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Trypanosoma brucei: Chemical evidence that cathepsin L is essential for survival and a relevant drug target

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ABSTRACT

The protozoan parasite causing human African trypanosomiasis, *Trypanosoma brucei*, displays cysteine peptidase activity, the chemical inhibition of which is lethal to the parasite. This activity comprises a cathepsin B (*Tb*CATB) and a cathepsin L (*Tb*CATL). Previous RNA interference (RNAi) data suggest that *Tb*CATB rather than *Tb*CATL is essential to survival even though silencing of the latter was incomplete. Also, chemical evidence supporting the essentiality of either enzyme which would facilitate a target-based drug development programme is lacking. Using specific peptidyl inhibitors and substrates, we quantified the contributions of *Tb*CATB and *Tb*CATL to the survival of *T. brucei*. At 100 μM, the minimal inhibitory concentration that kills all parasites in culture, the non-specific cathepsin inhibitors, benzyloxycarbonyl-phenylalanyl-arginyl-diazomethyl ketone (Z-FA-diazomethyl ketone) and (L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester (CA-074Me) inhibited *Tb*CATL and *Tb*CATB by >99%. The cathepsin L (CATL)-specific inhibitor, ((2*S*,3*S*)-oxirane-2,3-dicarboxylic acid 2-[((*S*)-1-benzylcarbamoyl-2-phenyl-ethyl)-amide] 3-{[2-(4-hydroxy-phenyl)-ethyl]-amide}) (CAA0225), killed parasites with >99% inhibition of *Tb*CATL but only 70% inhibition of *Tb*CATB. Conversely, the cathepsin B (CATB)-specific inhibitor, (L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074), did not affect survival even though *Tb*CATB inhibition at >95% was statistically indistinguishable from the complete inhibition by Z-FA-diazomethyl ketone and CA-074Me. The observed inhibition of *Tb*CATL by CA-074 and CA-074Me was shown to be facilitated by the reducing intracellular environment. All inhibitors, except the CATB-specific inhibitor, CA-074, blockaded lysosomal hydrolysis prior to death. The results suggest that *Tb*CATL, rather than *Tb*CATB, is essential to the survival of *T. brucei* and an appropriate drug target.

1. Introduction

The protozoan parasite *Trypanosoma brucei* is the causative agent of human African trypanosomiasis (HAT or sleeping sickness). The pathogen is transmitted by the bite of infected tsetse flies (*Glossina* sp.) and multiplies extracellularly in the blood and tissue fluids of the human host. *Trypanosoma brucei gambiense* is responsible for the chronic form of sleeping sickness in western and central Africa whereas *Trypanosoma brucei rhodesiense* causes the acute form of the disease in eastern and southern Africa (Barrett et al., 2003). Currently over 60 million people living in 36 sub-Saharan countries are at risk of contracting the disease (Barrett et al., 2003; Steverding, 2008; Brun et al., 2010). Due to reinforced surveillance, the number of new cases reported has fallen and at present the estimated number of infected patients is thought to be approximately 30,000 (World Health Organization, 2010).

If left untreated, HAT is fatal. Current chemotherapy is unsatisfactory as only four drugs (suramin, pentamidine, melarsoprol and eflornithine) are approved for use (Fairlamb, 2003; Steverding, 2010). Also, these drugs have toxic side effects and the occurrence of drug-resistant trypanosome strains is a growing problem (Matovu et al., 2001; Fairlamb, 2003; Delespaux and de Koning, 2007). Hence, there is an urgent need to develop new drugs for the treatment of HAT.

