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(54) Title: TRYPANOSOMA CRUZI TREATMENT WITH CATHEPSIN S INHIBITORS

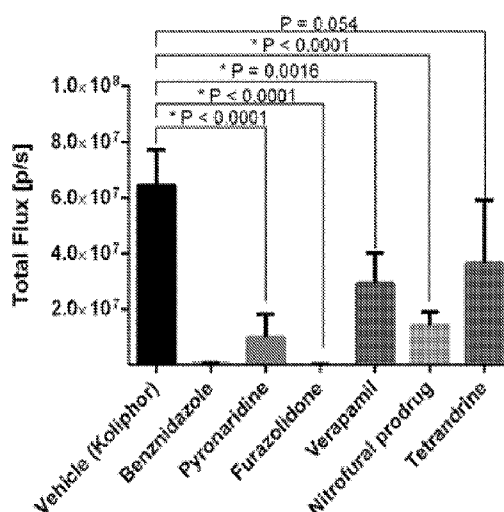


FIGURE 1

(57) Abstract: The disclosure provides for methods and compositions for treating a *T. cruzi* infection and/or Chagas Disease.

TRYPANOSOMA CRUZI TREATMENT WITH CATHEPSIN S INHIBITORS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119 from Provisional Application Serial No. 62/150,270, filed April 20, 2015, the disclosure of which is incorporated herein by reference.

TECHNICAL FIELD

[0002] The disclosure provides for a compositions and methods of treatment of *Trypanosoma cruzi*.

BACKGROUND

[0003] Chagas disease is a neglected tropical disease (NTD) caused by the eukaryotic parasite *Trypanosoma cruzi*. The disease is endemic to Latin America, but is increasingly found in North America and Europe, primarily through immigration. The spread of this disease is bringing new attention to the need for novel, safe, and effective therapeutics to treat *T. cruzi* infection.

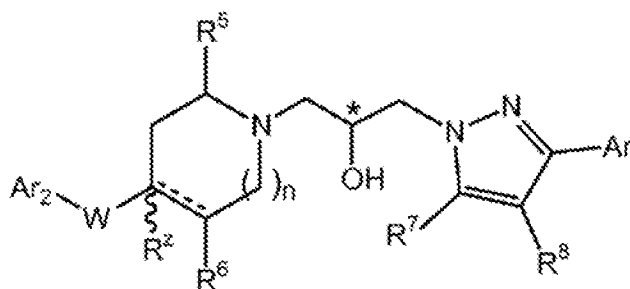
[0004] The current clinical and preclinical pipeline for *T. cruzi* treatments is extremely sparse and lacks drug target diversity. The most advanced product is the re-evaluation of a toxic general DNA damage agent benznidazole, approved for use in Chagas disease outside the U.S., but not by the U.S. Food and Drug Administration (FDA). Beznidazole requires dosing of sixty days or more and has significant toxicity.

SUMMARY

[0005] The disclosure provide methods and composition to inhibit a *T. cruzi* infection and/or inhibit Chagas Disease. The method includes administering a cysteine protease inhibitor. In one embodiment the cysteine protease inhibitor is a cathepsin S inhibitor. In another embodiment, the cysteine protease inhibitor is administered in combination benznidazole and/or nifurtimox. In another embodiment, the disclosure provides a therapy for *T. cruzi* infection comprising any of the compounds described herein showing a biological effect in inhibiting *T. cruzi* infection.

[0006] The disclosure provides a method of treating a *T. cruzi* infection comprising contacting a subject with the infection with an agent that inhibits a cathepsin S or a homolog having cysteine

protease activity. In one embodiment, the agent comprises a compound of Formula I:



Formula I

wherein

Ar₂ is a monocyclic or bicyclic ring system, unsaturated, saturated or aromatic, optionally fused, optionally including between 1 and 5 heteroatom ring moieties independently selected from the group consisting of O, S, N, SO₂ and C=O; wherein said Ar₂ ring system is optionally substituted with between 1 and 4 substituents;

W represents O, S, NR²⁷, C=O, (C=O)NH, NH(C=O)₁CHR²⁸, or a covalent bond;

