nature medicine

Identification of oxadiazoles as new drug leads for the control of schistosomiasis

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Treatment for schistosomiasis, which is responsible for more than 280,000 deaths annually, depends almost exclusively on praziquantel. Millions of people are treated annually with praziquantel, and drug-resistant parasites thus are likely to evolve. Phosphinic amides and oxadiazole 2-oxides, identified from a quantitative high-throughput screen, were shown to inhibit a parasite enzyme, thioredoxin glutathione reductase (TGR), with activities in the low micromolar to low nanomolar range. Incubation of parasites with these compounds led to rapid inhibition of TGR activity and parasite death. The activity of the oxadiazole 2-oxides was associated with a donation of nitric oxide. Treatment of schistosome-infected mice with 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide led to marked reductions in worm burdens from treatments against multiple parasite stages and egg-associated pathologies. The compound was active against the three major schistosome species infecting humans. These protective effects exceed benchmark activity criteria set by the World Health Organization for lead compound development for schistosomiasis.

Schistosomiasis is a chronic disease caused by trematode flatworms of the genus *Schistosoma*. The disease remains a major, neglected, poverty-related health problem in many tropical areas[1]. The health burden resulting from schistosomiasis is estimated to include more than 200 million people infected, 779 million at risk of infection, 280,000 deaths annually and more than 20 million individuals experiencing high morbidity[2–4]. Chemotherapy is the mainstay of schistosomiasis control and is carried out largely through the use of praziquantel (PZQ). The drug's low cost (<$0.25/dose) and efficacy against adult worms of all schistosome species that infect humans has led to its widespread use; tens of millions receive annual treatments of PZQ[5]. Because of high reinfection rates, PZQ must be administered on an annual or semiannual basis. Although vaccines are not yet available, if developed they would be best administered with drugs in an integrated approach to schistosomiasis management. The widespread reliance on PZQ for schistosomiasis control may hasten the selection of drug-resistant parasites. In fact, PZQ-resistant isolates of *Schistosoma mansoni* and *Schistosoma haematobium* have been identified[6–8], and PZQ-resistant parasites have been selected for in the laboratory[9]. Artemether has also shown promise for schistosomiasis therapy, although its use for schistosomiasis may be restricted in areas of malaria transmission to avoid putting its use as an antimalarial at risk[10]. The older drug oxamniquine is no longer manufactured and is effective only against *S. mansoni*, and resistance to it has been reported[11]. Protease inhibitors and 2-(alkylamino)-1-phenyl-1-ethanethiosulfuric acids have promising activity against schistosome infection[12,13] but have yet to reach clinical trials. The resulting dependence on a single drug for the treatment of schistosomiasis is not sustainable, and thus there is an urgent need to identify new targets and drugs for schistosomiasis treatment.

Schistosome parasites have a complex life cycle involving snail intermediate and human definitive hosts. Humans become infected by contacting water containing cercariae released by infected snails. After penetration, cercariae remain in the skin for several days, then enter the general circulation and are carried to the lungs, where they reside for several more days before finally entering the liver. Once in the liver, parasites undergo rapid growth, development and sexual differentiation and locate a mate. After pairing, adult parasites migrate to the mesenteric venules (*S. mansoni* and *Schistosoma japonicum*) or the venules of the urogenital system (*S. haematobium*) of their human host, where they commence egg production. Because schistosome parasites reside in an aerobic environment in their mammalian hosts, they must have means to minimize damage caused by reactive oxygen species produced by their own aerobic respiration as well as by the host immune assault. In vertebrates there are two largely independent systems to detoxify reactive oxygen species, one based on glutathione and the other based on thioredoxin. Each of these systems has a dedicated NADPH-dependent flavoenzyme to maintain glutathione or thioredoxin in the reduced state, glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively[14,15]. However, based on biochemical analyses, these two pathways in *S. mansoni* are dependent on a single multifunctional selenocysteine-containing flavoenzyme, thioredoxin-glutathione reductase (TGR), which replaces both GR and TrxR in the parasite[16]. Further support for the existence of a single, multifunctional enzyme comes from

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Received 17 September 2007; accepted 14 February 2008; published online 16 March 2008; doi:10.1038/nm1737

analysis of the *S. mansoni* genome assembly v. 4.0, which did not identify homologs of GR and TrxR. It has been shown that TGR is essential for parasite survival, is biochemically distinct from host enzymes and appears to be a molecular target of potassium antimonyl tartrate[17], which was used as schistosomiasis therapy for nearly 70 years.

