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# Aziridine-2,3-dicarboxylate inhibitors targeting the major cysteine protease of *Trypanosoma brucei* as lead trypanocidal agents

Radim Vicik,[a] Verena Hoerr,[a,b] Melanie Glaser,[b] Martina Schultheis,[d] Elizabeth Hansell,[c] James H. McKerrow,[c] Ulrike Holzgrabe,[a] Conor R. Caffrey,[c] Alicia Ponte-Sucre,[d,e] Heidrun Moll,[d] August Stich[b] and Tanja Schirmeister[a,*]

[a]*Institute of Pharmacy and Food Chemistry, University of Wuerzburg, Am Hubland, D-97074 Wuerzburg, Germany*
[b]*Medical Mission Institute Wuerzburg, Department of Tropical Medicine, Hermann-Schell-Str. 7, D-97074 Wuerzburg, Germany*
[c]*Sandler Center for Basic Research in Parasitic Diseases, QB3 Building, Box 2550, 1700 4th Street, University of California, San Francisco, CA 94143, USA*
[d]*Institute for Molecular Infection Biology, University of Wuerzburg, Roentgenring 11, D-97070 Wuerzburg, Germany*
[e]*Faculty of Medicine, Laboratory of Molecular Physiology, Universidad Central de Venezuela, Venezuela*

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**Abstract**—The protozoan parasite *Trypanosoma brucei* causes Human African trypanosomiasis, which is fatal if left untreated. Due to the toxicity of currently used drugs and emerging drug resistance, there is an urgent need for novel therapies. The major trypanosome papain-like cysteine protease expressed by the parasite (e.g., rhodesain in *T. b. rhodesiense*) is considered an important target for the development of new trypanocidal drugs. Series of aziridine-2,3-dicarboxylate-based cysteine protease inhibitors have been tested, most of them inhibiting rhodesain in the low micromolar range. Among these, only dibenzyl aziridine-2,3-dicarboxylates display trypanocidal activity being equipotent to the drug eflornithine. The Leu-Pro-containing aziridinyl tripeptides **13a**–**f** are the most promising as they are not cytotoxic to macrophages up to concentrations of 125 μM.


The burden of tropical diseases of humans caused by protozoan parasites is large, not just in terms of mortality and morbidity, but also by impeding economic growth and prosperity.[1] Diseases such as malaria (caused by various species of *Plasmodium*), Chagas' disease (*Trypanosoma cruzi*) and African trypanosomiasis (sleeping sickness in humans caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*) count as the most severe.[2,3] African trypanosomes are flagellated protozoa living extracellularly in blood and tissues of the mammalian hosts and are transmitted by the bite of tsetse flies (*Glossina* spp.). In late-stage African trypanosomiasis, the disease is characterized by somnolence and coma, and is invariably fatal, if untreated.

Chemotherapy depends on drugs developed decades ago that lack adequate efficacy and cause serious side effects. Further, the emergence of drug-resistant *Trypanosoma* strains has been reported.[2] Thus, new strategies to treat African trypanosomiasis are required.

One promising strategy to develop new anti-trypanosomal drugs has been to target the major cysteine protease of the parasite.[3] This enzyme is termed rhodesain[4] in *T. b. rhodesiense* and brucipain[5] in *T. b. brucei* (infective to animals). Both belong to the cathepsin L subfamily of the papain-like (clan CA, family C1) cysteine proteases[6] and are nearly identical (98.4%) in protein sequence. Cysteine protease inhibitors have been shown to kill

*Abbreviations*: Azet, azetidine-2-carboxylic acid; Azi, aziridine-2,3-dicarboxylic acid; Azy, aziridine-2-carboxylic acid; Bio, biotin; Caa, cyclic amino acid; Hxa, 6-amino hexanoic acid; Ini, isonipecotic acid; MIC, minimal inhibition concentration; Nip, nipecotic acid; Pip, pipecolic acid.

*Keywords*: Aziridine; Cysteine protease inhibitor; Rhodesain; Sleeping sickness; *Trypanosoma brucei*.

