Supplemental Material for Online Posting

Reversible Cysteine Protease Inhibitors Show Promise for a Chagas Cure

Chow Preparation of Cz007 and Cz008: The milled test article needed for the study was placed in a mortar (~3" diameter) and the same weight of powdered chow was added. The mixture was ground with a pestle. Once homogeneous, the volume was doubled with fresh chow and the mixture ground with a pestle again to homogeneity (e.g.) 100 mg of drug +100 mg of chow = 200 mg of material to which is added 200 mg of drug-free chow. The mixture was doubled again and ground with a pestle until homogeneous. The dilution process was repeated until the capacity of the mortar was reached. The chow containing the drug was then transferred in a larger mortar (~8" diameter) to be further diluted. To prevent any loss of drug, the small mortar and its corresponding pestle were rinsed twice with drug-free powdered chow that was ground for few seconds in the small mortar before being added to the larger mortar. The addition of chow was repeated as described earlier until the total amount of chow containing the drug reached ~100g. The chow containing the drug was then transferred to a stainless steel bowl (~20" diameter) and the mortar was rinsed as described earlier with drug-free chow. From that point, the chow was stirred with an electric egg beater at moderate speed for at least 2 minutes between each addition of drug-free chow. The volume was doubled with each addition of fresh chow. Once the capacity of the bowl was reached, the chow containing the drug was transferred to a plastic barrel and the mixing of the chow was done by rolling the barrel vigorously on the floor for 5 min between each addition of drug-free chow. Test article laden chow was stored at 4-6°C until use.

Cytotoxicity of CPIs in J774 Cells. J774 murine macrophages were seeded in 96-well flat bottom micro plates (Immunolon, Ottawa, ON) at 10^5 cells/well and were incubated at 37 ºC for 24 h with 200 µL RPMI without phenol red. After the incubation time, the media was replaced with fresh media containing the test compounds at final concentrations of 10, 30, 50 and 100 µM and the incubation continued at 37 ºC for 6, 12, 24 and 48 h. At required time points, 36 µL of tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyaniline (XTT) (Sigma) working solution was added to each well. The working solution was prepared immediately prior to culture application by mixing 1 mL of XTT (2 mg/mL in PBS stock solution with 20 µL of PMS (1.53 mg/mL in PBS). The microplates were incubated at 37ºC and optical density was read at 450 nm (Kc Junior, Winooski, VT) every hour for a period of 4 hours for each time point (Ref S1).

