH**:**$CHCl_3$**:**NH_3; 10**:**10**:**1). After completion of reaction, reaction mixture was washed with $NaHCO_3$ solution and then water. DCM layer was collected and dried over anhydrous sodium sulphate. On evaporation of organic layer an oily residue was obtained. The residue was dissolved in acetone and dry HCl gas was passed. Hydrochloride salts of amide derivatives were appeared as white precipitate. The product was further purified by recrystallization from ethanol-acetone.

White solid; mp-170-172 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 8.28–8.22 (m, 2H), 8.07–8.05 (d, 1H, *J* = 8.4 Hz), 7.98-7.96 (d, 1H, *J* = 8.4 Hz), 7.52-7.11 (m, 5H), 3.91-3.80 (m, 3H), 3.50-3.47 (m, 1H), 3.34-3.28 (m, 1H), 3.15 (s, 1H), 1.55-1.50 (m, 1H); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 165.39, 149.53, 140.13, 129.60, 129.10, 128.26, 126.49, 123.75, 47.28, 39.48, 34.35, 29.71; MS(EI): m/z 56, 76, 109, 137, 150, 193, 222. Anal. calcd for $C_{17}H_{19}N_3O_3S$: C, 59.11; H, 5.54; N, 12.17; S, 9.28. Found: C, 59.15; H, 5.55; N, 12.13; S, 9.25.

N-(2-Acetyl-phenyl)-3-phenyl-acrylamide (4). 2-aminoacetophenone 10 mmol (1.3 ml) was taken in round bottom flask and to this 20 ml dry DCM was added. Triethylamine 2 mmol (3 ml) was added slowly. The content was stirred till the clear solution was formed. After that the cinnamoyl chloride 11 mmol (1.1 g) in 30 ml of DCM was added slowly (in 2 h) at 20-25

°C. Reaction was monitored by GC-MS and TLC with hexane: ethylacetate (1:1) used as mobile phase. After completion, reaction mixture was washed with $NaHCO_3$ solution and then water. DCM layer was collected and dried over anhydrous sodium sulphate. On evaporation of organic layer, a solid residue was obtained and purified by recrystallization from ethanol.

White solid; mp- 89-90 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.04 (s, 1H), 8.92–8.90 (d, 1H, *J* = 8.4 Hz), 7.93-7.90 (m, 1H), 7.76-7.72 (m, 1H), 7.60-7.56 (m, 2H), 7.43-7.37 (m, 4H), 7.29-7.20 (m, 1H), 6.62 (d. *J* = 16 Hz, 1H), 2.68 (s, 3H); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 202.76, 164.67, 142.00, 141.14, 135.02, 134.47, 131.49, 129.75, 128.64, 127.84, 122.19, 121.92, 121.56, 120.75, 28.38; MS(EI): m/z 51, 77, 103, 131, 145, 222, 265(M^+). Anal. calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 76.93; H, 5.75; N, 5.26.

N-(2-Hydroxy-phenyl)-3-phenyl-acrylamide (5). 2-aminophenol 10 mmol (1.1 g) was taken in 100 ml of dichloromethane at room temperature. 12 mmol (2.0 g) of cinnamoylchloride was added in portions over a period of one hour. 10 mmol (1.5 ml) of triethylamine dissolved in dichloromethane (50 ml was then added in 3 h. Reaction mixture was further stirred for 1 h. On completion of the reaction contents were washed with 100 ml of 5 N HCl followed by 10% sodium carbonate solution. On evaporation of the solvent white solid was obtained which was purified by column chromatography.

Light brown solid; mp 156-158 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.17(s, 1H), 7.84-7.80 (d, 1H, *J* = 15 Hz), 7.57-7.55 (m, 3H), 7.42-7.40 (m, 3H), 7.18-7.14 (m, 1H), 7.07-7.03 (m, 2H), 6.90-6.87 (m, 1H), 6.65-6.60 (d, 1H, *J* = 16 Hz); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 165.27, 148.43, 142.34, 134.74, 129.94, 128.87, 127.98, 126.57, 126.04, 122.02, 120.46, 119.92, 118.25; MS(EI): m/z = 51, 77, 103, 131, 239(M^+). Anal. calcd for $C_{15}H_{13}NO_2$: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.33; H, 5.45; N, 5.82.

N-(2-Hydroxy-ethyl)-3-phenyl-acrylamide (6). Synthesised as **5** starting from 2-aminoethanol.

White solid; mp 100-102 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66-7.62 (d, 1H, *J* = 16 Hz), 7.50-7.48 (m, 2H), 7.38-7.35 (m, 3H), 6.45-6.41 (d, 1H, *J* = 16 Hz), 6.18 (s, 1H), 3.81 (s, 2H), 3.59-3.55 (m, 2H), 2.77 (s, 1H); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 167.13, 141.50, 134.57, 129.80, 128.81, 127.81, 120.16, 62.28, 42.65; MS (EI) m/z = 77, 102, 103, 115, 131, 132, 146, 148, 172, 173, 191(M^+). Anal. calcd for $C_{11}H_{13}NO_2$: C, 69.09; H, 6.85; N, 7.32. Found. C, 69.05; H, 6.87; N, 7.36.

