Identification of Novel Parasitic Cysteine Protease Inhibitors Using Virtual Screening. 1. The ChemBridge Database

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Trypanosomiasis, leishmaniasis, and malaria are major parasitic diseases in developing countries. The existing chemotherapy of these diseases suffers from lack of safe and effective drugs and/or the presence of widespread drug resistance. Cysteine proteases are exciting novel targets for antiparasitic drug design. Virtual screening was performed in an attempt to identify novel druglike nonpeptide inhibitors of parasitic cysteine proteases. The ChemBridge database consisting of approximately 241 000 compounds was screened against homology models of falcipain-2 and falcipain-3 in three consecutive stages of docking. A total of 24 diverse inhibitors were identified from an initial group of 84, of which 12 compounds appeared to be dual inhibitors of falcipain-2 and falcipain-3. Four compounds showed inhibition of both the malarial cysteine proteases as well as *Leishmania donovani* cysteine protease.

Introduction

Trypanosomiasis, leishmaniasis, and malaria are major parasitic diseases in developing countries caused by protozoa of the genus *Trypanosoma*, *Leishmania,* and *Plasmodium*, respectively. American trypanosomiasis (Chagas's disease) is an endemic disease prevalent in most of Latin America. It affects an estimated 16−18 million people and approximately 50 000 patients die each year of Chagas disease.[1] Leishmaniasis is endemic in 86 countries around the world and it afflicts an estimated 10−15 million people annually.[2] Moreover, *Leishmania*/HIV coinfection is emerging as an extremely serious, prevalent new disease.[3] At present, the chemotherapy of trypanosomiasis and leishmaniasis depends on a relatively small number of synthetic drugs and is associated with significant toxicity and the development of drug resistance.[4−6] Malaria is present in more than 90 countries and about 40% of the world's population is affected by the disease.[7] It is responsible for 1.5 to 2.7 million deaths and 300−500 million cases each year.[8] The increasing resistance of malaria parasites, in particular *Plasmodium falciparum*, to existing antimalarial drugs is a key factor in the persistence of this disease as a major worldwide public health threat. The ongoing discussion clearly suggests an urgent need for new drugs designed against novel targets in these organisms.

Cysteine proteases constitute an important class of enzymes that play vital roles in the life cycle of many parasites.[9] A possible strategy for combating parasitic infections is to inhibit cysteine proteases that are crucial to parasite metabolism and reproductive function. Papain-like cysteine proteases from *Leishmania donovani*, *Plasmodium falciparum* (falcipain-2 and falcipain-3), and *Trypanosoma cruzi* (cruzain) have been identified and functionally characterized.[9−11] Several studies have confirmed the efficacy of peptidyl cysteine protease inhibitors in arresting and killing parasites in tissue culture models of parasite replication or cell invasion and have also demonstrated their efficacy in vivo.[9] Vinyl sulfone-based peptides (Figure 1) are known to be irreversible inhibitors of cysteine proteases such as cruzain[9] and falcipains[12] forming covalent bonds with the thiolate of the catalytic cysteine. Although such irreversible inhibitors are quite potent with IC_{50} values in the nanomolar range, the poor selectivity for parasitic cysteine proteases over the human cysteine proteases remains a significant concern.[13] Also, it is desirable to design reversible inhibitors to minimize the potential toxicity that can be observed with irreversible inhibitors.[13] Recently several ketone-based peptides (Figure 1) have been reported to be potent and reversible inhibitors of cruzain.[14] These compounds are proposed

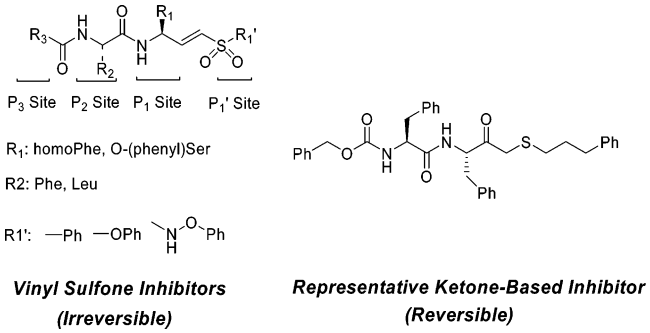

Figure 1. Representative structures of reported parasitic cysteine protease inhibitors.

