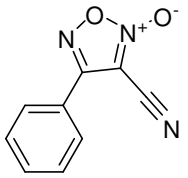
Project: Inhibitors of *Schistosoma Mansoni* Redox Cascade

Probes:

4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide



SID: 11111612
Furoxan

Probe Synonyms: Furoxan, Sigma P1726, MFCD00270898 (MDL ID), NCGC00015800 (internal ID), 4 Phenyl 3 furoxancarbonitrile

PubChem Bioassay Identifiers (AID): 448, 1011

SID	AC50 (μM)	Anti-target	Selectivity*
11111612	TGR IC_{50} = 9μM	human GR*	5.5
11111612	TGR IC_{50} = 9μM	*S. mansoni* LDH*	5.5

* Inactive @ 50 uM against human GR and *S. mansoni* LDH

Assigned Assay Grant #: MH076449-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: David Lee Williams, Illinois State University
Assay or Pathway Target: Thioredoxin/Glutathione Reductase (TGR) and Preoxiredoxin (PRX)

Assay provider information

Specific Aim: Schistosomiasis is one of the most important human parasitic diseases in terms of morbidity and mortality. Only one drug is currently available for treatment of schistosomiasis; new drugs are urgently needed. Studies on schistosome redox balance mechanisms indicate a distinct and compressed pathway in the parasite compared to its human host. *Schistosoma mansoni* peroxiredoxins (Prx) are important parasite antioxidant proteins that play a crucial role in redox balance mechanisms. Our data strongly suggest the possible use of Prx as novel drug targets. First, the proteins are essential for the parasite survival. Second, the proteins exhibits sufficient biochemical and structural differences from host Prx proteins. Third, the protein(s) is amenable for study at the molecular level and can be produced in bacteria in large quantities, in soluble and active form. Moreover, Prx activity can be screened in high throughput, inexpensive, and sensitive assays. Since no parasite-specific inhibitors of Prx are currently available, our overall goals can be summarized as: The identification of inhibitors of *Schistosoma mansoni* peroxiredoxins by conducting a high

throughput screen of the Small Molecule Repository of the Molecular Libraries Screening Center Network.

Significance: Schistosomiasis is an important, debilitating disease affecting ~250 million people in more than 70 countries. The annual mortality of this disease is estimated to be ~280,000 in sub-Saharan Africa, while 20 million individuals suffer from extreme disability (1). In the coming years, the Bill and Melinda Gates Foundation and endemic country programs will treat tens of millions of people with the single anti-schistosomiasis drug in widespread use, praziquantel (2). There is already clinical and laboratory evidence for the existence of praziquantel resistance parasites (3), and widespread use is expected to generate strong selective pressure for drug resistance. Clearly, there is an urgent need for new anti-schistosome drugs. Although hundreds of millions of people suffer from consequences of schistosome infections, schistosomiasis could easily be considered an orphan disease; since the vast majority of schistosome-infected individuals is poor and lives in the developing world, there is little incentive for the pharmaceutical industry to invest millions of dollars to identify and develop new anti-schistosome therapies. The ability to rapidly and efficiently screen libraries of potential inhibitors against critical schistosome target proteins would greatly accelerate progress to identify new lead compounds for anti-schistosomiasis therapies.

Rationale: Redox Balance in Schistosomes: *Schistosoma mansoni*, a causative agent of schistosomiasis, reside in the bloodstream of their host up to 30 years without being eliminated by the host immune attack. One proposed survival mechanism is the production of an antioxidant "firewall" that neutralizes the oxidative assault of the host's immune attack (4). Recent work strongly supports the hypothesis that this antioxidant firewall is a potential weakness of the organism. Schistosomes lack catalase, the main H_2O_2-neutralizing enzyme of many organisms, and their glutathione peroxidases (GPx) are in the phospholipid class, with poor reactivity toward H_2O_2. Peroxiredoxins appear to be the main parasite enzymes for reduction of hydrogen peroxide, and are biochemically distinct from human Prx (8). Moreover, Schistosomes lack two enzymes essential for redox metabolism in mammals, glutathione reductase (GR) and thioredoxin reductase (TrxR). Instead, Schistosomes utilize a novel enzyme, thioredoxin glutathione reductase (TGR), which provides both TrxR and GR enzymatic functions as well as a significant portion of the glutaredoxin activity in the worm (Figure 1, 2).