Synthesis of 3-phenyl-acrylic acid 4-oxo-2-phenyl-4 h-chromen-3-yl ester (7). 3-Hydroxyflavone 10 mmol (2.38 g) was taken in round bottom flask in 10 ml dry DCM and cinnamoyl chloride 10 mmol (1.67 gm was added. Triethylamine 12 mmol (1.80 ml) was added slowly at 30-35 °C. Reaction was monitored by GC-MS and TLC with mobile phase hexane: ethylacetate (1:1). After completion, reaction mixture was washed with $NaHCO_3$ solution and then water. DCM layer was collected and dried over anhydrous sodium sulphate. On evaporation of organic layer, a solid residue was obtained. The product was further purified by recrystallization from DCM hexane.

White solid; mp- 188-190 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30–8.26 (m, 1H), 7.93-7.88 (m, 3H), 7.52-7.71 (m, 1H), 7.59-7.41 (m, 10H), 6.71-6.67 (d, 1H, *J* = 16 Hz); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 172.31, 164.09, 156.39, 155.71, 147.80, 134.12, 133.97, 131.27, 130.87, 128.99, 128.75, 128.48, 128.35, 126.27, 125.22, 118.14, 116.17; MS(EI): m/z 77, 103, 131, 238. Anal. calcd for $C_{24}H_{16}O_4$: C, 78.25; H, 4.38. Found. C, 78.27; H, 4.33.

(E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (8). Prepared by the literature reported method[14]

Yellow solid; mp- 91-92 °C; ^{1}H-NMR (400M Hz, $CDCl_3$): δ 8.15–8.13 (d, 1H, *J* = 0.02 Hz), 7.93-7.89 (d, 1H, *J* = 0.04 Hz), 7.89-7.82 (m, 3H), 7.56-7.52 (m, 1H), 7.04-6.97 (m, 4H), 3.88 (s, 3H). MS (EI): m/z 65, 77, 89, 108, 121, 134, 147, 161, 237, 254(M^+).

3-(4-Chloro-phenyl)-1-(2-hydroxy-phenyl)-propenone (9). Prepared by the literature reported method[14]

Yellow solid; mp- 149-155 °C; ^{1}H-NMR: δ 12.737(s, 1H), 7.851-7.922 (m, 2H), 7.593-7.650 (m, 3H), 7.49-7.53 (t, 1H), 7.374-7.428 (d, 2H), 7.027-7.051 (dd, 1H, *J* = 8.4, 1.2 Hz), 6.933-6.974 (m, 1H). MS(EI): m/z = 65, 75, 92, 101, 120, 138, 147, 165, 223, 241, 257, 258 (M^+).

3-(2-Fluoro-phenyl)-1-(2-hydroxy-phenyl)-propenone (10). Prepared by the literature reported method[14]

Yellow solid; mp- 80 °C; ^{1}H-NMR: δ 12.753 (s, 1H), 7.983–8.02 (ds, 1H), 7.906-7.930 (dd, 1H, *J* = 8.0, 1.6 Hz), 7.770-7.809 (d, 1H), 7.637-7.679 (t, 1H), 7.490-7.5331 (t, 1H), 7.38-7.443 (m, 1H), 7.133-7.239 (m, 2H), 7.026-7.050 (dd, 1H, *J* = 8.8, 1.2 Hz), 6.934-6.976 (t, 1H). MS(EI): m/z = 65, 75, 92, 101, 121, 147, 222, 241,242(M^+).

1-(2-Hydroxy-phenyl)-3-thiophen-2-yl-propenone (11). Prepared by the literature reported method[14]

Yellow solid; mp- 96-98 °C; ^{1}H-NMR: δ 12.835(s, 1H), 8.035–8.073 (ds, 1H), 7.874-7.898 (dd, 1H, *J* = 8, 1.6 Hz), 7.461-7.518 (m, 2H), 7.405-7.423 (m, 1H), 7.108-7.130 (dd, 1H, *J* = 10, 0.8 Hz), 6.928-6.969 (t, 1H). MS(EI): m/z = 65, 97, 110, 121, 137, 146, 213, 230(M^+).