* Corresponding author. Tel.: +49 931 888 5440; fax: +49 931 888 5494; e-mail: schirmei@pzlc.uni-wuerzburg.de

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African trypanosomes in vitro and in animal models of the disease.[3] Examples of effective inhibitors include thiosemicarbazones,[7–9] isatins,[8] and peptides containing aldehydes,[10] diazomethyl ketones,[10,11] allyl sulfones,[12] vinyl sulfones,[3,13] and fluoromethyl ketones.[3] In addition, for the orthologous protease in *T. cruzi* (cruzain or cruzipain) peptidyl-α′,β′-epoxy ketones,[14] peptidyl vinyl sulfones,[3,13] epoxysuccinyl peptides,[15] peptidyl hydroxymethyl ketones,[16] chalcones, and acyl hydrazides,[17,18] as well as peptidyl fluoromethyl ketones,[3,19] are potent in vitro and in vivo inhibitors. Cruzain is the only trypanosomal cysteine protease for which an X-ray crystallographic structure has been solved.[16,20–22]

Whereas thiosemicarbazones are reversible inhibitors, vinyl sulfones, epoxy ketones, and epoxysuccinates are irreversible inhibitors containing an electrophilic moiety that is attacked by the negatively charged cysteine thiolate in the enzyme active site. Similarly, aziridine-2,3-dicarboxylates analogous to the epoxysuccinates, are also irreversible cysteine protease inhibitors.[23–25]

Recently we reported the syntheses of aziridine-2,3-dicarboxylates containing either a Boc-Leu(Gly)-Caa (Caa = cyclic amino acid) or a Boc-Phe-Ala sequence attached to the aziridine nitrogen (Fig. 1), and the activity of these compounds against the CAC1 cysteine proteases cathepsins L and B.[26]

The compound series (Table 1) consists of 50 aziridine-2,3-dicarboxylates, including 13 dipeptides of the general structure X-Caa-Azi(OEt)$_2$ (compds **1**–**6**), the dipeptide Cbz-(*S*)-Leu-(*R*,*R*)-Azi(OEt)$_2$ (**7c**) (for structures, see Supplementary data), 27 aziridinyl tripeptides of the general structure Boc-Leu(Gly)-Caa-Azi(OR)$_2$ (compds **8**–**18** and **21**–**25**), 8 Boc-Phe-Ala containing aziridine-2,3-dicarboxylates (Boc-Phe-Ala-Azi(OR)$_2$, compds **19**–**20**), as well as the biotinylated aziridine Bio-Hxa-(*R*,*R*)-Azi(OEt)$_2$ **(26)**.[27]

These compounds had activity against cathepsins L and B, but compounds containing a cyclic amino acid (Caa) displayed higher selectivities for cathepsin L over cathepsin B.[26] Using both biotinylated and desthiobiotinylated model compounds, we proved the irreversible and active site-directed inhibition mechanism.[26,27] Also, docking experiments proposed binding modes with cathepsin L in which the inhibitors span across both the prime and the non-prime substrate binding sites thereby explaining the high cathepsin L selectivity.[26]

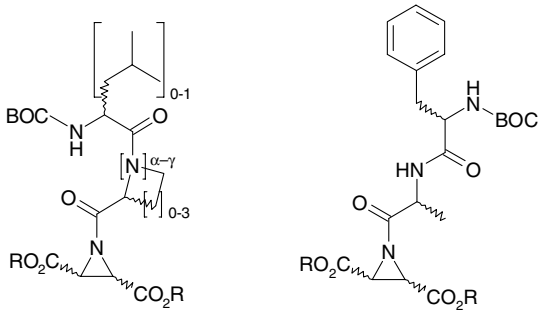


**Figure 1.** Schematic representation of the Boc-Gly-Caa-, Boc-Leu-Caa- (left), and Boc-Phe-Ala-containing (right) aziridinyl peptides; Caa, cyclic amino acid; Azy, aziridine-2-carboxylic acid, $n = 0$; Azet, azetidine-2-carboxylic acid, $n = 1$; Pro, proline, $n = 2$; Pip, pipecolic acid, $n = 3$, N-α; Nip, nipecotic acid, $n = 3$, N-β; Ini, isonipecotic acid, $n = 3$, N-γ; R = H, Et, Bn.

We now extend our inhibition studies to include the major cathepsin L-like protease of *T. b. rhodesiense*, rhodesain, and compare enzyme inhibition with the parasiticidal activity against the animal pathogenic model parasite *T. b. brucei* in vitro. Furthermore, the cytotoxicity of the inhibitors was studied on the macrophage cell line J774.1.

The data generated in vitro for the aziridine-2,3-dicarboxylates are compared to those of five drugs used in treatment of trypanosomiasis (pentamidine, suramin, eflornithine, melarsoprol, and nifurtimox).[28]

The inhibitors[29] were prepared as described recently.[26]

The activity of the inhibitors on rhodesain was evaluated in fluorometric microplate assays using the substrate Cbz-Phe-Arg-AMC. As found for other cysteine proteases,[26,27] in most cases only non-time-dependent inhibition could be observed (Fig. 2).

