

Chalcone, Acyl Hydrazide, and Related Amides Kill Cultured *Trypanosoma brucei brucei*

Linda Troeberg,¹ Xiaowu Chen,² Terrence M. Flaherty,² Rory E. Morty,¹ Maosheng Cheng,² Huiming Hua,² Clayton Springer,² James H. McKerrow,^{2,3} George L. Kenyon,² John D. Lonsdale-Eccles,¹ Theresa H. T. Coetzer,¹ and Fred E. Cohen,^{4,5}

¹School of Molecular and Cellular Biosciences, University of Natal (Pietermaritzburg), Scottsville, South Africa

²Departments of Pharmaceutical Chemistry and

³Pathology, Veterans Affairs Medical Center, University of California, San Francisco, California, U.S.A.

⁴Departments of Biochemistry and Biophysics and

⁵Pharmacology and Medicine, University of California, San Francisco, California, U.S.A.

Communicated by F. E. Cohen. Accepted May 12, 2000

Abstract

Background: Protozoan parasites of the genus *Trypanosoma* cause disease in a wide range of mammalian hosts. *Trypanosoma brucei brucei*, transmitted by tsetse fly to cattle, causes a disease (Nagana) of great economic importance in parts of Africa. *T. b. brucei* also serves as a model for related *Trypanosoma* species, which cause human sleeping sickness.

Materials and Methods: Chalcone and acyl hydrazide derivatives are known to retard the growth of *Plasmodium falciparum* in vitro and inhibit the malarial cysteine proteinase, falcipain. We tested the effects of these compounds on the growth of bloodstream forms of *T. b. brucei* in cell culture and in a murine trypanosomiasis model, and investigated their ability to inhibit trypanopain-Tb, the major cysteine proteinase of *T. b. brucei*.

Results: Several related chalcones, acyl hydrazides,

and amides killed cultured bloodstream forms of *T. b. brucei*, with the most effective compound reducing parasite numbers by 50% relative to control populations at a concentration of 240 nM. The most effective inhibitors protected mice from an otherwise lethal *T. b. brucei* infection in an in vivo model of acute parasite infection. Many of the compounds also inhibited trypanopain-Tb, with the most effective inhibitor having a K_i value of 27 nM. K_i values for trypanopain-Tb inhibition were up to 50- to 100-fold lower than for inhibition of mammalian cathepsin L, suggesting the possibility of selective inhibition of the parasite enzyme.

Conclusions: Chalcones, acyl hydrazides, and amides show promise as antitrypanosomal chemotherapeutic agents, with trypanopain-Tb possibly being one of their in vivo targets.

Introduction

Trypanosoma brucei brucei is the causative agent of bovine trypanosomiasis (also known as Na-

gana), a disease of great economic importance in many parts of Africa. It renders an estimated one quarter of Africa unsuitable for livestock farming (1), with the total economic impact of the disease in the range of US\$5 billion annually (2). *T. b. brucei* infection is also a suitable model for human trypanosomiasis (sleeping sickness) caused by the related parasites *T. b. gambiense* and *T. b. rhodesiense*. The emergence of drug-resistant

Address Correspondence to: Theresa H. T. Coetzer, Departments of Cellular and Molecular Pharmacology, Medicine, Pharmaceutical Chemistry and Biochemistry & Biophysics, University of California, San Francisco, CA 94143-0446. Phone: 415-476-8519; Fax: 415-476-6516; E-mail: cohen@cgl.ucsf.edu

trypanosome strains, coupled with the toxicity and limited efficacy of the current treatment methods, have prompted the search for more effective antitrypanosomal agents.

The cysteine proteinases of several parasites have received attention as potential targets for chemotherapeutic intervention. Previous studies have shown that *T. b. brucei* is killed in vitro by cysteine proteinase inhibitors (3,4), and *T. cruzi* (5) and *T. congolense* (6) are killed by cysteine proteinase inhibitors. Additionally, the growth and development of *Schistosoma mansoni* (7), *Plasmodium vinckei* (8), *P. falciparum* (9), *Leishmania major* (10) and *T. cruzi* (5,11,12) are all inhibited by cysteine proteinase inhibitors. We therefore sought to investigate the effects of new classes of cysteine proteinase inhibitors on *T. b. brucei*.

