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Improved trypanocidal activities of cathepsin L inhibitors

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Abstract

The major lysosomal cysteine proteinase of African trypanosomes is a candidate target for novel chemotherapy of sleeping sickness. This cathepsin L-like enzyme is termed rhodesain and brucipain in *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei brucei*, respectively. Three potent and selective dipeptidyl cathepsin L inhibitors have been investigated for their trypanocidal activities in vitro using culture-adapted bloodstream forms of *T. b. brucei*. Compared with general cysteine proteinase inhibitors used previously by ourselves and others, the present inhibitors had improved selectivity indices and, importantly, anti-trypanosomal activities comparable with those of commercial anti-sleeping sickness drugs. Using purified recombinant rhodesain, potent k_{inact}/K_i values of up to 2.3×10^6 M^{-1} s^{-1} were recorded with the inhibitors. Also, all inhibitors blocked proteinolysis in the lysosome consistent with the inhibition of rhodesain/brucipain. In conclusion, the data support the potential of cathepsin L inhibitors for rational anti-trypanosomal drug development.

Keywords: Sleeping sickness; *Trypanosoma brucei brucei*; *Trypanosoma brucei rhodesiense*; Cysteine proteinase; Cathepsin L inhibitors

1. Introduction

African trypanosomes, the causative agents of sleeping sickness in humans and nagana disease in cattle, are flagellated protozoa that live extracellularly in the blood and tissue fluids of their mammalian host and are transmitted by the bite of infected tsetse flies. Over 60 million people living in 36 countries are at risk of acquiring sleeping sickness and the estimated number of people thought to have the disease is between 300 000 and 500 000 [1]. In addition, 46 million cattle are exposed to the risk of contracting nagana and the disease costs an estimated US $1340 million per year [2]. Chemotherapy of African trypanosomiasis still relies on drugs developed decades ago and some of these show toxic side effects [3]. In addition, the emergence of drug-resistant trypanosome strains has been reported [4,5]. Thus, new strategies to treat African trypanosomiasis are required.

One promising line of research towards the establishment of new anti-trypanosomal chemotherapies has been the targeting of the major cysteine proteinase of the parasite [6–8]. In African trypanosomes, the major lysosomal cysteine proteinase has a primary sequence and biochemical characteristics broadly similar to mammalian cathepsin L [9–11]. The enzyme is termed rhodesain and brucipain in *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei brucei*, respectively. Recently, irreversible, dipeptidyl, substrate-analogue inhibitors of cysteine proteinases were shown to kill *T. b. brucei*, the model organism for human African trypanosomes, both in culture and experimentally infected animals [7,12,13]. Importantly, it was demonstrated that killing of trypanosomes was correlated with inhibition of the target enzyme brucipain [12]. However, so far, only dipeptidyl inhibitors with a broad activity against cysteine proteinases have been tested. For this

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report, therefore, we have tested the trypanocidal activities and inhibition efficacies of three commercially available cathepsin L inhibitors.

2. Materials and methods

2.1. Reagents

N-Benzyloxycarbonyl-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC), *N*-benzyloxycarbonyl-phenylalanyl-alanyl diazomethylketone (Z-Phe-Ala-CHN_2), *N*-benzyloxycarbonyl-phenylalanyl-tryrosinal (Z-Phe-Tyr-CHO), *N*-benzyloxycarbonyl-phenylalanyl-*t*-butyl-tryrosyl diazomethylketone (Z-Phe-Tyr(OtBu)-CHN_2), and 1-naphthalenylsulphonyl-isoleucyl-tryptophanal (1-NapSO_2-Ile-Trp-CHO) were from Bachem (Heidelberg, Germany or Torrance, CA, USA). 1,4-Diazabicyclo[2.2.2]octane (DABCO), 4,6-diamidino-2-phenylindole (DAPI), and bovine serum albumin (BSA) were from Sigma (Dorset, U.K.). FITC-labelled human transferrin was from Cambridge BioScience (Cambridge, UK).