Time-dependent inhibition (Fig. 3) of rhodesain was detected with the Boc-Phe-Ala-containing dibenzyl ester **19b** and the diacid **20e**. The following inhibition constants were obtained:

Compound **19b**: $k_i = 0.044 \pm 0.002\ \text{min}^{-1}$, $K_i = 1.2 \pm 0.3\ \mu\text{M}$, $k_{2nd} = 36{,}666\ \text{M}^{-1}\ \text{min}^{-1}$;

Compound **20e**: $k_i = 0.021 \pm 0.002\ \text{min}^{-1}$, $K_i = 0.11 \pm 0.03\ \mu\text{M}$, $k_{2nd} = 190{,}909\ \text{M}^{-1}\ \text{min}^{-1}$ (Fig. 3).

Very low first order rates of inhibition ($k_i$) were found in both cases, which are an intrinsic property of the electrophilic aziridine-2,3-dicarboxylate moiety.[31,32]

Comparable to results obtained with cathepsin L,[26] only few aziridinyl dipeptides inhibited rhodesain (data not shown, see Supplementary data), whereas most of the aziridinyl tripeptides show $K_i$ values in the low micromolar range (Table 1). Exceptions are the diacids **21a**, **22b**–**25a**, and compound **19e** which did not inhibit rhodesain. Interestingly, this latter compound exhibits trypanocidal activity and cytotoxicity against macrophages suggesting an unspecific mechanism of action. Within the diacid series only inhibitors derived from the peptide Phe-Ala (**20**) and inhibitor **22a** inhibited rhodesain. The latter, however, is not active against the parasite. The same holds true for most diethyl esters (**8a**–**10c**, **26**) whereas most dibenzyl esters display both enzyme inhibition and trypanocidal activity. Exceptions within the dibenzyl ester series are the Gly-Pro-containing inhibitors **14a** and **14b**.

In other words, only rhodesain inhibitors containing a dibenzyl aziridine-2,3-dicarboxylate moiety also exhibit anti-trypanosomal activity. This phenomenon is likely

**Table 1.** Rhodesain inhibition, trypanocidal activities, and cytotoxicity of protease inhibitors of the general structure X-Leu(Gly)-Caa-Azi $(OR)_2$ or Boc-Phe-Ala-Azi$(OR)_2$, and of standard drugs used to treat trypanosomiasis