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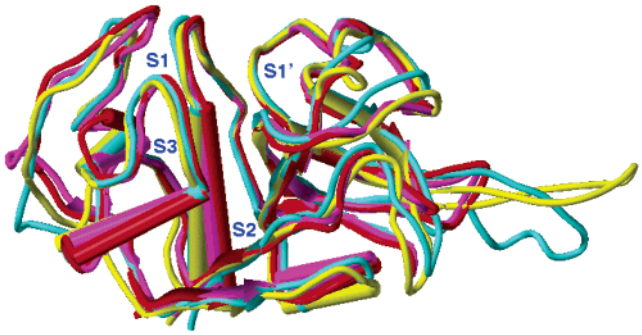

Figure 2. Homology models of falcipain-2 (yellow),[17] falcipain-3 (cyan),[18] and *L. donovani* cysteine protease (red; unpublished data) superimposed on the crystal structure of cruzain (magenta).[16] The subsites are labeled as S1, S1′, S2, and S3.

to form transition-state-like hemithioacetal complexes with cysteine proteases. However, it is beneficial to have nonpeptide inhibitors with more druglike properties since they impede degradation by proteases in the living system, retaining the potential for high in vivo activity and selectivity.

Falcipain-2, falcipain-3, cruzain, and the cysteine protease from *L. donovani* share more than 40% homology in the mature domain and about 90% for the binding site residues.[9,11] Crystal structures of cruzain with several peptide inhibitors have been reported[15,16] while homology models of falcipain-2[17] and falcipain-3[18] have been developed by us. As shown in Figure 2, the structure of the binding pocket appears to be highly conserved across the protozoal cysteine proteases. This suggests the possibility of development of common inhibitors of these enzymes to treat malaria, leishmaniasis, and trypanosomiasis.

In the recent past, virtual screening has emerged as a powerful tool for identification of novel and diverse lead structures.[19,20] The technique involves computational docking of compounds from large databases into the active site of a drug target to identify potential hits which can then be biologically evaluated. Thus, it helps to reduce the burden of high-throughput screening by reducing the number of compounds to be tested. In this paper, we report successful utilization of virtual screening to identify novel cysteine protease inhibitors as lead structures for development of broad spectrum antiprotozoal drugs.

Computational Tools. All calculations were performed on a Silicon Graphics Octane 2 workstation, equipped with two parallel R12000 processors, V6 graphics board, and 512 MB memory. GOLD[21] was utilized for docking while physicochemical parameters related to ADME were calculated using Cerius2 (Accelrys, San Diego, CA). Concord (Tripos Inc., St. Louis, MO) was used to generate 3D coordinates of the compounds.

Protein Preparation. Since falcipain-2 and falciapin-3 appear to be equally important for hemoglobin processing in *P. falciparum*,[11] both were considered in virtual screening to identify common inhibitors. A relatively common binding pocket in each was defined to make sure that the common residues lining the active site were all included in the binding pocket definitions. These enzymes act in the *P. falciparum* food vacuole, which is an acidic, lysosome-like organelle. For this reason, the protonation status of the Arg, Lys, Asp, Glu, His, and Cys residues was considered to be critical before docking the molecules into these enzymes. This was achieved by adding the hydrogen atoms at pH 5 in InsightII 2002 (Accelrys, San Diego, CA). To determine if the residues were assigned the correct protonation states, all the Arg, Lys, Asp, Glu, His, and Cys residues were visually inspected. Asp and Glu were treated as deprotonated unless they were located in a hydrophobic environment. Arg and Lys were treated as protonated unless hydrophobic residues surrounded them. For each target protein, the active site was defined as the collection of amino acids enclosed within a 12 Å radius sphere centered on the sulfur of the catalytic cysteine Cys 42 in falcipain-2[17] and Cys 51[18] in falcipain-3. Visual inspection of the binding pocket revealed that all the important interacting residues were included using this definition.

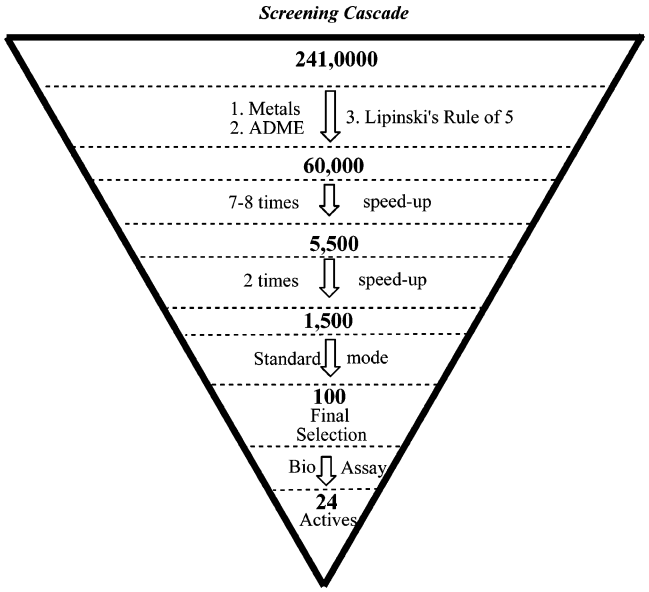

Figure 3. Protocol utilized for virtual screening. 7−8 times speed-up, 2-times speed-up and standard mode represent three different sets of default parameters used for docking.