4-Chloro-N-[2-(2-propylsulfanyl-ethylamino)-ethyl]-benzamide hydrochloride (12). Synthesized as **3** starting from N^1-(2-Propylsulfanyl-ethyl)-ethane-1, 2-diamine[23] and 4-chlorobenzoyl chloride

White solid; mp- 190-191 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ =7.91-7.89 (m, 2H), 7.55-7.53 (m, 2H), 3.75-3.73 (t, 2H, J = 6 Hz), 3.34-3.30 (m, 4H), 2.87-2.85 (t, 2H, *J* = 6 Hz), 2.63-2.60 (t, 2H, *J* = 6 Hz), 1.68-1.65 (m, 2H), 1.05-1.03 (t, 3H, *J* = 6 Hz); MS(EI): m/z 61, 75, 103,

111, 139, 181, 211;^{13}C NMR (150.9 MHz, DMSO) δ 166.13, 136.73, 133.14, 129.87, 128.81, 46.82, 36.32, 33.19, 26.70, 22.74, 13.61. Anal. calcd for $C_{14}H_{21}ClN_2OS$: C, 55.89; H, 7.04; N, 9.31; S, 10.66. Found: C, 55.84; H, 7.06; N, 9.28; S, 10.61.

2-Styryl-1H-quinolin-4-one (13). Under inert atmosphere **4 (**1 mmol, 0.265 g) was taken in 15 ml dry ^{t}BuOH in a round bottom flask and ^{t}BuOK (3.5 mmol) was added. Reaction mixture was heated to 80–85 °C. Reaction was monitored by TLC with mobile phase hexane: ethylacetate (2:8). After completion of reaction, reaction mixture was neutralized by 1 M HCl solution at 0-5°C. Solid precipitates appeared. Solid was filter off and wash three times with water and dried under vacuum. The product was further purified by recrystallization from ethanol.

Light yellow solid; mp- >255 °C; ^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD): 8.24–8.19 (m, 1H), 7.61-7.57 (m, 5H), 7.40-7.37 (m, 4H), 6.92 (d, J=16.2 Hz, 1H); ^{13}C NMR (150.9 MHz, DMSO) δ 147.52, 140.82, 135.99, 135.24, 132.30, 129.72, 129.49, 127.75, 125.60, 125.18, 123.46, 122.82, 118.83, 107.90; MS(EI): m/z 63, 89, 108, 190, 217, 230,246, 247(M^+). Anal calcd for $C_{17}H_{13}NO$: C, 82.57; H, 5.30; N, 5.66. Found: C, 82.53; H, 5.36; N, 5.64.

2-(2-Fluoro-phenyl)-1H-quinolin-4-one (14). Synthesized as **13** starting from N-(2-Acetyl-phenyl)-4-fluoro-benzamide.

Light brown solid; mp > 230 °C; ^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD): δ 8.34–8.29 (m, 1H), 7.69-7.61 (m, 3H), 7.56-7.53 (m, 1H), 7.45-7.41 (m, 1H), 7.36-7.33 (m, 1H), 7.29-7.25 (m, 1H), 6.52 (s, 1H); ^{13}C NMR (150.9 MHz, DMSO) δ 177.00, 160.07, 158.42, 145.41, 140.62, 132.66, 132.61, 132.28, 131.07, 125.31, 125.07, 123.69, 122.90, 122.81, 118.79, 116.69, 116.54, 110.08; MS(EI): m/z 63, 89, 105, 183, 190, 211, 222, 239(M^+). Anal. calcd for $C_{15}H_{10}FNO$: C, 75.30; H, 4.21; N, 5.85. Found: C, 75.34; H, 4.20; N, 5.87.

N-(2-Acetyl-phenyl)-2-fluoro-benzamide (15). Synthesized as **4** starting from 2-aminoacetophenone and 2- fluoro-benzoyl chloride.

White solid; mp- 87 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.50 (s, 1H), 8.97 (d, 1H, *J* = 8.0 Hz), 8.07–8.05 (m, 1H), 7.96 (dd, 1H, *J* = 4.0 Hz), 7.65-7.62 (m, 1H), 7.56-7.52 (m, 1H), 7.33-7.29 (m, 1H), 7.25-7.19 (m, 2H), 2.70 (s, 3H); ^{13}C NMR (150.9 MHz, DMSO) δ 203.09, 162.29, 160.43, 158.77, 139.19, 134.71, 134.31, 132.31, 131.04, 125.36, 124.07, 123.78, 123.07, 122.98, 121.08, 116.96, 116.80; MS(EI): m/z 95, 123, 214, 242, 257(M^+). Anal. calcd for $C_{15}H_{12}FNO_2$: C, 70.03; H, 4.70; N, 5.44. Found: C, 70.05; H, 4.74; N, 5.42.

N-(1-Methylene-3-phenyl-allyl)-N'-(2-propylsulfanyl-ethyl)-ethane-1,2-diamine (16). Synthesised as **3** starting from N^1-(2-Propylsulfanyl-ethyl)-ethane-1,2-diamine[23] and cinnamoyl chloride.