Li et al. (13) showed that chalcone (1,3-diphenyl-2-propen-one) derivatives inhibit the major cysteine proteinase of *P. falciparum* (falcipain) and inhibit growth of both chloroquine-resistant and -sensitive *P. falciparum*. Chalcones have also been shown to be active against various *Leishmania* species (10,14). Earlier studies by Ring et al. (15) and Li et al. (16) demonstrated the efficacy of bis aryl acyl hydrazides against *P. falciparum* and falcipain. Molecular modeling of falcipain allowed structure-based selection of potentially more effective chalcone and hydrazide derivatives guided by the program DOCK (17). Here we report that several members of these families of compounds are trypanocidal against cultured bloodstream forms of *T. b. brucei* and that those compounds most effective in vitro were able to combat experimental *T. b. brucei* infections in mice. Several of the compounds inhibited purified trypanopain-Tb, mirroring their effectiveness against falcipain (13,15,16). However, no direct correlation was observed between trypanocidal activity and inhibition of purified trypanopain-Tb, suggesting that these compounds may have additional targets within the parasite.

Materials and Methods

Minimal essential medium (MEM) with Earle's salts, bathocuproine disulfonic acid, penicillin/streptomycin mixture, dimethyl sulfoxide (DMSO), L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64), and carbobenzoxy (Z)-Phe-Arg-aminomethyl coumarin (AMC) were from Sigma Chemical Co. (St. Louis, Mo.).

Fetal calf serum was from Delta BioProducts (Kempton Park, South Africa). Cell culture pre-filters (0.45 μm) were from Corning (Staffordshire, U.K.) and 0.22- μm filter units were from Millipore (Bedford, MA). Sterilized cell culture pipettes (10 and 1 ml) and sterile Petri dishes (50-mm diameter) were from Bibby Sterilin (Staffordshire, U.K.). All aldehydes, methyl ketones, and acyl hydrazides were purchased from Adrich Chemical Co. (Milwaukee, WI) and used without further purification.

Chemical Syntheses and Calculation of Log P Values

General Procedure for the Preparation of Chalcones A substituted aldehyde (1 mmol) and a substituted methyl ketone (1 mmol) were dissolved in a minimal amount of methanol (3–5 ml). A sodium hydroxide pellet (approximately 100 mg) was added to the solution. Precipitates formed within a few minutes to 24 hr. The solution was filtered and the precipitate washed with cold methanol (3×10 ml). Products were recrystallized from appropriate solvents whenever necessary.

General Procedure for the Preparation of Acyl Hydrazides A substituted aldehyde (1 mmol) and a substituted acyl hydrazine (1 mmol) were dissolved in methanol (20 ml). The solution was heated at reflux. Precipitates formed within a few minutes to several hours. The solution was filtered and the precipitate washed with hot methanol (3×10 ml). Products were recrystallized from appropriate solvents whenever necessary. Chemical purity was confirmed by NMR.

General Procedure for the Preparation of α,β -Unsaturated Amides A 50-ml round bottom flask charged with an aldehyde (1.0 mmol), malonic acid (1.0 mmol), piperidine (0.1 mmol), and pyridine (15 ml) was heated at reflux for 4 hr. The solution was cooled and poured onto ice. The solid was filtered and washed with water to provide an α,β -unsaturated acid. To a stirred solution of the acid (1.0 mmol) in dimethyl formamide (10 ml) was added bezotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (1.1 mmol), diisopropylethylamine (2.0 mmol), and an amine (1.5 mmol). After 18 hr, the solution was partitioned between ethyl acetate and water.

The organic phase was collected, dried (on Na_2SO_4), and evaporated. The crude product was purified by flash chromatography using hexanes/acetone as the eluent.

Calculation of log P Values of Chalcones, Acyl Hydrazides, and Amides

The log of the partition coefficient, P , between octanol and water ($\log P$) for the compounds, which serves as an indication of their hydrophobicity, was calculated using the software CLogP 4.51 (Daylight Chemical Information Systems, Santa Fe, NM), which is an implementation of the prescription stated in (18).

Effect of Chalcones, Acyl Hydrazides, and Amides on Growth of Cultured T. b. brucei

Bloodstream forms of *T. b. brucei* (clone IL-Tat 1.1) were grown in Petri dishes in MEM with Earle's Salts supplemented with 0.25 mM cysteine, 0.01 mM bathocuproine disulfonic acid, 15% (v/v) heat-inactivated fetal calf serum, and penicillin/streptomycin in a 95% humidified atmosphere, containing 5% (v/v) CO_2 at 37°C (19). Various concentrations of each tested compound (in 2.5 μl DMSO, with final concentrations between 0.8–20 μM) were added to *T. b. brucei* in MEM (2.5 ml) in Petri dishes (duplicate dishes for each concentration of compound) and the number of motile trypanosomes counted (twice from each Petri dish) after 24 hr using a hemocytometer. Control trypanosome populations were treated with DMSO alone. Plots of compound concentration versus the number of live trypanosomes were prepared and the concentration reducing parasite numbers to 50% of that of the control population determined (defined as the IC_{50}). The error on the gradient of this line was calculated and was less than 10% in all cases.

