Synthesis and Structure−Activity Relationships of Parasiticidal Thiosemicarbazone Cysteine Protease Inhibitors against *Plasmodium falciparum, Trypanosoma brucei,* and *Trypanosoma cruzi*

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Received October 27, 2003

We have synthesized a library of thiosemicarbazones and screened them against three parasitic cysteine proteases, cruzain, falcipain-2, and rhodesain, and against the respective parasite sources of these three proteases, *Trypanosoma cruzi, Plasmodium falciparum,* and *Trypanosoma brucei*. The screens identified compounds that were effective against the enzymes and the parasites but also some compounds that were parasiticidal despite a lack of activity against the proteases. Several compounds were effective in killing all tested parasites. These promising lead compounds were tested for general toxicity in mice, and only one produced observable toxicity after 62 h. Our results suggest that thiosemicarbazones represent validated drug leads that kill several species of protozoan parasites through the inhibition of cysteine proteases as well as other novel targets.

Introduction

Thiosemicarbazones are a class of small molecules that have been evaluated over the last 50 years as antivirals[1,2] and as anticancer therapeutics,[3] as well as for their parasiticidal action against *Plasmodium falciparum*[4] and *Trypanosoma cruzi*,[5,6] which are the causative agents of malaria and Chagas's disease, respectively. Currently, a thiosemicarbazone, triapine, is being evaluated in human phase II trials as an antineoplastic therapeutic.[7]

A series of thiosemicarbazones was recently shown to inhibit a *Trypanosoma cruzi*-derived cysteine protease, cruzain.[6] This study generated basic structure−activity relationships (SAR) against cruzain around three positions, using the scaffold shown in Figure 1. The authors found that halides such as bromine and chlorine were tolerated at the 3′- and/or 5′-position on the aryl ring. In addition, changing the methyl substituent at the 2-position had little effect, and N1 NH_2-monosubstitution at position 3 was deleterious. Last, the sulfur and methine bonds were critical to the inhibition of cruzain. The authors concluded that the probable site of attack by the active site cysteine thiol on the thiosemicarbazone scaffold was the thiocarbonyl group and accordingly suggested binding modes using DOCK-based algorithms.[6] This model placed the 3′-substituent of the thiosemicarbazone into the main specificity S2 pocket of cruzain. Proteases bind peptide substrates in their active site cleft through backbone and side chain interactions that lie around several defined pockets in the enzyme termed S1, S2, S3, S4 for those pockets that bind to the corresponding amino acids that are N-terminal to the scissile amide bond while the S1′, S2′, S3′, S4′ pockets bind to the amino acids C-terminal to the scissile bond.

In view of these SAR results, we synthesized a second generation series of thiosemicarbazones. These compounds were tested in vitro against cruzain as well as the cysteine proteases falcipain-2 and rhodesain. In addition, we screened all compounds, whether active against the target enzyme or not, in culture against the respective parasites, *T.cruzi*, *P. falciparum*, and *T. brucei*. All three parasites are major public health problems in tropical and subtropical countries, and for which new chemotherapies are desperately needed.

In this synthetic design of the SAR library, attention was paid to further explore substituents around the aromatic ring as well as the previously unexplored N1 NH_2-monosubstitution of the original thiosemicarbazone scaffold. We reasoned that a diversity-oriented synthesis using Suzuki- or copper-mediated coupling of a range of commercially available boronic acids could be efficiently employed for optimization of interactions with the S2 pocket. In addition, we expanded the SAR to include possible prime side interactions at the S1′ or S2′ positions by exploring N1 NH_2-disubstitution. It is noteworthy that thiosemicarbazones have only recently been reported to inhibit falcipain-2 and rhodesain, and these reported compounds are based on the isatin scaffold. Their antiparasitic activities in cell culture have not been determined.[8]

Chemistry

Biphenyl thiosemicarbazones, **1a**−**c**, were prepared in high yield (80−90%) from biphenyl acetophenones

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10.1021/jm030549j CCC: $27.50
Published on Web 05/04/2004

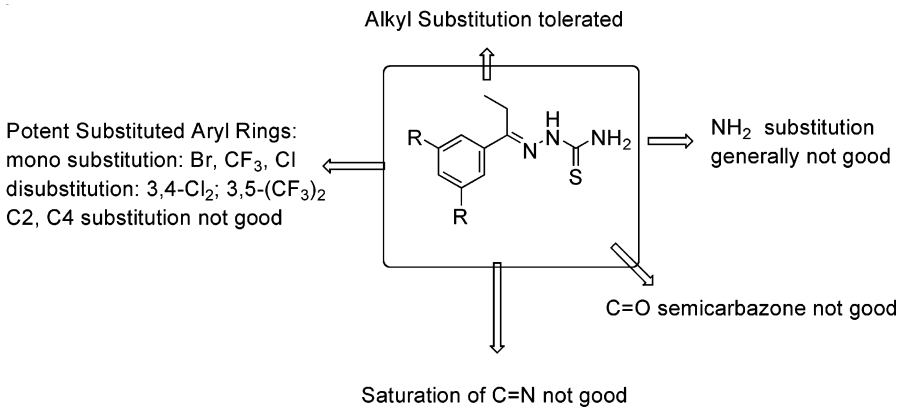
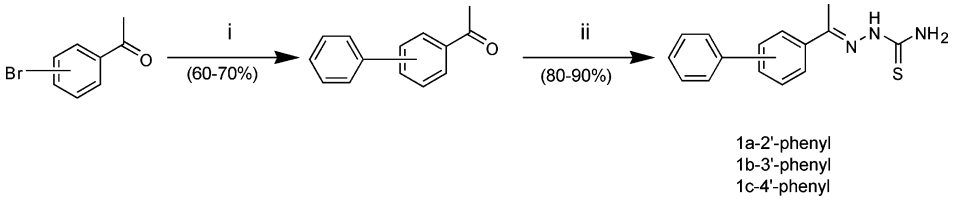