Because of the unusual organization of the schistosome defense against oxygen radicals, we hypothesized that the parasite redox pathway would be an effective target for the development of new antischistosomal chemotherapies. As part of the NIH Molecular Libraries Initiative, we have recently completed a quantitative high-throughput screen (qHTS)[18] of the Molecular Libraries Small Molecule Repository and NIH Chemical Genomics Center libraries. The screen, followed by confirmatory and target deconvolution experiments, identified several promising series, notably phosphinic amides and oxadiazole 2-oxides, that were active against the *S. mansoni* antioxidant pathway[19]. Here we investigated the activities of oxadiazole 2-oxides and phosphinic amides in detail against the following systems: TGR, the molecular target of the compounds, live cultured worms of different developmental stages and from all major species infecting humans, and *S. mansoni*–infected mice.

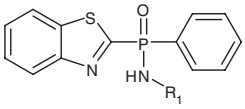
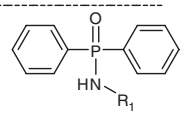
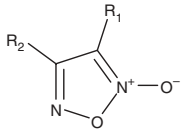
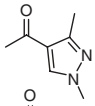
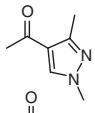
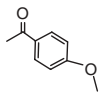
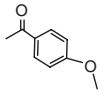
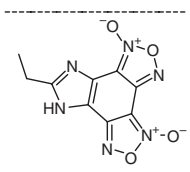
RESULTS

Inhibition of TGR by phosphinic amides and oxadiazole 2-oxides

The activities of phosphinic amides and oxadiazole 2-oxides were examined against both the GR and the TrxR activities of recombinant schistosome TGR (**Table 1**). The compounds inhibited both TGR activities, with most showing greater inhibition of the TrxR activity. The inhibitory activities of compounds **3**, **5** and **7** were in the low nanomolar range and near the limit imposed by the concentration of TGR (15 nM) in the assays. Chemical structures of the compounds used in this study are shown in **Table 1**.

We investigated the action of two compounds, *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**), an oxadiazole 2-oxide, in greater detail. Both compounds showed time-dependent inhibition of TGR (**Supplementary Fig. 1** online). Compound **3** was found to be a reversible inhibitor of TGR, whereas compound **9** was found to be an irreversible inhibitor. However, inhibition of TGR by compound **9** was partially reversible by strong thiol-reducing agents, suggesting that the inhibition of TGR occurs through the modification of one or more cysteine or selenocysteine residue(s) in the protein.

Table 1 Structure and potency of phosphinic amides and oxadiazole 2-oxides used in this study

	Analog no.	R_1	R_2	GSSG	$Trx(S)_2$
Phosphinic amide					
	1		NA	1.54	0.789
	2		NA	20.3	15.1
	3		NA	0.121	0.021
	4		NA	>50	>50
Oxadiazole 2-oxide					
	5			0.082	0.038
	6			4.08	1.29
	7			0.51	0.042
	8			2.44	0.105
	9	-CN	-Ph	0.32	1.67
	10	NA	NA	12.5	6.68

The potency (IC_{50} in μM) of the compounds against the glutathione reductase (GSSG) and thioredoxin reductase ($Trx(S)_2$) activities of *Schistosoma mansoni* thioredoxin glutathione reductase are as indicated. Assays used were as described in Methods. IC_{50} values >50 μM signify lack of fitted curve through the dose-response data—that is, flat response within the range tested. NA, not applicable.