In Vivo Testing of Chalcones and Hydrazides

Three compounds showing greatest trypanocidal effectiveness *in vitro* were further tested *in vivo*. The toxicity of the chalcone (MC161) and hydrazides (ZLIII43A and ZLIV44A) was first evaluated by injecting adult Balb/c mice ($n = 3$) intraperitoneally with a single dose of 100 μl of phosphate/saline/glucose (PSG; 57 mM NaH_2PO_4 , 57 mM Na_2HPO_4 , 46 mM NaCl, 56 mM glucose, 0.1 mM hypoxanthine, pH 8.0) and 25 μl

of DMSO containing 2 mg (100 mg/kg body mass) of compound. Subsequently, another group of mice ($n = 3$) were injected daily for 5 days with 100 μl PSG and 25 μl of DMSO containing 0.5 mg of compound (25 mg/kg body mass).

The effect of the compounds on experimental *T. b. brucei* infection was investigated by injecting mice ($n = 8$ for each group) intraperitoneally with trypanosomes (5,000 parasites in 100 μl of PSG) and immediately afterward with 25 μl of compound (0.5 mg, 50 mg/kg body mass) in DMSO. Control mice were injected with 100 μl trypanosomes (5,000 trypanosomes) and 25 μl of DMSO alone.

The effects of delayed administration of the compounds were tested by injecting mice ($n = 8$ in each group) intraperitoneally with *T. b. brucei* (5,000 parasites in 100 μl of PSG) and 3 hr later with compound (0.5 mg in 25 μl DMSO, 25 mg/kg body mass). Controls received trypanosomes and 25 μl of DMSO alone.

In each case, parasitemia was monitored twice daily by examination of wet blood films of blood taken from the tail vein.

Enzyme Isolation and Assay

Trypanopain-Tb was purified from bloodstream *T. b. brucei* (clone ILTat 1.1) using three-phase partitioning and ion exchange chromatography on Q-Sepharose (20). The enzyme was assayed at 25°C in 200 mM sodium phosphate buffer, pH 7.0, containing 2 mM Na_2EDTA and 30 mM cysteine (20) with Z-Phe-Arg-AMC (final concentration 5 μM , Ex 370 nm, Em 460 nm) as the substrate.

Sheep cathepsin L, purified from sheep liver (21), was assayed against 5 μM Z-Phe-Arg-AMC (22) using 340 mM sodium acetate buffer, pH 5.5, containing 60 mM acetic acid, 4 mM Na_2EDTA , 4 mM dithiothreitol, pH 5.5.

The active concentrations of both enzymes were determined by titration against E-64 (23).

Determination of Equilibrium Constant for Inhibition (K_i)

Initial studies showed that the activity against the fluorogenic substrate Z-Phe-Arg-AMC of both trypanopain-Tb and cathepsin L was reduced by micromolar concentrations of chalcones, hydrazides, and amides. Additionally, this inhibition was shown to be reversible because dilution reduced the observed percentage of inhibition (results not shown). K_i was

thus calculated from three separate continuous assays by comparing the rates of Z-Phe-Arg-AMC hydrolysis in the absence and presence of chalcones, hydrazides, or amides. Enzyme (either trypanopain-Tb or cathepsin L, 3 ng, 0.1 pmol, 240 μ l) was activated in prewarmed assay buffer (25°C, 5 min, 500 μ l), Z-Phe-Arg-AMC (5 μ M, 250 μ l) added and the initial rate of substrate hydrolysis (v_o) determined. Inhibitor (0.1–10 nmol in 10 μ l DMSO, 0.1–100 μ M final concentration) was added at several thousand-fold molar excess over the enzyme (first-order conditions; 24) and the inhibited reaction rate (v_i) determined. Mean $K_i \pm$ SD ($n = 3$) was then calculated from the equation,

$$K_i = \frac{[1]}{\frac{S_o}{v_i} - 1} \bigg/ 1 + \frac{[S]}{k_m} I$$

Because chalcones, acyl hydrazides, and amides are competitive inhibitors, the concentration of both the substrate (S) and the inhibitor (I) were considered. The affinity between substrate and enzyme was accounted for by inclusion of the Michaelis constant for hydrolysis of Z-Phe-Arg-AMC (K_m), which is 1.2 μ M for trypanopain-Tb (20) and 6.81 μ M for cathepsin L (25).