Preliminary Docking Study. A preliminary docking study was carried out using the crystal structure of cruzain with a vinyl sulfone inhibitor resolved at 1.60 Å (PDB id: 1F2A)[16] to establish the protocol. Docking studies were performed using three different settings: standard mode, 2-times speed-up mode and 7−8 times speed-up mode. The default GOLD parameters for these settings were used except that the 'flood fill radius' was set to 12 Å and the 'number of allowed ligand bumps' to 3. The ligands were scored based on the 'GoldScore'. GOLD was run to save up to 10 top-ranked docking solutions for the ligands. Interestingly, the pose that corresponded to the highest fitness was the closest to the experimental binding mode. The two poses differed by only 1.44 Å RMSD for the standard mode. At 2-times speed-up docking, the RMSD was 1.8 Å and at 7−8 times speed-up setting an RMSD of 2.1 Å was obtained suggesting that the protocols provided a reasonable prediction of the experimental binding mode of the vinyl sulfone ligand.

Database Filtration. A flowchart depicting various stages of the virtual screening including the database filtration and subsequent docking studies is shown in Figure 3. The virtual screening was performed using

the ChemBridge database (Express Pick, October 2001: ChemBridge Corporation, San Diego, CA) containing a library of about 241 000 compounds. The database was filtered to collect only druglike molecules for docking using the following protocol. Molecules with metals were removed and in case of salts the counterions were stripped and the anions were neutralized using Sybyl (Tripos Inc., St. Louis, MO) SPL scripts. Good ADME properties are just as critical as the activity against the given target. Hence the database was filtered based on ADME related parameters to eliminate non-drug-like molecules. *ADME_absorption* parameters as available in Cerius2 were calculated. The predicted absorption level for a particular molecule depends on the polar surface area (PSA) and AlogP98 values.[22] The molecules were sorted as per their absorption levels, and only those that had absorption level 0 (zero: good absorption) were extracted. An average PSA value of 52 and AlogP98 value of 3.2 were calculated for this set of compounds. The molecules were then subjected to the next filter based on solubility related parameters. About 38% of the molecules classified as having '*good to very soluble*' levels were retained. In the next stage, molecules were subjected to the Lipinski's rule of five.[23] Only those molecules having molecular weight less than 500, number of H-bond donors less than 5, and acceptors less than 10 were selected. The filtered database consisting of approximately 60 000 compounds was then subjected to Concord to generate 3D coordinates and subsequently used for docking.

Docking. Docking was carried out in three consecutive stages employing three different settings in GOLD, as described earlier. The protocol helped to optimize the balance between the quality of docking and the time required for the process. Falcipain-2 and falcipain-3 are highly homologous, having more than 92% identity in the binding pockets, with comparable polarities.[18] In fact, homology modeling of the two proteases has suggested similar structures for the binding sites (Figure 2).[17,18] Hence, it was decided to perform the first two stages of the docking studies for only one of these proteases. Among the two falcipains, the distal end of the S2 pocket in falcipain-3 is proposed to be narrower than that in falcipain-2.[18] Thus it appears that compounds binding to falcipain-3 are likely to fit falcipain-2 as well, while the reverse may not be always true. Hence for the first two stages, the database was docked in falcipain-3. Molecules retained at the end of the second stage were finally docked in both the falcipains using the standard mode settings in GOLD. For all the docking stages, 10 representative vinyl sulfone inhibitors of falcipains[12] were included as positive controls. These inhibitors were placed among the top 20 ranked ligands by the docking protocol.

As shown in Figure 3, starting with about 60 000 molecules for the first stage of docking, a total of 5500 top ranking molecules were selected for the next stage. Next, the top 1500 molecules selected based on the second stage of docking were screened against both falcipain-2 and falcipain-3. It is interesting to note that the top 100 molecules in the falcipain-3 run were among the top 200 molecules in the falcipain-2 run, with 78% of molecules in the top 100. The top 200 common hits for the two enzymes were visually inspected based on the following criteria: (1) reasonable internal geometry of the ligand in the binding pose; (2) proximity of electrophilic center of the ligand (if any) to the catalytic cysteine in the proposed binding mode; (3) complementarity between ligand and protein surfaces in terms of spatial occupancy and contacts in hydrophilic/hydrophobic regions. Of the 100 compounds so selected, only 84 compounds could be procured and subjected to biological evaluations. The structures of the procured compounds were verified by ^{1}H NMR and more than 90% purity for all compounds was estimated based on the ^{1}H NMR data. The average pair wise Tanimoto similarity index[24] of 0.29 ± 0.09 for the selected structures clearly suggested that the compounds were structurally diverse. These compounds were then examined for in vitro enzyme inhibition in assays against falcipain-2 and falcipain-3. Since *L. donovani* cysteine protease is highly homologous to the malarial cysteine proteases, as mentioned earlier, the compounds were also tested for their ability to inhibit this enzyme. In addition, in vitro parasite growth inhibition assays were conducted against cultured *P. falciparum* and against *L. donovani* promastigotes.