| Compound | Peptide sequence | Configuration Azi | R | Rhodesain $K_i$[a] (μM) | *T.b.b.* $IC_{50}$[b] (μM) | Cytotoxicity (J774.1) $IC_{50}$[b] (μM) | SI[c] |
|---|---|---|---|---|---|---|---|
| **8a** | Boc-(*S*)-Leu-(*S*)-Pro | *S*,*S* | Et | 3.1 ± 1.6 | >100 | >100 | |
| **9a+b** | Boc-(*S*)-Leu-(*R*+*S*)-Nip | *S*,*S* | Et | 1.0 ± 0.1 | >100 | >100 | |
| **9c+d** | Boc-(*S*)-Leu-(*R*+*S*)-Nip | *R*,*R* | Et | 1.9 ± 0.4 | >100 | >100 | |
| **10a** | Boc-(*S*)-Leu-Ini | *S*,*S* | Et | 1.4 ± 0.2 | >100 | >100 | |
| **10c** | Boc-(*S*)-Leu-Ini | *R*,*R* | Et | 3.3 ± 0.2 | >100 | >100 | |
| **11c+d** | Boc-(*S*)-Leu-(*R*+*S*)-Azet | *R*,*R* | Et | 3.1 ± 0.2 | 20.6 ± 12.4 | >100 | >4.8 |
| **12a+b** | Boc-(*S*)-Leu-(*R*+*S*)-Azet | *S*,*S* | Bn | 0.8 ± 0.2 | 30.8 ± 1.2 | 37.2 ± 0.29 | 1.2 |
| **13a** | Boc-(*S*)-Leu-(*S*)-Pro | *S*,*S* | Bn | 2.3 ± 0.3 | 11.1 ± 0.2 | >125[f] | >11.3 |
| **13b** | Boc-(*S*)-Leu-(*R*)-Pro | *S*,*S* | Bn | 1.2 ± 0.2 | 31.4 ± 1.2 | >100 | >3.2 |
| **13c** | Boc-(*S*)-Leu-(*S*)-Pro | *R*,*R* | Bn | 0.5 ± 0.1 | 10.9 ± 4.0 | >125[f] | >11.5 |
| **13e** | Boc-(*R*)-Leu-(*S*)-Pro | *S*,*S* | Bn | 1.1 ± 0.2 | 34.3 ± 5.2 | >100 | >2.9 |
| **13f** | Boc-(*R*)-Leu-(*R*)-Pro | *S*,*S* | Bn | 2.8 ± 0.3 | 35.2 ± 7.0 | >100 | >2.8 |
| **14a** | Boc-Gly-(*S*)-Pro | *S*,*S* | Bn | 3.1 ± 0.8 | >100 | >100 | |
| **14b** | Boc-Gly-(*R*)-Pro | *S*,*S* | Bn | 1.6 ± 0.3 | >100 | >100 | |
| **15a** | Boc-Gly-(*S*)-Pip | *S*,*S* | Bn | 0.7 ± 0.1 | 27.6 ± 3.3 | nd | |
| **16a+b** | Boc-(*S*)-Leu-(*R*+*S*)-Nip | *S*,*S* | Bn | 0.6 ± 0.1 | 25.6 ± 3.0 | >100 | >3.9 |
| **16e+f** | Boc-(*R*)-Leu-(*R*+*S*)-Nip | *S*,*S* | Bn | 0.7 ± 0.1 | 34.0 ± 9.7 | >100 | >2.9 |
| **17a+b** | Boc-Gly-(*R*+*S*)-Nip | *S*,*S* | Bn | 1.1 ± 0.1 | 36.4 ± 17.8 | >100 | >2.7 |
| **18a** | Boc-(*S*)-Leu-(*S*)-Azy | *S*,*S* | Bn | 0.3 ± 0.1 | 25.7 ± 1.2 | 50.0 ± 12.0 | 1.9 |
| **18e** | Boc-(*R*)-Leu-(*S*)-Azy | *S*,*S* | Bn | 1.6 ± 0.3 | 28.3 ± 3.3 | >100 | >3.5 |
| **19a** | Boc-(*S*)-Phe-(*S*)-Ala | *S*,*S* | Bn | 1.9 ± 0.1 | 26.7 ± 4.6 | >100 | >3.7 |
| **19b** | Boc-(*S*)-Phe-(*R*)-Ala | *S*,*S* | Bn | 1.2 ± 0.3 | 30.5 ± 3.5 | >100 | >3.2 |
| **19e** | Boc-(*R*)-Phe-(*S*)-Ala | *S*,*S* | Bn | ni | 34.3 ± 3.2 | 43.7 ± 0.0[d] | 1.3 |
| **19f** | Boc-(*R*)-Phe-(*R*)-Ala | *S*,*S* | Bn | 3.9 ± 1.6 | 25.8 ± 4.8 | >100 | >3.8 |
| **20a** | Boc-(*S*)-Phe-(*S*)-Ala | *S*,*S* | H | 0.5 ± 0.1 | nd | nd | |
| **20b** | Boc-(*S*)-Phe-(*R*)-Ala | *S*,*S* | H | 0.8 ± 0.2 | nd | nd | |
| **20e** | Boc-(*R*)-Phe-(*S*)-Ala | *S*,*S* | H | 0.1 ± 0.03 | nd | nd | |
| **20f** | Boc-(*R*)-Phe-(*R*)-Ala | *S*,*S* | H | 5.0 ± 0.3 | nd | nd | |
| **21a** | Boc-(*S*)-Leu-(*S*)-Pro | *S*,*S* | H | ni | nd | nd | |
| **22a** | Boc-Gly-(*S*)-Pro | *S*,*S* | H | 0.9 ± 0.1 | >100 | >100 | |
| **22b** | Boc-Gly-(*R*)-Pro | *S*,*S* | H | ni | >100 | >100 | |
| **23a+b** | Boc-(*S*)-Leu-(*R*+*S*)-Nip | *S*,*S* | H | ni | nd | nd | |
| **23e+f** | Boc-(*R*)-Leu-(*R*+*S*)-Nip | *S*,*S* | H | ni | nd | nd | |
| **24a+b** | Boc-Gly-(*R*+*S*)-Nip | *S*,*S* | H | ni | nd | nd | |
| **25a** | Boc-(*S*)-Leu-Ini | *S*,*S* | H | ni | nd | nd | |
| **26** | Bio-Hxa | *R*,*R* | Et | 0.6 ± 0.1 | >100 | >100 | |
| **S1** | Pentamidine diisethionate | | | 27.9 ± 1.6[e] | 0.0029 ± 0.0002 | 41.6 ± 11.1 | 14,345 |
| **S2** | Suramin Na | | | 31.5 ± 10.3[e] | 0.31 ± 0.05 | >1000 | >3,225 |
| **S3** | Eflornithine HCl | | | 30.7 ± 0.5[e] | 22.9 ± 4.6 | >1000 | >44 |
| **S4** | Melarsoprol | | | 94.3 ± 1.5[e] | 0.0026 ± 0.0004 | 4.3 ± 1.0 | 1,653 |
| **S5** | Nifurtimox | | | nd | 3.4 ± 0.4 | >200[f] | >58 |