Action of compounds against *ex vivo* parasites

Adult *S. mansoni* worms were cultured in the presence of inhibitors and mobility and parasite death were monitored. The oxadiazole 2-oxides had similar effects on worm survival, with activity at concentrations as low as 5 μM (**Fig. 1a**). Compound **9** was the most active compound tested: 10 μM of compound **9** resulted in 100% parasite death within 24 h and 2 μM killed worms in 120 h. Compound **9** was active against all stages of *S. mansoni* tested: mechanically transformed skin larva, lung-stage schistosomula, juvenile liver worms and adult worms (**Fig. 1b**). Furthermore, compound **9** was active against cultured adult *S. japonicum* and *S. haematobium* worms (**Fig. 1b**). Worm mortality was assessed by lack of movement, gross morphological changes or both. In contrast to the morphological changes seen by others after treatment of cultured worms with a variety of compounds[20], no gross changes in worm structure or tegument were seen after treatment with compound **9** (**Supplementary Fig. 2** online). The morphological effects of compound **9** treatment were similar to those seen after RNA interference to silence TGR expression[17]. When the drug was withdrawn from the worms and fresh, drug-free medium was added, the worms showed no recovery. The activities of the phosphinic amides were markedly less than those of the oxadiazoles: compound **3** at 50 μM and 25 μM resulted in 100% worm death in 24 h and 96 h, respectively, whereas lower concentrations of compound **3**, and all other phosphinic amides, were inactive against the worms (**Fig. 1c**).

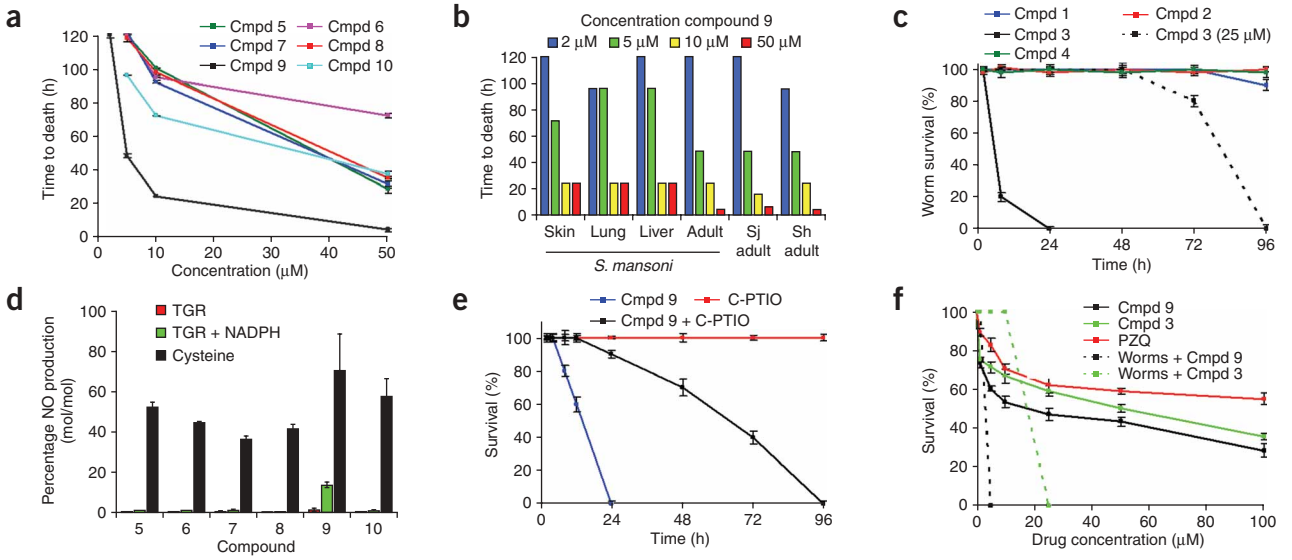


Figure 1 Activity of oxadiazole 2-oxides and phosphinic amides against thioredoxin glutathione reductase and cultured *Schistosoma mansoni* worms. (**a**) Survival of cultured adult *S. mansoni* worms treated with oxadiazole 2-oxides. Cultured worms were treated with compounds at the indicated concentrations and the time to 100% parasite death was recorded. (**b**) Survival of cultured *S. mansoni* worms at different developmental stages and of adult *S. japonicum* (Sj) and *S. haematobium* (Sh) worms after treatment with the indicated concentrations of compound **9**. (**c**) Survival of cultured adult *S. mansoni* worms treated with several phosphinic amides at 50 μM and with compound **3** at 25 μM. (**d**) NO release by oxadiazole 2-oxides (10 μM) after incubation with 15 nM *S. mansoni* TGR, with 15 nM *S. mansoni* TGR plus 100 μM NADPH, or with 5 mM cysteine alone. (**e**) Survival of adult worms in 10 μM compound **9**, 10 μM compound **9** plus 100 μM carboxy-PTIO (C-PTIO), and drug carrier plus 100 μM carboxy-PTIO–treated control. (**f**) Cytotoxicity after 5 d of culture at the indicated concentrations of compounds **3** and **9** against mouse myeloma line SP2/0 and adult *Schistosoma mansoni* worms and praziquantel against mouse myeloma. Error bars (**a**,**c**–**f**) represent the ± s.d. from three independent experiments.