Biological Evaluation. In vitro inhibition studies for falcipain-2 and falcipain-3 were conducted in a similar fashion as described previously.[12] Equal amounts of recombinant enzymes were incubated for 30 min at room temperature in 100 mM sodium acetate, pH 5.5, 10 mM dithiothreitol with different concentrations of tested inhibitors diluted from stocks 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-methyl-coumarin) in the same buffer was added to final concentration of 25 μM. Fluorescence was monitored for 15 min at room temperature in a Labsystems Fluoroskan Ascent spectrofluorometer. IC_{50} values were determined from plots of percents of activity over the compound concentration using GraphPad Prism software.

Antimalarial activity of the compounds was determined in vitro on chloroquine sensitive (D6, Sierra Leone) and resistant (W2, IndoChina) strains of *P. falciparum*. The 96-well microplate assay is based on evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity.[25] The appropriate dilutions of the compounds were prepared in DMSO and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set up in clear flat-bottomed 96-well plates. The plates were placed into a humidified chamber and flushed with a gas mixture of 90% N_2, 5% CO_2, and 5% O_2. The cultures were incubated at 37 °C for 48 h. Growth of the parasite in each well was determined by the pLDH assay using the Malstat reagent.[25] Controls containing only medium and only erythrocytes were also setup in each plate. The standard antimalarial agents, chloroquine and artemisinin, were used as the positive controls while DMSO was tested as the negative control.

For the in vitro testing of the compounds against leishmania cysteine protease (LCP) a semipurified preparation of leupeptin/E64 sensitive cysteine protease from *L. donovani* promastigotes was used. The enzyme was assayed spectrophotometrically by activity toward

Table 1. Biological Evaluation of Compounds Selected Based on Virtual Screening[a]

compound	FP-2[b] IC_{50} (μM)	FP-3[c] IC_{50} (μM)	D6[d] EC_{50} (μM)	W2[d] EC_{50} (μM)	LCP[e] IC_{50} (μM)	*L. donovani*[f] IC_{50} (μM)	cytotoxicity[g] IC_{50} (μM)
1	1.0	4.9	34.8	41.0	72.0	NA	NC
2	2.1	7.5	12.3	20.9	65.3	NA	NC
3	2.2	27.8	31.1	34.8	NA	NA	NC
4	3.9	13.9	63.9	63.9	NA	NA	NC
5	4.6	5.8	6.9	6.6	112.9	8.8	28.9
6	6.2	12.0	61.1	61.1	NA	NA	NC
7	6.2	11.8	>60.0	>60.0	NA	NA	NC
8	7.0	10.5	3.7	6.2	NA	NA	NC
9	7.5	5.4	13.5	12.1	NA	NA	NC
10	7.5	5.4	62.7	50.1	NA	NA	NC
11	12.4	29.6	>62.2	>62.2	NA	NA	NC
12	40.9	62.2	56.3	59.6	62.6	NA	NC
13	5.7	>50.0	59.3	67.2	NA	NA	NC
14	20.8	>50.0	46.6	74.9	NA	NA	NC
15	22.5	>50.0	52.0	50.1	NA	11.5	NC
16	26.6	>50.0	68.9	68.9	NA	NA	NC
17	27.3	>50.0	68.5	43.2	129.5	NA	NC
18	31.1	>50.0	>59.9	>59.9	NA	NA	NC
19	53.4	>50.0	52.7	40.9	NA	NA	NC
20	54.6	>50.0	4.8	5.3	NA	3.6	NC
21	59.9	>50.0	>78.4	>78.4	NA	NA	NC
22	63.4	>50.0	32.5	67.3	NA	NA	NC
23	>50.0	>50.0	2.5	3.3	NA	1.9	NC
24	>50.0	>50.0	35.1	37.7	NA	0.3	NC
chloroquine	-	-	0.009	0.28	-	-	-
artemisinin	-	-	0.012	0.017	-	-	-
pentamidine	-	-	-	-	-	2.0	-
amphotericine b	-	-	-	-	-	0.16	-
pepstatin	-	-	-	-	0.16	-	-
E-64	0.015	0.035	-	-	-	-	-

[a] NA: Not active, NC: Not cytotoxic at the highest concentration tested (23.8 μg/mL). [b] Falcipain-2. [c] Falcipain-3. [d] Chloroquine-sensitive (D6) and -resistant (W2) strains of *Plasmodium falciparum*. [e] *Leishmania donovani* cysteine protease. [f] Antileishmanial activity against *Leishmania donovani* promastigotes. [g] Cytotoxicity in VERO (monkey kidney fibroblast) cells.