R²⁷ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, naphthyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R²⁹OC=O, R³⁰R³¹NC=O, R²⁹SO, R²⁹S, R²⁹SO₂ or R³⁰R³¹NSO₂; or alternatively, R²⁷ and part of Ar₂ can be taken together to form an optionally substituted 5- to 6-membered heterocyclic ring with optionally 1 to 3 additional heteroatom moieties in the ring selected from O, NR⁹, NR¹⁰, N, SO₂, C=O and S; which ring may be saturated, unsaturated or aromatic; wherein R⁹ and R¹⁰ are independently selected from the group consisting of H, C₁₋₃ alkyl, and -CH₂CO₂(C₁₋₄alkyl);

R²⁸ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, hydroxy, phenyl, benzyl, C₁₋₅ heterocyclyl, R²⁹O, R³⁰R³¹NC=O, R²⁹S, R²⁹SO, R²⁹SO₂ or R³⁰R³¹NSO₂;

R²⁹ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl;

R³⁰ and R³¹ are each independently selected from the group consisting of hydrogen, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl, phenethyl, naphthyl, and C₁₋₅ heteroaryl; alternatively R³⁰ and R³¹ can be taken together to form an optionally substituted 4- to 7-membered ring carbocyclic or heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^z is H or OH and the dashed line is absent; or R^z is absent where the dashed line is an sp^2 bond;

R^5 and R^6 are each independently selected from the group consisting of hydrogen and C_{1-5} alkyl;

n is an integer selected from 0, 1 or 2;

R^7 and R^8 are each independently hydrogen, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} alkoxy, C_{1-5} alkylthio, halogen, or a 4-7 membered carbocyclyl or heterocyclyl; alternatively, R^7 and R^8 can be taken together to form an optionally substituted 5- to 7-membered carbocyclic or heterocyclic ring, which ring may be unsaturated or aromatic, and may be optionally substituted with between one and three substituents independently selected from the group consisting of halo, cyano, amino, hydroxy, nitro, R^4 , R^4O- , R^4S- , $R^4O(Ci-5alkylene)-$, $R^4O(C=O)-$, $R^4(C=O)-$, $R^4(C=S)-$, $R^4(C=O)O-$, $R^4O(C=O)(C=O)-$, R^4SO_2 , $NHR^{44}(C=NH)-$, $NHR^{44}SO_2-$, an $NHR^{44}(C=O)-$;

R^4 is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl) $N-$, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

R^{44} is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl) $N-$, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

Ar represents a monocyclic or bicyclic aryl or heteroaryl ring, optionally substituted with between 1 and 3 substituents independently selected from the group consisting of halogen, C_{1-5} alkoxy, C_{1-5} alkyl, C_{2-5} alkenyl, cyano, azido, nitro, $R^{22}R^{23}N$, $R^{24}SO_2$, $R^{24}SO$, $R^{24}OC=O$, $R^{22}R^{23}NC=O$, C_{1-5} haloalkyl, C_{1-5} haloalkoxy, C_{1-5} haloalkylthio and C_{1-5} alkylthio;

R^{22} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, phenethyl, benzyl, C_{1-5} heterocyclyl, C_{2-8} acyl, aroyl, $R^{38}OC=O$, $R^{25}R^{26}NC=O$, $R^{38}SO$, $R^{38}SO_2$, $R^{38}S$, or $R^{25}R^{26}NSO_2$;

R²³ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; alternatively, R²² and R²³ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R²⁴ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl, or C₁₋₅ heterocyclyl;

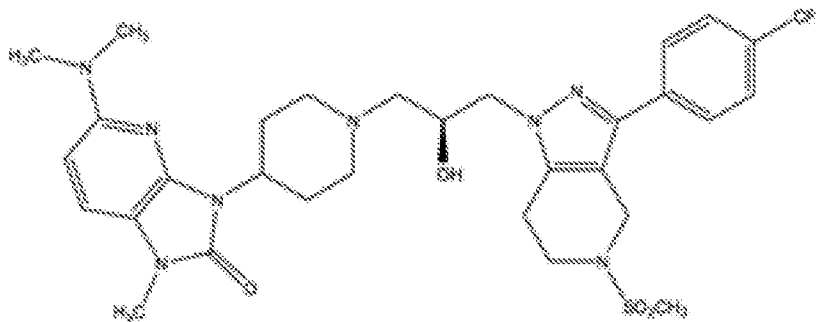
R²⁵ and R²⁶ independently are hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; or alternatively, R²⁵ and R²⁶ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R³⁸ is H, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl, phenethyl or C₁₋₅heterocyclyl;