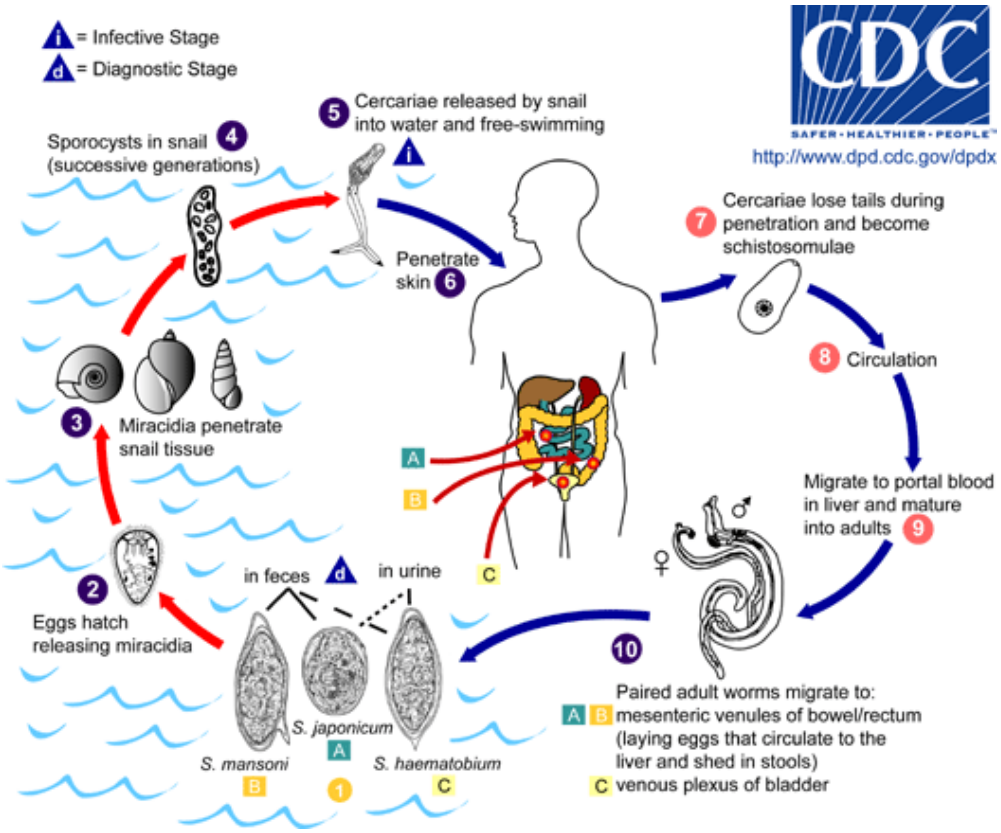


Figure 1: Targeted redox cascade in *S. mansoni*. *S. mansoni* depends on its peroxiredoxin "TGR/Prx" pathway (antioxidant "firewall") to prevent being destroyed by human immune system oxidative assault. Unlike humans, *S. mansoni* maintains a single enzyme (TGR) to serve as both glutathione and thioredoxin reductase. Blockade of this pathway is a promising approach to treating schistosomal infection.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Names:
AID 448: *Schistosoma Mansoni* Peroxiredoxins (Prx2)
AID 1011: Confirmation Concentration-Response Assay for Inhibitors of the *Schistosoma mansoni* Redox Cascade.

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
448	PRX2 – TGR	57μM – 3 nM[a]	Primary qHTS
1011	TGR	57μM – 3 nM[a]	Confirmation assay

[a]Seven point concentration-titration series was used covering the indicated range; 1:5 dilutions.

Primary Assay Description as defined in PubChem:

Overview:

Schistosoma mansoni, a causative agent of schistosomiasis, reside in the bloodstream of their host up to 30 years without being eliminated by the host immune attack. One proposed survival mechanism is the production of an antioxidant "firewall" that neutralizes the oxidative assault of the host's immune attack. *Schistosoma mansoni* peroxiredoxins (Prx) are important parasite antioxidant proteins that play a crucial role in redox balance mechanisms. Data strongly suggest the possible use of Prx as novel drug targets. First, the proteins are essential for the parasite survival. Second, the proteins exhibit sufficient biochemical and structural differences from host Prx proteins. Third, the protein(s) is amenable for study at the molecular level and can be produced in bacteria in large quantities, in soluble and active form. Inhibition of Prx activity was screened in a coupled-enzyme format with reducing equivalents being transferred from NADPH to glutathione intermediate via thioredoxin/glutathione reductase (TGR)-catalyzed reaction, then from thioredoxin to hydrogen peroxide via Prx-catalyzed electron transfer. A decrease in the fluorescence intensity of NADPH was used to measure the enzyme activity. Purified *S. mansoni* TGR and Prx were provided by Professor David L. Williams, Illinois State University.