BenzPFRNan as described earlier.[26] Antileishmanial activity of the compounds was tested in vitro on a culture of *L. donovani* promastigotes. In a 96-well microplate assay compounds with appropriate dilution were added to the leishmania promastigotes culture (2×10^6 cell/mL). The plates were incubated at 26 °C for 72 h and growth of leishmania promastigotes was determined by Alamar blue assay.[27] Pentamidine and amphotericin B were tested as the standard antileishmanial agents. All the compounds were simultaneously tested for cytotoxicty in VERO (monkey kidney fibroblast) cells by Neutral Red assay.[28] The IC_{50} value for each compound was computed from the growth inhibition curve.

Results and Discussion

The results of the biological evaluations are presented in Table 1. Structures of the active compounds are shown in Figure 4. A total of 24 interesting compounds were identified showing inhibition of one or more of the cysteine proteases tested along with antiplasmodial and/or antileishmanial activity.

In the case of the falcipains, a total of 22 compounds appear to inhibit either or both of the enzymes with IC_{50} values ranging from 1 to 63 μM, with 12 compounds showing dual activity. When results differed, inhibition was almost always greater against falcipain-2, as predicted by modeling (see above). In view of the fact that the crystal structures of the enzymes were not available and the virtual screening was performed using homology models, the results are quite positive, with a success rate of 29% (24 active compounds out of 84 tested). Also, the strategy of screening the database only against falcipain-3 in the first two stages followed by both the enzymes in the last stage appears to be appropriate in this case. It provides an efficient approach in terms of computation time to identify broad spectrum inhibitors of homologous proteins.

As an example, the proposed binding mode of compound **1** in the homology model of falcipain-2 as obtained by the docking study is illustrated in Figure 5. The inhibitor appears to fit the binding pocket well, showing strong *van der Waals* interactions with the binding site residues and $\pi-\pi$ interactions with Trp 206 in the S1′ pocket. One of the carboxylate groups of the inhibitor shows hydrogen bonding interactions with the backbone amide proton of Ile 85 in the S2 pocket. Most other inhibitors also appear to form one or more hydrogen bonds with the binding site residues. One of the possible approachs to enhance the cellular permeability of compound **1** would be its conversion into a prodrug by esterification of the two carboxylic acid groups. This in turn might improve its antiplasmodial activity.

It is interesting to note that compounds **1**, **2**, **5**, and **12** show broad spectrum cysteine protease inhibition. It is encouraging that inhibitors of *L. donovani* cysteine protease could be identified in this study primarily on the basis of screening against the homologous plasmodial cysteine proteases. This also supports the proposition that the cysteine protease from *L. donovani* is structurally similar to the plasmodial cysteine proteases.

A total of 20 compounds exhibit reasonable antiplasmodial activity and five compounds show antileishmanial activity. It is surprising that the antilplasmodial

