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INTRODUCTION

This contracted project is aimed at designing, synthesizing, and testing small molecule inhibitors of the cytotoxin ricin. Ricin is a class B biological agent which is known to be in the possession of terrorist groups (Loyd and Fletcher, 2001). Although not as menacing as infectious agents, ricin is of great concern because of the ease with which large amounts of semi pure material can be produced (Wellner, et al, 1995). The Army is proceeding with vaccine development for key military personnel (Olson et al, 2004). However, wide spread vaccination of the military or civilian population is not practical or desirable, and so there is a need for an efficacious antidote. In addition to its utility such a compound could reduce panic that arises from a relatively minor terrorist incident. Our area of expertise is the rational design of inhibitors of enzymes like ricin A chain, RTA (Miller et al, 2002). We have elucidated the three-dimensional structure of ricin and this model serves as a template for the design of small molecules that can bind tightly and inactivate the toxin. These inhibitor compounds should also incorporate elements of drug design, including solubility, stability, and low toxicity. We have used computer searches to identify classes of inhibitors that act as "platform" molecules. These platforms have been modified and appended creating novel inhibitors for RTA. This particular project is a collaboration between structural biologists (Robertus group) and synthetic chemists (Kerwin group) to extend our previous research efforts on antidotes. It is a step-wise process, beginning with modest inhibitors, which are then sequentially improved after analysis, to produce ever more potent compounds; the program is scheduled to last three years. Our overall goal is to create a ricin inhibitor which is efficacious at inhibiting ricin intoxication of cultured cells, and itself is non-toxic. Since an aerosol dispersion is considered the most likely form of attack we (Kehrer group) will use cultured human lung cells for this test.

BODY

The original Statement of Work (SOW) is as follows:

Task 1: Design improved specificity pocket (months 1 - 12)
- a. Prepare 9-oxaguaninine
- b. Prepare other related heterocycles

Task 2: Identify ligands for second ricin binding pocket (months 1 - 18)
- a. Use computer searches based on ricin structure
- b. Apply crystallographic screening of shape-diverse sets of commercial compounds

Task 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

Task 4: Use iterative crystallographic algorithm to refine tripartite inhibitors (months 12-36)
- a. Modify compound shape to fit enzyme contours
- b. Design compounds for maximum water solubility and biological uptake
- (This task (Task 4) is not reported in this report).

Task 5: Test biological efficacy of inhibitors as ricin antidotes (months l-36)
- a. Candidate inhibitors will be tested against ricin enzyme activity
- b. Ricin inhibitors will be tested for protective action in cultured human lung cells
- c. The most promising ricin antidotes will be sent to a commercial testing facility for initial human safety tests using a panel of enzyme and receptor assays.

ACCOMPLISHMENTS FOR (CALENDAR) YEAR 3

TASK 1: Design improved specificity pocket ligands (months 1-12)

Work carried out in the past year has elucidated a problem with 9-deaza-guanine (9DG), a specificity pocket ligand which we viewed as our best platform for drug design. Figure 1 helps illustrate the problem. Based on our observations of the binding of pterins, and adenines (Yan et al, 1997) and on ab initio energy calculations (Yan et al, 1998), we anticipated that the guanine ring would bind as seen in panel A. that is, N1 (labeled with *) would be protonated while N3 would be a hydrogen bond acceptor. This is a slightly higher energy tautomer, but it allowed two additional hydrogen bonds to form, and this tautomer had been seen in the crystal structure of the pterins. Last year we synthesized a potentially useful, synthetically facile, platform 9DG. It was an inhibitor, but our X-ray structure revealed it bound as shown in panel B. That is, it retained the low energy tautomer and optimized hydrogen bonding by rotating the heterocyclic ring roughly 180° around an axis normal to the rings. It also formed a long, and weak, hydrogen bond between the amido N of Tyr 123 and the exocyclic oxygen off C6; we had predicted this would interacted with R180. Based on this structure we felt that pendant groups should be added to the platform off of the nitrogen at position 7, pointing into the active site cleft and heading towards other potential binding partners for larger pendant groups. As a first step we synthesized 7-methyl-9deaza-guanine. It was an inhibitor (Ki ~ 2 mM). This year we solved the X-ray structure. The result is indicted in panel C. In this case, the high energy tautomer is seen and the carbonyl oxygen does indeed receive a hydrogen bond from R180. The added methyl side chain is accommodated near R180, but there is very little room to add any larger pendants. It is apparent that the orientation of the 9dG ring is difficult to predict. It has a low

degree of rotational symmetry regarding hydrogen bond donors and acceptors, along the C8 C2 axis. Very subtle effects can tip the preferred binding either way. For now, it seems best to add pendants to C8, which should accommodate either major ring orientation