Center Summary of the Primary Screen:

Assay principle and protocol:

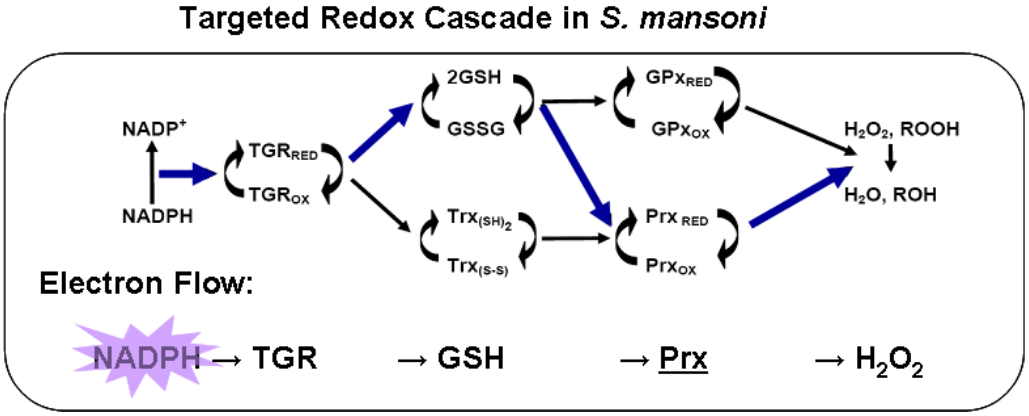


Figure 2: Top, The targeted redox cascade in *S. mansoni*. Bottom, qHTS Protocol for PRX2-TGR assay. Inhibition of thioredoxin-glutathione reductase TGR/Prx activity was screened in a coupled-enzyme format with reducing equivalents being transferred from NADPH to hydrogen peroxide (main ROS) via TGR- and Prx-catalyzed steps. A decrease in the fluorescence intensity of NADPH (Ex 365nm /Em 450nm) was used to measure the enzyme activity.

Protocol:

***S. Mansoni* redox cascade assay protocol**			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3uL	Enzyme and no-enzyme control solutions
2	Library compounds	23 nL	57 uM to 2.9 nM titration series
3	Control compound	23 nL	PAT titration
4	Incubation time	15 min	Compound interaction with target
5	Reagent	1 uL	Second equivalent of NADPH
6	Reagent	1 uL	H2O2 substrate addition to initiate reaction
7	Detector	Ex 365 Em 450 nm	Fluorescence intensity kinetic read

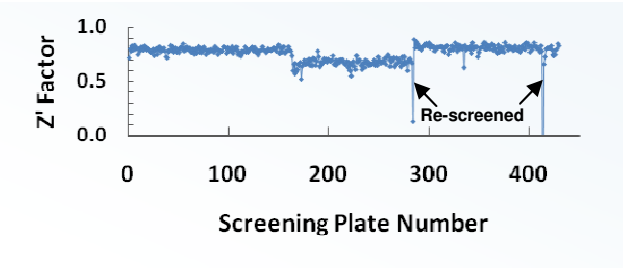
Step	**Notes**
1	Black solid bottom plates, single-tip dispense, 100 uM NADPH/25 nM TGR/700 uM GSH/50 nM Prx2 mixture in columns 1, 2, 5-48, no-enzyme, 100 uM NADPH only in columns 3 and 4. Buffer: 100 mM phosphate buffer, pH 7.4, 10 mM EDTA, 0.01% Tween-20.
2	Pintool transfer of library to columns 5-48 of the assay plate
3	PAT titration from 1 mM, then 1:5 dilutions pin-transferred to lower half of column 2. Column 1 and upper half of column 2 are neutral (100% activity). Columns 3 and 4, negative control (fully inhibited, zero % activity).
4	Room temperature incubation in auxiliary hotel
5	Solution of 400 uM NADPH and 700 uM GSH
6	Hydrogen peroxide addition via 8-tip dispense
7	ViewLux CCD imager, one read per 30 seconds for 8 min