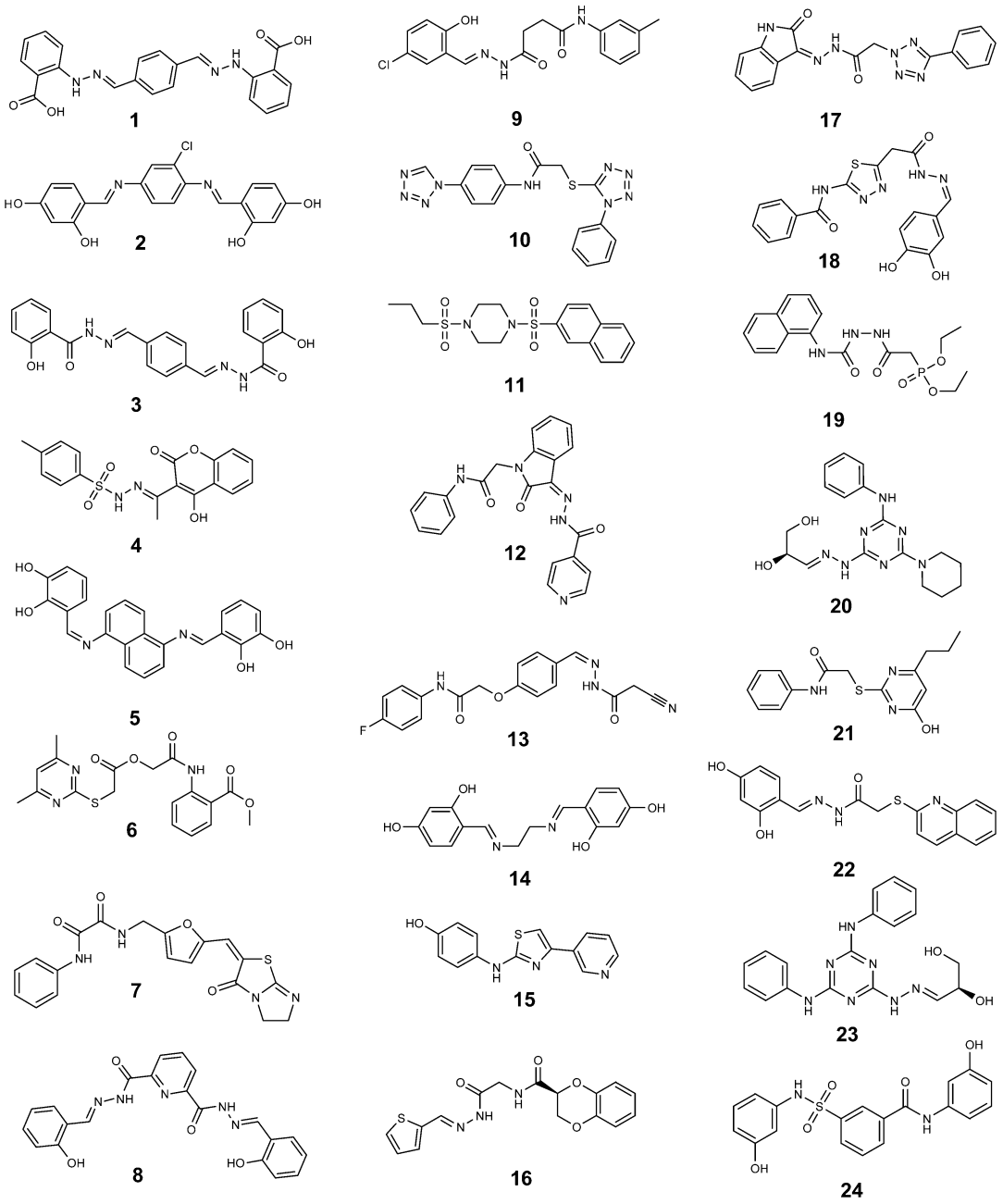

Figure 4. Structures of parasitic cysteine protease inhibitors identified using virtual screening.

EC_{50} value for compound **8** is lower than the IC_{50} value for falcipain-2 and falcipain-3 inhibition. This might be due to selective uptake of the compound by the organism or involvement of some additional unknown mechanisms of action. Also, compounds **20**, **23**, and **24** show antiplasmodial and antileishmanial activity without significantly inhibiting the corresponding cysteine proteases. Once again this suggests a possibility of alternate mechanism of action for these compounds. It is also noteworthy that except for compound **5**, none of the compounds show cytotoxicity.

It appears that 16 out of the 24 active compounds possess bioreactive moieties such as hydrazones or imines. Although not intentional, it was not surprising to see such functional groups in the active compounds since compounds with electron-deficient centers such as the vinyl sulfones and the reversible ketone-based inhibitors (Figure 1) have been shown to be potent cysteine protease inhibitors.[9,12,14] At the same time, this observation might also raise a question: "Is the activity of the hydrazone and the imine containing compounds merely due to the presence of such reactive species?" Analysis of the activity data of the 84 compounds revealed that out of 48 compounds having such bioreactive species, 32 were inactive. Thus, it appears that the activity of the hydrazone and imine compounds is not merely due to the presence of these potentially reactive moieties but is a result of overall interaction and shape complementarity of the compounds with the active site of the enzyme. Additionally, some of the entropy and solvent related issues, which could not be accounted for in the present study, may also play an important role in governing the activity of such compounds. It should also be stated here that hydrazone containing compounds have been shown to be orally bioavailable in animal models.[29]

One interesting outcome of the present study is the finding that it is possible to identify common nonpeptide