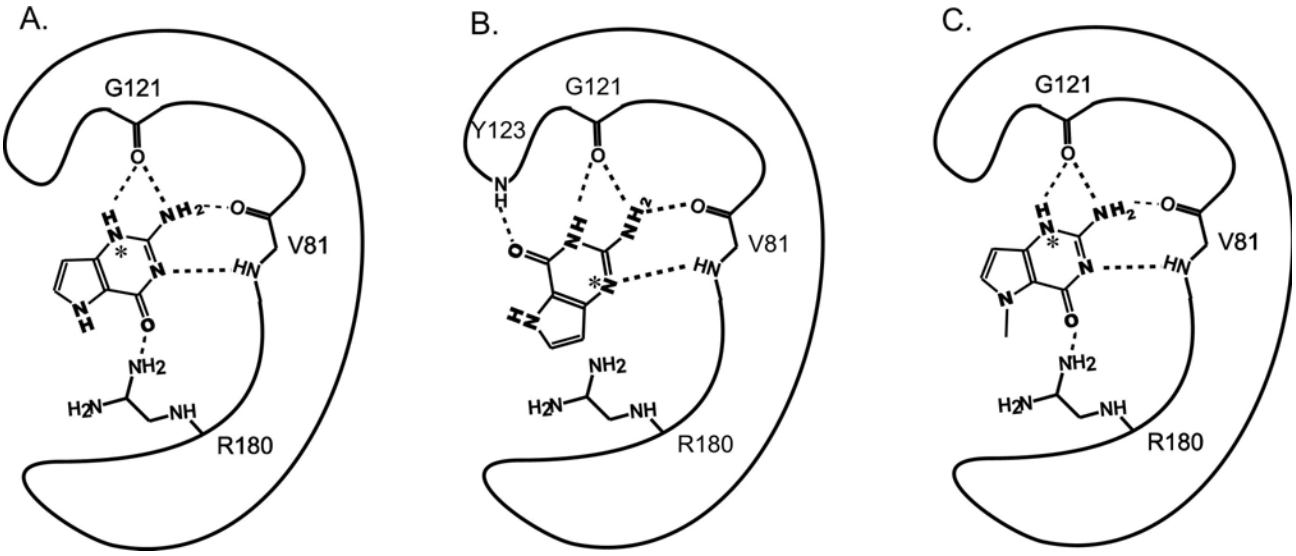


Figure 1: Cartoon of 9DG binding to RTA. A. the predicted mode of 9DG; B. the observed mode of binding;C. the observed binding of the 7-methyl derivative.

Because difficulties remain with the specificity pocket platform molecule, we felt it was prudent to test new in silico methods of screening. To this end we acquired two commercial virtual screening programs, eHiTS (SimBioSys), and GOLD (). We used RTA as a receptor and began testing by docking known inhibitors via the two programs. The results were mixed, some results are shown in Figure 2. Both programs did a good job of docking PTA, a reasonably large inhibitor, shown in panel A. They also did a good job with 7-Me-9DG as seen in B. However both programs missed the actual binding of 9DG itself. As seen in panel C the key exocyclic amine is bound correctly, but the rings are flipped around this vector so that the modeled carbonyl oxygens, Om, are on the opposite side from that observer by X-ray (Ox). The binding