Assay Steps: Three uL of reagents were dispensed to 1536-well Greiner black plates. Compounds and controls (23 nL) were transferred via Kalypsys PinTool. The plates were incubated for 15 min at room temperature, and then a 1 uL aliquot of 400 uM NADPH/700 uM GSH was added, immediately followed by a 1uL aliquot of 2.5 mM H2O2 (to start the reaction). The plate was transferred to ViewLux (Perkin-Elmer) High-throughput CCD imager where kinetic measurements (every 30 sec, for a total of 8 min) of NADPH fluorescence were taken using 365 nm excitation/450 nm emission filter set. During dispense, enzyme-containing reagent bottles were kept submerged into 4 deg C water bath to minimize degradation.

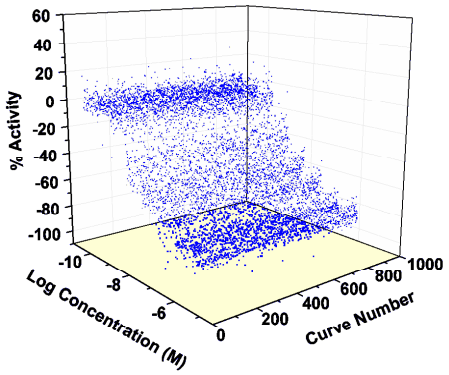
qHTS Summary of Assay results:

A fully-automated qHTS experiment[1-2] was performed against the entire collection plated as seven- to fifteen-point concentration series. In order to generate a robust data set and to minimize the effect of compound auto fluorescence, apparent reaction rates derived from an eight-minute time course were utilized instead of end-point readouts.

a



b



c

qHTS Performance Summary	
Parameter	**Value**
System	Kalypsys robot
Plates Screened	453 (437 passed qc)
Plates Failed QC	16 (Rescreened)
Samples (total wells)	597,652
Number of Data Points	9,562,432
Compounds	71,028
% 6 or 7 pt-titrations	86%
% 14 or 15 pt-titrations	14%
Data Layers	16 Kinetic Reads, Calculated Slope, Intercept, Delta Activity
Z'	0.76

d

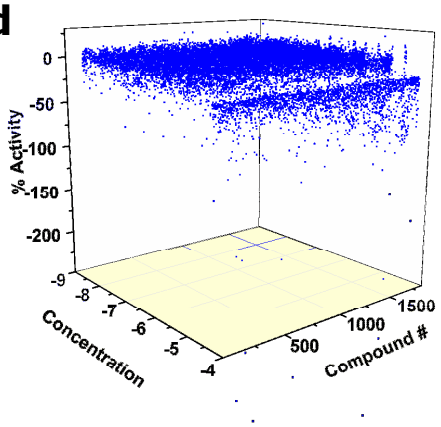


Figure 3: a) Z' plot of the qHTS. Z' remained flat with the screen progression, with minor shifts tracking the introduction of new batches of the two enzymes. b) The intraplate potassium antimonyl tartrate control titration was stable throughout the screen progression, resulting in average IC50 of 14 nM. c) Summary of the PRX2-TGR screen of 71,028 compounds each. Data were collected in multiple time points and all time courses were processed and stored for detailed examination of compound behavior. The delta of the last and first time points was used for activity calculations. d) 3D activity plot of compounds showing signal decrease. Many of these compounds were false positives due to auto-fluorescence in the assay. Time course data collected for each sample during qHTS was used to find and eliminate these false positives in the assay follow-up stages.

Identification of Active Clusters: Following the qHTS, the concentration response curve (CRC) data were subjected to a classification scheme to rank the quality of the CRCs as described by Inglese and co-workers[1]. Briefly, CRCs are placed into four classes. Class 1 contains complete CRCs showing both upper and lower asymptotes and r^2 values > 0.9. Class 2 contains incomplete CRCs lacking the lower asymptote and shows r2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. For this assay, compounds that showed 'activation' were regarded as active due to fluorescence and were thus filtered out. Additional compounds that showed high intercept in kinetic response were also considered active due to fluorescence and were eliminated. Remaining compounds that showed signal decrease were considered apparent inhibitors of the assay. Once this active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed by using Leadscope (Leadscope Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were

abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.