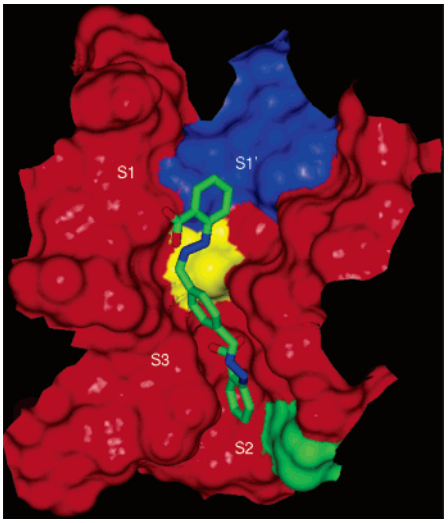

Figure 5. Proposed binding mode of compound **1** in homology model of falcipain-2.[17] The compound is shown as sticks colored by atom type while the binding site of the enzyme is shown as a Connolly surface. The surface of the catalytic cysteine (Cys 42) is colored yellow, Gln 36 and Trp 206 blue, and Asp 234 green. The subsites are labeled as S1, S1′, S2, and S3.

inhibitors of several homologous parasitic cysteine proteases, and that this can be achieved without individually screening the entire database against each of the proteases. Thus, this offers an efficient, time saving approach based on virtual screening. It should also be noted that the inhibitors identified in this study have IC_{50} values in the micromolar range and thus may not be suitable for use as drugs themselves. However, these compounds do provide much needed druglike nonpeptide leads against parasitic cysteine proteases. Another issue that remains to be addressed is the selectivity of these inhibitors for the parasitic cysteine proteases over the human cysteine proteases such as cathepsin B, cathepsin K, and cathepsin L. Further studies are underway to assess the enzyme isoform selectivity of the current hits. On the basis of these results, the hits would be further optimized to obtain potent and selective nanomolar inhibitors of protozoal cysteine proteases for development into broad spectrum antiprotozoal drugs. For this purpose, we also plan to develop focused libraries of some of the broad spectrum inhibitors identified in the study.

Conclusion

In summary, the ChemBridge database consisting of some 241 000 compounds was screened against homology models of plasmodial cysteine proteases to identify broad spectrum parasitic cysteine protease inhibitors. A total of 24 druglike nonpeptide inhibitors were identified of which twelve compounds showed dual activity against falcipain-2 and falcipain-3. Four compounds were found to inhibit both the plasmodial cysteine proteases as well as the *L. donovani* cysteine protease. The leads identified in the present study can be optimized into broad spectrum antiprotozoal drugs.