White solid; mp- 157-158 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ =7.65-7.60 (m, 3H), 7.45-7.42 (m, 3H), 6.68-6.66 (d, 1H, *J* = 12 Hz), 3.67-3.65 (t, 2H, *J* = 6 Hz), 3.30-3.22 (m, 2H), 2.88-2.85 (t, 2H, *J* = 6 Hz), 2.63-2.61 (m, 2H), 1.69-1.66 (m, 2H), 1.35-1.33 (t, 2H, *J* = 6 Hz), 1.06-1.03 (m, 3H); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 167.50, 141.57, 134.59, 129.85, 128.79, 128.03, 120.34, 48.35, 47.61, 45.99, 36.43, 34.10, 27.43, 22.76, 13.32, 8.67; MS (EI): m/z 61, 77, 103, 131, 174, 203. Anal. calcd for $C_{16}H_{24}N_2OS$: C, 65.71; H, 8.27; N, 9.58; S, 10.96. Found: C, 65.65; H, 8.29; N, 9.56; S, 10.99.

2-(3-Methyl-thiophen-2-yl)-1H-quinolin-4-one (17). Synthesized as **13** starting from 3-Methyl-thiophene-2-carboxylic acid (2-acetyl-phenyl)-amide.

White solid; mp- >235 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.27–8.25 (d, 1H, J = 8 Hz), 7.76-7.73 (m, 1H), 7.69-7.67 (d, 1H, J = 8.4 Hz), 7.60-7.59 (d, 1H, J = 5 Hz), 7.47-7.45 (m, 1H), 7.08-7.07 (m, 1H), 6.39 (s, 1H), 2.41 (s, 3H); MS(EI): m/z 77, 121, 213, 224, 24. Anal calcd for $C_{14}H_{11}NOS$: C, 69.68; H, 4.59; N, 5.80; O, 6.63; S, 13.29. Found: C, 69.63; H, 4.52; N, 5.88; O, 6.60; S, 13.23.

***N*-(2-{[2-(phenylsulfanyl)ethyl] amino}ethyl) cyclohexane carboxamide hydrochloride (18).** Synthesised as **3** starting from N^1-(2-Phenylsulfanyl-ethyl)-ethane-1, 2-diamine[23] and cyclohexanecarbonyl chloride.

White solid; mp- 148 °C; ^{1}H NMR (400 MHz, CD_3OD): δ =7.49-7.47 (m, 2H), 7.38-7.34 (m, 2H), 7.30-7.26 (m, 1H), 3.47-3.44 (t, 2H, *J* = 6.0Hz), 3.31-3.18 (m, 4H), 3.16-3.13 (t, 2H, *J* = 6.0 Hz), 2.24-2.17 (m, 1H), 1.83-1.77 (m, 4H), 1.70-1.68 (m, 1H), 1.46-1.37 (m, 5H). ^{13}C NMR (100 MHz, CD_3OD): δ 180.65, 134.82, 131.59, 130.45, 128.35, 37.23, 30.63, 30.53, 26.82, 26.69. EIMS: m/z 55, 77, 83, 109, 137, 154, 183, 233; ^{13}C NMR (150.9 MHz, DMSO): δ 175.99, 134.27, 129.43, 128.57, 126.50, 46.47, 45.95, 44.05, 35.21, 29.18, 27.73, 25.57, 25.35; MS(EI): m/z 55, 83, 109, 137, 154, 183. Anal. calcd for $C_{17}H_{26}N_2OS$: C, 66.62; H, 8.55; N, 9.14; S, 10.46. Found: C, 66.60; H, 8.53; N, 9.17; S, 10.43.

N-(2-Acetyl-phenyl)-4-nitro-benzamide (19). Synthesized as **4** starting from 2-aminoacetophenone and 4- nitro-benzoyl chloride.

Yellow solid; mp- 183 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.92 (s, 1H), 8.94 (d, 1H, *J* = 8.4 Hz), 8.37 (d, 2H, *J* = 8.4 Hz), 8.23 (d, 2H, *J* = 8.8 Hz), 8.00 (d, 1H, *J* = 8.0 Hz), 7.68-7.64 (m, 1H), 7.26-7.21 (m, 1H), 2.74 (s, 3H); ^{13}C NMR (150.9 MHz, DMSO) δ 203.80, 163.70, 149.94, 140.41, 139.86, 135.11, 132.52, 129.02, 124.42, 123.96, 120.91, 29.05; MS(EI): m/z 75, 76, 92, 104, 120, 134, 150, 241, 269, 284(M^+). Anal. calcd for $C_{15}H_{12}N_2O_4$: C, 63.38; H, 4.25; N, 9.85. Found: C, 63.36; H, 4.28; N, 9.83.

N-(2-Acetyl-phenyl)-4-fluoro-benzamide (20). Synthesized as **4** starting from 2-aminoacetophenone and 4- fluoro-benzoyl chloride.