2.2. Enzyme assay

The substrate Z-Phe-Arg-AMC and inhibitors were prepared as 10 and 20 mM stock solutions, respectively, in DMSO. Assays were performed at room temperature and incorporated either recombinant rhodesain [8], or, as a comparison, recombinant cruzain [14] derived from the South American trypanosome, *T. cruzi*. Rhodesain (8.3 nM) or cruzain (7.5 nM) was pre-incubated for 20 min at room temperature in 100 μl of 100 μM sodium acetate, pH 5.5, 5 mM DTT, in the presence of inhibitor (0.001–60 μM) or DMSO alone added as a 1 μl aliquot. For rhodesain, the incubation buffer also contained 0.01% Triton X-100. Substrate (10 μM), in 100 μl of the appropriate buffer, was then added. Release of free AMC was measured at emission and excitation wavelengths of 355 and 460 nm, respectively, in a Molecular Devices Flex-Station fluorometer. Measurements were taken every 1.52 s. The rate of inhibition (k_{obs}) was determined at each inhibitor concentration according to $[P] = v_o/k_{obs} \times \{1-\exp(-k_{obs} \times t)\}$, where $[P]$ is the concentration of product formed over time t and v_o the initial rate, using non-linear regression analysis (GRAPHPAD PRISM 3.0a). If the k_{obs} versus inhibitor concentration could reliably fit a two-step irreversible mechanism ($r^2 > 0.9$), the inhibition constant K_i and the inactivation constant k_{inact} were determined by non-linear regression analysis according to $k_{obs} = k_{inact} \times [I]_o/\{[I]_o + K_i^* \times (1+[S]_o/K_m)\}$ where $[I]_o$ and $[S]_o$ are the concentrations of inhibitor and substrate, respectively, and $K_i^* = K_i \times (1+[S]_o/K_m)$. For those inhibitors with which k_{obs} was linear with increasing concentrations of inhibitor, linear regression analysis was used to obtain the association constant k_{ass} using $k_{obs} = k_{ass} \times [I]_o/(1+[S]_o/K_m)$. Multiple determinations at each inhibitor concentration were performed.

2.3. Cell culture

Bloodstream forms of the monomorphic *T. b. brucei* clone 427-221a [15] were grown in Baltz medium [16] supplemented with 16.7% (v/v) heat-inactivated foetal bovine serum. Human HL-60 cells (DSMZ, Braunschweig, Germany) were propagated in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum. All cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.4. Toxicity assays

Toxicity studies were performed as previously described [12]. Cells were seeded into 24-well tissue culture plates in 1 ml medium containing various concentrations (10^{-4}–10^{-12} M) of cathepsin L inhibitors dissolved in 100% DMSO. The controls contained DMSO alone. In all experiments, the final DMSO concentration was 1%, which had no effect on cell growth [17]. To ensure that the cells were in logarithmic growth phase during the entire experiment, they were seeded at an initial density of 1×10^4 trypanosomes ml^{-1} and 5×10^4 HL-60 cells ml^{-1}, respectively. After 48 h of incubation, the cells were counted using a Neubauer haemocytometer. Each experiment was repeated three times. The 50% effective dose value (ED_{50}), i.e. the inhibitor concentration necessary to reduce the growth rate of the cells to 50% of that of controls, was determined by linear interpolation as previously described [18]. The minimum inhibitory concentration (MIC), i.e. the lowest concentration of an inhibitor at which all cells were killed, was determined microscopically.

2.5. Fluorescence microscopy

Bloodstream forms of *T. b. brucei* 427-221a (5×10^6 per ml) were incubated with 100 μg ml^{-1} FITC-labelled human transferrin in Baltz medium supplemented with 2% BSA in the presence or absence of cathepsin L inhibitors. After 2 h, cells were fixed with 2% formaldehyde/0.05% glutaraldehyde in PBS, applied to poly-L-lysine coated microscope slides, and treated with 0.0001% DAPI in PBS. The slides were mounted in Mowiol 4-88 [19] containing 25 mg ml^{-1} DABCO as anti-fade reagent. Cells were examined with a Leica DMR microscope using a PL Fluotar 100× oil objective. Images were recorded using a CCD camera.