Results

Effect of Chalcones, Hydrazides, and Amides on Growth of Cultured T. b. brucei

Several chalcones, hydrazides and amides reduced growth of bloodstream forms of *T. b. brucei* in culture at micromolar concentrations (Tables 1, 2, and 3). A comparison of the IC_{50} for each inhibitor, defined as the inhibitor concentration that reduces parasite numbers to 50% of that of the control population, identified the most active compounds in the chalcone, acyl hydrazide, and amide series. MC161 was the most trypanocidal in the chalcone series with an IC_{50} value of 0.24 μ M, while in the hydrazide series, MC357 (0.42 μ M), TF-1-52 (0.58 μ M), and ZLIV44A (0.72 μ M) exhibited IC_{50} values below 1 μ M. The amide series was less effective with the best IC_{50} values that of BG31A02 (1.82 μ M), BG21A11, HH27A43 and HH29A43 (4.4 μ M).

The most active chalcones and acyl hydrazides ($IC_{50} < 1 \mu$ M) and amides ($IC_{50} < 4.5 \mu$ M) have CLogP values below 5 (Tables 1, 2 and 3); that is, MC161 (3.05), MC375 (4.296), TF-1-52 (3.865), ZLIV44A (3.317), BG31A02 (4.721), BG21A11 (3.203), HH27A43 (4.101), HH29A43 (1.610), and HH25A43 (1.104), suggesting that they will have good absorption properties and membrane permeability (26).

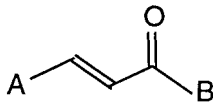
Effect of Chalcones and Hydrazides on T. b. brucei Infection in Mice

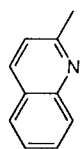
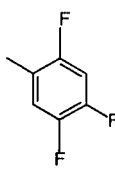
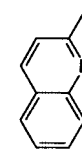
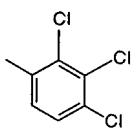
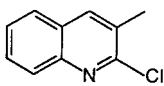
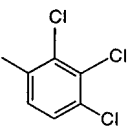
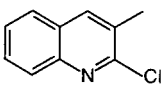
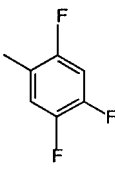
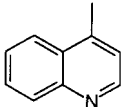
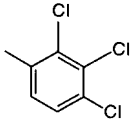
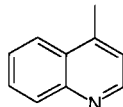
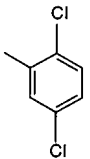
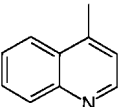
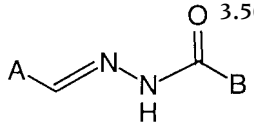
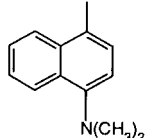
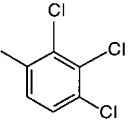
Three of the compounds with highest trypanocidal activity were chosen for testing in vivo, against a murine *T. b. brucei* infection. All three tested compounds have CLogP values below 5 and would therefore be sufficiently membrane soluble to act as drugs in vivo. The toxicity of the chalcones and hydrazides was first evaluated by injecting adult Balb/c mice intraperitoneally with a single dose of compound of 2 mg (100 mg/kg body mass). Subsequently, another group of mice were injected with 0.5 mg compound daily for 5 days (25 mg/kg body mass). Both experiments showed the compounds to be nonlethal to adult Balb/c mice, although mice appeared slightly bloated and photosensitive for 1 week following administration.

The effect of these compounds on experimental *T. b. brucei* infection was initially investigated by injecting mice intraperitoneally with a lethal dose of trypanosomes and immediately afterward with 0.5 mg of tested compound (50 mg/kg body mass). Control mice were injected with trypanosomes alone and died on day 7.17 ± 1.33 . All mice treated with MC161 and ZLIII43A survived, with no parasites evident in their bloodstream a month after infection. Of those treated with ZLIV44A, five survived and three died (on day 5.33 ± 0.56).