wherein each of the above hydrocarbonyl or heterocarbonyl groups, unless otherwise indicated, and in addition to any specified substituents, is optionally and independently substituted with between 1 and 3 substituents selected from the group consisting of methyl, halomethyl, hydroxymethyl, halo, hydroxy, amino, nitro, cyano, C₁₋₅ alkyl, C₁₋₅ alkoxy, -COOH, C₂₋₆ acyl, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkylene, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkyl-NH-CO-, and C₁₋₅ haloalkoxy;

or a pharmaceutically acceptable salt, amide or ester thereof; or a stereoisomeric form thereof.

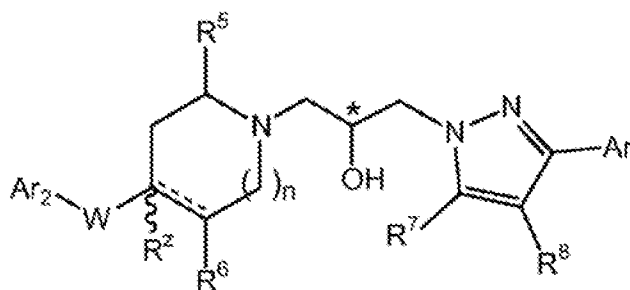
[0007] In another embodiment, the agent comprises a compound having the general formula C₃₁H₃₉F₃N₈O₄S or salt thereof. In still another embodiment, the agent comprises a compound of Formula II:



Formula II.

In another embodiment, the homolog having cysteine protease activity comprises cruzipain. In yet another embodiment, the agent inhibits cruzipain activity.

[0008] The disclosure also provides a method of treating Chagas Disease comprising contacting a subject with Chagas Disease with an agent that inhibits a cathepsin S or a homolog having cysteine protease activity. In one embodiment, the agent comprises a compound of Formula I:



Formula I

wherein

Ar₂ is a monocyclic or bicyclic ring system, unsaturated, saturated or aromatic, optionally fused, optionally including between 1 and 5 heteroatom ring moieties independently selected from the group consisting of O, S, N, SO₂ and C=O; wherein said Ar₂ ring system is optionally substituted with between 1 and 4 substituents;

W represents O, S, NR²⁷, C=O, (C=O)NH, NH(C=O)₁CHR²⁸, or a covalent bond;

R²⁷ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, naphthyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R²⁹OC=O, R³⁰R³¹NC=O, R²⁹SO, R²⁹S, R²⁹SO₂ or R³⁰R³¹NSO₂; or alternatively, R²⁷ and part of Ar₂ can be taken together to form an optionally substituted 5- to 6-membered heterocyclic ring with optionally 1 to 3 additional heteroatom moieties in the ring selected from O, NR⁹, NR¹⁰, N, SO₂, C=O and S; which ring may be saturated, unsaturated or aromatic; wherein R⁹ and R¹⁰ are independently selected from the group consisting of H, C₁₋₃ alkyl, and -CH₂CO₂(C₁₋₄alkyl);

R²⁸ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, hydroxy, phenyl, benzyl, C₁₋₅ heterocyclyl, R²⁹O, R³⁰R³¹NC=O, R²⁹S, R²⁹SO, R²⁹SO₂ or R³⁰R³¹NSO₂;

R²⁹ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl;

R^{30} and R^{31} are each independently selected from the group consisting of hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl, phenethyl, naphthyl, and C_{1-5} heteroaryl; alternatively R^{30} and R^{31} can be taken together to form an optionally substituted 4- to 7-membered ring carbocyclic or heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^z is H or OH and the dashed line is absent; or R^z is absent where the dashed line is an sp^2 bond;

R^5 and R^6 are each independently selected from the group consisting of hydrogen and C_{1-5} alkyl;

n is an integer selected from 0, 1 or 2;