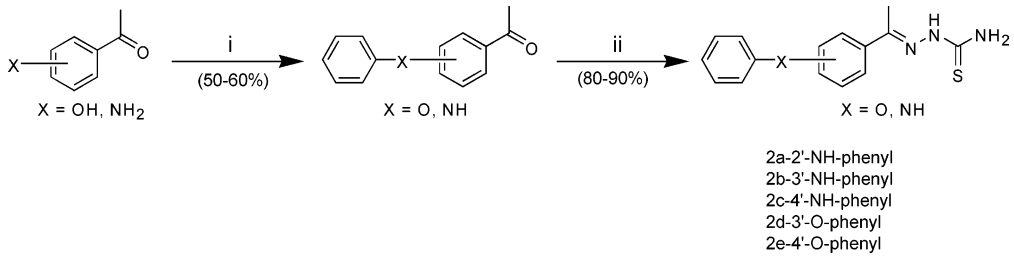
Figure 1. Summary of known thiosemicarbazone structure−activity relationships with cruzain.

Scheme 1[a]



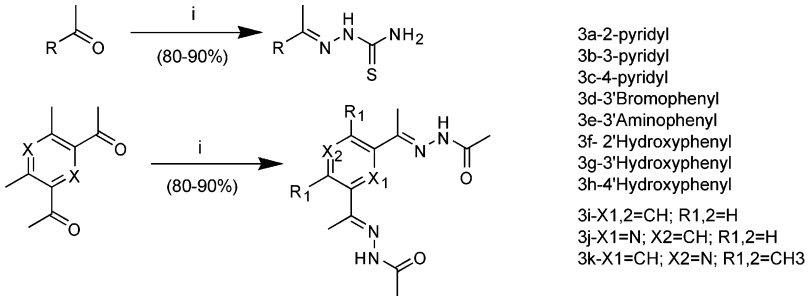
[a] Reagents and conditions: (i) $Pd(Ph_3P)_4$, K_2CO_3(aq), PhMe, reflux, phenylboronic acid. (ii) thiosemicarbazide, MeOH, AcOH, reflux.

Scheme 2[a]



[a] Reagents and conditions: (i) $Cu(OAc)_2$ (xs), pyridine, CH_2Cl_2, rt, phenylboronic acid. (ii) thiosemicarbazide, MeOH, AcOH, reflux.

Scheme 3[a]



[a] Reagents and conditions: (i) Thiosemicarbazide, MeOH, AcOH, reflux.

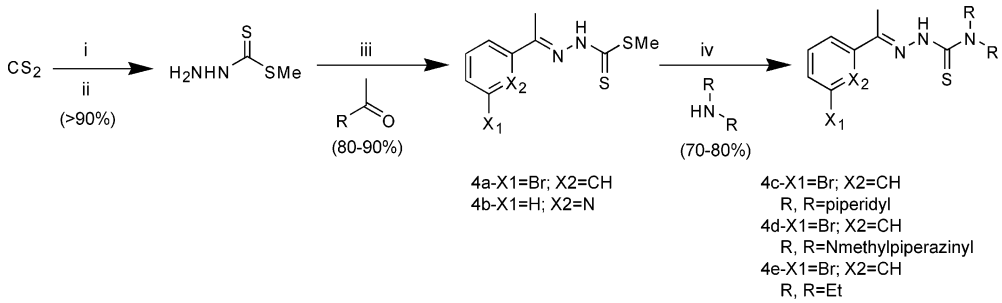
which were in turn prepared in good yield (60−70%) via a Suzuki cross-coupling procedure from the corresponding bromoacetophenones and phenylboronic acid (Scheme 1). The biarylamine and ether derivatives, **2a**−**e**, were also prepared in high yield (80−90%) via moderate yielding (50−60%) N- and O-arylation, respectively (Scheme 2). Treatment of the intermediate biphenyl, biarylamine, and biaryl ether acetophenones with thiosemicarbazide afforded the title compounds in high yield (80−90%). Thiosemicarbazones **3a**−**h** were prepared with high yield (80−90%) from commercially available starting materials by reaction with thiosemicarbazide (Scheme 3). On the other hand, N,N-disubstituted derivatives **4a**−**e** were synthesized in good yield (70−80%) by reacting thiosemicarbazone thioesters with selected secondary amines (Scheme 4).

In theory, two geometrical isomers (*E* and *Z*) about the imine (C═N) double bond are possible for the above thiosemicarbazones. However, analysis of the ^{1}H NMR spectra of the target compound indicated one predominant isomer. This predominant isomer is presumed to have the *E*-configuration for the aryl-based thiosemicarbazones and the *Z*-configuration for the 2-acetylpyridine-based thiosemicarbazones.

Biological Results

Cruzain and *T. cruzi* SAR. Previously, Du et al.[6] showed that the best thiosemicarbazone inhibitor against

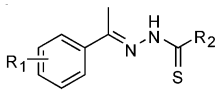
Scheme 4[a]



[a] Reagents and conditions: (i) NH_2NH_2 (xs), KOH i-PrOH. (ii) MeI. (iii) i-PrOH. (iv) i-PrOH.