Research over the last decade has shown that targeting Clan CA Family C1 cysteine peptidase (CP) activity (Rawlings et al., 2010)

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with small-molecule inhibitors is trypanocidal both in vitro and in vivo (Ashall et al., 1990; Scory et al., 1999; Troeberg et al., 1999; Caffrey et al., 2000, 2001; Nkemngu et al., 2003; Vicik et al., 2006; Jaishankar et al., 2008; Mallari et al., 2008a, 2009; Bryant et al., 2009), offering the hope that CP inhibitors might form the basis of a new chemotherapy for HAT. First characterised in the 1980s, the CP activity comprises a major cathepsin L activity (North et al., 1983, 1990; Lonsdale-Eccles and Grab, 1987; Robertson et al., 1990), termed *Tb*CATL (earlier referred to as rhodesain, brucipain or Trypanopain-Tb depending on the report and parasite sub-species; Caffrey and Steverding, 2009). *Tb*CATL is expressed in both mammalian and insect life cycle stages of *T. brucei* (Pamer et al., 1989; Caffrey et al., 2001) and is localised to the lysosomes, at least in bloodstream forms (Lonsdale-Eccles and Grab, 1987; Scory et al., 1999).

Studies with small-molecule CP inhibitors to kill trypanosomes (e.g., Scory et al., 1999; Troeberg et al., 1999; Nkemngu et al., 2003) were originally thought to target only *Tb*CATL. However, the discovery and RNA interference (RNAi) validation of a minor (in terms of overall CP activity), yet essential, *T. brucei* cathepsin B (*Tb*CATB) in vitro (Mackey et al., 2004) and in vivo (Abdulla et al., 2008) raised the possibility that it was the chemical inhibition of *Tb*CATB, rather than *Tb*CATL, that was responsible for lethality. The suggestion was supported by the finding that RNAi of *Tb*CATL produced no in vitro phenotype (Mackey et al., 2004) and that in vivo, although RNAi of *Tb*CATL prolonged the survival of infected mice, it was insufficient for final rescue (Abdulla et al., 2008). As a consequence, subsequent medicinal chemistry efforts have gravitated toward improving inhibitor specificity for *Tb*CATB (Mallari et al., 2008a, 2009).

Scrutiny of the previous data (Mackey et al., 2004; Abdulla et al., 2008) reveals that RNAi of *Tb*CATL in vitro and in vivo never resulted in complete silencing but approximately a 60–65% reduction of protein. Accordingly, suggestions that *Tb*CATL is not essential to the parasite (Mackey et al., 2004; Abdulla et al., 2008) are open to re-interpretation. This incomplete RNAi of *Tb*CATL and the absence of rigorous chemical evidence for or against the enzyme's essential nature (even though complete or partial loss of CATL activity using CP inhibitors is consistently associated with parasite death, as referenced above) impede crucial insight into its utility as a drug target and perhaps the possibility that targeting both *Tb*CATL and *Tb*CATB with small-molecule inhibitors may prove additively or even better, synergistically, trypanocidal.

Here, we employ a number of small-molecule CP inhibitors and peptidyl substrates with varying specificities for mammalian CATL or CATB in order to chemically correlate parasite killing with inhibition of *Tb*CATL or *Tb*CATB. We show that (i) the specificities understood for the inhibitors and peptidyl substrates of human enzymes also hold for the trypanosome orthologs and (ii) inhibitor potency can be modulated by the presence or absence of a reducing environment. For those trypanocidal inhibitors, cell death is always preceded by the accumulation of fluorescently-labelled transferrin in the lysosome (Scory et al., 1999; Nkemngu et al., 2003). Based on our findings, we offer a brief perspective on the future of anti-trypanosomal CP inhibitor development that attempts to balance the time and resources required to improve on-target inhibitor specificity with the pertinent need for pharmaceutical optimisation.

2. Materials and methods

2.1. CP substrates and inhibitors

Benzyloxycarbonyl-phenylalanyl-arginyl-diazomethyl ketone (Z-FA-DMK) was from Bachem, (Germany). L-3-*trans*-Propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) and the methyl ester version of CA-074 (CA-074Me) were from Sigma–Aldrich, (UK). The fluorogenic substrates, benzyloxycarbonyl-arginyl-arginyl-7-amido-4-methyl coumarin (Z-RR-AMC) and benzyloxycarbonyl-phenylalanyl-arginyl-7-amido-4-methyl coumarin (Z-FR-AMC), were purchased from BIOMOL, (UK).

