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Identification of small-molecule inhibitors of ricin and shiga toxin using a cell-based high-throughput screen

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ABSTRACT

The Category B agents, ricin and shiga toxin (Stx), are RNA *N*-glycosidases that target a highly conserved adenine residue within the sarcin-ricin loop of eukaryotic 28S ribosomal RNA. In an effort to identify small-molecule inhibitors of these toxins that could serve as lead compounds for potential therapeutics, we have developed a simple Vero cell-based high-throughput cytotoxicity assay and have used it to screen ~81,300 compounds in 17 commercially available chemical libraries. This initial screen identified ~300 compounds with weak (≥30 to <50%), moderate (≥50 to <80%), or strong (≥80%) ricin inhibitory activity. Secondary analysis of 244 of these original "hits" was performed, and 20 compounds that were capable of reducing ricin cytotoxicity by >50% were chosen for further study. Four compounds demonstrated significant dose-dependent ricin inhibitory activity in the Vero cell-based assay, with 50% effective inhibitory concentration (EC_{50}) values ranging from 25 to 60 μM. The same 20 compounds were tested in parallel for the ability to inhibit ricin's and Stx1's enzymatic activities in an *in vitro* translation reaction. Three of the 20 compounds, including the most effective compound in the cell-based assay, had discernible anti-toxin activity. One compound in particular, 4-fluorophenyl methyl 2-(furan-2-yl)quinoline-4-carboxylate ("compound **8**"), had 50% inhibitory concentration (IC_{50}) of 30 μM, a value indicating >10-fold higher potency than is the case for previously described ricin–Stx1 inhibitors. Computer modeling predicted that compound **8** is capable of docking within the ricin active site. In conclusion, we have used a simple high-throughput cell-based method to identify several new small-molecule inhibitors of ricin and Stx.

1. Introduction

Ricin and shiga toxins (Stx) are members of the A–B family of toxins whose enzymatic A subunits are RNA *N*-glycosidases that target a highly conserved adenine residue within the sarcin-ricin loop of eukaryotic 28S ribosomal RNA (Endo and Tsurugi 1987, 1988). Both toxins are of public health concern, and they are classified by the Centers for Disease Control and Prevention (CDC) as Category B agents Mantis, 2005. Ricin is found in the seeds of castor bean plant (*Ricinus communis*) and is known to have been weaponized for chemical warfare purposes (Centers for Disease Control and Prevention, 2000, 2003; Crompton and Gall, 1980). The toxin is potentially lethal to humans if inhaled, ingested or injected (Audi et al., 2005). By the injection route, the lethal dose in humans is estimated at 0.1–1.0 μg/kg (Miller et al., 2002). The manifestations of ricin poisoning vary depending on the mode of exposure, but may include fever, gastroenteritis, fluid and electrolyte depletion, hypoglycemia, circulatory collapse, and multi-organ failure (Audi et al., 2005). Stx is produced by *Shigella dysenteriae* type 1 (Strockbine et al., 1988) and by certain strains of *Escherichia coli* (Calderwood et al., 1987). The A subunit (StxA) shows limited homology with the A subunit of ricin (RTA), although

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the two proteins catalyzes the same depurination reaction (Calderwood et al., 1987; Endo et al., 1988; Strockbine et al., 1988). Stx-producing *E. coli* (STEC) strains such as O157:H7, cause gastrointestinal illnesses including bloody diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (HUS). For either ricin or Stx exposure, treatment is strictly supportive; there are currently no specific antidotes against these toxins (Audi et al., 2005; Challoner and McCarron, 1990; Mantis, 2005; Quiñones et al., 2009; Serna and Boedeker, 2008).

RTA is linked via a single disulfide bond to the B subunit (RTB), a galactose-specific lectin that facilitates binding of ricin to host cell surfaces (Baenziger and Fiete, 1979). On binding to cognate cellular glycoprotein and glycolipid receptors, ricin is internalized by endocytosis and then trafficked via the retrograde pathway to the Golgi apparatus and the endoplasmic reticulum (ER) (Sandvig and van Deurs, 2000; Sandvig et al., 2002). The toxin is processed in the ER, and RTA is translocated to the cytoplasm, where a fraction of it escapes degradation by proteosomes and is able to target the host protein biosynthetic machinery (Sandvig and van Deurs, 2000; Sandvig et al., 2002). Stx, following association with its cognate receptor globotriaosylceramide (Gb3), follows a similar intracellular route. Once in the cytoplasm, both StxA and RTA selectively inactivate 28S rRNA (Sandvig and van Deurs, 2000). Ricin's cytoxicity is due to a combination of protein synthesis arrest and triggering of intracellular stress-activated pathways; the result is the induction of apoptosis, with the release of pro-inflammatory mediators (Gonzalez et al., 2006; Hughes et al., 1996; Yoder et al., 2007). Because all of these effects are initiated following ribosome arrest, the most effective inhibitors of ricin and Stx are likely to be those that directly interfere with the toxins' active sites.