White solid; mp- 92-93 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.74 (s, 1H), 8.99–8.98 (m, 1H), 8.13–8.11 (m, 2H), 8.01–8.00 (m, 1H), 7.68-7.65 (m, 1H), 7.24-7.20 (m, 3H), 2.76 (s, 3H). ^{13}C NMR (150.9 MHz, DMSO) δ 203.51, 165.21, 163.94, 163.55, 139.61, 134.63, 132.15, 130.85, 129.85, 123.46, 123.16, 120.30, 116.06, 115.91, 28.68; MS (EI): m/z 75, 95, 123,

214, 242, 257(M^+). Anal. calcd for $C_{15}H_{12}FNO_2$: C, 70.03; H, 4.70; F, 7.38; N, 5.44; O, 12.44. Found: C, 70.05; H, 4.72; N, 5.47.

3. Shiga Toxin

3.1. Prepararion of Shiga toxin

Standard culture of *S. dysenteriae* type 1 was obtained from National Institute of Cholera and Enteric Diseases (NICED), Kolkata. Pure colony of S. *dysenteriae* type 1 was inoculated in 5 ml of Brain heart infusion (BHI) broth and incubated overnight at 37 °C with shaking at 200 rpm. BHI broth (200 ml) was inoculated with 1% of the mother culture and incubated at 37°C, with shaking at 200 rpm for 24 h. The overnight grown culture was centrifuged at 10,000 ×g for 10min at 4 °C. The pellet was re-suspended in 50 mM Tris-Cl buffer (pH 8.0), lysozyme (20 μg/ml) and incubated at 4 °C for 1 h. The re-suspended solution was subjected to sonication (5 cycles of 9s on & 9s off), followed by centrifugation. The supernatant obtained was filtered with 0.22 μm syringe filter (Millipore) and after protein estimation (micro-BCA, Pierce) aliquots were stored in –80 °C. These aliquot were further used for cytotoxicity assay and other experiments. This lysate was referred as Stx.

3.2. Preparation of Recombinant Shiga Toxin A Subunit (rStxA)

Expression, purification and immunological characterization of recombinant Shiga Toxin A subunit was done in laboratory as described earlier.[24] Purified recombinant StxA subunit was used for the thermal shift assay and SPR studies.

4. Screening of Small Molecule Inhibitors against Stx

4.1 *in silico* Screening Using AutoDock 4.2

Small molecule structures in ligand.pdb format were prepared using ArgusLab 4.0.1 (http://www.arguslab.com). The 1DM0.pdb file of Stx A subunit was obtained from RCSB (http://www.ncbi.nlm.nih.gov/pubmed/7656009). AutoDock 4.2 is used for docking studies.[22] The Grid box was centred on the active site amino acid residues Ser112, Tyr77,

Val78, Tyr114, Glu167, Ala168, Trp203 and Arg170. AutoDock was launched and resulting docking conformations compared to determine similarities and then they are clustered accordingly. The root-mean-square deviation (RMSD) is used to determine whether two docked conformations are similar enough to be in the same cluster. Then these clusters are ranked from the lowest energies to highest.

4.2. Screening of Small Molecule using Fluorescence-Based Thermal Shift Assay (FTS)

Thermal shift assay is the primary method of identification of interaction between protein and small molecule inhibitors. Sypro orange is a fluorescent dye used to monitor protein unfolding. Melting of the globular proteins exposes the hydrophobic region of proteins that results in large increase in fluorescence.[15] The shift in temperatures obtained in presence of ligands as compared to absence of ligands of unfolded protein is used for estimation of change in transition temperature (ΔTm). Fluorescence based thermal shift assay (FTS) was conducted on Step One real time PCR detection system (Applied Biosystems, Life Technologies, USA). The device is made of heating/cooling system for temperature control and a detector based charge coupled device to detect fluorescence change in the wells in real time. The 48 well microtiter plate was used for FTS assay. Total of 20 µl/well solution was prepared containing 1.76 µM rStxA, 5x sypro orange and small molecules at 10 µM concentration in PBS. The plate was heated at 1% of ramp rate (~5 $^{\circ}$C/min) from 25 to 90 $^{\circ}$C and Rox is selected as reporter for detection of fluorescence intensity.

4.3. Surface Plasmon Resonance Analysis

The interaction between small molecules inhibitors and rStxA was further explored using a ProteOn XPR36 instrument (Bio-Rad, Hercules, CA). This instrument provides real-time data of 36 simultaneous interactions in one experiment. The instrument features six injection needles with the ability to use the horizontal for six ligand immobilization conditions and vertical for six concentrations/analytes orientations of the fluidics of the sensor chip.

Standard amine coupling chemistry was used to immobilize recombinant StxA (25 μg/ml in 10 mM sodium acetate buffer, pH 5.0) on the EDAC/Sulfo-NHS [1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDAC) and N-hydroxysulfosuccinimide (Sulfo-NHS)] activated GLH sensor chip at a density of ~6000 resonance units (RU) as per the manufacturer's instructions. Small molecules were prepared in phosphate buffered saline-tween 20 (PBST and compounds were injected at 100 μl/min for 200s at concentrations of 25.00–1.625 μM. The dissociation was monitored for 600s. Chip surfaces were regenerated by injecting 50 mM NaOH, for 30s for reuse. All the SPR experiments were performed at 25 °C. The sensogram data obtained was analyzed using ProteOn Manager 3.1 software and fitted to the simplest Langmuir 1:1 interaction model.