R^7 and R^8 are each independently hydrogen, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} alkoxy, C_{1-5} alkylthio, halogen, or a 4-7 membered carbocyclyl or heterocyclyl; alternatively, R^7 and R^8 can be taken together to form an optionally substituted 5- to 7-membered carbocyclic or heterocyclic ring, which ring may be unsaturated or aromatic, and may be optionally substituted with between one and three substituents independently selected from the group consisting of halo, cyano, amino, hydroxy, nitro, R^4 , R^4O- , R^4S- , $R^4O(Ci-5alkylene)-$, $R^4O(C=O)-$, $R^4(C=O)-$, $R^4(C=S)-$, $R^4(C=O)O-$, $R^4O(C=O)(C=O)-$, R^4SO_2 , $NHR^{44}(C=NH)-$, $NHR^{44}SO_2-$, an $NHR^{44}(C=O)-$;

R^4 is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl) $N-$, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

R^{44} is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl) $N-$, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

Ar represents a monocyclic or bicyclic aryl or heteroaryl ring, optionally substituted with between 1 and 3 substituents independently selected from the group consisting of halogen, C_{1-5} alkoxy, C_{1-5} alkyl, C_{2-5} alkenyl, cyano, azido, nitro, $R^{22}R^{23}N$, $R^{24}SO_2$,

$R^{24}SO$, $R^{24}OC=O$, $R^{22}R^{23}NC=O$, C_{1-5} haloalkyl, C_{1-5} haloalkoxy, C_{1-5} haloalkylthio and C_{1-5} alkylthio;

R^{22} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, phenethyl, benzyl, C_{1-5} heterocyclyl, C_{2-8} acyl, aroyl, $R^{38}OC=O$, $R^{25}R^{26}NC=O$, $R^{38}SO$, $R^{38}SO_2$, $R^{38}S$, or $R^{25}R^{26}NSO_2$;

R^{23} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl or C_{1-5} heterocyclyl; alternatively, R^{22} and R^{23} can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^{24} is C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl, or C_{1-5} heterocyclyl;

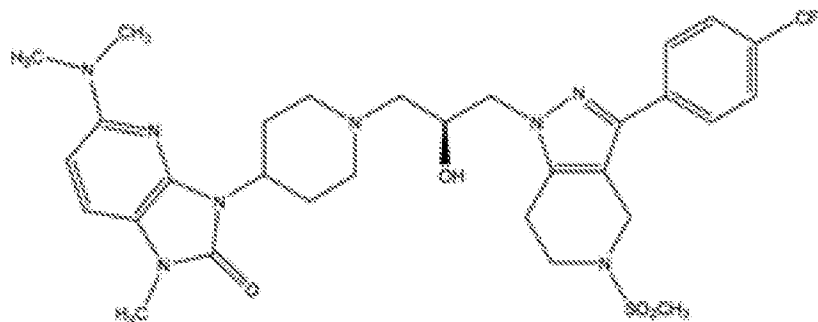
R^{25} and R^{26} independently are hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl or C_{1-5} heterocyclyl; or alternatively, R^{25} and R^{26} can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^{38} is H, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl, phenethyl or C_{1-5} heterocyclyl;

wherein each of the above hydrocarbonyl or heterocarbonyl groups, unless otherwise indicated, and in addition to any specified substituents, is optionally and independently substituted with between 1 and 3 substituents selected from the group consisting of methyl, halomethyl, hydroxymethyl, halo, hydroxy, amino, nitro, cyano, C_{1-5} alkyl, C_{1-5} alkoxy, $-COOH$, C_{2-6} acyl, $[di(C_{1-4}$ alkyl)amino] C_{2-5} alkylene, $[di(C_{1-4}$ alkyl)amino] C_{2-5} alkyl-NH-CO-, and C_{1-5} haloalkoxy;

or a pharmaceutically acceptable salt, amide or ester thereof; or a stereoisomeric form thereof.

[0009] In another embodiment, the agent comprises a compound having the general formula $C_{31}H_{39}F_3N_8O_4S$ or salt thereof. In still another embodiment, the agent comprises a compound of Formula II:



Formula II.