To test the effects of delayed administration of these compounds, mice were injected intraperitoneally with a lethal dose of *T. b. brucei* and 3 hr later with compound (0.5 mg, 25 mg/kg body mass). Controls received trypanosomes alone and died on day 4.43 ± 1.13 . ZLIII43A was ineffective, with treated mice dying at the same time as the controls (day 4.38 ± 0.74). Mice treated with ZLIV44A lived for 18% longer than the controls, dying on day 5.22 ± 0.83 , while mice treated with MC161 lived 47% longer than the controls, dying on day 6.5 ± 2.07 .

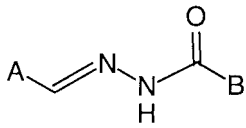
Table 1. Effects of chalcones on cultured *T. b. brucei*, purified trypanopain-Tb, and sheep cathepsin L

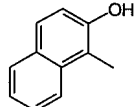
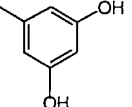
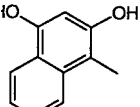
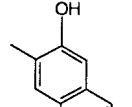
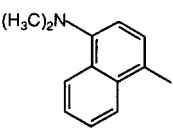
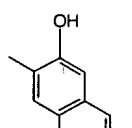
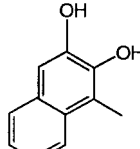
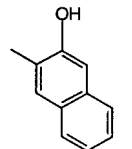
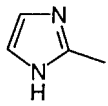
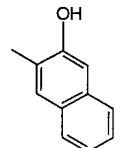
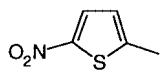
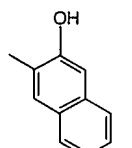
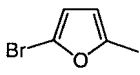
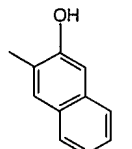
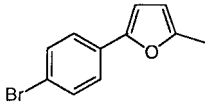
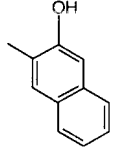
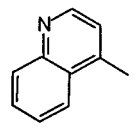
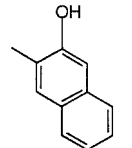


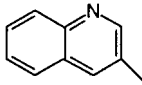
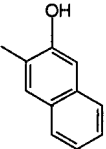
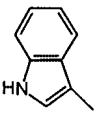
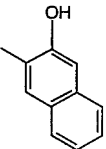
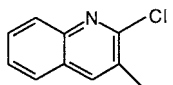
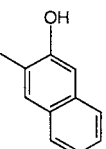
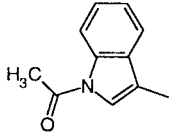
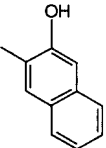
Compound	A	B	CLogP	<i>T. b. brucei</i> IC_{50} (μM)	Trypanopain K_i (μM) ^a	Cathepsin L K_i (μM) ^a
MC153			3.295	4.79	NI ^b	12.11 \pm 1.41
MC135			4.245	5.49	NI	9.55 \pm 1.42
MC131b			5.252	13.80	0.37 \pm 0.06	7.16 \pm 0.62
MC151			4.302	9.07	9.65 \pm 0.91	9.48 \pm 1.59
MC159			4.455	3.52	NI	4.57 \pm 0.55
JD159			4.115	1.88	17.14 \pm 1.76	6.95 \pm 0.80
MC161			3.505	0.24	NI	40.72 \pm 4.81
MC143			5.937	3.50	2.25 \pm 0.29	2.66 \pm 0.62

^a Data reflect the mean $K_i \pm$ SD ($n = 3$).^b No inhibition.

Table 2. Effects of hydrazide derivatives on cultured *T. b. brucei*, purified trypanopain-Tb, and sheep cathepsin L



Compound	A	B	CLogP	<i>T. b. brucei</i> IC ₅₀ (μM)	Trypanopain K _i (μM) ^a	Cathepsin L K _i (μM) ^a
ZLIV44A			3.317	0.72	1.31 ± 0.20	9.48 ± 0.52
ZLIII43A			4.226	1.53	0.027 ± 0.002	1.49 ± 0.17
ZLIII115A			5.755	1.01	0.34 ± 0.04	0.90 ± 0.14
MC357			4.296	0.42	0.64 ± 0.08	1.07 ± 0.14
TF-1-58			2.215	1.44	1.20 ± 0.01	6.47 ± 1.18
TF-1-52			3.865	0.58	1.12 ± 0.24	1.59 ± 0.31
TF-1-54			4.255	2.34	3.45 ± 0.27	3.18 ± 0.48
TF-1-56			6.523	1.66	0.22 ± 0.06	1.90 ± 0.10
TF-1-51			4.273	1.55	4.47 ± 0.64	1.52 ± 0.28