Four top series were selected out of the primary qHTS active clusters: Phophinic amides, Oxadiazole 2-oxides, Isoxazolones, and Phosphoramidites (Table 3). Top compounds from qHTS and newly purchased analogues from each series were tested in a qHTS confirmation assay. All retested compounds were found to be inactive against PRX, leaving TGR as the sole target for all confirmed actives.

Target Deconvolution: Confirmed compounds (see Table 1) were assayed against TGR and PRX (Figure 4). TGR activity was determined via DTNB reduction by following ↑ A410 nm due to the production of 2-nitro-5-thiobenzoic acid. PRX2 activity was based on the reduction of H_2O_2 by PRX2 in the presence of GSH, measured by the reduction of the GSSG produced in a coupled assay with yeast glutathione reductase (GR).

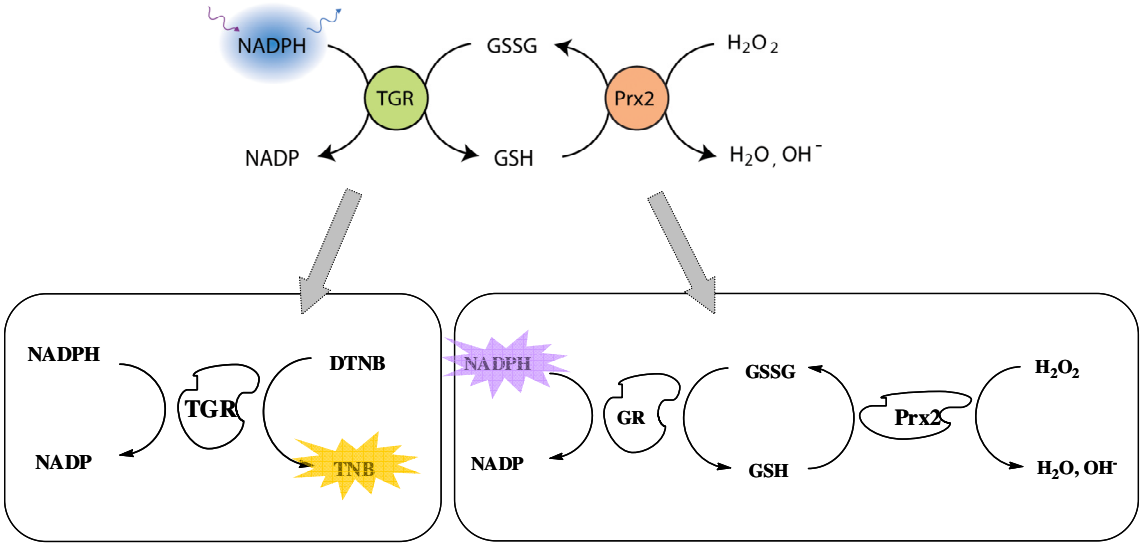
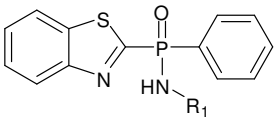
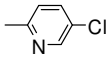
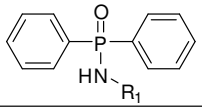
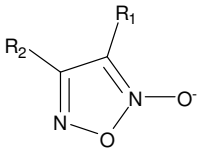
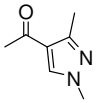
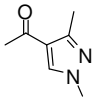
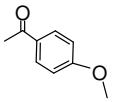
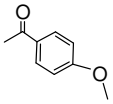
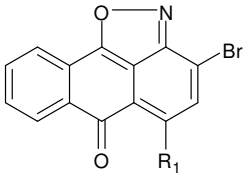
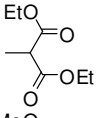
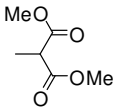
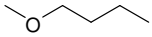
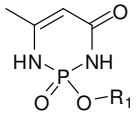


Figure 4: Confirmation and target deconvolution of redox cascade.

Table 1: Structure and Potency (IC_{50} in uM) of Actives and Series Expansion Analogues. IC50 values obtained from the primary screen; 'confirmation': IC50 values obtained using the identical assay as in the qHTS from the compounds identified in qHTS and expansion series; 'TGR Assay' : IC50 values obtained in an assay of TGR activity using DTNB as a substrate. NS, not screened (compound not in the screening collection), ND, not determined, NA, not applicable. IC50 > 50 or 57 μM signify lack of fitted curve through the concentration-response data, i.e. flat response within the range tested.