In one embodiment, the homolog having cysteine protease activity comprises cruzipain. In another embodiment, the agent inhibits cruzipain activity.

[0010] The disclosure also provides a combination therapy comprising: an agent that inhibits cathepsin S and at least one agent of benznidazole and/or nifurtimox.

DESCRIPTION OF DRAWINGS

[0011] **Figure 1** shows *in vivo* efficacy of test compounds (50mg/kg b.i.d.) in a 4-day mouse model of infection by transgenic *T. cruzi* Brazil luc strain 35 expressing firefly luciferase.

[0012] **Figure 2** shows broad Chagas *T. cruzi* dose response: good features from FCFP_6.

[0013] **Figure 3** shows broad Chagas *T. cruzi* dose response: bad features from FCFP_6.

[0014] **Figure 4** shows broad Chagas *T. cruzi* dose response and cytotox: good features from FCFP_6.

[0015] **Figure 5** shows broad Chagas *T. cruzi* dose response and cytotox: bad features from FCFP_6.

[0016] **Figure 6** shows a dose response curve for Formula II *in vitro*.

DETAILED DESCRIPTION

[0017] As used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a parasite" includes a plurality of such parasites and reference to "the enzyme" includes reference to one or more enzymes known to those skilled in the art, and so forth.

[0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

[0019] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0020] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0021] Any publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0022] Chagas disease is a vector-transmitted tropical disease caused by *Trypanosoma cruzi*.

[0023] Historically, Chagas Disease has been regarded as largely affecting people living in rural areas of Latin America, primarily rural areas of Central and South America. The symptoms of the disease are silent and can appear many years after infection.

[0024] Chagas Disease is expanding beyond its endemic area as a result of migration from and to the endemic countries (Hotez, 2008; Schofield and Kabayo, 2008). In addition, the World Health Organization (WHO) reports that blood donations and poor safety in blood banks have led to infections with Chagas Disease in countries outside Latin America as some people who may be unaware they carry the infection have donated their blood to the national blood supply. As a result, the disease has now appeared in several countries in Europe and various parts of the United States of America.

[0025] A hematophagous insect (such as a triatomine insect) takes a blood meal from a vertebrate host infected with *Trypanosoma cruzi* and becomes a vector for Chagas Disease. The insect ingests trypomastigotes from an infected vertebrate host, which proliferate and transform into the epimastigote form and then transform into the metacyclic trypomastigote form. The feces from the vector contain metacyclic trypomastigotes and can contaminate a bite or wound and pass into a vertebrate host (a mammalian "reservoir," which can be a human). The metacyclic trypomastigotes penetrate various cells at the bite or wound site and transform into amastigotes, which multiply by binary fission in cells of infected tissue. The intracellular amastigotes transform into trypomastigotes that then burst from the cells into the bloodstream. Clinical manifestations can result from the repetitive infective cycle in an infected vertebrate host.

[0026] Once an individual has contracted Chagas Disease, the infection may remain relatively dormant, in some cases for decades. Many people who have the disease do not know they are infected. Chagas Disease is a silent killer that causes the slow swelling of its victims' internal organs causing their eventual death. Most people later develop cardiac complications, resulting in disability and even death. Intestinal complications are also known to develop in patients resulting in an enlarged esophagus or colon which make it difficult for the person to eat normally or pass stool.

[0027] There are currently two drugs used in the treatment of Chagas Disease, benznidazole and nifurtimox. Where Chagas Disease is endemic one of the two is used to treat disease victims. WHO has reported the two drugs (nifurtimox and benznidazole) are currently used to treat early stages of Chagas Disease and that studies are being conducted for efficacy in treating later states of the disease. More recent literature reports the two drugs may be used to treat the acute phase of the infection where parasites (trypomastigotes) are detectable in the peripheral blood (Andrade et al., 2004; Schofield and Kabayo, 2008).

[0028] Nifurtimox is a 5-membered nitrofuran compound that is orally administered for 30 to 60 days. Benznidazole is an orally administered antiparasitic medication formerly marketed under the

brand names ROCHAGAN and RADANIL. Benznidazole is reportedly effective in the acute or early chronic stage of infection with decreased effectiveness during late chronic phase. Recently, the emergence of *Trypanosoma cruzi* strains resistant to benznidazole have been reported. Both drugs have gastrointestinal and neurological side effects which may worsen as the patient ages. There are problems of non-compliance and there is no prescribed approved pediatric formulation.