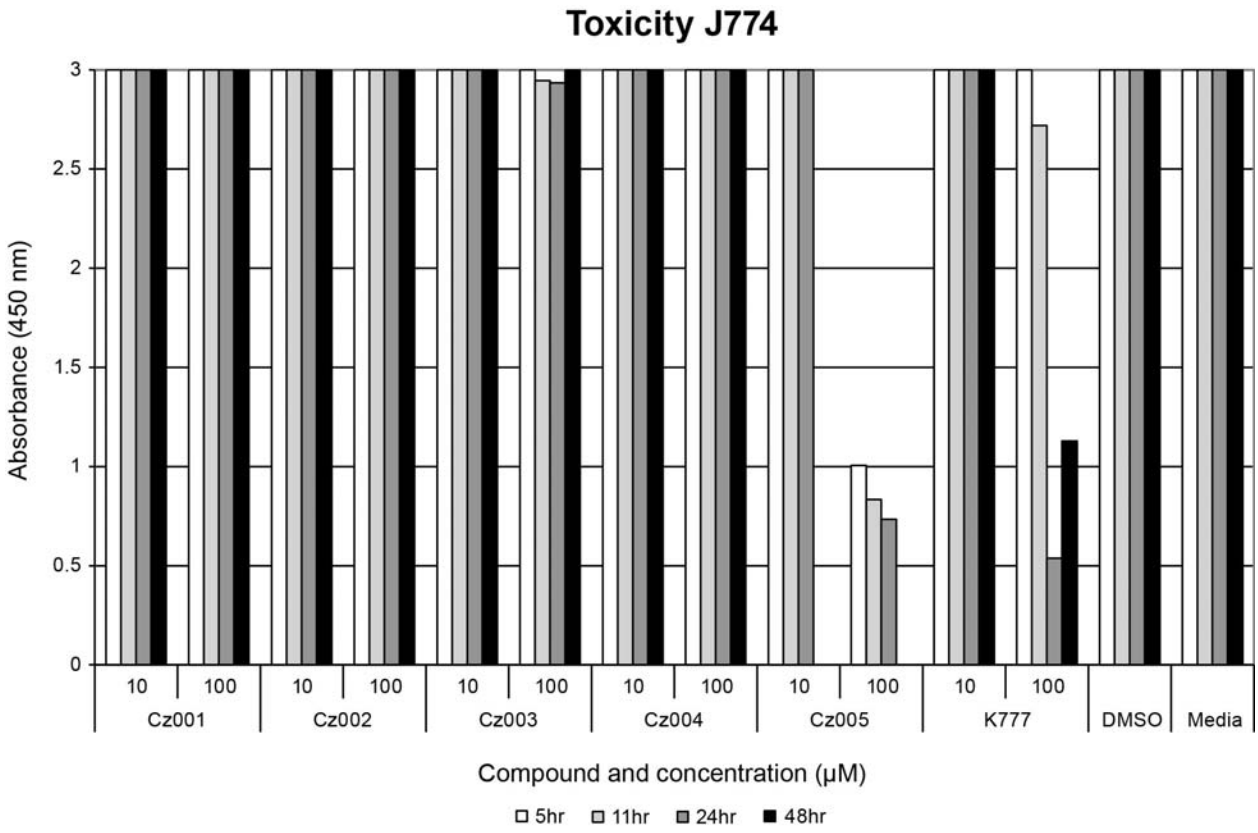

Figure S1. Toxicity testing of cruzipain inhibitors in macrophage cell line J774.

Murine Pilot Inoculation Study: Ten male CD-1 mice 6-8 weeks old and weighing an average of 30g were infected with 10^4 trypomastigotes of *T. cruzi*. The trypomastigotes were harvested as described above. The course of infection was followed for 54 days, with body weight and body temperature measured every day. For parasitemia assessment, a 10 µL blood sample was taken from the tail vein three times a week. Parasitemia was determined by manual count using a Neubauer Chamber. The limit of detection was 1 parasite in 20 fields which corresponds to $2.5x10^5$ parasites per mL of blood as described by (Ref S2). On Day 54, the mice were euthanized and tissues such as the heart, spleen, esophagus and liver were harvested to determine the presence of parasites by qualitative PCR. The blood also was collected for qualitative PCR and ELISA.

ELISA Assay. Antibody titers against *T. cruzi* were detected by coating 96-wells plates (Immulon 2; Thermo Labsystems, Franklin, Mass) overnight with 10^6 epimastigotes (100 µL per well), in 1M sodium carbonate buffer (pH 9.6). Plates were blocked with PBS-5% bovine serum albumin-0.1 %Tween [Sigma] for 1 hour at 37°C. Serum samples were diluted in blocking buffer (1:400), added in duplicate and incubated for 1 hour at 37 °C. An Antimouse IgG was diluted at 1:2,000 and added for 30 min at 37°C. 3,3',5,5'-tetramethylbenzidine (Millipore) substrate was added for 10 min and the colorimetric reaction was stopped with 1N sulfuric acid (Sigma). The optical density (OD) values were read at 450nm in an ELISA reader (Kc Junior).

Results of Pilot Study. In the pilot inoculation study, peak blood parasitemia was seen over days 8-20 post-inoculation and the average peak was ~$3x10^6$ parasites/mL of blood (range $1x10^5$-$1x10^7$). No mortality was observed and body temperatures were in the normal range,

typically 35.5 to 37°C. Mice weights increased an average of 23% over the study, from 30 to 37 g, and no mortality was observed. Every mouse showed a positive PCR signal in blood at 54 days and the uninfected mice showed no signal. Trypomastigotes were also visually observed in the peripheral blood from day 35 to 54. Qualitative PCR results from tissues only showed positive results in the esophagus, spleens and hearts of 10-20% of the mice. This is in accordance with previous findings (Ref S3). The livers were all negative. The ELISA analysis and antibody titers in the blood at sacrifice were positive for all mice, with an OD range of ~1.0-2.5 at 450 nM.