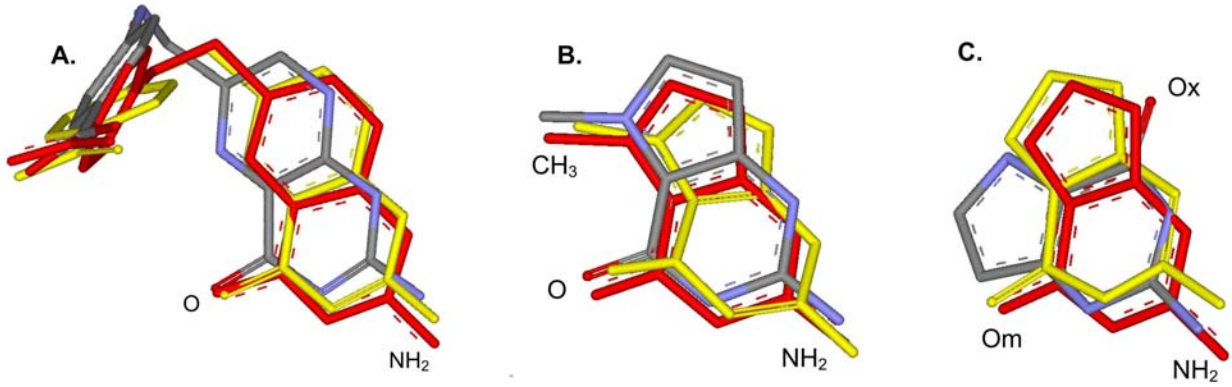


Figure 2: Three test dockings into RTA. In each case the binding observed by X-ray is in atom coded colors, mostly light blue. The mode predicted by eHiTS is red, and that by GOLD is in yellow. Certain key groups are labeled.

of two other large inhibitors, neopterin and FMP, were also accurately predicted by both programs. This suggests, based on a limited data set, that binding of mid to large compounds with a defined set of markers for the receptor may be predicted by these programs.

To follow up on this experiment, we began to screen a large commercial data base, the 50,000 compounds from the Sigma catalog, which has been made available as a molecular file (sdf format). We recently set up a collaboration with Dr. Chandra Bajaj to use his 128 processor computer cluster to initiate parallel screens. So far we have been screening with a single computer and have examined only 2000 compounds. Even so, our search has revealed some potential new platform molecules. Figure 3 shows the docking predicted by eHiTS and GOLD for two compounds that each rank in the top 5% and for which the two programs agree well on the orientation. The compound in panel A places a pyrrolidine ring in the specificity pocket of RTA. The carboxylate makes strong interactions with R180. The carbonyl oxygen, labeled O, receives a hydrogen bond from the amido of Tyr 123 and the large fluorene ring binds in a key hydrophobic patch, particularly with the side chain of Trp 211. The compound in panel B is somewhat similar, placing an oxazoline ring in the specificity pocket and a smaller benzene ring in the hydrophobic patch. We are now acquiring these, and other, commercial compounds for kinetic testing.

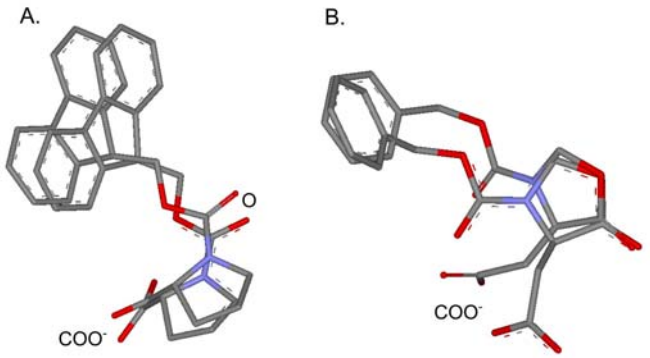


Figure 3: Two candidate inhibitors from virtual screening. Each panel shows the docking by eHiTS and GOLD.

TASK 2: Identify ligands for second ricin binding pocket

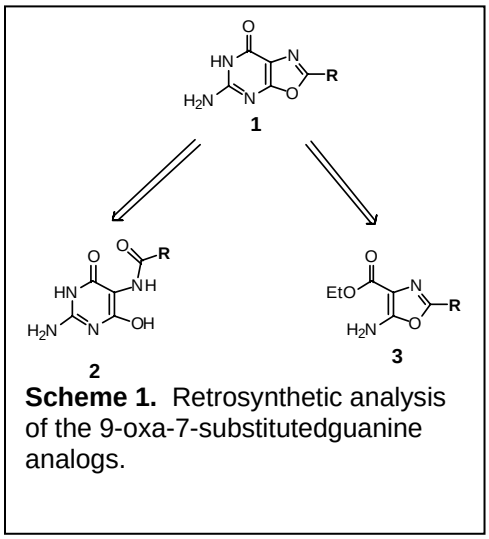
All efforts to identify ligands binding to the second pocket by X-ray diffraction have failed. A major problem appears to be that most of the candidates have poor solubility. We know that the Kd values for specific active site molecules, like adenine and pterins, are 1 -2 mM. It is likely the Kd for this other site, when taken alone, is at least that high. The screening of shape diverse libraries requires a good deal of organic solvent to dissolve the panel of compounds and this has interfered with crystal screening, as it does with kinetic screening.