Table 1
Effects of cathepsin L inhibitors on *T. b. brucei* 427-221a bloodstream forms and human HL-60 cells

Inhibitor	*T. b. brucei*		HL-60 cells		SI
	ED_{50} (nM)	MIC (μM)	ED_{50} (nM)	MIC (μM)	
Z-Phe-Tyr-CHO	45	100	21 500	>100	482
Z-Phe-Tyr(OtBu)-CHN_2	126	10	17 000	>100	141
1-NapSO_2-Ile-Trp-CHO	18	10	21 000	>100	1150

Values are the means of three independent experiments. The standard error never exceeded 30%. ED_{50}, 50% effective dose value (inhibitor concentration necessary to reduce the growth rate of cells by 50%); MIC, minimum inhibitory concentration (lowest concentration at which all cells were killed); SI, selectivity index (ratio of ED_{50} for HL-60 cells to ED_{50} for *T. b. brucei*).

3. Results

Three potent cathepsin L inhibitors, Z-Phe-Tyr-CHO [20], Z-Phe-Tyr(OtBu)-CHN_2 [21], and 1-NapSO_2-Ile-Trp-CHO [22] were tested for their anti-trypanosomal activities. All three compounds showed a dose-dependent effect on the proliferation of bloodstream-form trypanosomes with ED_{50} values in the mid nanomolar range and MIC values between 10 and 100 μM (Table 1). In contrast, the cytotoxicities of the inhibitors towards mammalian HL-60 cells were much lower with ED_{50} values in the mid micromolar range and MIC values >100 μM (Table 1). As a result, the selectivity index (SI), i.e. the ED_{50} value obtained with HL-60 cells divided by that found for *T. b. brucei*, was >100 for all inhibitors (Table 1). The most active compound was 1-NapSO_2-Ile-Trp-CHO with an SI of 1150. Compared with previously tested dipeptidyl cysteine proteinase inhibitors [7,12,13], the present cathepsin L inhibitors exhibit at least a 10-fold higher trypanocidal activity against in vitro-cultured *T. b. brucei* bloodstream forms with respect to ED_{50} values.

Inhibition kinetics of the putative target rhodesain were measured with the three cathepsin L inhibitors and the general cysteine proteinase inhibitor, Z-Phe-Ala-CHN_2, one of the most trypanocidal compounds previously tested [7,12]. Data were compared with those obtained for cruzain, the related cysteine proteinase of *T. cruzi*. All three cathepsin L inhibitors were potent inhibitors of rhodesain and cruzain with inactivation rates ranging from 1.5×10^4 to 4.3×10^6 M^{-1} s^{-1} (Table 2). Two of the cathepsin L inhibitors, namely Z-Phe-Tyr-CHO and 1-NapSO_2-Ile-Trp-CHO, yielded better inactivation rates (42- and 11-fold, respectively, with rhodesain) than Z-Phe-Ala-CHN_2. For both enzymes, each inhibitor was similarly potent and the same rank order of inhibition was found.

Transferrin has been shown to be a lysosomal marker for bloodstream-form trypanosomes [23–25]. To determine whether the three cathepsin L inhibitors block proteinolysis in lysosomes, *T. b. brucei* bloodstream forms were incubated with FITC-labelled human transferrin. In the presence of 100 μM Z-Phe-Tyr-CHO, 10 μM Z-Phe-Tyr(OtBu)-CHN_2, or 10 μM 1-NapSO_2-Ile-Trp-CHO, the respective concentrations of the inhibitors at which all parasites were killed in vitro (see Table 1), a fluorescent spot was observed between the kinetoplast and the nucleus (Fig. 1). No labelling was detected in the absence of inhibitor (Fig. 1). The labelled region corresponded to the location of the lysosome in bloodstream-form trypanosomes [23–25]. This result demonstrates that the presence of cathepsin L inhibitors in trypanosome culture leads to accumulation of undegraded transferrin in the lysosome most likely through inhibition of brucipain activity.