[0029] Treatment is complicated due to high costs and side effects. Therapy mostly depends on the two known drugs that require long term administration, and are not available to all patients due to their high cost.

[0030] Consequently, there is a search for alternative drugs with efficacy against *Trypanosoma cruzi* and, in particular, drugs having a more selective mode of action.

[0031] Cathepsins belong to the papain superfamily of cysteine proteases. These proteases function in the normal physiological as well as pathological degradation of connective tissue. Cathepsins play a major role in intracellular protein degradation and turnover and remodeling. To date, a number of cathepsins have been identified and sequenced from a number of sources. These cathepsins are naturally found in a wide variety of tissues. For example, cathepsin B, C, F, H, L, K, O, S, V, W, and Z have been cloned. Cathepsin K (which is also known by the abbreviation cat K) is sometimes also referred to as cathepsin O and cathepsin O2. Cathepsin S is implicated in Alzheimer's disease, asthma, atherosclerosis, chronic obstructive pulmonary disease and certain autoimmune disorders, including, but not limited to juvenile onset diabetes, multiple sclerosis, pemphigus vulgaris, Graves' disease, myasthenia gravis, systemic lupus erythematosus, rheumatoid arthritis and Hashimoto's thyroiditis, allergic disorders - including, but not limited to asthma, and allogenic immune responses - including, but not limited to, rejection of organ transplants or tissue grafts.

[0032] Cruzipain is a cysteine protease enzyme present in *Trypanosoma cruzi* and is thought to play an important role in all stages of the parasite's life cycle. The enzyme is highly expressed in the epimastigote stage where it is primarily a lysosomal enzyme

and may be involved in protein digestion during differentiation to the infective metacyclic trypomastigote stage. Identification of cruzipain in the membrane of the trypomastigote implicates this enzyme in the penetration of the parasite into the host cell. Cruzipain is also found in the membranes of the amastigote form of the parasite, see Cazzulo, J. J., et al., Current Pharmaceutical Design, 7:1143-1156, 2001. Cruzipain efficiently degrades human IgG, which may play a protective role for the parasite by preventing antigen presentation and thus reducing the host immune response. Thus, cruzipain is a valid drug target for drug therapy of Chagas Disease.

[0033] Cruzipain has been reported to exist in at least two polymorphic sequences, known as cruzipain 1 and cruzipain 2, both of which may be involved in the viability of *Trypanosoma cruzi* (Lima, et al., Molecular & Parasitology 114:41-52, 2001). Cruzain is a C-terminally truncated recombinant species of cruzipain.

[0034] As used herein, "Chagas Disease" refers to a parasitic disease associated with or caused by infection with the protozoan parasite *Trypanosoma cruzi*. Chagas Disease can comprise an acute phase which typically last from weeks or months and is often symptom-free. When symptoms do occur they can include swelling at the infection site, fever, fatigue, rash, body aches, eyelid swelling, headache, loss of appetite, nausea, diarrhea, vomiting, swollen glands and enlargement of the liver or spleen. The disease can also include a chronic phase that typically occurs 10 to 20 years after initial infection. In the chronic stage symptoms can includes irregular heartbeat, congestive heart failure, sudden cardiac arrest, difficulty swallowing due to enlargement of the esophagus, and/or abdominal pain or constipation due to enlargement of the colon.

[0035] As used herein, "cruzain," (also known as cruzipain or gp 57/51), refers to the major cysteine protease of *T. cruzi*. Cruzain is a 60 kDa high-mannose type glycoprotein.

[0036] The term "inhibitor of Cathepsin S" should be understood broadly and encompasses inhibitors of cathepsin S activity and inhibitors of cathepsin S expression. An "inhibitor of expression" refers to a compound (e.g., synthetic) that has a biological effect

to inhibit or significantly reduce the expression of a gene. Thus, an inhibitor of cathepsin S includes inhibitor nucleic acids (e.g., RNAi molecules such as siRNA, shRNA, miRNA and the like) as well as small molecule inhibitors that can inhibit the biological activity of the cathepsin S polypeptide. Consequently an "inhibitor of Cathepsin S expression" refers to a natural or synthetic compound that has a biological effect to inhibit or significantly reduce the expression of the gene encoding for the Cathepsin S gene.