Because oxadiazoles are known to be nitric oxide (NO) donors in the presence of physiological levels of thiols[21], we assessed whether NO release played a part in their mechanism of action against worms. All oxadiazoles produced NO at approximately the same efficiency when incubated in 5 mM cysteine (**Fig. 1d**). There was a highly significant difference between NO production from the reaction of compound **9** with TGR + NADPH and compound **9** with TGR alone and between that from the reaction of compound **9** and all other oxadiazole 2-oxides with TGR + NADPH (**Fig. 1d**, $P < 0.0001$).

Given that compound **9** both was the most efficient NO donor in the presence of TGR and was more active against cultured worms than the other oxadiazoles, we hypothesized that NO production may be involved in the action of compound **9** against cultured worms. To investigate this, we cultured adult *S. mansoni* worms with 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), a specific tool for NO purging and scavenging[22], in the presence or absence of 10 μM of compound **9**. Worm viability in the presence of compound **9** plus the NO scavenger was extended and was similar to that seen for worms cultured with the other oxadiazoles (**Fig. 1e**). Survival of worms in exogenously generated NO (sodium nitroprusside + 5 mM cysteine) was not affected by concentrations of up to 1 mM sodium nitroprusside. This corresponds to a concentration of 0.72 mM NO (under these conditions sodium nitroprusside + cysteine generates 72.52% NO (mol/mol)). The lethal effect of sodium nitroprusside on worm survival was seen only at concentrations of 2 mM sodium nitroprusside or higher, or about a 1,000 times higher concentration than the effective dose of compound **9**.

To examine if compounds **3** and **9** are tolerated by mammalian cells, we incubated the mouse myeloma cell line SP2/0 with the

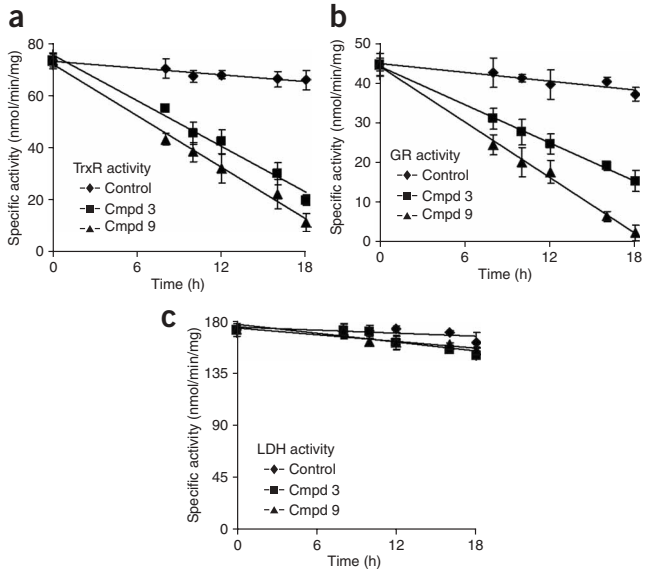


Figure 2 The action of compounds **3** and **9** on thioredoxin glutathione reductase activities in extracts prepared from cultured *Schistosoma mansoni* worms. (**a**) The specific thioredoxin reductase (TrxR) activity of TGR in worm homogenates from control worms and worms treated with 50 μM compound **3** or 10 μM compound **9**. (**b**) The specific glutathione reductase (GR) activity of TGR in worm homogenates from control worms and worms treated with 50 μM compound **3** or 10 μM compound **9**. (**c**) The specific lactate dehydrogenase (LDH) activity in worm homogenates from control worms and worms treated with 50 μM compound **3** or 10 μM compound **9**. The error bars represent ± s.d. from the average of three independent experiments.