4. Discussion

The major proteinolytic activity in the lysosome of bloodstream-form trypanosomes derives from a cathepsin L-like cysteine proteinase termed brucipain in *T. brucei* [9,11,26] or rhodesain in *T. b. rhodesiense* [8]. Previous studies have shown that dipeptidyl cysteine proteinase inhibitors exhibiting ED_{50} values in the low to mid micromolar range kill *T. b. brucei* bloodstream forms in vitro [7,12,13]. Also, only cysteine proteinase inhibitors having alanine, phenylalanine, homophenylalanine or valine at the P_1 position (in the nomenclature of Schechter and Berger [27]) have been investigated for their anti-trypanosomal activities so far [13]. On the other hand, it has been shown that dipeptidyl com-

Table 2
Inhibition of rhodesain and cruzain by cysteine proteinase inhibitors

Inhibitor	k_{inact}/K_i value (M^{-1} s^{-1})	
	Rhodesain	Cruzain
Z-Phe-Tyr-CHO	2.3×10^6	4.3×10^6
Z-Phe-Tyr(OtBu)-CHN_2	2.9×10^4	1.5×10^4
1-NapSO_2-Ile-Trp-CHO	5.9×10^5	4.0×10^{5a}
Z-Phe-Ala-CHN_2	5.5×10^4	2.3×10^4

Data are the mean of three independent experiments. k_{inact}, inactivation constant; K_i, inhibition constant. Standard error values were less than 20 and 45% for the k_{inact} and K_i values, respectively.

[a] k_{ass}, association constant.

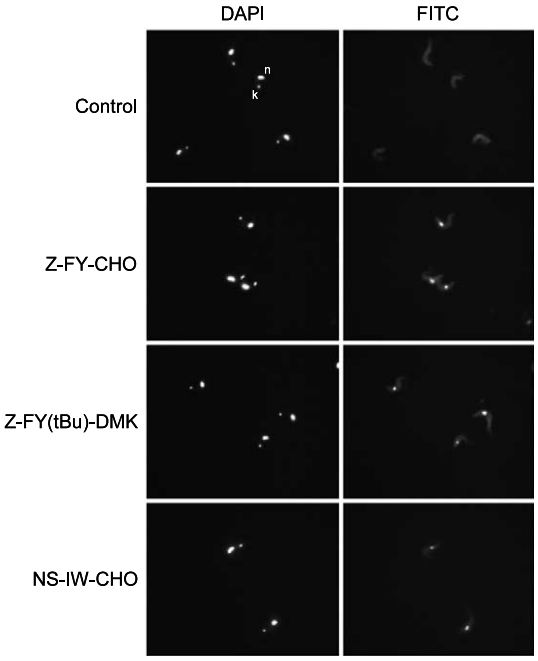

Fig. 1. Accumulation of FITC-labelled human transferrin in the lysosome upon incubation of *T. b. brucei* 427-221a bloodstream forms with cathepsin L inhibitors. Trypanosomes were incubated with 100 μg ml^{-1} FITC-labelled human transferrin in the absence (control) or presence of 100 μM Z-Phe-Tyr-CHO (Z-FY-CHO), or 10 μM Z-Phe-Tyr(OtBu)-CHN_2 (Z-FY(tBu)-DMK), or 10 μM 1-NapSO_2-Ile-Trp-CHO (NS-IW-CHO) for 2 h. FITC fluorescence representing accumulated transferrin in the presence of inhibitors is located between the kinetoplast (k) and nucleus (n) and corresponds to the location of the lysosome.