	Analogue #	R_1	R_2	qHTS	Confirmation	TGR Assay
Phosphinic Amide 	**1**		NA	NS	0.25	0.80
	2		NA	NS	23	12
	3		NA	0.037	0.025	0.025
	**4**		NA	>57	>57	>50
Oxadiazole 2-oxide 	**5**			NS	1.5	0.025
	6			24	ND	2.0
	7			9.2	1.5	0.020
	8			10	4.7	0.060
SID: 11111612 Furoxan	**9***	-CN	-Ph	8.7	ND	1.8
Isoxazolone 	**10**		NA	0.53	>57	9.0
	11		NA	NS	0.12	0.080
	12		NA	NS	0.84	0.15
Phosphoramidite 	**13**	4-chlorophenyl	NA	NS	0.42	0.55
	14	naphthyl	NA	0.56	0.12	0.20
	15	phenyl	NA	NS	2.0	4.0
	16	4-ethoxyphenyl	NA	NS	0.18	0.6

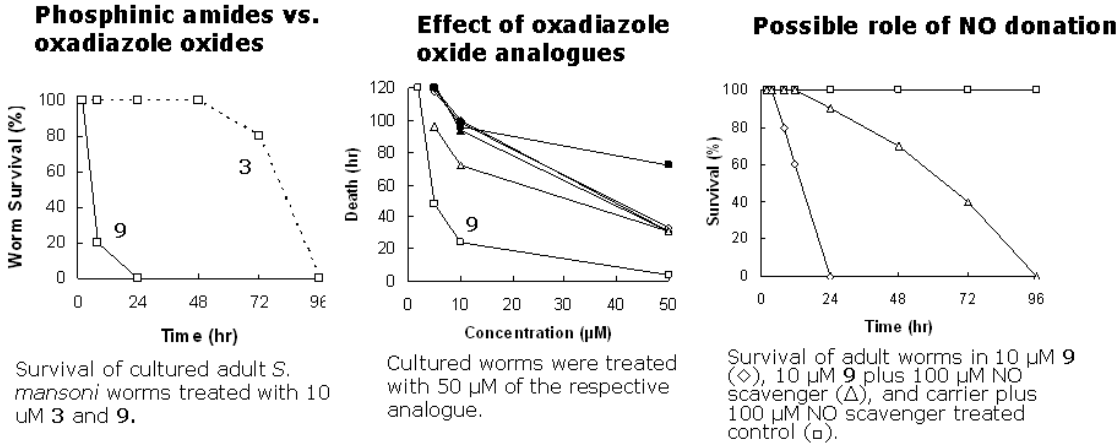
Probe Characterization

Prior Art for PRX/TGR: No parasite-specific inhibitors of Prx or TGR were available before the discovery of this probe.

Mode of action of probe ***SID: 11111612***: The action of two compounds were further investigated: N-(benzothiazol-2-yl-phenyl-phosphoryl)-1,3-thiazol-2-amine (compound 3), a phosphinic amide, and 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide (commonly 4-

phenyl-3-furoxancarbonitrile or furoxan) (compound 9, probe **SID: 11111612**). Adult *S. mansoni* worms were cultured in the presence of inhibitors and mobility and parasite death were monitored (Figure 5). While the phosphinic amide (compound 3) was the most potent active identified from the screen, its activity against worms was lower than that of the oxadiazole 2-oxide (**SID: 11111612**). The anti-schistosomal activity of **SID: 11111612** is concomitant with NO production; however, other unrelated NO donors did not have an effect against Schisto worms[3].

Figure 5: Two actives tested against cultured worms:



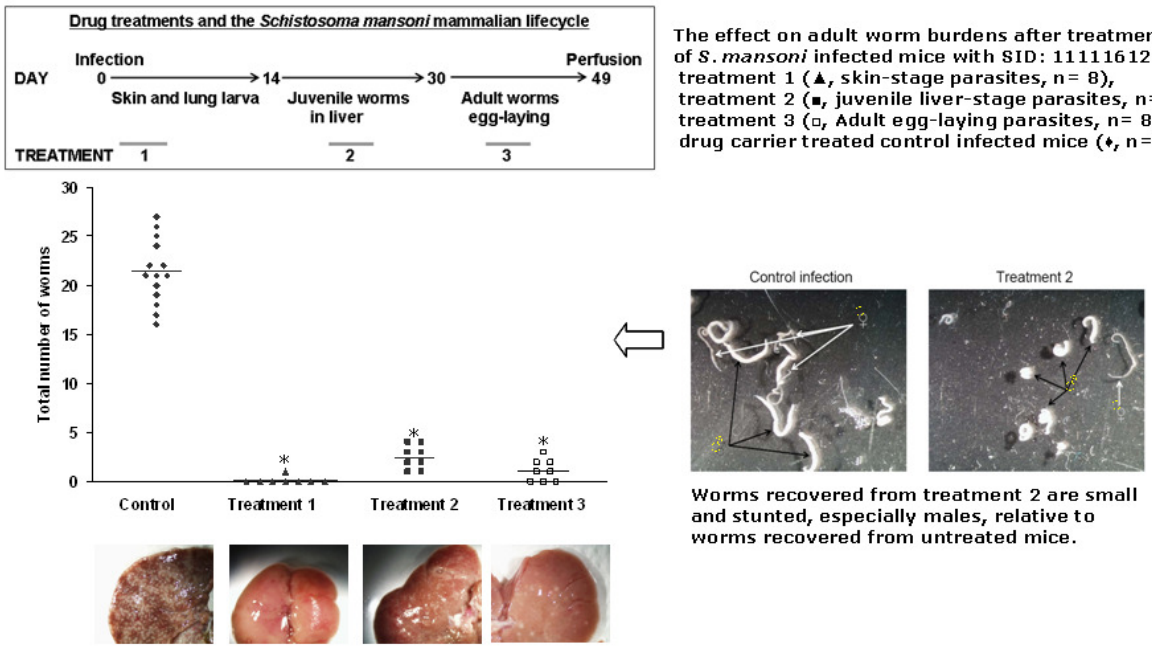
Survival of cultured adult *S. mansoni* worms treated with 10 uM **3** and **9**.

Cultured worms were treated with 50 μM of the respective analogue.

Survival of adult worms in 10 μM **9** (◇), 10 μM **9** plus 100 μM NO scavenger (Δ), and carrier plus 100 μM NO scavenger treated control (□).

In vivo drug treatment with probe: The probe compound **SID: 11111612** was dissolved in DMSO and administrated by intraperitoneal injection at 10 mg/kg daily for five consecutive days during the development of *S.mansoni* in the mouse (Figure 6). Large and highly significant reductions in worm burdens were observed from all experimental treatments.

Figure 6: In vivo drug treatment with **SID: 11111612**



The effect on adult worm burdens after treatment of *S. mansoni* infected mice with SID: 11111612 in: treatment 1 (▲, skin-stage parasites, n= 8), treatment 2 (■, juvenile liver-stage parasites, n=8), treatment 3 (□, Adult egg-laying parasites, n= 8); drug carrier treated control infected mice (♦, n=16)

Worms recovered from treatment 2 are small and stunted, especially males, relative to worms recovered from untreated mice.

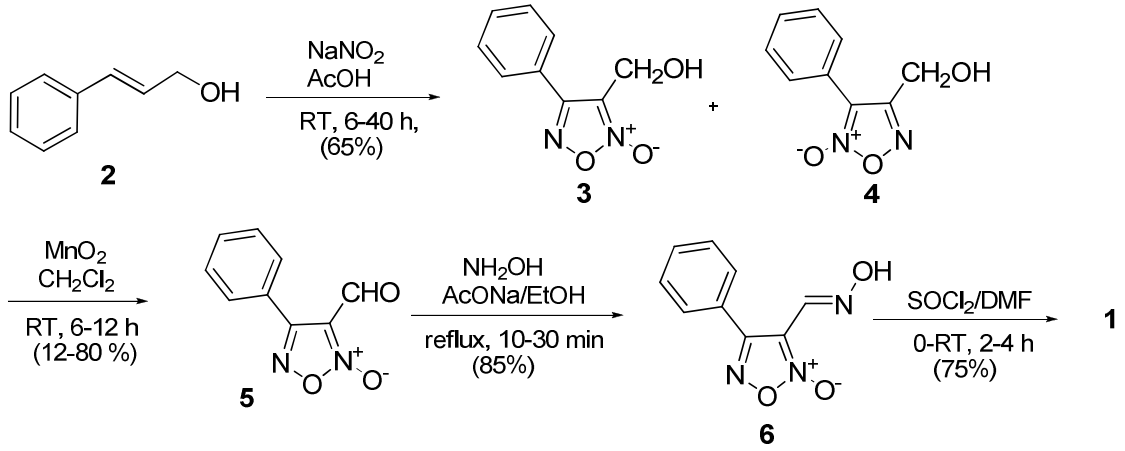
The white dots from the collected mice livers are liver granulomas resulting from host immunological reaction against trapped parasite eggs.