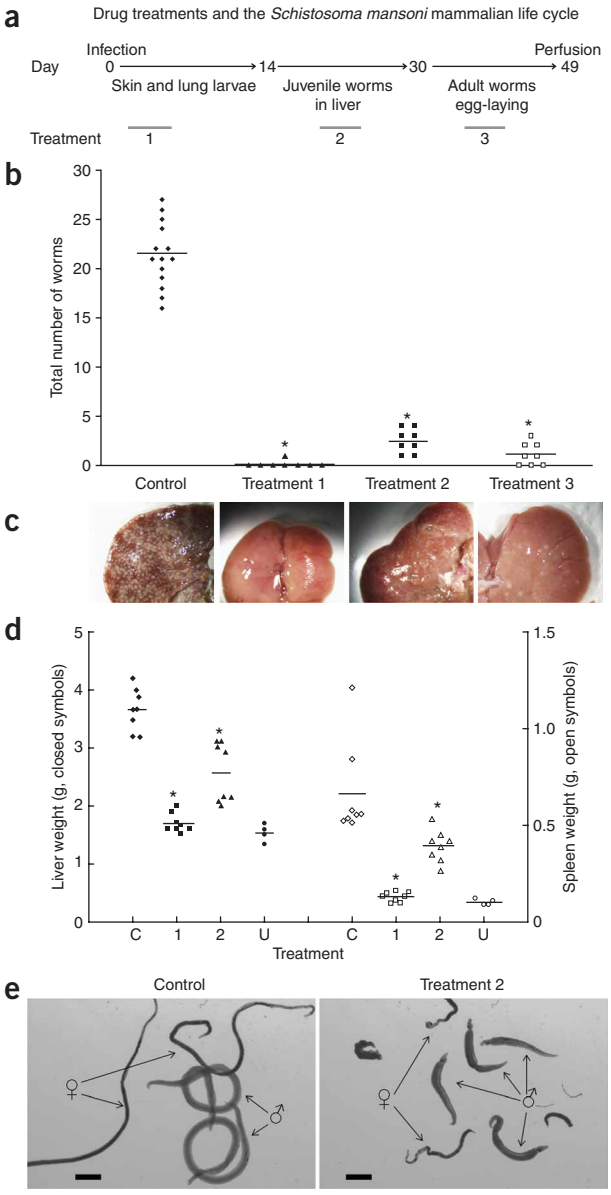


Figure 3 *In vivo* drug treatment with compound **9**. (**a**) Scheme for the administration of compound **9** at 10 mg/kg during the development of *Schistosoma mansoni* in the mouse. (**b**) Effect on adult worm burdens after treatment of *S. mansoni*–infected mice with compound **9**. Points represent data from individual mice; $*P < 0.0001$. The horizontal bars represent the average worm burdens of each treatment. (**c**) Pictures of the livers collected from the different treatments; the white dots are liver granulomas resulting from host immunological reaction against trapped parasite eggs. (**d**) Anti-pathologic effects of treatment with compound **9**. Liver and spleen weights from infected control mice (C), treatment 1 (1), treatment 2 (2) and uninfected age matched control mice (U); $* P < 0.0001$. (**e**) Photo-micrographs of worms recovered from Treatment 2– and control-infected mice. Worms recovered from mice subjected to Treatment 2 are stunted relative to worms recovered from untreated mice. Scale bars, 1.0 mm.

compounds and monitored cell viability after 120 h. All worms were killed by concentrations of compound **9** as low as 5 μM and by compound **3** at 25 μM (**Fig. 1f**). At 25 μM of compound **3**, compound **9** or PZQ, the viability of the myeloma cells was 59%, 54% and 62%, respectively (**Fig. 1f**). Although compound **3** and compound **9** both appear to be more toxic to mammalian cells than PZQ, the cells tolerated concentrations as high as 100 μM (**Fig. 1f**).