pounds with tyrosine or tryptophan in the P_1 position preferentially inhibit cathepsin L over other cysteine proteinases [20–22]. The results of this study seem to support this observation. Z-Phe-Tyr-CHO, Z-Phe-Tyr(OtBu)-CHN_2 and 1-NapSO_2-Ile-Trp-CHO, three dipeptidyl compounds selected for potent inhibitory activity against cathepsin L [20–22] are, on average, 10–100 fold more potent than inhibitors previously tested [7,12,13]. Their ED_{50} values of 18–126 nM compare well with those of the commercial anti-trypanosomal drugs suramin (130–360 nM [17]) and diminazene aceturate (20–100 nM [17]). Though the MIC values of the present inhibitors (10–100 μM) are higher than those of suramin (1.4–2.3 μM [17]) and diminazene aceturate (0.5–0.92 μM [17]), the SI values of 141–1150 approach those of the same commercial drugs (1900 for suramin and 3600 for diminazene aceturate [17]).

A perfect correlation between anti-trypanosomal activity and inactivation rates of rhodesain was not observed for the cathepsin L inhibitors. However, the data indicate that the peptidyl aldehydes are more potent against both the target enzyme and parasites in culture. Discrepancies between enzyme inhibition and trypanocidal activity may be ascribed to differences in the uptake of the inhibitors by the parasite. Interestingly, 1-NapSO_2-Ile-Trp-CHO has as N-terminal substituent (P_3 position) a naphthyl group which recently has been suggested to facilitate the uptake of respective acyl hydrazide proteinase inhibitors by bloodstream forms of *T. b. brucei* [28].

Treatment of *T. b. brucei* bloodstream forms in vitro with the cathepsin L inhibitors elicited an accumulation in the lysosome of undigested transferrin, which is an established lysosomal marker of African trypanosomes [23–25]. Given that brucipain is the predominant cysteine proteinase in *T. b. brucei* and is localised to the lysosome [8], it is reasonable to consider that brucipain is the critical target of the cathepsin L inhibitors.

In conclusion, the results suggest that inhibitors of cathepsin L are a rational choice for future anti-trypanosomal drug development. For instance, it would be interesting to test the three compounds coupled to the reactive vinyl sulphone group rather than to the aldehyde or diazomethylketone groups. Vinyl sulphones are relatively novel inhibitors [29,30] and are considerable less toxic than aldehyde or diazomethylketones. For example, vinyl sulphone inhibitors have previously been shown to kill bloodstream forms of *T. b. brucei* in vivo [7] and to cure experimental infections of mice with *T. cruzi* without undue toxicity to the host [31].

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References

[1] WHO. African trypanosomiasis or sleeping sickness. World Health Org. Fact Sheet 2001;259 (http://www.who.int/inf-fs/en/fact259.html).
[2] Kristjanson PM, Swallow BM, Rowlands GJ, Kruska RL, de Leeuw PN. Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. Agric Syst 1999;59:79–98.
[3] Croft SL. The current status of antiparasitic chemotherapy. Parasitology 1997;114(Suppl.):S3–S15.
[4] Geerts S, Holmes PH, Diall O, Eisler MC. African bovine trypanosomiasis: the problem of drug resistance. Trends Parasitol 2001;17:25–8.
[5] Matovu E, Seebeck T, Enyaru JCK, Kaminsky R. Drug resistance in *Trypanosoma brucei* ssp., the causative agents of sleeping sickness in man and nagana in cattle. Microbes Infect 2001;3:763–70.
[6] McKerrow JH, Engel JC, Caffrey CR. Cysteine protease inhibitors as chemotherapy for parasitic infections. Bioorg Med Chem 1999;7:639–44.