Compound	A	B	CLogP	<i>T. b. brucei</i> IC ₅₀ (μM)	Trypanopain K _i (μM) ^a	Cathepsin L K _i (μM) ^a
TF-1-59			4.063	2.14	1.38 ± 0.14	3.81 ± 0.59
TF-1-53			4.376	2.70	2.74 ± 0.44	3.39 ± 0.31
TF-1-55			5.070	1.91	4.06 ± 0.66	2.32 ± 0.16
TF-1-60			4.632	1.02	0.39 ± 0.12	1.35 ± 0.21

^a Data reflect the mean K_i ± SD (n = 3).

Inhibition of Purified Trypanopain-Tb and Cathepsin L

Chalcones, hydrazides and amides effectively inhibited trypanopain-Tb and cathepsin L, with K_i values in the low micromolar and high nanomolar range (Tables 1, 2, and 3). The rate of association (k_{ass}) between the enzymes and chalcones, hydrazides, or amides was too fast for accurate determination without a stopped-flow apparatus. Inhibition of trypanopain-Tb, together with their antiparasitic action, mirrors these compounds' previously reported effectiveness against *Leishmania* (10) and *Plasmodium* (15).

Discussion

Several of the tested chalcones, hydrazides, and amides effectively killed cultured *T. b. brucei* at micromolar concentrations. Taken in conjunction with previous reports of their effectiveness against *Leishmania* (10,14) and *Plasmodium* (13,15,16), this report suggests that these compounds have potential as broad-range antiparasitic agents. We also show in this study that these compounds can act systemically, combating

an experimental trypanosome infection, if given at the time of infection. The reduction in efficacy upon delayed administration is thought to be a function of concentration. That is, when the compounds are co-administered with the trypanosomes into the peritoneum, the effective concentration of compound reaching the parasites is higher than if the compounds are administered when the parasites have already migrated from the peritoneum into the bloodstream. Future developments in formulation will include enhancing aqueous solubility of the compounds and optimizing the dosing regimen.

It is not clear how chalcones, hydrazides, and amides kill *T. b. brucei* or related parasites. Because they inhibit purified trypanopain-Tb (this study) and falcipain (13,15,16) in vitro, it is tempting to speculate that they kill *T. b. brucei* by inhibiting this enzyme. However, because they are reversible inhibitors of trypanopain-Tb, it is not possible to confirm trypanopain-Tb as an intracellular target by labeling the inhibitors and identifying the covalently linked enzyme/inhibitor complex. Additionally, chalcones have also been shown to inhibit glutathione reductase (27) and have been suggested as possible trypanothione reductase

Table 3. Effects of amide derivatives on cultured *T. b. brucei*, purified trypanopain-Tb, and sheep cathepsin L

Compound	A	B	CLogP	<i>T. b. brucei</i> IC_{50} (μM)	Trypanopain K_i (μM) ^a	Cathepsin L K_i (μM) ^a
MC24A24			5.276	28.56	0.61 ± 0.09	11.52 ± 1.38
MC24A4I			5.746	11.82	1.06 ± 0.15	3.25 ± 0.62
MC23A24			5.276	10.00	0.26 ± 0.04	1.42 ± 0.14
MC08A27			5.498	13.50	0.66 ± 0.11	13.01 ± 1.49
BG31A02			4.721	1.82	0.50 ± 0.08	52.17 ± 3.43
BG21A11			3.203	4.44	NI ^b	61.07 ± 7.54
HH27A43			4.101	4.40	1.49 ± 0.24	16.09 ± 3.94
HH29A43			1.610	4.40	NI	20.49 ± 1.87
HH25A43			1.140	4.72	NI	49.70 ± 11.32

^a Data reflect the mean K_i ± SD ($n = 3$).^b No inhibition.

inhibitors (14). We observed no direct correlation between trypanocidal activity and inhibition of purified trypanopain-Tb, supporting the suggestion that trypanopain-Tb is not the major target of these compounds in vivo. Thus, although MC161 has the lowest IC_{50} value against cultured *T. b. brucei*, it does not inhibit trypanopain-Tb at 100 μM .

Inhibition of host cathepsins has been one of the predicted major stumbling blocks to the therapeutic use of cysteine proteinase

inhibitors. For in vivo use, a cysteine proteinase inhibitor should ideally be entirely specific for its target enzyme and not cross-react with related host enzymes. Although this has been difficult to achieve in practice, the inhibitors tested in this study were able to discriminate between trypanopain-Tb and cathepsin L to some degree. The amide BG31A02 and the acyl hydrazide ZLIII43A were 104 and 55 times more effective against trypanopain-Tb than against cathepsin L, respectively.