Because both compounds **3** and **9** inhibit recombinant parasite TGR and cause rapid death of cultured parasites, we examined if the mechanism of action of these compounds in living worms occurred, at least in part, through inhibition of TGR activity. We cultured freshly perfused adult *S. mansoni* worms with 10 μM of compound **9** or 50 μM of compound **3**. We observed a progressive decrease in TGR activity after treatment with either compound **3** or compound **9**, and after 18 h, TrxR and GR activities were reduced by 83% and 93% for compound **9** and 69% and 59% for compound **3**, respectively, as compared to those in carrier-treated control worms (**Fig. 2a**,**b**). The activity of parasite lactate dehydrogenase in homogenates from compound **3**– and compound **9**–treated worm showed no significant difference from that in homogenates from carrier-only treated worms (**Fig. 2c**).

In vivo action of 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide

In all treatments, compound **9** was administrated once daily for five consecutive days by intraperitoneal injection at 10 mg/kg to *S. mansoni* infected mice; treatment 1 started 1 d after infection (skin-stage parasites), treatment 2 started 23 d after infection (juvenile, liver-stage parasites) and treatment 3 started 37 d after infection (adult, egg-laying parasites) (**Fig. 3a**). In all treatments, adult worms were quantified after perfusion of mice 49 d after infection.

We observed large and highly significant reductions in worm burdens from all experimental treatments. Treatment 1 resulted in a decrease in worm burden of >99% ($P < 0.0001$) compared to the control *S. mansoni*–infected mice (**Fig. 3b**). The protective effect of compound **9** was also seen in a significant reduction of hepato- and splenomegaly ($P < 0.0001$, **Fig. 3c**,**d**) as compared to control infected mice; indeed, the weights of the spleens and livers after this treatment were not statistically different from those of livers from uninfected age-matched mice ($P > 0.1$, **Fig. 3d**) and there were no signs of egg-induced liver granulomas. Treatment 2 resulted in a decrease of 89% ($P < 0.0001$) in worm burdens (**Fig. 3b**) and marked reductions in hepato- and splenomegaly relative to control infected mice ($P < 0.0001$, **Fig. 3c**,**d**). The number of liver granulomas was also considerably lower than that from the control infections (**Fig. 3c**). Moreover, the worms that recovered were distinctly smaller after this treatment (**Fig. 3e**) and appeared similar to adult parasites obtained after treatment of infected mice with a protease inhibitor[12]. An equal number of male and female worms were recovered from this treatment, indicating that compound **9** was equally active against both worm sexes. Treatment 3 resulted in a decrease of 94% ($P < 0.0001$) in worm burdens relative to control infected mice (**Fig. 3b**). The livers collected from this treatment also had much lower numbers of egg-induced granulomas than the control group (**Fig. 3c**) and also showed reduced hepatomegaly (mean liver weight in untreated control infections 4.5 g compared to 2.6 g in Treatment 3; $P < 0.0001$).

DISCUSSION

In this study we have identified oxadiazole 2-oxides as new lead compounds for schistosomiasis chemotherapy. Compound **9**, the most active in the series, produces parasitological cures of infected animals when given at any point during the infection. Furthermore, we have shown that the primary target of compound **9** in cultured worms is the parasite redox protein TGR. The anti-schistosome activity of compound **9** is associated with a donation of NO through the action of the target enzyme.

The nearly complete reliance on PZQ for schistosomiasis treatment will jeopardize control efforts should resistance arise. Therefore, it is important to identify drugs to replace PZQ or, preferably, to be used in combination with PZQ to prevent resistance from developing. The development of drugs for neglected tropical diseases (NTDs) is hampered by the general lack of interest from the pharmaceutical industry in this class of diseases, and fewer than 1% of all new drugs target NTDs[23]. Partnerships to develop drugs for NTDs (as well as rare and orphan diseases) include the NIH Molecular Libraries Initiative (MLI), which was started in 2004 to provide academic investigators with the infrastructure and technologies necessary to discover both chemical probes for investigating fundamental biological phenomena and starting points for development of new therapeutics for human disease[24]. The work described here is the product of the first assay officially accepted for screening by the MLI.