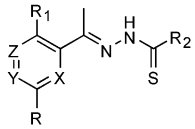
Chart 1. Structure−Activity Relationships against Cruzain and *T. cruzi*

A)



Cmpd#	R1	R2	Cruzain IC50(μM)	Infected Host Cell Survival (days@5 μM)	Host Toxicity
1b	3'-phenyl	NH_2	8	43 days	none
1c	4'-phenyl	NH_2	5	8 days	toxic
2b	3'-NH-phenyl	NH_2	>20	43 days	none
2c	4'-NH-phenyl	NH_2	>20	43 days	none
2d	3'-O-phenyl	NH_2	10	27 days	toxic
2e	4'-O-phenyl	NH_2	10	28 days	toxic
3d	3'-Br	NH_2	0.06	43 days	none
4a	3'-Br	SMe	>20	7 days	toxic
4c	3'-Br	piperidyl	5	5 days	none
4e	3'-Br	NEt_2	5	5 days	none
4d	3'-Br	N-methylpiperazinyl	5	14 days	toxic

B)



Cmpd#	R	R1	R2	X	Y	Z	Cruzain IC50(μM)	Infected Host Cell Survival (days@5 μM)	Host Toxicity
3a	H	H	NH_2	N	CH	CH	>20	43 days	none
3i	CH(Me)NNHC(S)NH_2	H	NH_2	CH	CH	CH	5	5 days	none

cruzain contained a bromo substituent at the 3′-position (depicted as compound **3d** in Chart 1a, 2a, and 3a) or 3′,5′-bis(trifluoromethyl) substituents on the aryl ring. Du et al.[6] proposed a reversible covalent interaction between thiosemicarbazones and cruzain in which the aryl group lies in the S2 pocket of the enzyme. From this we elaborated a series of biphenyl, biarylamine, and biaryl ether derivatives with differing positions around the aryl ring. Thus, the rationale for our choice of these derivatives is based on both experimental and modeling results.

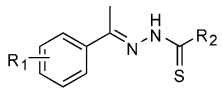
Against recombinant cruzain, a number of compounds with a substituent at the 3′- (**1b**, **2d**, **3d**, **3i**, **4d**, **4e**, **4c**) and 4′- (**1c**, **2e**) positions on the aromatic ring were active in the mid-nanomolar to low micromolar range (Chart 1a). The best compound, the parent molecule **3d**, contained a bromine at the 3′-position. Thus, the 3′- and 4′-positions appear to be promising areas for further expansion. Overall, there was no significant advantage in cruzain inhibition between the biaryls (**1b**, **1c**) and aryl ethers (**2d**, **2e**), while the arylamines (**2b**, **2c**) were ineffective. Replacing the terminal thioamide amino group with disubstituted derivatives (**4c**, **4d**, **4e**) lowered potency by 2 orders of magnitude compared to the parent compound (**3d**).

Seven compounds (**1b**, **2b**, **2c**, **2d**, **2e**, **3d**, **4d**) were effective at protecting macrophage host cells against *T.cruzi* (Chart 1). Two compounds (**1b**, **3d**) were effective against both cruzain and parasites, both of which contain a 3′-substituent on the aromatic ring. Interestingly, two compounds with 3′- and 4′-substituents (**2b**, **2c**) did not inhibit cruzain in vitro but were successful in killing parasites in culture (Charts 1a and 1b). This suggests that the biarylamine substituents at the 3′- and 4′-positions may be important for binding to both cruzain as well as another protease or an unrelated target. Finally, the differences in cell culture activity between 2-, 3-, and 4-acetylpyridine thiosemicarbazones **3a**, **3b**, and **3c** is noteworthy (Chart 1 and Supporting Information Chart 1S). Compound **3a**, acetylpyridine thiosemicarbazone, is likely a tridentate metal chelator in which the pyridine ring nitrogen is involved in chelation and may act by binding endogenous iron and/or copper.

Rhodesain and *T. brucei* SAR. Many more compounds were effective against rhodesain as compared to cruzain although the basic SAR was similar. This may indicate that the S2 pocket of rhodesain is larger than that of cruzain. Those compounds with substitu-

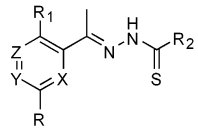
Chart 2. Structure−Activity Relationships against Rhodesain and *T. brucei*

A)



Cmpd#	R1	R2	Rhodesain IC50(μM)	*T.brucei* ED50(μM)
1b	3'-phenyl	NH_2	1.1	12
1c	4'-phenyl	NH_2	0.09	10
2b	3'-NH-phenyl	NH_2	0.8	8
2c	4'-NH-phenyl	NH_2	18	4.5
2d	3'-O-phenyl	NH_2	0.55	6
2e	4'-O-phenyl	NH_2	8	3
3d	3'-Br	NH_2	0.05	>20
4a	3'-Br	SMe	>20	10
4c	3'-Br	piperidyl	4	3
4d	3'-Br	N-methylpiperazinyl	4	2
4e	3'-Br	NEt_2	1.8	3

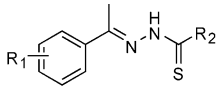
B)



Cmpd#	R	R1	R2	X	Y	Z	Rhodesain IC50(μM)	*T.brucei* ED50(μM)
3a	H	H	NH_2	N	CH	CH	>20	4
4b	H	H	SMe	N	CH	CH	>20	0.3
3i	$CH(Me)NNHC(S)NH_2$	H	NH_2	CH	CH	CH	0.33	>20

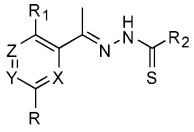
Chart 3. Structure−Activity Relationships against Falcipain and *P. falciparum*

A)



Cmpd#	R1	R2	Falcipain 2 IC50(μM)	*P.falciparum* ED50(μM)
1c	4'-phenyl	NH_2	10	>20
2c	4'-NH-phenyl	NH_2	>20	9.9
4d	3'-Br	N-methylpiperazinyl	>20	4

B)



Cmpd#	R	R1	R2	X	Y	Z	Falcipain 2 IC50(μM)	*P.falciparum* ED50(μM)
3a	H	H	NH_2	N	CH	CH	>20	0.08
4b	H	H	SMe	N	CH	CH	>20	0.03
3j	$CH(Me)NNHC(S)NH_2$	H	NH_2	N	CH	CH	>20	0.03

ents at the 3′- (**1b**, **2b**, **2d**, **3d**, **3i**, **4c**, **4d**, **4e**) and 4′- (**1c**, **2c**, **2e**) position were effective in the mid-nanomolar to low micromolar range (Charts 2a and 2b). This agrees with the SAR data for cruzain. In contrast to the cruzain SAR, the biarylamine inhibitors (**2b**, **2c**) were effective against rhodesain, which indicates that these compounds can, in fact, bind to a cysteine protease. This further indicates that substituents at these positions are important for binding to cysteine proteases.