Table S1. qPCR at Day 90 for mice that died after cyclophosphamide treatment in in vivo Experiment 2.

Animal Code	Treatment (Compound and Dose)	Day of Death: (Cyclophosphamide Treatment was Day 90)	Blood qPCR on Day 90 (Parasites per mL)
4A2-2	Vehicle (No treatment)	106	<5
3A2-1	BNZ 50 mg/kg	104	<5
3A2-4	BNZ 50 mg/kg	105	<5
3A2-5	BNZ 50 mg/kg	105	<5
2B1-4	Cz007 10 mg/kg	106	<5
2B2-4	Cz007 10 mg/kg	109	100
2B2-5	Cz007 10 mg/kg	109	<5
2A1-3	Cz007 50 mg/kg	104	100
1B2-5	Cz008 3 mg/kg	105	100
1A1-2	Cz008 10 mg/kg	104	<5
1A2-4	Cz008 10 mg/kg	104	<5

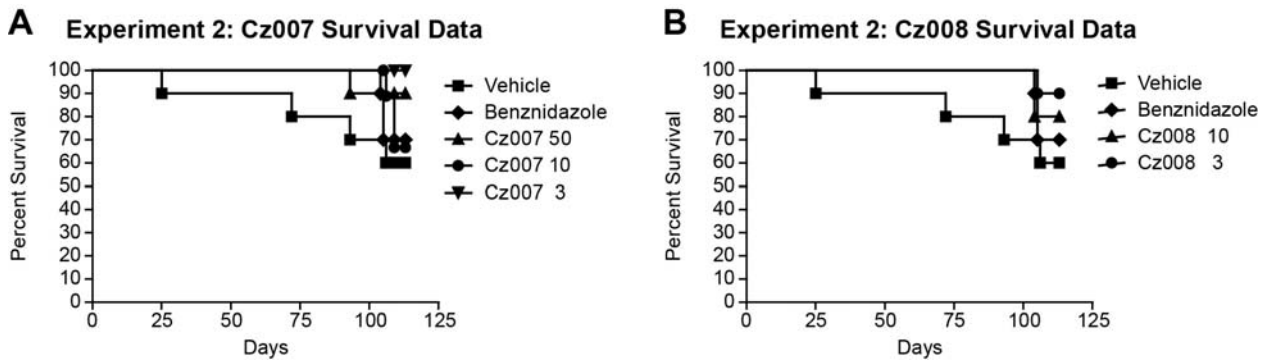

Figure S2. Survival curves of in vivo murine Experiment 2. Cyclophosphamide treatment was administered at Day 90. Panel A is Cz007 and panel B is Cz008 for clarity of viewing.

References for Supplementary Material Section

S1. **Korhonen, R., H. Kankaanranta, A. Lahti, M. Lahde, R. G. Knowles, and E. Moilanen**. 2001. Bi-directional effects of the elevation of intracellular calcium on the expression of inducible nitric oxide synthase in J774 macrophages exposed to low and to high concentrations of endotoxin. Biochem.J **354**:351-358.

S2. **Herbert, W. J. and W. H. Lumsden**. 1976. Trypanosoma brucei: a rapid "matching" method for estimating the host's parasitemia. Exp.Parasitol. **40**:427-431.

S3. **Guarner, J., J. Bartlett, S. R. Zaki, D. G. Colley, M. J. Grijalva, and M. R. Powell**. 2001. Mouse model for Chagas disease: immunohistochemical distribution of different stages of Trypanosoma cruzi in tissues throughout infection. Am.J Trop.Med.Hyg. **65**:152-158.