It may be useful to use the virtual screening software to search for groups with an affinity for the second pocket. This will be easy to do once the parallel screening capabilities are brought online.

TASK 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

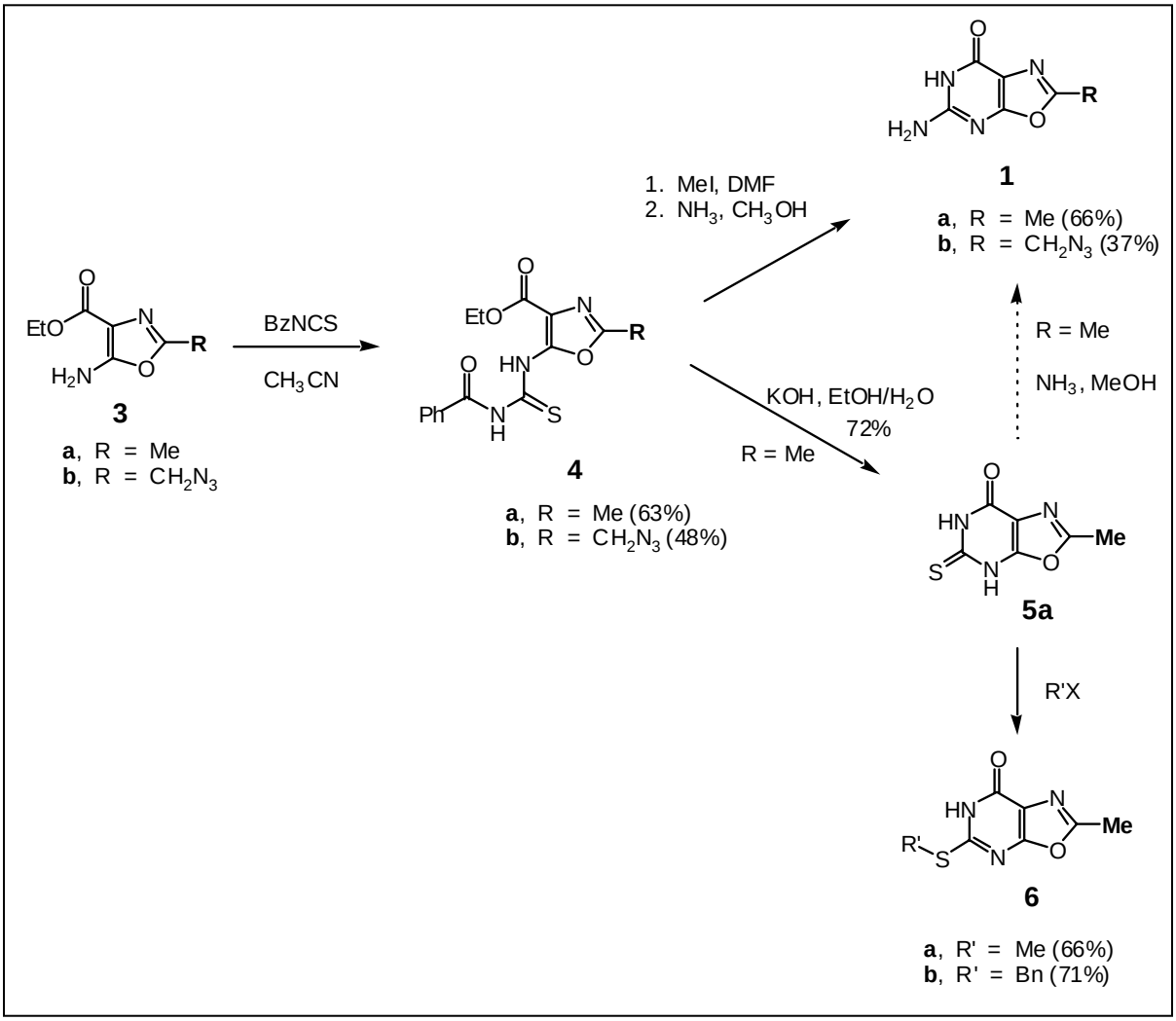
Synthetic approaches to tripartite inhibitors have focused on elaborating two specificity pocket-binding scaffolds, 9-oxoguanine and 9-deazaguanine. Although progress has been made in developing synthetic approaches, no potent inhibitors have been identified to date.