ni, no inhibition at 140 μM inhibitor concentration; nd, not determined.
*T.b.b.*, *Trypanosoma brucei brucei*; Azi, aziridine-2,3-dicarboxylate; Bio, biotin; Hxa, 6-amino hexanoic acid.
[a] Mean values of at least two independent assays.
[b] Values at 48 h, those at 72 h do not differ significantly, mean values of at least two independent assays.
[c] Selectivity index = $IC_{50}$ macrophages/$IC_{50}$ *T.b.b.*
[d] Value after 72 h, after 48 h: $IC_{50}$ > 100 μM.
[e] S1–S5, standard drugs used against trypanosomiasis, % inhibition of rhodesain at 200 μM concentration of standard drug, mean values of at least 2 independent assays.
[f] Higher inhibitor concentrations lead to precipitation.

connected to the compounds' lipophilicity leading to enhanced cell permeability. This correlation has also been observed with biotinylated aziridines:[27] although the diethyl (compd. **26**) and the related dibenzyl ester of this biotinylated aziridine-2,3-dicarboxylate show equipotent inhibition of the *P. falciparum* cysteine protease falcipain 2, only the dibenzyl ester exhibits anti-plasmodial activity.[27]

The trypanocidal activity of the rhodesain inhibitors ranges from $IC_{50}$ = 11 μM (**13a** and **13c**) to 37 μM (**17a+b**), which correspond to the concentration obtained with eflornithine (**S3**, $IC_{50}$ = 22.9 μM), the drug currently used as a first-line therapeutic agent. The most active trypanocidal compounds (**13a** and **13c**) exhibit $IC_{50}$ values of approximately 11 μM. In contrast to eflornithine (MIC > 1000 μM) which displays trypanostatic activity,[33] **13a** and **13c** are trypanocidal (MIC = 125 μM). With a $K_i$ value of 0.5 μM, **13c** is also one of the best rhodesain inhibitors. Higher activity against *T.b.b.* is displayed by the standard drugs **S1** (pentamidine), **S2** (suramin, MIC = 1 μM), **S4** (melarso-

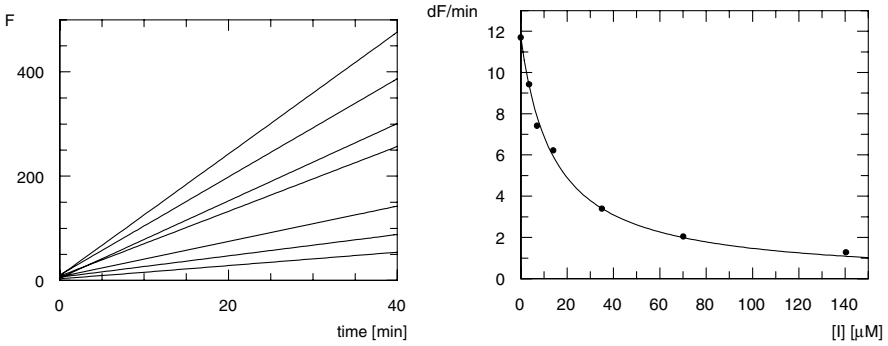


**Figure 2.** Non-time-dependent inhibition of rhodesain by **18a**; left, progress curves of substrate hydrolysis in the absence or presence of inhibitor; from top to bottom: [I] = 0–3.51–7.02–14.04–35.1–70.2–140.4 μM; right, non-linearised Dixon-plot; the $K_i$ value was determined to be 0.30 ± 0.1 μM with a substrate concentration of 81.0 μM, and 0.36 ± 0.015 μM with a substrate concentration of 31.6 μM, the diagrams are shown for the assays where [S] = 31.6 μM.[30] *F*, fluorescence units.