Salvesen and Nagase (24), however, suggest that to suppress the activity of a single proteinase in vivo, the selective inhibitor should have a K_i for the enzyme 1000-fold less than for any other enzyme in the mix, or a k_{ass} 1000-fold greater than for any other proteinase in the mix. The inhibitors we tested in vivo clearly do not fit these criteria, but nevertheless showed limited toxicity in vivo. *T. b. brucei* parasites live in the bloodstream of the host and therefore are more accessible to inhibition by blood-borne inhibitors than mammalian cysteine proteinases that reside in the lysosomes of cells. *T. cruzi* is also known to selectively accumulate fluoromethyl peptide inhibitors (28). Furthermore, because of host cysteine proteinase redundancy, cathepsin L inhibition may not be lethal to mammalian cells. Using ZLIII43A as a starting point, compounds with increased selectivity are currently being investigated.

Although trypanopain-Tb has not been unequivocally identified as the intracellular target of the tested compounds, they do inhibit the purified enzyme and are effective antitrypanosomal agents in vivo and in culture. Chalcones, hydrazides, and amides have potential as antitrypanosomal agents. They are inexpensive to synthesize, and thus potentially of use in the developing world. The major disadvantage of these inhibitors is their low water solubility, but attempts to improve this are under way. Other cysteine proteinase inhibitors, namely fluoromethylketones, diazomethylketones, and chloromethylketones, are known to kill parasites (4,7-9,12,29,30), but any potential clinical use of these inhibitors is limited by their toxicity and short in vivo half lives. The potential of less toxic cysteine proteinase inhibitors as antiparasitic agents has recently been clearly demonstrated by Engel et al. (5) who cured an experimental *T. cruzi* infection in mice using vinyl sulfones. Chalcones, hydrazides, and amides are not peptide based and are thus likely to have good in vivo half lives. Their in vitro antileishmanial (10,14) and antimalarial (15) activity suggest that these compounds may have wide-ranging antiparasitic action in addition to their trypanocidal activity described here.

Acknowledgments

This work was supported by grants from the South African Foundation for Research Devel-

opment and the University of Natal Research Fund (THTC), a Tropical Disease Research Unit Grant from the U.S. National Institute of Allergy and Infectious Diseases, and the Burroughs Wellcome Molecular Parasitology Scholar Award (JHMK).

References

- Hall HTB. (1977) *Disease and Parasites of Livestock in the Tropics*. Longman, Harlow.
- International Laboratory for Research on Animal Diseases. (1994) ILRAD 1993/4: Annual Report of the International Laboratory for Research on Animal Diseases. International Laboratory for Research on Animal Diseases, Nairobi, pp. 21-29.
- Troeberg L, Morty RE, Pike RN, et al. (1999) Cysteine proteinase inhibitors kill cultured bloodstream forms of *Trypanosoma brucei brucei* *Exp. Parasitol.* **91**: 349-355.
- Ashall F, Angliker H, Shaw E. (1990) Lysis of trypanosomes by peptidyl fluoromethyl ketones. *Biochim. Biophys. Res. Comm.* **70**: 923-929.
- Engel JC, Doyle PS, Hsieh I, McKerrow JH. (1998) Cysteine proteinase inhibitors cure an experimental *Trypanosoma cruzi* infection. *J. Exp. Med.* **188**: 725-734.
- Mbawa ZR, Gumm ID, Shaw E, Lonsdale-Eccles JD. (1992) Characterisation of a cysteine protease from bloodstream forms of *Trypanosoma congolense*. *Eur. J. Biochem.* **204**: 371-379.
- Wasilewski MM, Lim KC, Phillips J, McKerrow JH. (1996) Cysteine protease inhibitors block schistosome hemoglobin degradation *in vitro* and decrease worm burden and egg production *in vivo*. *Mol. Biochem. Parasitol.* **81**: 179-189.
- Rosenthal PJ, Lee GK, Smith RE. (1993) Inhibition of a *Plasmodium vinckei* cysteine proteinase cures murine malaria. *J. Clin. Invest.* **91**: 1052-1056.
- Rockett KA, Playfair JHL, Ashall F, Targett GAT, Angliker H, Shaw E. (1990) Inhibition of intraerythrocytic development of *Plasmodium falciparum* by proteinase inhibitors. *FEBS Lett.* **259**: 257-259.
- Selzer PM, Chen X, Chan VJ, et al. (1997) *Leishmania major*: molecular modeling of cysteine proteases and prediction of new non-peptide inhibitors. *Exp. Parasitol.* **87**: 212-221.
- Harth G, Andrews N, Mills AA, Engel JC, Smith R, McKerrow JH. (1993) Peptide-fluoromethyl ketones arrest intracellular replication and intercellular transmission of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **58**: 17-24.
- Mierelles MNL, Juliano L, Carmona E, et al. (1992) Inhibitors of the major cysteinyl proteinase