A. Prepare elaborated 9-oxaguanine analogues



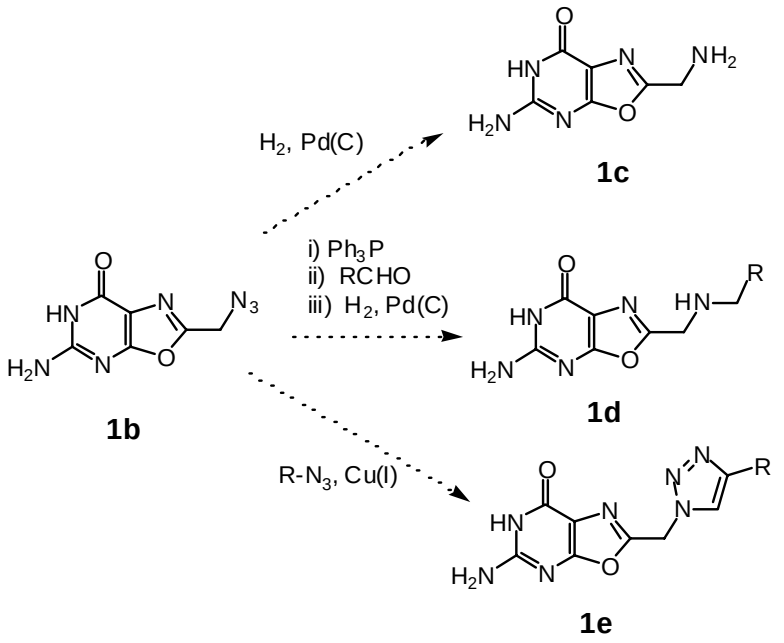
Scheme 1. Retrosynthetic analysis of the 9-oxa-7-substitutedguanine analogs.

Work continues on constructing inhibitors based on the 8-methyl-9-oxa-guanine (**1,** R = Me, Scheme 1) scaffold; this has been shown to be a soluble and relatively potent platform inhibitor (Miller et al, 2002). Two potential approaches to the 9-oxa-guanine ring system were proposed (Scheme 1). In one approach, an acylated diaminohydroxypyrimidine (**2**) undergoes cyclodehydration to form the oxazole ring of the 9-oxa-guanine heterocycle. Unfortunately, this route is only successful for preparing the 8-methyl substituted analog. The alternative approach, in which the oxazole ring is constructed first (**3**), and the pyrimidine ring is subsequently elaborated was initially explored during the previous funding period, in which work focused on a direct conversion of **3** to **1**. Although this work was not successful, it led to the hypothesis that the conversion of **3** to **1** would require initial acylation with an isothiocyanate followed by cyclization to the 9-oxopurine ring system.



The previously prepared4-amino-oxazole derivative **3a** (R = Me) and a newly prepared azidomethyl analog **3b** (R = CH_2N_3) were treated with benzoylisothiocyanate to afford the corresponding thioureas 4 (Scheme 2). These could be converted to the 9-oxoguanines **1** by a two-step procedure involving methylation on sulfur followed by ring closure in methanolic ammonia. Our observation that **4** was rather unstable led us to explore a modified procedure in which **4** is treated with base to afford the 2-thio-9-oxo-xanthine **5**, which may be converted to **1** by treatment with ammonia.

The preparation of **1b** is significant because this is the first 9-oxoguanine derivative bearing a functionalized 8-position substituent that we have been able to prepare. Azide **1b** can serve as the precursor to a wide variety of more complex 9-oxoguanine compounds (Scheme 3), including the primary amine **1c**, and the tripartite inhibitors **1d** and **1e**, the later derived from "click" chemistry (Hartmuth et al, 2001; Demko and Sharpless, 2002) (see below).