In screens against *T. brucei* in culture, there were many inhibitors that were able to inhibit both rhodesain and *T. brucei* (**1b**, **1c**, **2b**, **2c**, **2d**, **2e**, **4c**, **4d**, **4e**). This indicates that inhibition of rhodesain may be the primary action of these inhibitors. On the other hand, the most potent compound in the present series against *T. brucei* is a potential metal chelator (**4b**) with no ability to inhibit the enzyme rhodesain. Thus, compound **4b** may be killing *T. brucei* via a protease-independent mechanism. We have investigated the possibility of metal chelation as a route by which some of these inhibitors act on *T. brucei*. Inhibitors were assayed for the ability to kill parasites in the presence or absence of $FeCl_3$. There was no significant effect (data not shown), although these results are complicated by the fact that the compounds can exchange precomplexed iron for endogenous metals.

Falcipain 2 and *P. falciparum* SAR. The SAR against falcipain-2 is striking in that most compounds failed to inhibit the target enzyme. The exception (**1c**) was a weak inhibitor with no efficacy against the parasite. These data give some insight into the significant differences in the active site topology between all three closely related target proteases. On the basis of the data for all three enzymes, rhodesain is by far the

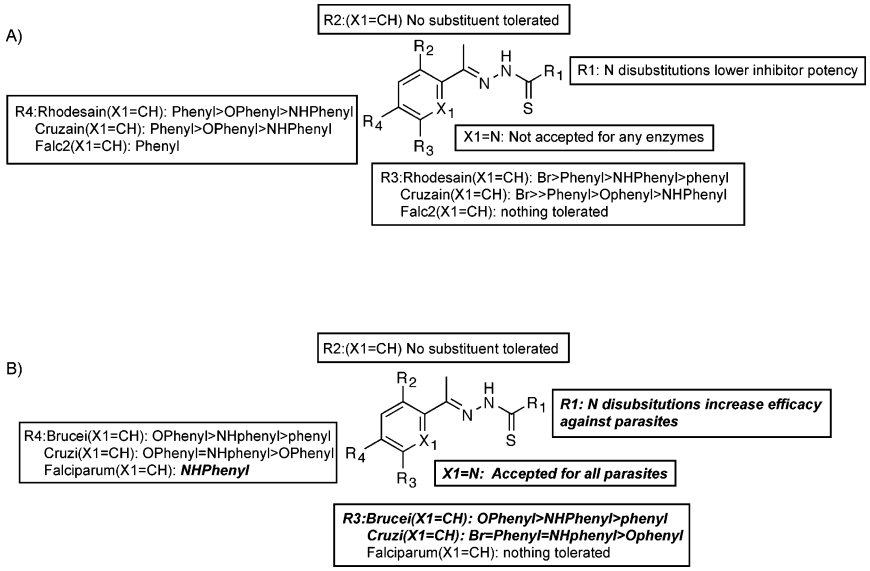


Figure 2. Summary of (A) protease and (B) parasite structure−activity relationships. Bold lettering indicates significant differences between parasite and enzyme SAR.

most promiscuous enzyme, while falcipain-2 is extremely restrictive.

However, with *P. falciparum* in culture, there were five inhibitors that were able to kill or halt growth in live *P.falciparum* cultures. The best inhibitors (**3a**, **3j**, **4b**) were potent having mid-nanomolar ED_{50}s. While the antimalarial activity of compound **3a** has been previously reported,[4] its protein target(s) in *P. falciparum* is unknown. Once again, these inhibitors have the ability to chelate a transition metal, and it is likely that this mechanism of action is responsible for the activity of these compounds.

Discussion

In this study we endeavored to expand the structure−activity relationships of thiosemicarbazones against parasitic cysteine proteases. To do this we took an unbiased approach to screening these compounds against both the target enzymes and the appropriate parasites. Our screening strategy allowed us to analyze SAR across protease targets as well as parasites. The protease SAR revealed that substituents at the 3′- or 4′-positions on the aryl ring are most favored to bind the protease targets and merit expansion (Figure 2a). The parasite SAR corroborates these data, indicating that most thiosemicarbazones with substituents at the 3′- and 4′-positions are acting via a protease-dependent mechanism. In contrast to the protease SAR, the parasite SAR shows that the pyridine thiosemicarbazones, which do not inhibit the target cysteine proteases, are excellent parasiticidal compounds (Figure 2b; **3a** and **4b**). Therefore, these types of compounds must be acting either through inhibition of another cysteine protease or by a protease-independent mechanism. Last, N1 NH_2-disubstitution as in compound **4d** lowered potency against target proteases but enhanced killing of *T. brucei* and *P. falciparum*, especially when a protonatable terminal nitrogen is present as in compound **4d**. This may be due to increased uptake of the compound across the cell membranes or enhanced binding to another target protein.

In addition to the primary SAR, the results from our unbiased screening approach uncovered four scaffolds that represent more general parasiticidal compounds capable of killing all parasites. Two of these are non-metal chelators (**2c**, **4d**) and two are potential metal chelators (**3a**, **4b**). The mechanism of action of these compounds appears to be complex. Compounds **2c** and **4d** can inhibit proteases, although **2c** inhibits only rhodesain, while **4d** inhibits both rhodesain and cruzain. Yet both compounds can kill all three organisms. This highlights the fact that there must be other targets that may or may not include cysteine proteases. On the other hand, compounds **3a** and **4b** do not inhibit the target cysteine proteases but are very effective at killing at least two or all three tested parasites. Clearly these inhibitors work through a different target and may chelate endogenous metals.

