Table 2: Number of *T. brucei* bloodstream forms purified from Z-Phe-Ala-CHN_2-treated and control mice with two kinetoplasts.

	Two kinetoplast configuration (%) *	
	Z-Phe-Ala-CHN_2-exposed trypanosomes	Control trypanosomes
Experiment 1	3.4	14.9
Experiment 2	4.8	16.4
Average	4.1	15.7

* Analysis of DAPI stained trypanosomes.

Discussion

Previously, we demonstrated that small molecule inhibitors of cysteine proteinases kill *T. brucei* in culture and experimentally-infected mice [4,6]. We now report that upon treatment of infected mice with the diazomethyl ketone inhibitor, Z-Phe-Ala-CHN_2, parasite death is preceded by an increase in cell body mass and enlargement of constituent organelles (lysosome and mitochondrion) with a predominance (>90%) of trypanosomes displaying a "stumpy-like" morphology. Swelling of the cell body prior to cell lysis has been reported previously for bloodstream forms of *T. brucei* and *T. cruzi* after incubation with peptidyl fluoromethyl ketones *in vitro* [15]. The mechanism proposed involved inhibition of cysteine proteinase activity.

Treatment with Z-Phe-Ala-CHN_2 elicited a striking enlargement of the lysosome of trypanosomes coincident with the appearance of the same organelle after staining with May-Grünwald's solution. This suggests that the inhibitor prevents normal proteolysis in the lysosome thereby allowing the accumulation of undegraded proteins and the consequent increase in parasite weight (Table 1). The alteration in lysosomal size and function is consistent with the previous finding that co-incubation of cultured *T. brucei* bloodstream forms with Z-Phe-Ala-CHN_2 and FITC-labelled transferrin prevented degradation of the latter in the lysosome [4]. However, the lack of increased electron density in the enlarged lysosome, as would normally be expected upon accumulation of undegraded proteins, may suggest an increased water permeability of the organelle.

While it is formally possible that Z-Phe-Ala-CHN_2 exerts its trypanocidal action through one or more off-target mechanisms, one likely molecular target responsible for the enlarged lysosome phenotype is brucipain given that it is localized in the lysosome [9] and that exposure to the inhibitor *in vivo* results in a marked decrease (92%) in cellular cysteine protease activity, most of which is due to brucipain [4]. It is also possible that the phenotype was a result of inhibition of tbcatb by Z-Phe-Ala-CHN_2, even though a sub-cellular localization of this enzyme consistent with the phenotype is as yet unknown [8]. Interestingly, tetracycline-induced RNAi of tbcatB, but not brucipain, induced a lethal phenotype prefaced by an enlarged endosome/lysosome compartment [8] similar to that consequent on exposure to Z-Phe-Ala-CHN_2. The conclusions were that tbcatb, not brucipain, was essential to *T. brucei* survival and that tbcatb was the most likely target of the inhibitor [8]. However, with respect to brucipain, both of these judgments are open to reinterpretation given the available data. First, fully 35% of rhodesain activity remained in the presence of tetracycline-induced RNAi [8], possibly sufficient to allow for normal cell function and the lack of an obvious phenotype. Therefore, it is still unclear what a total knock-down of brucipain might yield in terms of the parasite's ability to survive. Secondly, Z-Phe-Ala-CHN_2 is chemically reactive with both mammalian cathepsins B and L [16,17] and there is no quantitative data to suggest that tbcatb is preferentially inhibited by this compound. Indeed, it has been shown that, in *T. brucei* lysates, both brucipain and a 34 kDa proteinase species (consistent with the molecular weight of tbcatb) are inhibited by Z-Phe-Ala-CHN_2 [9].

For other protozoan parasites, morphological aberrations, consistent with the prevention of normal proteinolysis, have been noted upon application of cysteine proteinase inhibitors. Thus, incubation of *T. cruzi* epimastigotes with the cysteine proteinase inhibitor morpholinourea-phenylalany-homophenylalanine vinylsulfone phenyl (K11777) led to enlarged intracellular organelles (endoplasmatic reticulum, nuclear membrane, mitochondrion) and morphological alterations of the Golgi complex [18]. Likewise, for *Plasmodium falciparum* trophozoites, cysteine proteinase inhibitors disrupted the morphology of the food vacuole and prevented degradation of haemoglobin [19,20].

In addition to the morphological changes, the "stumpy-like" nature of trypanosomes exposed to Z-Phe-Ala-CHN_2 was substantiated by the low number of dividing parasites. Only 4% of the parasites were proliferating which is close to the number of dividing cells (long-slender forms) of about 2% found in natural short-stumpy enriched pop-