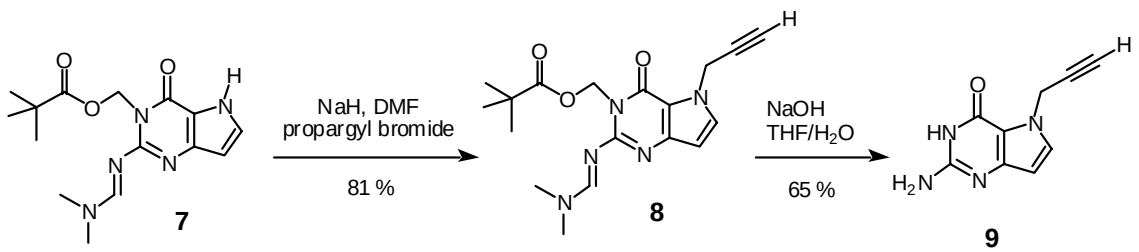


Scheme 3. Elaboration of 9-oxogunaine **1b**.

While thioxanthine **5** is not an inhibitor of RTA, the corresponding thiomethyl derivative **6a** is a weak inhibitor (IC_{50} ~ 4.8 mM). Based on this observation, analogs of **6a** in which the 2-thiomethyl group are elaborated (e.g., **6b**, Scheme 2) are also being prepared.

B. Prepare 7-substituted-9-deazaguanine derivatives.

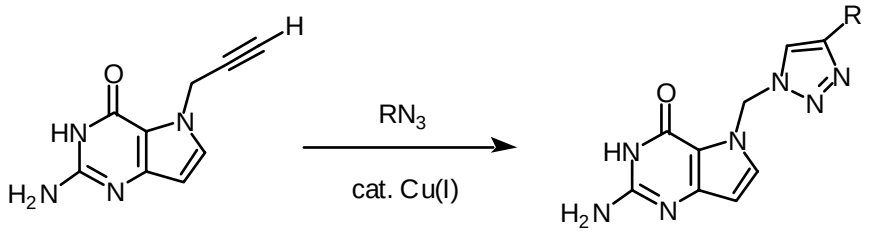
Our continuing work preparing tripartate inhibitors based on the 9-deazaguanine scaffold has focused on compounds containing a triazole linking group derived from 7-propargyl-9-deazagunaine (**9**, Scheme 4), which is a weak inhibitor of RTA (IC_{50} = 1.6 mM). These triazoles (**10**) are obtained by coupling **9** and a variety of azides via "click" chemistry, a versatile copper-catalyzed version of the Huisgen thermal [3+2] cycloaddition reaction



Scheme 4. Synthesis of 7-propargyl-9-deazaguanine.

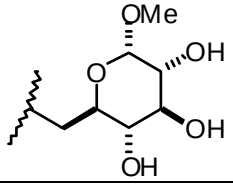
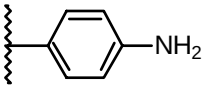
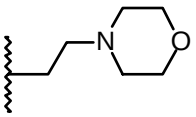
(Table 1). The advantages of this click chemistry approach include the potential for favorable dipole interactions between the triazole linker and RTA, the regiochemical control leading to 1,4-

substituted triazoles exclusively, and the ability to carry out the coupling in aqueous solution. This last point is particularly important because it leads to the possibility that inhibitors might be simultaneously selected for and synthesized via in situ click chemistry, as has been reported for other enzyme targets (Whiting, et al., 2006; Lee, et al., 2003). In order to address this possibility, a virtual library of triazoles **10** was constructed using ~100 commercially available azides. Docking of this library against RTA with eHiTs was carried out in order to select the five highest scoring library members for synthesis.



9 **10**

Table 1. Design of tripartate 9-deazaguanine inhibitors via click chemistry.

Entry	R =	eHiTs Score[a]	Yield of 8	RTA Inhibition
a	Bn	-38.2	81 %	nd[b]
b		-34.5	48 %	na[c]
c	$-CH_2CO_2H$	-33.4	62 %	na[c]
d		-32.9	36 %	na[c]
e		-31.9	51 %	-*-[d]

a. predicted RTA binding energy of **10**. b. not determined due to insolubility of this inhibitor. c. no activity observed at any concentration. d. inhibition of protein synthesis observed in the absence of RTA at the highest inhibitor concentration examined (1.9 mM).