5. Cell Culture and Evaluation of Small Molecule Inhibitors

On the basis of molecular docking studies twenty molecules were selected for *in vitro* screening. The short listed molecules consisted of amides, chalcones, esters and quinolones. We have also evaluated twelve compounds which were previously reported against Stx for its efficacy.[11]

5.1. Cell Maintenance

Vero cells were obtained from National Centre for Cell Science, Pune and being maintained in Dulbeco's Modified Eagle Medium (DMEM) containing 10% FBS in an incubator at 37 °C humidified atmosphere with 5% CO_2 and 95% air.

5.2. CC_{50} Determination of Toxin

The concentration of Stx, toxic to 50% of the cells (CC_{50}) was determined from the plots of cell viability by the neutral red (NR) uptake method and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical, St. Louis, Missouri, USA). Vero cells were dispensed in flat-bottomed 96-well microtiter plates (Nunc, Denmark) at a density 10^4 cells/well and maintained overnight at 37 °C. Then the cells were incubated with

various concentration of Stx (20 ng/ml to 120 ng/ml) for further 24 h. For NR assay after 24 h of treatment, 0.33% neutral red solution (10 µl/well) was added to cells and incubated for 4h. Incorporated dye was solubilized in 50% methanol containing 1% acetic acid and absorbance was read at 540 nm (Biotek Instruments, USA). For MTT assay, MTT (5 mg/ml, 20 µl/well) solution was added to each well and incubated for 4 h. Formazan crystals were solubilized with DMSO and absorbance was measured at 570 nm using a multiscanner. Data were normalized to the measurement from control cultures which were considered 100% survival.

5.3. Toxicity Measurements of the Compounds

Similar culture conditions as described above were used for the toxicity measurements of the small molecules. Small molecules used for this study listed in table1, were dissolved in sterile filtered absolute DMSO and 20 mM stock solution was prepared. Compounds were tenfold serially diluted (1000 to 0.0001 µM) in media for the CC_{50} determination. We have also determined highest concentration of compounds which is non-toxic to cells known as maximum tolerable dose (MTD).

5.4. Determination of Efficacy of Compounds against Stx

For screening of efficacy and determination of IC_{50} (concentration of compounds reversing 50% toxicity of Stx, assuming 100% toxicity of Stx) of compounds against Stx, cells were treated with varying concentrations of compounds for 1 h prior to Stx treatment. Cells were also treated with DMSO as a vehicle control and no toxicity was observed when administered alone (data not shown). The cell viability assay was performed at 24 h post treatment employing neutral red dye uptake assay. Efficacies of promising compounds were also determined by MTT assay. We have observed the dose dependent efficacy of compounds which offered protection against Stx. Selectivity index (SI) as an estimate of therapeutic window, of compounds was calculated from value of CC_{50} and IC_{50} of compounds ($SI = CC_{50}/IC_{50}$).

5.5. Lactate Dehydrogenase Release Assay

Efficacy of compounds that offered protection against Stx by MTT and NR assay was further validated by LDH release assay. LDH activity in the culture media was measured spectrophotometrically at 490 nm according to manufacturer's protocol (Sigma Chemical, St. Louis, Missouri, USA) as an index of plasma membrane damage and membrane integrity loss. Enzyme activity was expressed as the percentage of extra cellular LDH activity of the total LDH activity of the cells.

6. Animal Treatment Strategy and Husbandry

Balb/c male mice were randomly bred in institute's animal facility, weighing between 22-24 g were used in this study. The animals were housed in standard conditions of temperature and humidity. The animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India). Food and water were provided *ad libitum*. The animals were used according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, and Institutional Animal Ethics Committee approved the experiment (BT/01//DRDE/2012).

Compounds that offered protection in *in vitro* study were further evaluated in mice for their efficacy against Stx toxicity. Animals were divided into five groups of eight animals each. For treatment, median lethal dose of Stx, required to kill half the members of a tested population (LD_{50}), was determined for 7 days using reported method.[25] For all treatment intraperitoneal route (i.p.) of administration was used. Toxin alone treated group was administered a single dose of 5x LD_{50} of Stx by i.p. route. In second group mice were treated with the toxin that was pre incubated with compounds for 1 h. In third group animals were treated with compounds 1 h prior to toxin injection. In fourth group toxin was injected 1h prior to the treatment by compound. Control animals received the same volume of PBS as the experimental group. All the mice were treated with 20 mM compounds at fixed dose of 100 µl.

