molecule inhibitors of Clan CA cysteine proteinases [2,3] kill *T. brucei in vitro* and alleviate parasitiemia in mouse models of the disease [4-7]. As possible targets for these inhibitors, two cysteine proteinases have been identified. The first, an ortholog of mammalian cathepsin B (tbcatB), is a single copy gene and expressed in both procyclic and bloodstream forms, but with greater detectable mRNA levels in the latter stage [8]. As yet, its sub-cellular localization is unclear but may be in either the endosome and/or lysosome. Tetracycline-induced RNAi of tbcatB resulted in dysmorphic parasites leading to cell death [8], raising the possibility that tbcatB may be a useful molecular target for disease intervention.

The second potential target for cysteine proteinase inhibitors, termed trypanopain-Tb [5], brucipain [6] or rhodesain [9], is a cathepsin L-like cysteine proteinase [10,11] encoded by 11 gene copies [12] and predominant in terms of enzymatic activity [9]. Inhibition of brucipain by the small molecule inhibitor, carbobenzoxy-phenylalanyl-alanine-diazomethyl ketone (Z-Phe-Ala-CHN_2), correlated with the compound's trypanocidal action *in vivo* [4]. Also, this and other peptidyl inhibitors blocked proteinolysis in the lysosome as evidenced by the accumulation of undigested FITC-transferrin [4,7], data consistent with the lysosomal localization of brucipain using specific antibodies [9,13]. Brucipain is developmentally expressed, with approximately five-fold more protein found in short-stumpy forms than in either long-slender or procyclic forms [9].

Here, we demonstrate that Z-Phe-Ala-CHN_2 when administered to mice infected with *T. brucei* results in parasites with altered cell morphology, a decreased capacity to degrade intracellular protein and an inability to mitotically replicate. We discuss these findings with respect to the parasite proteases targeted by Z-Phe-Ala-CHN_2.

Results

To study the effect of Z-Phe-Ala-CHN_2 on the cell morphology and cell division activity of bloodstream-form trypanosomes *in vivo*, mice infected with *T. brucei* were injected i.p. once daily on days 3 and 4 p.i. with 250 mg kg^{-1} of the inhibitor or vehicle alone. On day 5 p.i., blood smears were prepared and parasites were isolated from infected blood.

For examining the cell morphology of the parasites by light microscopy, blood smears were stained with May-Grünwald dye. In the blood of control mice, a mixed population of dividing long-slender forms and cell-arrested short-stumpy forms was found (Fig. 1b), with significantly (four times) more long-slender forms. In contrast, the blood of Z-Phe-Ala-CHN_2-treated mice contained few long-slender forms and almost all trypanosomes (>90%) appeared as stumpy-like forms (Fig. 1a). In addition, a large blue-stained region was observed between the kinetoplast and the nucleus, i.e., in a position consistent with that of the lysosome (Fig. 1a). That this is the lysosome is corroborated by the fact that the May-Grünwald dye stains acidic cell components. Long-slender and short-stumpy forms from control mice did not contain this structure (Fig. 1b).

Upon electron microscopy, trypanosomes from Z-Phe-Ala-CHN_2-treated mice were considerably larger than those from control mice (Fig. 2). Also, the lysosomes of trypanosomes exposed to the inhibitor were significantly larger than those of short-stumpy forms from control mice (Fig. 2). The enlargement of the lysosome may also explain why this organelle could be easily observed by light microscopy after May-Grünwald staining. In addition, the mitochondrion were also enlarged (Fig. 2).

Next, the protein content of trypanosomes purified from Z-Phe-Ala-CHN_2-treated and control mice was compared. Trypanosomes exposed to the inhibitor had 65% more

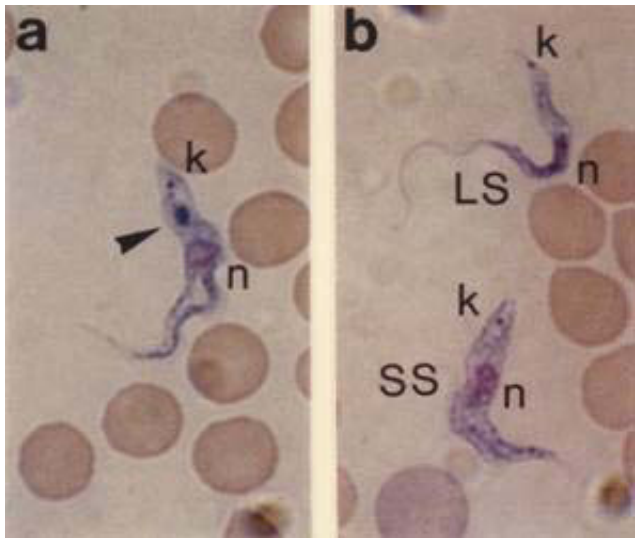

Figure 1
Effect of Z-Phe-Ala-CHN_2 on the morphology of *T. brucei* bloodstream forms *in vivo*. Mice that had been infected with the pleomorphic variant clone AnTat 1.1 were injected intraperitoneally with 250 mg kg^{-1} of Z-Phe-Ala-CHN_2 or vehicle alone on days 3 and 4 p.i. On day 5 p.i., blood smears were prepared and stained with May-Grünwald's stain solution. Representative examples from Z-Phe-Ala-CHN_2-treated mice (**a**) and control mice (**b**) are shown. Trypanosomes exposed to the inhibitor appeared stumpy-like with a blue-stained region (arrowhead) between the kinetoplast and the nucleus, a location that is consistent with that of the lysosome in bloodstream forms. k, kinetoplast; n, nucleus; LS, long-slender forms; SS, short-stumpy forms.