Secondary assay description: Inhibitor studies on cultured worms: Compounds were dissolved in dimethylsulfoxide (DMSO) and added at concentrations indicated to freshly perfused worms in RPMI 1640 containing 25 mM HEPES, pH 7, 150 units/ml penicillin, 125 mg/ml streptomycin and 10% FCS (Cell Grow, Fisher). Media were replaced every 2 d with fresh media with the compounds added at the designated concentrations. Control worms were treated with equal amounts of DMSO alone. Worms were subsequently observed for motility and mortality and collected at the indicated times for analysis. Worms were homogenized by sonication in PBS, and homogenates were assayed for enzyme activities as described. To assess the importance of NO production, the potassium salt of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, Invitrogen), a NO scavenger, was dissolved in water and incubated with freshly perfused *S. mansoni* worms at 100 mM in the presence or absence of 10 mM **SID: 11111612**.

Compound preparation: Compound is prepared in DMSO at 10 mM stock concentration.

The synthesis of 4-phenyl-3-cyano-furoxan (**1**, Figure 7) was accomplished by known methods reported by Gasco and co-workers.[5-6] Cyclization of commercially available alcohol **2** with $NaNO_2$ in glacial acetic acid provided the required 4-phenyl-3-furoxanmethanol derivative as a mixture of regioisomers **3** and **4**, favoring the desired **3**. The structural assignments were based on known differences in ^{1}H and ^{13}C NMR resonances of the methylene group in the 3-position as a result of shielding from the N-oxide moiety. Allylic oxidation of **3** provided aldehyde **5**, which could then be easily separated from the undesired isomer (from the previous reaction) by column chromatography. Formation of the oxime **6** was achieved using hydroxylamine hydrochloride in the presence of sodium acetate in good yield. Subsequent dehydration of **6** provided the target compound **1**.

Figure 7: Synthetic scheme for **SID: 11111612**:



Project related direct publications:
See references 2, 3 for further information.

Known probe properties:

Properties of probe compounds as reported in PubChem:

SID: 11111612:

Molecular Weight	187.1549 [g/mol]
Molecular Formula	$C_9H_5N_3O_2$
H-Bond Donor	0
H-Bond Acceptor	4
Rotatable Bond Count	1
Exact Mass	187.038176
MonoIsotopic Mass	187.038176
Topological Polar Surface Area	76.8
Heavy Atom Count	14

Canonical SMILES: C1=CC=C(C=C1)C2=NO[N+](=C2C#N)[O-]

InChi: InChI=1/C9H5N3O2/c10-6-8-9(11-14-12(8)13)7-4-2-1-3-5-7/h1-5H

Has this compound been provided to the MLSMR:

No. This compound is part of the SigmaAldrich LOPAC collection.

Probe availability: Compound is available from SigmaAldrich, www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/P1726. Probe has also been resynthesized at NCGC, aliquots available upon request.

Appendices: N/A

Bibliography:

(1) Inglese, J. et al. (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. Proc Natl Acad Sci U S A 103 (31), 11473-11478

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(3) Sayed, A.A. et al. (2008) Identification of oxadiazoles as new drug leads for the control of schistosomiasis. Nat Med 14 (4), 407-412

(4) Gasco, A. M., Fruttero, R., Sorba, G. & Gasco, A. (1991). Unsymmetrically substituted furoxans, XIII. Phenylfuroxancarbaldehydes and related compounds. *Liebigs Ann. Chem.* 1991 (11) 1211-1213.

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(6) Medana, C et al (1994). Furoxans as Nitric Oxide Donors. 4-Phenyl-3-furoxancarbonitrile: Thiol-Mediated Nitric Oxide Release and Biological Evaluation**.** *J Med Chem* 37(5): 4412 – 4416.