Ultimately, a compound that can kill parasites is necessary but not sufficient to represent a good drug lead unless it also demonstrates low or no toxicity among other criteria. Thus the most promising lead compounds (**1b**, **2c**, **3a**, **3j**, **4b**, **4d**) were tested for general toxicity in mice (Supporting Information Chart 4). Compounds **1b**, **2c**, **3j**, **4b**, and **4d** generated no toxicity in mice after 62 h while compound **3a** was toxic. In addition to having no obvious toxicity, the four best general scaffolds (**2c**, **4d**, **3a**,**4b**) are nonpeptidic and achiral and utilize chemistries that are relatively inexpensive and amenable to chemical library generation. For example, the synthesis of compound **2c** utilizes copper-mediated coupling chemistry using both commercially and synthetically accessible boronic acids as diversity elements. Thus, we are pursuing the generation of a second library based on this scaffold. These results are encouraging, and we will conduct further studies on their mechanism of action as well as efficacy studies using these inhibitors in mouse models of African sleeping sickness, Chagas's disease, and malaria.

The detailed mechanism of action of thiosemicarbazones has been elusive. The antimalarial 2-acetylpyridine thiosemicarbazones (**3a**, **4b**, and **3j)** may be acting

as iron chelators in *P. falciparum*. Tridentate chelating thiosemicarbazones have been shown to inhibit ribonucleotide reductase, RR, an enzyme essential for DNA synthesis.[9,10] Tridentate chelating thiosemicarbazones have also been proposed to act by inhibiting dihydrofolate reductase.[11,12] Last, there is evidence that copper complexes of thiosemicarbazones produce significant oxidative stress by binding endogenous reducing agents such as glutathione.[13] Our work suggests that the mechanism of action of thiosemicarbazones is indeed complex and may be mediated through inhibition of multiple targets.

Finally, we hope that these results encourage the screening of all compounds in a SAR series against both the protein target as well as the relevant organism since the combined data may reveal hidden target complexity. If we had tested, in culture, only compounds in this series that inhibited the target proteases, several very potent parasiticidal compounds would have been overlooked. Furthermore both types of data, against target enzyme and parasite, help to optimize the next round of library synthesis to optimize the properties necessary for both inhibiting the target as well as killing the organism.

Conclusion

We identified several thiosemicarbazones that were effective against parasite-derived cysteine proteases and the appropriate parasites. Among the most effective compounds, some were parasiticidal without activity against the cognate protease. Four compounds were effective in killing all tested parasites, and these compounds showed no obvious toxicity in mice after 62 h. These results indicate that thiosemicarbazones represent promising drug leads capable of killing multiple protozoal parasites through the inhibition of cysteine proteases as well as other novel targets.

Experimental Procedures

Protease Inhibition and Parasite Cell Culture Assays. Recombinant cruzain (from *T. cruzi*) and rhodesain (from *T. brucei rhodesiense*) were recombinantly expressed as described previously.[14,15] Cruzain (2 nM) or rhodesain (3 nM) was incubated with 0.5 to 10 μM inhibitor in 100 mM sodium acetate, pH 5.5, containing 5 mM DTT (buffer A), for 5 min at room temperature. Then buffer A containing Z-Phe-Arg-AMC (Bachem, $K_m = 1$ μM) was added to enzyme inhibitor to give 20 μM substrate in 200 μL, and the increase in fluorescence (excitation at 355 nM and emission at 460 nM) was followed with an automated microtiter plate spectrofluorimeter (Molecular Devices, Flex station). Inhibitor stock solutions were prepared at 20 mM in DMSO, and serial dilutions were made in DMSO (0.7% DMSO in assay). Controls were performed using enzyme alone and enzyme with DMSO. IC_{50} values were determined graphically using inhibitor concentrations in the linear portion of a plot of inhibition versus log [I] (seven concentrations tested with at least two in the linear range).

IC_{50} values against recombinant Falcipain-2 were determined as described previously.[16] Enzyme was incubated for 30 min at room temperature in 100 mM sodium acetate, pH 5.5, 10 mM DTT, with different concentrations of tested inhibitors. Inhibitor solutions were prepared from stock in DMSO (maximum concentration of DMSO in the assay was 1%).

After 30 min incubation, the substrate Z-Leu-Arg-AMC (benzoxycarbonyl-Leu-Arg-7-amino-4-methylcoumarin) in the same buffer was added to a final concentration of 25 μM. Fluorescence was monitored for 15 min at room temperature in a Fluoroskan Ascent spectrofluorometer (Labsystems). IC_{50} values were determined from plots of percents of activity over the compound concentration using the data analysis program Prism (GraphPad software).

***T. cruzi* Culture Assay.** Mammalian cells were cultured in RPMI-1640 medium supplemented with 5−10% heat-inactivated fetal calf serum (FCS) at 37 °C in 5% CO_2. The Y strain of *T. cruzi* was maintained by serial passage in bovine embryo skeletal muscle (BESM) cells. Infectious trypomastigotes are collected from culture supernatants. For drug assays, J774 macrophages were irradiated (5000 rad) and plated onto six-well tissue culture plates 24 h prior to infection with about 106 trypomastigotes/well. Parasites were removed 2 h postinfection, and the medium was supplemented with the appropriate inhibitor (10 μM). Inhibitor stocks (10 mM) in DMSO were stored at −20 °C. J774 monolayers treated with a blank containing DMSO were used as a negative control. RPMI medium with or without inhibitor was replaced every 48 h. Cultures were maintained for up to 46 days and monitored daily by contrast phase microscopy. *T. cruzi* completed the intracellular cycle in 5−6 days in the untreated controls. The comparative effectiveness of each inhibitor was estimated from plots of the duration of the intracellular cycle of *T. cruzi* (days) in treated vs untreated control wells.