[7] Caffrey CR, Scory S, Steverding D. Cysteine proteinase of trypanosome parasites: novel targets for chemotherapy. Curr Drug Targets 2000;1:155–62.
[8] Caffrey CR, Hansell E, Lucas KD, et al. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. Mol Biochem Parasitol 2001;118:61–73.
[9] Lonsdale-Eccles JD, Grab DJ. Lysosomal and non-lysosomal peptidyl hydrolases of the bloodstream forms of *Trypanosoma brucei brucei*. Eur J Biochem 1987;169:467–75.
[10] Authié E. Trypanosomiasis and trypanotolerance in cattle: a role for congopain. Parasitol Today 1994;10:360–4.
[11] Troeberg L, Pike RN, Morty RE, Berry RK, Coetzer THT, Lonsdale-Eccles JD. Proteases from *Trypanosoma brucei brucei*. Purification, characterisation and interactions with host regulatory molecules. Eur J Biochem 1996;238:728–36.
[12] Scory S, Caffrey CR, Stierhof Y-D, Ruppel A, Steverding D. *Trypanosoma brucei*: killing of bloodstream forms in vitro and in vivo by the cysteine proteinase inhibitor Z-Phe-Ala-CHN_2. Exp Parasitol 1999;91:327–33.
[13] Troeberg L, Morty RE, Pike RN, et al. Cysteine proteinase inhibitors kill cultured bloodstream forms of *Trypanosoma brucei brucei*. Exp Parasitol 1999;91:349–55.
[14] Eakin AE, Mills AA, Harth G, McKerrow JH, Craik CS. The sequence, organization, and expression of the major cysteine protease (cruzain) from *Trypanosoma cruzi*. J Biol Chem 1992;267:7411–20.
[15] Hirumi H, Hirumi K, Doyle JJ, Cross GAM. In vitro cloning of animal-infective bloodstream forms of *Trypanosoma brucei*. Parasitology 1980;80:371–82.
[16] Baltz T, Baltz D, Giroud C, Crockett J. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. EMBO J 1985;4:1273–7.
[17] Merschjohann K, Sporer F, Steverding D, Wink M. In vitro effect of alkaloids on bloodstream forms of *Trypanosoma brucei* and *T. congolense*. Planta Med 2001;67:623–7.
[18] Huber W, Koella JC. A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. Acta Trop 1993;55:257–61.
[19] Rodriguez J, Deinhardt F. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 1960;12:316–7.
[20] Woo J-T, Sigeizumi S, Yamaguchi K, et al. Peptidyl aldehyde derivatives as potent and selective inhibitors of cathepsin L. Biorg Med Chem Lett 1995;5:1501–4.
[21] Shaw E, Mohanty S, Colic A, Stoka V, Turk V. The affinity-labelling of cathepsin S with peptidyl diazomethyl ketones. Comparison with the inhibition of cathepsin L and calpain. FEBS Lett 1993;334:340–2.
[22] Yasuma T, Oi S, Choh N, et al. Synthesis of peptide aldehyde derivatives as selective inhibitors of human cathepsin L and their inhibitory effect on bone resorption. J Med Chem 1998;41:4301–8.
[23] Grab DJ, Wells CW, Shaw MK, Webster P, Russo DCW. Endocytosed transferrin in African trypanosomes is delivered to lysosomes and may not be recycled. Eur J Cell Biol 1992;59:398–404.
[24] Steverding D, Stierhof Y-D, Fuchs H, Tauber R, Overath P. Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. J Cell Biol 1995;131:1173–82.
[25] Kabiri M, Steverding D. *Trypanosoma evansi*: demonstration of a transferrin receptor derived from expression site-associated genes 6 and 7. J Parasitol 2001;87:1189–91.
[26] Robertson CD, North MJ, Lockwood BC, Coombs GH. Analysis of the proteinase of *Trypanosoma brucei*. J Gen Microbiol 1990;136:921–5.
[27] Schechter I, Berger A. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 1967;27:157–62.
[28] Caffrey CR, Schanz M, Nkemgu-Njinkeng J, et al. Screening of acyl hydrazide proteinase inhibitors for antiparasitic activity against *Trypanosoma brucei*. Int J Antimicrob Agents 2002;19:227–31.
[29] Hanzlik RP, Thompson SA. Vinylogous amino acid esters: a new class of inactivators for thiol proteases. J Med Chem 1984;27:711–2.
[30] Palmer JT, Rasnick D, Klaus JL, Brömme D. Vinyl sulfones as mechanism-based cysteine protease inhibitors. J Med Chem 1995;38:3193–6.
[31] Engel JC, Doyle PS, Hsieh I, McKerrow JH. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. J Exp Med 1998;188:725–34.