7. Statistical Analysis

The complete data was expressed as a mean ±SEM of three or more independent experiments with triplicate samples. Statistical comparisons between groups were performed using one-way ANOVA followed by Dunnett's test or Student's *t*-test or Student-Newman-Keuls multiple comparison test. Differences with *p* value less than 0.05 were taken as significant.

Corresponding Author

Ram Kumar Dhaked, email: ramkumardhaked@hotmail.com

Division of Biotechnology, Defence Research Development & Establishment, Defence Research & Development Organization, Gwalior-474002, MP, India, Fax +91-751-2341148, Phone: +91-751-2390274.

Abbreviations

Stx, Shiga toxin; IC_{50}, half maximal inhibitory concentration; CDC, Centers for Disease Control and Prevention; HUS, hemorrhagic uremic syndrome; RIPs, ribosome inactivating proteins; FTS, Fluorescent based thermal shift assay; SPR, surface plasmon resonance; rStx A, recombinant Stx A subunit; ΔTm, change in transition temperature; CC50, 50% cytotoxic concentration; MTT, 3-(4, 5- 82 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NR, Neutral red; LDH, lactate dehydrogenase; SI, Selectivity index; RU, resonance units; KD, dissociation constant; ka, association rate; kd, dissociation rate; LD50, median lethal dose; PAINS, pan assay interference compounds; RMSD, root-mean-square deviation; NICED, National Institute of Cholera and Enteric Diseases; BHI, brain heart infusion; DMEM, Dulbeco's Modified Eagle Medium; EDAC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; Sulfo-NHS, N-hydroxysulfosuccinimide; SI, selectivity index; LDH, lactate dehydrogenase; FTS, fluorescence-based thermal shift assay.

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Supporting Information

The supporting information is available free of charge on the ACS publication website at DOI: . Data in the form of figures and tables that includes the structures of compounds docked into StxA and binding energy, ki and ΔTm of reported molecules. Dose dependent response of compound **3** against Shiga toxin. SPR sensograms for compounds **4, 15** and **16**.

Authors Contributions

R.K.D. and N.S. conceptualized the study. V.C., D.C., and N.S. performed the experiments.

U.P. synthesized and characterized the compounds.

References

(1) Strockbine, N.A.; Jackson, M.P.; Sung, L.M.; Holmes, R.K.; O'Brien, A.D. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. *J. Bacteriol*. **1988**, *170*, 1116-1122.

(2) Calderwood, S.B.; Auclair, F.; Donohue-Rolfe A.; Keusch. G.T.; Mekalanos, J.J. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1987,** *84,* 4364-4368.

(3) Rahal, E.A.; Fadlallah, S.M.; Nassar, F.J.; Kazzi, N.; Matar, G.M. Approaches to treatment of emerging Shiga toxin-producing *Escherichia coli* infections highlighting the O104:H4 serotype. *Front. Cell Infect. Microbiol*. **2015,** *5,* 24.

(4) Beddoe, T.; Paton, A.W.; Le Nours. J.; Rossjohn, J.; Paton, J. C. Structure, biological function ns and applications of the AB5 toxins. *Trends Biochem. Sci*. **2010,** *35*, 411-418.

(5) Mukhopadhyay, S.; Linstedt, A.D. Retrograde trafficking of AB_5 toxins: mechanisms totherapeutics. *J. Mol. Med.* **2013,** *91*, 1131-1141.

(6) Stechmann, B.; Bai, S.K.; Gobbo, E.; Lopez, R.; Merer, G.; Pinchard, S.; Panigai, L.; Tenza, D.; Raposo, G.; Beaumelle, B.; Sauvaire, D.; Gillet, D.; Johannes, L.; Barbier, J. Inhibition of retrograde transport protects mice from lethal ricin challenge. *Cell* **2010,** *141,* 231-242.

(7) Saenz, J.B.; Doggett, T.A.; Haslam, D.B. Identification and characterization of small molecules that inhibit intracellular toxin transport. *Infect. Immun*. **2007,** *75,* 4552-4561.

(8) Wahome, P.G.; Bai, Y.; Neal. L.M.; Robertus, J.D.; Mantis, N. J. Identification of small-molecule inhibitors of ricin and Shiga toxin using a cell-based high-throughput screen. *Toxicon* **2010,** *56,* 313-323.

(9) Noel, R.; Gupta, N.; Pons, V.; Goudet, A.; Garcia-Castillo, M. D.; Michau, A.; Martinez, J.; Buisson, D.A.; Johannes, L.; Gillet, D.; Barbier, J.; Cintrat, J.C. N-

methyldihydroquinazolinone derivatives of Retro-2 with enhanced efficacy against Shiga toxin. *J. Med. Chem*. **2013,** *56,* 3404-3413.

(10) Endo, Y. Mechanism of action of ricin and related toxins on the inactivation of eukaryotic ribosomes, *Cancer Treat. Res.* **1988,** *37*, 75-89.