***T. brucei* Culture Assay.** *T. brucei rhodesiense* were grown to 10^6 cells/mL at 37 °C with 5% CO_2 in complete HMI-9 medium containing 10% FBS, 10% Serum Plus, 1× penicillin/streptomycin. To carry out drug screens, parasites were diluted to 10^4 cells/mL in complete HMI-9 medium and aliquoted into 5 mL for growth in culture flasks or 100 mL for growth in 96-well cultures plates. Each inhibitor was added to the appropriate flask or well containing cultured parasites beginning at the highest concentration. The inhibitors were then directly diluted in the cultured parasites by serial dilutions until the concentration of the inhibitors reached 1 nM. Parasites were then incubated in the presence of each inhibitor for 48 h at 37 °C with 5% CO_2 before monitoring viability. To assay for viability after treatment with inhibitors, parasites were tested for the production of ATP.[17] To do this, 100 mL of parasites from each flask were transferred to 96-well plates. An equal volume of CellTiter-Glo (Promega) was added to each well of the transferred parasites or parasites originally grown in 96-well plates separately. The mixture was then shaken at room temperature for 5 min before reading the plates using a SpectraFluor Plus multidetection plate reader (Tecan). Alternatively, the treated parasites were counted by hemacytometer 48 h after incubating them with inhibitors.

***P. falciparum* Culture Assay.** W2-strain *P. falciparum* parasites (1% parasitemia, 2% hematocrit) were cultured in 0.5 mL of medium in 48-well culture dishes.[18] Appropriate inhibitors from 10 mM stocks in DMSO were added to cultured parasites to a final concentration of 20 μM. From 48-well plates, 125 μL of culture was transferred to two 96-well plates (duplicates). Serial dilutions (1:5) of inhibitors were made to final concentrations of 10 μM, 2000 nM, 400 nM, 80 nM, 16 nM, and 3.2 nM. Cultures were maintained at 37 °C for 2 days. The parasites were washed and fixed with 1% formaldehyde in PBS. After 2 days, parasitemia was measured by flow cytometry using the DNA stain YOYO-1 as a marker for cell survival.

Mouse Toxicity Assay. C3H female mice (mean weight, 18 g) (Jackson Laboratories) were injected daily via ip with 20 mg/kg weight or 5 mg/kg weight of selected compounds ($n = 1$ per treatment). Compounds were resuspended in 100 μL [70% DMSO (Sigma):30% ddH_20] per dose and injected twice daily for 48 h. Animals were monitored for signs of toxicity, including behavior and feeding, and sacrificed 14 h after the last treatment for necropsy. Major organs were submitted for histological analysis.

Chemistry. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Varian Inova-400 MHZ spectrometer with Me_4Si(TMS) as the internal reference. Coupling constants (J) are given in hertz. Elemental and mass spectra analyses were performed at the Micro-Mass facility, University

of California, Berkeley. Thin layer chromatography (TLC) was carried out on aluminum-backed Merck silica gel 60 F_{254} using the same solvent systems as those used in column chromatography. Column chromatography was performed on silica gel (70−230 mesh). Final products usually precipitated out and were either rinsed with, and/or recrystallized from, methanol. Yields for Suzuki cross-couplings and N- and O-arylation reactions were in the range of 60−70% and 50−60%, respectively, while yields for all thiosemicarbazone-forming reactions were 80−90%.

General Procedure for the Preparation of Compounds. Suzuki Coupling (Scheme 1): 3 mmol of bromoacetophenone and 3 mmol of boronic acid were dissolved in a mixture of toluene (30 mL) and ethanol (12 mL). Tetrakistriphenylphosphinepalladium(0) (100 mg) was added. After addition of a saturated solution of potassium carbonate (30 mL), the mixture was heated under reflux for 5 h. Water was added, and the mixture was extracted with dichloromethane. Organic layers were combined and dried ($MgSO_4$), and the solvent was removed. Products were obtained by preparative TLC.

Arylation (Scheme 2): A slurry of the substrate, phenylboronic acid (2−3 equiv), anhydrous $Cu(OAc)_2$ (1−2 equiv), and pyridine (2−3 equiv) in methylene chloride (10 mL/0.5 g of substrate) was stirred at room temperature for 24−72 h. Products were isolated by direct loading of the crude reaction mixtures on silica gel.

Thiosemicarbazone derivatives **1**−**4** were prepared essentially as reported.[6]

1-Biphenyl-2-ylethanone thiosemicarbazone (1a): ^{1}H NMR δ_H (400 MHz; DMSO-d_6) 1.77 (s, 3H), 7.25 (s, 1H), 7.31 (d, 2H, J = 7.6,), 7.36 (d, 1H, J = 2.0), 7.39 (d, 2H, J = 9.2), 7.43 (s, 1H), 7.45 (s, 1H), 7.55 (d, 1H, J = 8.4), 8.12 (s, 1H), 10.13 (s, 1H), 11.95 (s, 1H); MS (EI) m/z 270.3 (MH^+). Anal. ($C_{15}H_{15}N_3S$) C, H, N, S.

1-Biphenyl-3-ylethanone thiosemicarbazone (1b): ^{1}H NMR δ_H (400 MHz; DMSO-d_6) 2.37 (s, 3H), 7.38 (t, 2H, J = 7.2), 7.48 (t, 2H, J = 8.0), 7.67 (d, 1H, J = 7.6), 7.74 (d, 2H, J = 7.2), 7.91 (d, 1H, J = 8.0), 8.02 (s, 1H), 8.11 (s, 1H), 10.23 (s, 1H); MS (EI) m/z 270.3 (MH^+). Anal. ($C_{15}H_{15}N_3S$) C, H, N, S.

1-Biphenyl-4-ylethanone thiosemicarbazone (1c): ^{1}H NMR δ_H (400 MHz; DMSO-d_6) 2.34 (s, 3H), 7.39 (t, 1H, J = 7.2), 7.49 (t, 2H, J = 8.0), 7.69 (dd, 4H, J = 7.6), 7.99 (s, 1H), 8.03 (d, 2H, J = 8.8), 8.30 (s, 1H), 10.25 (s, 1H); MS (EI) m/z 270.3 (MH^+). Anal. ($C_{15}H_{15}N_3S$) C, H, N, S.