- (GP57/51) impair host cell invasion and arrest the intracellular development of *Trypanosoma cruzi* *in vitro*. *Mol. Biochem. Parasitol.* **52**: 175–184.
13. Li R, Kenyon GL, Cohen FE, et al. (1995) *In vitro* antimalarial activity of chalcones and their derivatives. *J. Med. Chem.* **38**: 5031–5037.
 14. Torres-Santos EC, Moreira DL, Kaplan MAC, Meirelles MN, Rossi-Bergman B. (1999) Selective effect of 2', 6'-dihydroxy-4'-methylchalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. *Antimicrob. Agents Chemother.* **43**: 1234–1241.
 15. Ring CS, Sun E, McKerrow JH, et al. (1993) Structure-based inhibitor design by using protein models for the development of antiparasitic agents. *Proc. Natl. Acad. Sci. U. S. A.* **90**: 3583–3587.
 16. Li Z, Chen X, Davidson E, et al. (1994) Antimalarial drug development using models of enzyme structure. *Chem. Biol.* **1**: 31–37.
 17. Kuntz ID. (1992) Structure-based strategies for drug design and discovery. *Science* **257**: 1078–1082.
 18. Hansch C, Leo AJ. (1979) In: *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley Interscience, New York.
 19. Hesse F, Selzer PM, Mühlstädt K, Duszenko M. A novel cultivation technique for long-term maintenance of bloodstream form trypanosomes *in vitro*. *Mol. Biochem. Parasitol.* **70**: 157–166.
 20. Troeberg L, Pike RN, Morty RE, Berry RK, Coetzer THT, Lonsdale-Eccles JD. (1996) Proteases from *Trypanosoma brucei brucei*: purification, characterisation and interactions with host regulatory molecules. *Eur. J. Biochem.* **238**: 728–736.
 21. Pike RN, Coetzer THT, Dennison C. (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*. *Arch. Biochem. Biophys.* **294**: 623–629.
 22. Barrett AJ, Kirschke H. (1981). Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymol.* **80C**: 535–561.
 23. Barrett AJ, Kembhavi AA, Brown MA, et al. (1982) L-trans-epoxysuccinyl-leucylamido(4-guanidino) butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **201**: 189–198.
 24. Salvesen G, Nagase K. (1989). Inhibition of proteolytic enzymes, p. 83–104. In: Benyon RJ, Bond JS (eds.) *Proteolytic Enzymes: A Practical Approach*. IRL Press, Oxford.
 25. Dehrmann FM, Coetzer THT, Pike RN, Dennison C. (1995) Mature cathepsin L is substantially active in the ionic milieu of the extracellular medium. *Arch. Biochem. Biophys.* **324**: 93–98.
 26. Lipinski CA, Lombardo F, Dominy BW, Feeny PJ. (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **23**: 3–25.
 27. Zhang K, Yang EB, Tang YW, Wong KP, Mack P. (1991) Inhibition of glutathione reductase by plant polyphenols. *Biochem. Pharmacol.* **54**: 1047–1053.
 28. McKerrow JH, McGrath ME, Engel JC. (1995) The cysteine protease of *Trypanosoma cruzi* as a model for antiparasitic drug design. *Parasitol. Today* **11**: 279–282.
 29. de Cazzulo F, MartInez BMJ, North MJ, Coombs MH, Cazzulo J-J. (1994) Effects of proteinase inhibitors on growth and differentiation of *Trypanosoma cruzi*. *FEMS Microbiol. Lett.* **124**: 81–86.
 30. Rosenthal PJ, McKerrow JH, Rasnick D, Leech JH. (1989) *Plasmodium falciparum*: inhibitors of lysosomal cysteine proteinases inhibit a trophozoite proteinase and block parasite development. *Mol. Biochem. Parasitol.* **35**: 177–184.