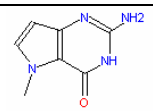
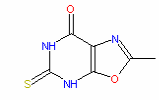
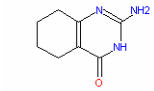
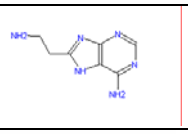
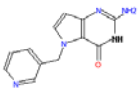
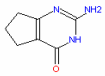
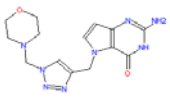
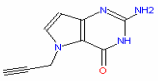
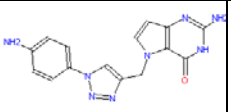
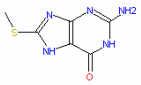
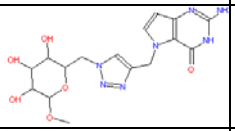
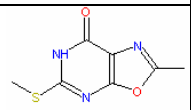
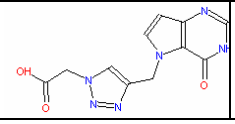
As shown in Table 1, despite favorable predicted binding energies, none of the triazole compounds were found to inhibit RTA. The failure of this approach to a tripartite 9DG-based inhibitor may be related to the difficulties associated with this scaffold discussed above under Task 1. Alternatively, the triazole linker may preclude the binding of these compounds to RTA, despite predictions of favorable binding orientation and interaction by eHiTs.

Task 5: Test biological efficacy of inhibitors as ricin antidotes (months l-36)

a. Candidate inhibitors will be tested against ricin enzyme activity

Over the past year we have tested the inhibition of RTA be a number of novel compounds. This is summarized in Table 2.

Table 2: List of Compounds Tested for RTA Inhibition. Res refers to the resolution of the X-ray crystal structure of the complex.

Compound	ID	IC_{50}	Res	Compound	ID	IC_{50}	Res
	KeRCN 088 (7-Me-9DG)	2.2 mM	1.8		WT063	4.7 mM	N/A
	THQ	None	N/A		purine-8-ethanamine 6-amino	None	N/A
	SM93	None	N/A		1-aminoisoquinoline	None	N/A
	SM92	None	N/A		KeRCN109	None	N/A
	SM91	1.6 mM	2.1		KeRCN110	None	N/A
	KeRCN 098	None	N/A		KeRCN112	None	N/A
	WT062	None	N/A		KeRCN111	None	N/A

KEY RESEARCH ACCOMPLISHMENTS

- A new route to the 9-oxoguanine scaffold has been developed which allows access to 8-position substituents suitable for construction of tripartite inhibitors.
- A series of 9-deazaguanine-based triazoles has been prepared via "click" chemistry. Although none of these are inhibitors, the success of this chemistry indicates that this will be a versatile approach to tripartite inhibitors when applied to alternative specificity pocket ligands and linkers.
- A new potential specificity pocket scaffold ligand, the 2-thioalkyl-9-oxoxanthine ring system was prepared.
- Initial tests on two virtual screening programs show encouraging results in tests of known inhibitors. Potential platform molecules have been identified.

REPORTABLE OUTCOMES

No papers from year one work have yet been prepared, but publishable work has been accomplished. We plan to submit an manuscript describing the new route to 9-oxoguanine derivatives during the next funding period.

CONCLUSIONS

The proposed inhibitor design project is far more difficult than originally envisioned. The active site of RTA is large and polar, evolved to accommodate a large number of weak interactions from a large RNA. Finding small molecules that make strong and specific interactions to compete with this large scale substrate binding is taxing. The 9DG platform molecule is an inhibitor, as are the 7-methyl and 7-propargyl groups. All can be seen in complex by X-ray. However, the new triazole constructs of 9-deaza guanine appear to be problematic - the larger triazole "click" compounds fail to inhibit. Even so, the diversity of subsites within the RTA target suggest that it should be possible to create tight binding inhibitors – it will simply require very skillful chemistry. We are also reasonable sanguine about the utility of in silico virtual screening. Tests recently completed suggest that the latest algorithms have merit, and may lead to identification of novel platform compounds and point to useful pendant groups as well.

"So what?": The novel synthesis of triazole inhibitors, arising from the versatile "click" chemistry, has encountered a number of difficulties as well. Because the chemistry is so facile, we are loath to abandon the idea and plan to add a methylene group between the 9DG and triazole groups. A second strategy is the use of the 9-oxa guanines, outlined above, which can be derivatized at the C8 position. Given the vagaries of the guanine ring orientations, adding pendants at this position may be the safest strategy. Several new compounds were tested and shown to be inhibitors, but they have not improved in Kd. It is essential to link larger pendants to the largely optimized platforms in order to improve binding by several logs.

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