1-(2-Phenylaminophenyl)ethanone thiosemicarbazone (2a): ^{1}H NMR δ_H (400 MHz; $CDCl_3$) 2.25 (s, 1H), 2.35 (s. 3H), 7.05 (m, 6H), 7.28 (m, 4H), 7.41 (d, 1H, J = 7.2), and 8.73 (s, 1H); MS (EI) m/z 285.3 (MH^+).

1-(3-Phenylaminophenyl)ethanone thiosemicarbazone (2b): δ_H (400 MHz; $CHCl_3$-d) 2.65 (s, 3H), 6.35 (broad s. 1H), 6.99 (t, 1H, J = 7.2), 7.11 (t, 2H, J = 8.4), 7.27 (m, 6H), 7.43 (s, 2H), 8.73 (s, 1H); MS (EI) m/z 285.3 (MH^+). Anal. ($C_{15}H_{16}N_4S$) C, H, N.

1-(4-Phenylaminophenyl)ethanone thiosemicarbazone (2c): δ_H (400 MHz; $CHCl_3$-d) 2.27 (s, 3H), 6.25 (broad s. 2H), 7.04 (d, 2H, J = 8.8), 7.15 (d, 1H, J = 8.0), 7.26 (s, 2H), 7.32 (t, 2H, J = 7.6), 7.63 (d, 2H, J = 8.8), 8.62 (broad s, 1H); MS (EI) m/z 285.3 (MH^+).

1-(3-Phenoxyphenyl)ethanone thiosemicarbazone (2d): δ_H (400 MHz; DMSO-d_6) 2.28 (s, 3H), 6.99 (t, 2H, J = 8.4), 7.13 (t, 1H, J = 6.8), 7.39 (dd, 3H, J = 7.2), 7.69 (s, 2H), 7.97 (s, 1H) 8.27 (s, 1H), 10.23 (s, 1H); MS (EI) m/z. 286.2 (MH^+). Anal. ($C_{15}H_{15}N_3OS$) C, H, N, S.

1-(4-Phenoxyphenyl)ethanone thiosemicarbazone (2e): δ_H (400 MHz; $CHCl_3$-d) 2.28 (s, 3H), 6.98 (d, 2H, J = 8.4), 7.02 (d, 2H, J = 8.4), 7.14 (t, 1H, J = 7.6), 7.34 (d, 2H, J = 7.6), 7.37 (s, 1H), 7.67 (s, 2H), 8.82 (s, 1H); MS (EI) m/z 286.2 (MH^+). Anal. ($C_{15}H_{15}N_3OS$) H, N, S.

3′-Bromoacetophenone thiosemicarbazone (3d): δ_H (400 MHz; DMSO-d_6) 2.28 (s, 3H), 7.34 (t, 1H, J = 8), 7.57 (d, 1H, J = 8.4), 7.89 (d, 1H, J = 7.6), 8.11 (s, 1H), 8.19 (s, 1H), 8.32 (s, 1H), 10.25 (s, 1H); MS (EI) m/z 273.2 (MH^+). Anal. ($C_9H_{10}BrN_3S$) C, H, N.

1-(3-Aminophenyl)ethanone thiosemicarbazone (3e): δ_H (400 MHz; DMSO-d_6) 2.22 (s, 3H), 5.04 (s, 2H), 6.60 (d, 1H, J = 4.4), 7.02 (d, 2H, J = 4.4), 7.09 (s, 1H), 7.67 (s, 1H), 8.29 (s, 1H), and 10.18 (s, 1H); MS (EI) m/z 209.0 (MH^+). Anal. ($C_9H_{12}N_4S$) C, H, N, S.

1-(2-Hydroxyphenyl)ethanone thiosemicarbazone (3f): δ_H (400 MHz; DMSO-d_6) 2.31 (s, 3H), 4.49 (s, 1H), 6.84 (s, 1H), 6.86 (t, 2H, J = 8.4), 7.25 (t, 1H, J = 7.6), 7.53 (s, 2H); MS (EI) m/z 210.2 (MH^+).

1-(3-Hydroxyphenyl)ethanone thiosemicarbazone (3g): δ_H (400 MHz; DMSO-d_6) 2.25 (s, 3H), 6.79 (d, 1H, J = 8.0), 7.19 (t, 1H, J = 8.0), 7.24 (s, 1H), 7.33 (d, 1H, J = 7.6), 7.78 (s, 1H), 8.27 (s, 1H) 9.43 (s, 1H), 10.21 (s, 1H); MS (EI) m/z. 210.2 (MH^+). Anal. ($C_9H_{11}N_3OS$) C, H, N, S.

1-(4-Hydroxyphenyl)ethanone thiosemicarbazone (3h): δ_H (400 MHz; DMSO-d_6) 2.23 (s, 3H), 6.75 (d, 2H, J = 8.8), 7.76 (d, 2H, J = 8.4), 7.80 (s, 1H), 8.16 (s, 1H), 9.72 (s, 1H), and 10.06 (s, 1H); MS (EI) m/z. 210.2 (MH^+). Anal. ($C_9H_{11}N_3OS$) C, H, N, S.

Methyl 3-[1-(3′-bromophenyl)ethylidene]hydrazinecarbodithioate (4a): δ_H (400 MHz; DMSO-d_6) 2.38 (s, 3H), 2.52 (s, 3H), 7.42 (t, 1H, J = 8), 7.65 (d, 1H, J = 7.6), 7.85 (d, 1H,J = 8.0), 8.02 (s, 1H), 12.53 (s, 1H); MS (EI) m/z 304.3 (MH^+). Anal. ($C_{10}H_{11}BrN_2S_2$) C, H, N, S.