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References

(1) Godal, T.; Nagera, J. Tropical diseases. *WHO Division of Control in Tropical Disease*; World Health Organisation: Geneva, Switzerland, 1990; pp 12−13.
(2) Ashford, R., The leishmaniases as emerging and reemerging zoonoses. *Int. J. Parasitol*. **2000**, *30*, 1269−1281.
(3) WHO. The leishmaniases and *Leishmania*/HIV co-infections. *World Health Organisation. Fact Sheet Number 116*, 2000 (http://www.who.int/mediacentre/factsheets/fs116/en/).
(4) Kirchhoff, L. V. Current Concepts: American Trypanosomiasis (Chagas' Disease) − A Tropical Disease Now in the United States. *N. Engl. J. Med*. **1993**, *329*, 639−644.
(5) Loiseau, P. M.; Bories, C. Recent strategies for the chemotherapy of visceral leishmaniasis. *Curr. Opin. Infect. Dis*. **1999**, *12*, 559−564.
(6) Brost, P.; Ouellette, M. New Mechanisms of Drug Resistance in Parasitic Protozoa. *Annu. Rev. Microbiol*. **1995**, *49*, 427−460.
(7) Breman, J. G. CR1 density polymorphism on erythrocytes of falciparum malaria patients in Thailand. *Am. J. Trop. Med. Hyg*. **2001**, *64*, 1−5.
(8) Butler, D.; Maurice, J.; O'Brien, C. Time to put malaria control on the global agenda. *Nature* **1997**, *386*, 535−536.
(9) Sajid, M.; McKerrow, J. Cysteine proteases of parasitic organisms. *Mol. Biochem. Parasitol*. **2002**, *120*, 1−21.
(10) Shenai, B. R.; Sijwali, P. S.; Singh, A.; Rosenthal, P. J. Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. *J. Biol. Chem*. **2000**, *275*, 29000−29010.
(11) Sijwali, P. S.; Shenai, B. R.; Gut, J.; Singh, A.; Rosenthal, P. J. Expression and characterization of the Plasmodium falciparum hemoglobinase falcipain-3. *Biochem. J*. **2001**, *360*, 481−489.
(12) Shenai, B. R.; Lee, B. J.; Alvarez-Hernandez, A.; Chong, P. Y.; Emal, C. D.; Neitz, R. J.; Roush, W. R.; Rosenthal, P. J. Structure−Activity Relationships for Inhibition of Cysteine Protease Activity and Development of Plasmodium falciparum by Peptidyl Vinyl Sulfones. *Antimicrob. Agents Chemother*. **2003**, *47*, 154−160.
(13) Engel, J. C.; Doyel, P. S.; Hseih, I.; McKerrow, J. H. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. *J. Exp. Med*. **1998**, *188*, 725−734.
(14) Huang, L.; Lee, A.; Ellman, J. A. Identification of potent and selective mechanism-based inhibitors of the cysteine protease cruzain using solid-phase parallel synthesis. *J. Med. Chem*. **2002**, *45*, 676−684.
(15) Cazzulo, J. Proteinases of Trypanosoma Cruzi: Potential Targets for the Chemotherapy of Chagas Disease. *Curr. Top. Med. Chem*. **2002**, *2*, 1261−1271.
(16) Brinen, L. S.; Hansell, E.; Cheng, J.; Roush, W. R.; McKerrow, J. H.; Fletterick, R. J. A target within the target: probing cruzain's P1′ site to define structural determinants for the Chagas disease protease. *Struct. Fold. Des*. **2000**, *8*, 831−840.
(17) Sabnis, Y.; Rosenthal, P. J.; Desai, P.; Avery, M. A. Homology modeling of falcipain-2: validation, de novo ligand design and synthesis of novel inhibitors. *J. Biomol. Struct. Dyn*. **2002**, *19*, 765−774.
(18) Sabnis, Y.; Desai, P.; Rosenthal, P. J.; Avery, M. A. Probing the structure of falcipain-3, a cysteine protease from Plasmodium falciparum: comparative protein modeling and docking studies. *Prot. Sci*. **2003**, *12*, 501−509.
(19) Walters, P. W.; Stahl, T. M.; Murcko, M. A. Virtual screening − an overview. *Drug Discovery Today* **1998**, *3*, 160−178.
(20) Bissantz, C.; Folkers, G.; Rognan, D. Protein-Based Virtual Screening of Chemical Databases. 1. Evaluation of Different Docking/Scoring Combinations. *J. Med. Chem*. **2000**, *43*, 4759−4767.
(21) Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *J. Med. Chem*. **1997**, *267*, 727−748.
(22) Egan, W. J.; Merz, K. M., Jr.; Baldwin, J. J. Prediction of drug absorption using multivariate statistics. *J. Med. Chem*. **2000**, *43*, 3867−3877.
(23) Lipinski, C.; Lombardo, F.; Dominy, B.; Feeney, P. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev*. **1997**, *23*, 3−25.

(24) Patterson, D. E.; Cramer, R. D.; Ferguson, A. M.; Clark, R. D.; Weinberger, L. E. Neighborhood Behavior: A Useful Concept for Validation of Molecular Diversity Descriptors. *J. Med. Chem*. **1996**, *39*, 3049−3059.
(25) Makler, M. T.; Ries, J. M.; Williams, J. A.; Bancroft, J. E.; Piper, R. C.; Gibbins, B. L.; Hinriches, D. J. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am. J. Trop. Med. Hyg*. **1993**, *48*, 739−741.
(26) Sanderson, S. J.; Pullock, K. G. J.; Hilley, J. D.; Meldal, M.; Hilaire, P. S.; Juliano, M. A.; Juliano, L.; Mottram, J. C.; Coombs, G. H. Expression and characterization of a recombinant cysteine proteinase of Leishmania mexicana. *Biochem J.* **2000**, *347*, 383−388.
(27) Mikus, J.; Steverding, D. A simple colorimetric method to screen drug cytotoxicity against Leishmania using the dye Alamar Blue. *Parasitol Int.* **2000**, *48*, 265−269.
(28) Babich, H.; Borenfreund, E. Cytotoxicity of T2 toxin and its metabolites with the neutral red cell viability assay. *App. Environ Microbiol*. **1991**, *57*, 2101−2103.
(29) Madsen, P.; Ling, A.; Plewe, M.; Sams, C. K.; Knudsen, L. B.; Sidelmann, U. G.; Ynddal, L.; Brand, C. L.; Andersen, B.; Murphy, D.; Teng, M.; Truesdale, L.; Kiel, D.; May, J.; Kuki, A.; Shi, S.; Johnson, M. D.; Teston, K. A.; Feng, J.; Lakis, J.; Anderes, K.; Gregor, V.; Lau, J. Optimization of alkylidene hydrazide based human glucagon receptor antagonists. Discovery of the highly potent and orally available 3-cyano-4-hydroxybenzoic acid [1-(2,3,5,6-tetramethylbenzyl)-1H-indol-4-ylmethylene]hydrazide. *J. Med. Chem.* **2002**, *45*, 5755−5775.

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