The most potent compound investigated in this study was an oxadiazole 2-oxide, a member of a class that has been of pharmacological interest because of the compounds' ability to donate NO and whose members have been investigated for vasodilating, platelet antiaggregating, anti-infective and other properties[25]. The death of treated cultured worms is preceded by the inhibition of the GR and TrxR activities of TGR, providing evidence that the antihelminthic effects of compounds **3** and **9** are due to the inhibition of TGR. No inhibition of an abundant and essential enzyme, lactate dehydrogenase, was seen. It is likely that the action on worms of compound **9** is intricately associated with NO donation and TGR inhibition. Nitric oxide produced by human white cells has been shown to kill larval schistosome parasites[26]. The release of NO by compound **9** has been demonstrated to be contingent upon a ligation event with free thiols[27]. The combined role of TGR inhibition by covalent modification of a cysteine or selenocysteine and the subsequent targeted release of NO are undoubtedly associated with worm killing, as NO scavenging decreased the activity of compound **9**. However, we found that NO was well tolerated by adult worms when generated exogenously. Production of NO was found to be enhanced by the addition of NADPH, which was in indication of an enzymatic NO release by TGR. Inhibition of TGR activity would be expected to lead directly to the inactivation of both the thioredoxin- and glutathione-based defenses and the accumulation of reactive oxygen species, and concomitant production of NO by TGR would result in increased nitrosative stress. Furthermore, it has been shown that inhibition of the related mammalian enzyme TrxR leads to an increase in its NADPH oxidase, resulting in the production of superoxide[28]. It is likely that inhibition of TGR will also increase its NADPH oxidase activity.

The activity of compound **9** surpasses criteria established by the World Health Organization (WHO) for potential lead compounds for schistosomiasis, whereby active 'hits' should result in 100% inhibition of motility of adult parasites at 5 μg/ml and highly active lead compounds are defined as those producing an 80% reduction in worm burdens after five injections at 100 mg/kg[29]. At 1.87 μg/ml, compound **9** produces 100% inhibition of motility of adult parasites in 24 h, and concentrations as low as 0.38 μg/ml are active. All trials in mice resulted in at least an 88% reduction in worm burdens when compound **9** was administered in five doses at 10 mg/kg. Furthermore, because schistosome TGR is not inhibited by PZQ[17], compound **9** and PZQ have different mechanisms of action and selection of cross-resistant parasites is highly unlikely; therefore, there is great potential in using the candidate compound or its derivatives in combination therapy with praziquantel.

It is significant to note that compound **9** is highly active against all intramammalian life cycle stages of *S. mansoni*, with at least an 88% and as high as a 99% reduction in worm burdens depending on the life cycle stage present during the treatment—numbers equal to or better than those for PZQ and artemether. In schistosomiasis, reductions in worm burden are associated with reduced pathology, and there is no concern about relapse because schistosome parasites do not multiply in the mammalian host. Compound **9** seems to be particularly active against early stages of the infection, as only one adult parasite was recovered from the eight mice infected. PZQ is much less effective against juvenile liver parasites than against adult parasites, producing only a 25–30% reduction in worm burdens[30]. Although artemether affords ~80% reduction if juvenile parasites are targeted, it is less effective against adult schistosome parasites, resulting in <50% reduction in worm burden[31]. In addition, compound **9** was found to be active against adults of the other main schistosome species infecting humans, *S. japonicum* and *S. haematobium*. Therefore, compound **9**, or its derivatives, could be useful as a prophylactic treatment (it is effective against skin and migrating parasites) and would kill adult parasites, which trigger disease by egg production and are typically the diagnostic stage.

In this study we have identified oxadiazole 2-oxides as new lead compounds for schistosomiasis chemotherapy. The level of activity of compound **9** was equal to or better than that of the currently used drug, PZQ, and surpasses all landmarks for new lead compounds for schistosomiasis control. The low cytotoxicity and high bioactivity and tolerance by mice of compound **9** support the potential of this compound as a highly effective lead compound for human schistosomiasis. Compound **9** is amenable to derivatization, and efforts are underway to identify selective and active derivatives and uncover the mechanism of inhibition of TGR.

METHODS

Active compounds. The phosphinic amides and oxadiazole 2-oxides were identified from a quantitative high-throughput screen against the TGR-peroxiredoxin redox cascade of *S. mansoni*[18,19]. Active compounds identified from the screen, as well as untested analogs, were purchased as powders of >90% purity from the respective primary vendors. Confirmatory studies, target deconvolution studies and the present studies were performed using these separately sourced samples.