Piperidine-1-carbothioic acid [1-(3-bromophenyl)ethylidene] hydrazide (4c): δ_H (400 MHz; DMSO-d_6) 1.61 (s, 6H), 2.27 (s, 3H), 3.83 (s, 4H), 7.38 (t, 1H, J = 7.6), 7.58 (d, 1H, J = 7.6), 7.55 (d, 1H, J = 7.6), 7.93 (s, 1H), 9.66 (s, 1H); MS (EI) m/z 340.3 (MH^+). Anal. ($C_{14}H_{18}BrN_3S$) C, H, N.

4-Methylpiperazine-1-carbothioic acid [1-(3-bromophenyl)ethylidene] hydrazide (4d): δ_H (400 MHz; DMSO-d_6) 2.21 (s, 3H), 2.28 (s, 3H), 2.39 (s, 4H), 3.86 (s, 4H), 7.39 (t, 1H, J = 7.2), 7.59 (d, 1H, J = 6.8), 7.55 (d, 1H, J = 7.6), 7.94 (s, 1H), 9.86 (s, 1H); MS (EI) m/z 355.5 (MH^+). Anal. ($C_{14}H_{19}BrN_4S$) C, H, N.

3′-Bromoacetophenone 4,4-diethyl-3-thiosemicarbazone (4e): δ_H (400 MHz; DMSO-d_6) 1.20 (t, 6H), 2.28 (s, 3H), 3.73 (q, 4H), 7.39 (t, 1H, J = 8.0), 7.59 (d, 1H, J = 7.6), 7.76 (d, 1H, J = 8.0), 7.95 (s, 1H), 9.45 (s, 1H); MS (EI) m/z 328.3 (MH^+)

1-Pyridin-2-ylethanone thiosemicarbazone (3a): δ_H (400 MHz; DMSO-d_6) 2.39 (s, 3H), 7.38 (dt, 1H, J = 1.2), 7.79 (dt, 1H, J = 2.4), 8.13 (s, 1H), 8.42 (t, 2H, J = 8.0), 8.57 (d, 1H, J = 4.4), 10.31 (s, 1H); MS (EI) m/z 195.3 (MH^+). Anal. ($C_8H_{10}N_4S.0.5H_2O$) C, H, N, S.

1-Pyridin-3-ylethanone thiosemicarbazone (3b): δ_H (400 MHz; DMSO-d_6) 2.28 (s, 3H), 7.35 (dd, 1H, J = 4.4), 8.04 (s, 1H), 8.29 (d, 2H,J = 6.8), 8.51 (d, 1H, J = 3.6), 9.06 (s, 1H), 10.28 (s, 1H); MS (EI) m/z 0.195.3 (MH^+). Anal. ($C_8H_{10}N_4S$) C, H, N, S.

1-Pyridin-4-ylethanone thiosemicarbazone (3c): δ_H (400 MHz; DMSO-d_6) 2.30 (s, 3H), 7.90 (dd, 2H, J = 2.0), 8.14 (s, 1H), 8.44 (s, 1H), 8.58 (dd, 2H, J = 1.6), 10.42 (s, 1H); MS (EI) m/z 195.3 (MH^+). Anal. ($C_8H_{10}N_4S$) C, H, N, S.

***N′*-(1-Pyridin-2-ylethylidene)hydrazinecarbodithioic acid methyl ester (4b):** δ_H (400 MHz; DMSO-d_6) 2.26 (s, 3H), 2.50 (s, 3H), 7.44 (t, 1H, J = 8.4), 7.67 (d, 1H, J = 8.0), 7.90 (d, 1H,J = 8.0), 8.65 (s, 1H); MS (EI) m/z 226.3 (MH^+). Anal. ($C_9H_{11}N_3S_2$) C, H, N, S.

1-(3-Acetylphenyl)ethanone thiosemicarbazone (3i): δ_H (400 MHz; DMSO-d_6) 2.35 (t, 6H), 7.40 (t, 1H, J = 7.6), 7.97 (dd, 4H, J = 1.6), 8.19 (s, 1H), 8.28(s, 2H), 10.22 (s, 2H); MS (EI) m/z 309.4 (MH^+). Anal. ($C_{12}H_{16}N_6S_2{\cdot}H_2O$) H, N, S.

1-(6-Acetylpyridin-2-yl)ethanone thiosemicarbazone (3j): δ_H (400 MHz; DMSO-d_6) 2.44 (s, 6H), 7.78 (t, 1H, J = 7.6), 8.16 (broad s, 2H), 8.43 (d, 4H, J = 8.4), 10.31 (s, 2H); MS (EI) m/z 310.3 (MH^+). Anal. ($C_{11}H_{15}N_7S_2.1.5H_2O$) C, H.

1-(5-Acetyl-2,6-dimethylpyridin-3-yl)ethanone thiosemicarbazone(3k): δ_H (400 MHz; DMSO-d_6) 2.21 (s, 6H), 2.29 (t, 6H, J = 6.8), 7.68 (d, 1H, J = 2.8), 8.24 (broad s, 4H), 10.23 (s, 2H); MS (EI) m/z 338.1 (MH^+). Anal. ($C_{13}H_{19}N_7S_2$) H, N, S.

Acknowledgment. This research was supported by the Sandler Family Support Foundation and NIH grant AI35707. D.C.G. was also supported by a NIH Postdoctoral Training Fellowship (CA 09270-27). K.C. gratefully acknowledges the award of a Sandler Sabbatical Fellowship from the Sandler Centre For Basic Research In Parasitic Diseases (University of California—San Francisco). The University of Cape Town and National Research Foundation of South Africa are thanked for approving leave of absence and additional support, respectively (K.C.).

Supporting Information Available: Charts for compounds with low efficacy against enzymes and organisms (1S, 2S, 3S). Chart showing toxicity of select compounds (4S). Tables of elemental analyses and mass spectrometric and ^{1}H NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM030549J