((2*S*,3*S*)-Oxirane-2,3-dicarboxylic acid 2-[((*S*)-1-benzylcarbamoyl-2-phenyl-ethyl)-amide] 3-{[2-(4-hydroxy-phenyl)-ethyl]-amide}) (CAA0225; Tamai et al., 1987; Takahashi et al., 2009), was synthesised according to a synthetic strategy previously reported by James et al. (2004) for structurally related aza-peptide epoxides. In Fig. 1 step 1, the central synthetic building block, (2*S*,3*S*-diethyl-oxirane-2,3-dicarboxylate), was mono-deprotected with 1 M KOH in ethanol. The resulting oxirane-2-carboxylic acid was converted into the corresponding amide using the mixed anhydride method (Fig. 1 step 2). Deprotection of the remaining ester group and coupling of the resulting carboxylic acid to L-phenylalanine benzylamide (Ramalingam et al., 2007) afforded CAA0225 as a white solid with moderate yield. The molecular structure of CAA0225 was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) and high-resolution mass spectrometry (HRMS).

2.2. Recombinant TbCATB and T. b. rhodesiense CATL (TbrCATL)

Recombinant *Tb*CATB was expressed in *Pichia pastoris* and purified from growth medium using Mono Q anion-exchange

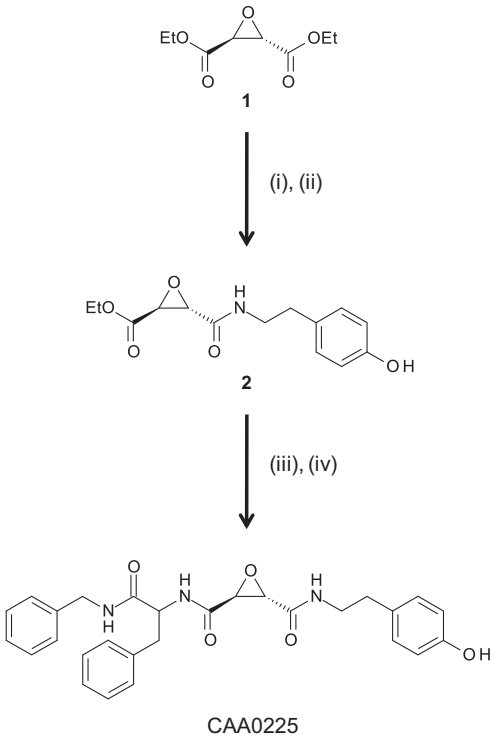

Fig. 1. Synthetic route for ((2*S*,3*S*)-oxirane-2,3-dicarboxylic acid 2-[((*S*)-1-benzylcarbamoyl-2-phenyl-ethyl)-amide] 3-{[2-(4-hydroxy-phenyl)-ethyl]-amide}) (CAA0225). In the first step (i), one of the ester groups in 1 was hydrolysed under basic conditions using KOH as the base. In the next step (ii), standard amino acid coupling conditions with tyramine, hydroxybenzotriazole (HOBt), *N*-methylmorpholine (NMM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) gave the protected intermediate 2. Finally, CAA0225 was obtained from 2 by removal of the second ester group (iii), followed by coupling to L-phenylalanine benzylamide under standard amino acid coupling conditions (iv). *Conditions:* (i) KOH (1.0 eq., 0.1 M in ethanol), 4 °C, 3 h, 82%; (ii) tyramine (1.2 eq.), HOBt (1.0 eq.), EDC (1.2 eq.), NMM (1.0 eq.), $CHCl_3$, 0 °C to room temperature, 16 h, 60%; (iii) KOH (1.2 eq., 0.1 M in ethanol), 4 °C, 3 h, 40%; (iv) L-phenylalanine benzylamide (1.1 eq.), HOBt (1.0 eq.), EDC (1.2 eq.), $CHCl_3$, 0 °C to room temperature, 16 h, 17%.