Parasite preparation. Mice (NIH-Swiss) were infected by percutaneous tail exposure[32] to *S. mansoni* cercariae (NMRI strain) for 2 h. Parasites were obtained from mice by perfusion with RPMI 1640 medium[32]. *Biomphalaria glabrata* infected with *S. mansoni*, mice infected with *S. japonicum* (Chinese strain) and Syrian hamsters infected with *S. haematobium* (Egyptian strain) were obtained from the Biomedical Research Institute. This study was approved by the Institutional Animal Care and Use Committee of Illinois State University (05-2006 and 23-2007; US Department of Health and Human Services animal welfare assurance number A3762-01).

Enzyme assays. Enzyme preparation and TrxR and GR assays were as described[17]. Activity of the control enzyme lactate dehydrogenase was determined with 10 μM sodium pyruvate and 100 μM NADPH as described[33]. Inhibitor reversibility was determined after overnight dialysis against 2,000 volumes of PBS (without inhibitor) of a TGR sample treated with inhibitor plus NADPH and compared to an aliquot of uninhibited TGR dialyzed in the same manner. Each assay was done in triplicate and each experiment was repeated three times.

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 μg/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 μM in the presence or absence of 10 μM compound **9** as described.

***In vivo* drug treatments.** Compound **9** was dissolved in DMSO and administrated by intraperitoneal injection at 10 mg/kg daily for five consecutive days following the schedule in **Figure 3a**. Compound **9** at this dosage has been shown to be well tolerated by mice[34]. Control *S. mansoni*–infected mice were administrated a corresponding amount of DMSO on the same timetable. Age-matched uninfected mice were used as reference group.

Cytotoxicity assay. Cytotoxicity assays were performed using sulforhodamine B to determine cellular protein content as described[35]. Mouse myeloma cell line SP2/0 was cultured in 96-well microtiter plates containing 0.2 ml of RPMI 1640 per well at a cell density of 900 per well at 37 °C in 5% CO_2. After treatment, cells were fixed with 10% TCA at 4 °C for 1 h. Fixed cells were rinsed to remove fixative and then stained in 0.4% (wt/vol) sulforhodamine B (Sigma) in 1% acetic acid for 35 min. After washing with 1% acetic acid and dye extraction in 10 mM Tris (pH 10.5), plates were read at $A_{564\ nm}$ and compared to carrier only–treated cells.

Determination of NO release. NO release was determined by the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) oxidation method[36]. Oxadiazoles at 10 μM were incubated at room temperature for 50 min with either 5 mM cysteine or 15 nM recombinant TGR ± 100 μM NADPH. The reaction mixture was then added to aerobic PBS, pH 7.4, containing 5 mM ABTS. The A_{660} of the resulting green $ABTS^+$ was determined. The conversion from 1 ml reaction to 96-well plate format used was performed by plotting the concentration of the oxidized form of ABTS ($ABTS^+$) subtracted from the reduced form ABTS ($ABTS^+$ – ABTS) versus different concentrations of tested ABTS. The slope of the regression line was calculated and used to recalculate the data at A_{660} in a 200-μl reaction. The concentrations of the released NO were calculated as a percentage of the added oxadiazoles. Each reaction was done in triplicate and the data presented are the average of three independent experiments.

Statistical analysis. Statistical significance of the difference between experimental and control groups was determined by the two-tailed Student's *t*-test.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported in part by US National Institutes of Health (NIH)/National Institute of Mental Health grant R03MH076449 (D.L.W.) and by NIH/National Institute of Allergy and Infectious Diseases (NIAID) grant R01AI065622 (D.L.W.), and in part by the NIH Roadmap for Medical Research Molecular Libraries Program. Schistosome life stages used in this research were supplied in part by the NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (Rockville, Maryland, USA) through NIAID Contract N01-AI-30026.

AUTHOR CONTRIBUTIONS

A.A.S., A.S. and D.L.W. designed the research. A.A.S. performed enzyme, cultured worm and *in vivo* experiments. A.A.S. and D.L.W. analyzed the data. C.J.T. and A.S. contributed oxadiazole 2-oxide reagents. A.A.S., D.L.W., A.S., J.I., C.J.T. and C.P.A. discussed the results and wrote the paper.

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