(11) Brigotti, M.; Carnicelli, D.; Accorsi, P.; Rizzi, S.; Montanaro, L.; Sperti, S. 4-Aminopyrazolo[3,4-d] pyrimidine (4-APP) as a novel inhibitor of the RNA and DNA depurination induced by Shiga toxin 1. *Nucleic Acids Res.* **2004,** *28,* 2383-2388.

(12) Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E.; UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004,** *25*, 1605-1612.

(13) Chimenti, F.; Maccioni, E.; Secci, D.; Bolasco, A.; Chimenti, P.; Granese, A.; Befani, O.; Turini, P.; Alcaro, S.; Ortuso, F.; Cirilli, R.; La Torre, F.; Cardia, M.C.; Distinto, S. Synthesis, molecular modeling studies, and selective inhibitory activity against monoamine oxidase of 1-thiocarbamoyl 1-3,5-diaryl-4-5-dihydro-(1H)- pyrazole derivatives. *Bioorg. Med. Chem. Lett*. **2005,** *48,* 7113-7122.

(14) Kamboj, R.C.; Arora, R.; Sharma, G.; Kumar, D.; Sharma, C.; Joshia, R.; Anejaa, K.R. Eco-friendly synthesis and antimicrobial activity of chalcones. *Der. Pharma. Chemica.* **2010,** *2,* 157-170.

(15) Pantoliano, M.W.; Petrella, E.C.; Kwasnoski, J.D.; Lobanov, V.S.; Myslik, J.; Graf, E.; Carver, T.; Asel, E.; Springer, B.A.; Lane, P.; Salemme, F.R. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J. Biomol. Screen.* **2001,** *6,* 429-440.

(16) Bai, Y.; Monzingo, A.F.; Robertus, J.D. The X-ray structure of ricin A chain with a novel inhibitor. *Arch. Biochem. Biophys.* **2009,** *483,* 23–28.

(17) Miller, D.J.; Ravikumar, K.; Shen, H.; Suh, J.K.; Kerwin, S.M.; Robertus, J.D. Structure-based design and characterization of novel platforms for ricin and Shiga toxin inhibition. *J. Med. Chem.* **2002,** *45,* 90–98.

(18) Monzingo, A.F.; Robertus, J.D. X-ray analysis of substrate analogs in the ricin A-chain active site. *J. Mol. Biol.* **1992,** *227,* 1136–1145.

(19) Pang Y.P.; Park, J.G.; Wang, S.; Vummenthala, A.; Mishra, R.K.; McLaughlin, J.E.; Di, R.; Kahn, J.N.; Tumer, N.E.; Janosi, L.; Davis, J.; Millard, C.B. Small-molecule inhibitor leads of ribosome-inactivating proteins developed using the doorstop approach. *PLoS One* **2011,** *6,* e17883.

(20) Shoichet, B.K. Virtual screening of chemical libraries. *Nature* **2004,** *432,* 862–865.

(21) Baell, J.B.; Holloway, G.A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem*. *53,* 2719−2740.

(22) Morris, G.M.; Huey, R.; Lindstrom, W.; Sanner, M.F.; Belew, R.K.; Goodsell, D.S.; Olson, A.J. Autodock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009,** *16,* 2785-2791.

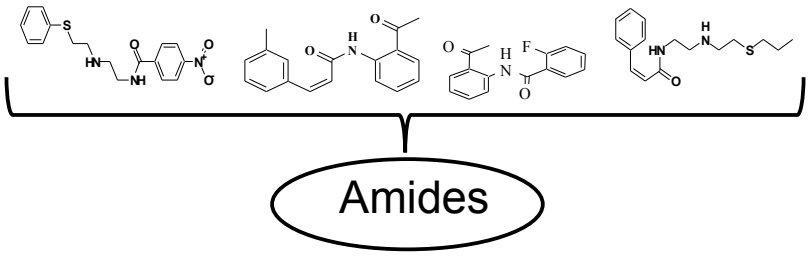
(23) Pathak, U.; Raza, S.K.; Kularni, A.S.; Kumar, P.; Vijayaraghavan, R.; Jaiswal, D. K. Novel S-substituted aminoalkylamino ethanethiols as potential antidotes against sulfur mustard toxicity. *J. Med. Chem.* **2004,** *47,* 3817-3822.

(24) Chauhan, V.; Chaudhary, D.; Rao, M.K.; Dhaked, R.K. Expression, purification and immunological characterization of recombinant Shiga toxin A subunit. *Prot. Pept. Lett.* **2015,** *22*, 844-852.

(25) Gad, S.C.; Weil, C.S. Statistics for toxicologists. In *Principles and methods of toxicology* 2nd ed.; Hayes, A.W., Ed.; Raven Press: New York, **1989**; pp. 435-483.

Graphic for manuscript

Surface image of active site of Shiga toxin with docked ligands (IC_{50} at 7.96 μM and SI of 22.23)



Amides