The X-ray structure of RTA was solved to resolutions of 2.8 Å and 2.5 Å by Montfort et al. (1987) and Rutenber et al. (1991), respectively. Those studies, in combination with site-directed mutagenesis experiments, enabled the identification of the key active site residues, including Glu177, Arg180, Tyr80, Tyr123, and Typ211. Monzingo and Robertus proposed that depurination of adenine involves: Protonation of adenine (N3) by Arg180; delocalization of ring electrons, causing cleavage of C1′—N9 glycosidic bond; and generation of an oxacarbenium ion at C1′. The latter is stabilized by a hydroxide ion that is generated when Glu177 abstracts a proton from a free water molecule in the active site (Monzingo and Robertus, 1992). The authors also reported the crystal structures of RTA bound to two substrate analogues, formycin monophosphate (FMP) and a dinucleotide ApG. The structures of these complexes revealed important ionic and hydrophobic interactions that promote binding of the substrate and its analogues in the active site of RTA (Monzingo and Robertus, 1992). The active site of Stx has key residues that are conserved within the family of ribosome inactivating protein (RIP) and is analogous to the active site of RTA (Fraser et al., 1994; Katzin et al., 1991, Monzingo and Robertus, 1992).

There have been numerous attempts to identify active-site inhibitors of RTA, with the long-term goal of using these molecules as therapeutics against both ricin and Stx. Virtual screening (Shoichet, 2004) has identified substrate analogues and derivatives of pterin, pyrimidine, and guanine as weak to modest RTA inhibitors, with IC_{50} values ranging from 200 to >2000 μM (Bai et al., 2009; Monzingo and Robertus, 1992; Robertus et al., 1996; Yan et al., 1997). For example, pteroic acid (PTA) and 8-methyl-9-oxaguanine were identified using this method and were confirmed by kinetic measurements to be modest inhibitors of RTA, with respective IC_{50} values of ~0.6 and 0.4 mM (Miller et al., 2002; Yan et al., 1997). These two bicyclic inhibitors mimic substrate binding in the active site by displacing the side chain of Tyr 80 from a position that makes it partially block the "mouth" of the active site. Occupancy of the active site by adenine or a substrate analogue causes rotation of Tyr 80 by 45°, enabling the phenyl ring of Tyr 80 to π-stack with the ring moiety of the substrate (Miller et al., 2002; Yan et al., 1997). Other inhibitors of RTA have been identified that bind the closed form of RTA. For example, 2,5-diamino-4-6-dihydroxypyridine (DDP), a monocyclic inhibitor with an IC_{50} of 2.2 mM, was found to stack against the side chain of Tyr80 in its apoenzyme conformation, and failed to enter the RTA specificity pocket (Bai et al., 2009; Miller et al., 2002). Other active-site inhibitors identified thus far include stem-loop oligonucleotides (aptamers) that mimic the oxacarbenium transition state at the RTA depurination site. While these inhibitors form high-affinity complexes with RTA at low pH (~4) (Chen et al., 1998), they do not appear to bind RTA at physiologic pH and likely have limited applicability as drug candidates, due to poor cellular uptake and their inherent susceptibility to degradation *in vivo* (Bai et al., 2009).

While advances in *in silico* screening technology will undoubtedly lead to the identification of additional ricin and Stx inhibitors, there is clearly an urgent need for alternative approaches that can accelerate the drug discovery process. Toward this end, Haslam et al. recently developed and employed a quantitative luciferase-based assay to identify inhibitors of Stx; positive candidates were then tested against ricin (Saenz et al., 2007; Zhao and Haslam, 2005). The methodology involved transfection of cell lines with cDNA encoding a destabilized derivative luciferase with a short half life. Therefore, cells treated with protein synthesis inhibitors, including Stx and ricin, demonstrated a rapid decline of luciferase activity. Saenz et al. (2007) exploited the latter finding to screen ~14,000 compounds, in an HTS format for small-molecule inhibitors of Stx. Those investigators identified two inhibitors of Stx that also showed activity against ricin. Subsequent analysis of the two compounds revealed that they affected intracellular toxin transport. While these particular compounds are unlikely to be developed as therapeutics due to their global effect on intracellular trafficking, they did demonstrate the feasibility of using cell-based HTS to screen for inhibitors of ricin and Stx.

In the present study, we have developed a simplified cell-based luciferase assay that does not require transfection of cells or handling of radioactive material; the assay was easily adapted to an HTS format. Using this method, we screened 17 chemical libraries (>81,000 compounds) and identified a number of compounds that showed weak, moderate, or strong anti-ricin activity. The method was validated through identification of known inhibitors of ricin. Due to the close similarity of the active sites of ricin and Stx, it is likely that