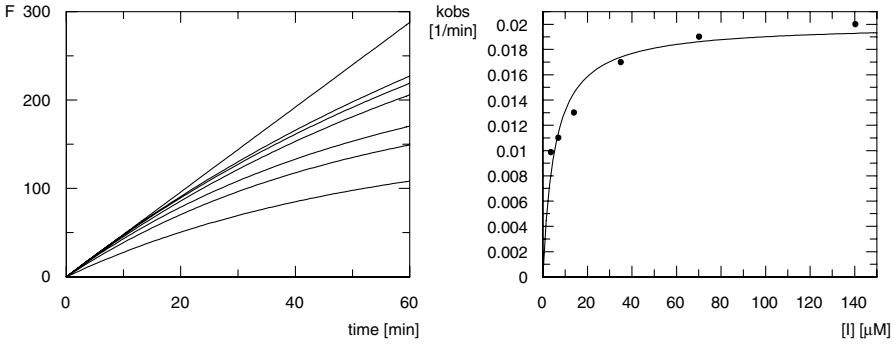


**Figure 3.** Time-dependent inhibition of rhodesain by **20e**; left, progress curves of substrate hydrolysis in the absence or presence of inhibitor; from top to bottom, [I] = 0–3.51–7.02–14.04–35.1–70.2–140.4 μM; right, plot of pseudo-first order inactivation rates $k_{obs}$ versus inhibitor concentrations [I]; $K_i = 0.11 \pm 0.03$ μM, $k_i = 0.021 \pm 0.002$ min$^{-1}$, and $k_{2nd} = 190{,}909$ M$^{-1}$ min$^{-1}$, a substrate concentration of [S] = 31.6 μM was used. *F*, fluorescence units.

prol), and **S5** (nifurtimox, MIC = 200 μM), however, **S1** and especially **S4** are highly toxic to host cells. With the exception of melarsoprol which is well known to unspecifically react with thiol groups in proteins these drugs only weakly inhibited rhodesain (ca. 30% inhibition at 200 μM).

In general, the aziridinyl peptides are not toxic to macrophages up to concentrations of 125 μM. Exceptions are the compounds **12a+b**, **18a**, and **19e**, whose $IC_{50}$ values against macrophages are in the same range as for the standard drug **S1**, pentamidine ($IC_{50}$ = 37–50 μM).

These results demonstrate that toxicity depends on the inhibitors' peptide sequences and not the aziridine ring. In this respect, the properties of aziridinyl peptides are similar to other irreversible anti-protozoal cysteine protease inhibitors, many of which are also non-toxic in vitro.[19,34]

In conclusion, we have identified Leu-Pro-containing dibenzyl aziridine-2,3-dicarboxylates (**13**) as the most promising in a series of aziridine-2,3-dicarboxylate cysteine protease inhibitors studied. In addition to their low micromolar $K_i$ values against the target enzyme, rhodesain, the compounds exhibit $IC_{50}$ values in the range of 11 to 30 μM against *T. b. brucei* while remaining non-toxic to host-derived J744.1 macrophages up to concentrations of 125 μM. Furthermore, the same inhibitors display antileishmanial[35] and anti-plasmodial activity[36] suggesting their potential as novel broad-spectrum anti-parasitic lead compounds. Accordingly, we will investigate their potential toxicity with other host cell lines and evaluate their efficacy in animal models of the disease.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.02.026.

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| Xaa | Caa | Azi | Descriptor |
|---|---|---|---|
| | *S* | *S*,*S* | **a** |
| | *R* | *S*,*S* | **b** |
| | *S* | *R*,*R* | **c** |
| | *R* | *R*,*R* | **d** |
| *S* | *S* | *S*,*S* | **a** |
| *S* | *R* | *S*,*S* | **b** |
| *S* | *S* | *R*,*R* | **c** |
| *S* | *R* | *R*,*R* | **d** |
| *R* | *S* | *S*,*S* | **e** |
| *R* | *R* | *S*,*S* | **f** |


30. Inhibition assays were carried out at different substrate concentrations (12.4, 31.6, and 81.0 μM) to evaluate whether inhibition is competitive with respect to the substrate. Because the $K_i$ values (i.e., the $K_i^{app}$ values which were corrected to zero substrate concentration) determined at different substrate concentrations are in the same range, inhibition is therefore competitive with respect to the substrate.
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