[0037] Particularly, a "selective inhibitor of cathepsin S expression" refers to a compound that inhibits Cathepsin S expression more strongly than that of Cathepsins L or K expression in the sense that the inhibitor is at least 10 times, more preferably at least 100 times and most preferably at least 1000 times stronger inhibitor of the Cathepsin S expression.

[0038] An "inhibitor of cathepsin S activity" has its general meaning in the art, and refers to a compound (natural or not) which has the capability of reducing or suppressing the activity of a protein. It can be an antibody which binds the cathepsin S enzyme or a small molecule agent or other biological molecule that binds to or inhibits, e.g., the active site of cathepsin S and thus inhibits its activity.

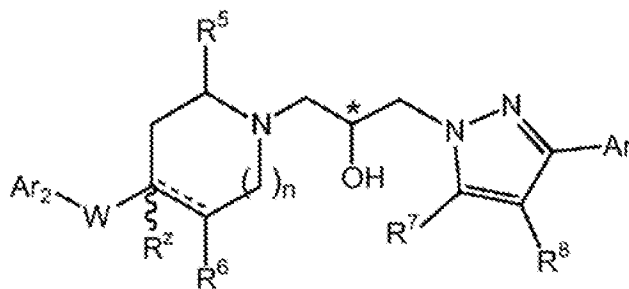
[0039] Particularly, a "selective inhibitor of cathepsin S activity" refers to a compound that inhibits Cathepsin S activity more strongly than that of, for example, Cathepsins L and K activity in the sense that the inhibitor is at least 10 times, more preferably at least 100 times and most preferably at least 1000 times stronger inhibitor of the cathepsin S activity.

[0040] As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Typically, a subject according to the disclosure is a human.

[0041] In one embodiment, the disclosure provides the use of inhibitors of Cathepsin S activity for the treatment of an infection with *Trypanosoma cruzi* or Chagas Disease and associated disorders. Particularly, the disclosure relates to the use of selective inhibitors of Cathepsin S activity for the treatment of an infection by *T. cruzi* and/or the treatment of Chagas Disease.

[0042] In one embodiment, the inhibitor of Cathepsin S activity can be a small molecule agent. Several molecules have been described as inhibitors of Cathepsin S activity.

[0043] In one embodiment, the small molecule agent comprises a structure having formula I:



Formula I

wherein

Ar₂ is a monocyclic or bicyclic ring system, unsaturated, saturated or aromatic, optionally fused, optionally including between 1 and 5 heteroatom ring moieties independently selected from the group consisting of O, S, N, SO₂ and C=O; wherein said Ar₂ ring system is optionally substituted with between 1 and 4 substituents;

W represents O, S, NR²⁷, C=O, (C=O)NH, NH(C=O)₁CHR²⁸, or a covalent bond;

R²⁷ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, naphthyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R²⁹OC=O, R³⁰R³¹NC=O, R²⁹SO, R²⁹S, R²⁹SO₂ or R³⁰R³¹NSO₂; or alternatively, R²⁷ and part of Ar₂ can be taken together to form an optionally substituted 5- to 6-membered heterocyclic ring with optionally 1 to 3 additional heteroatom moieties in the ring selected from O, NR⁹, NR¹⁰, N, SO₂, C=O and S; which ring may be saturated, unsaturated or aromatic; wherein R⁹ and R¹⁰ are independently selected from the group consisting of H, C₁₋₃ alkyl, and -CH₂CO₂(C₁₋₄alkyl);

R²⁸ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, hydroxy, phenyl, benzyl, C₁₋₅ heterocyclyl, R²⁹O, R³⁰R³¹NC=O, R²⁹S, R²⁹SO, R²⁹SO₂ or R³⁰R³¹NSO₂;

R²⁹ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl;

R³⁰ and R³¹ are each independently selected from the group consisting of hydrogen, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl, phenethyl, naphthyl, and C₁₋₅ heteroaryl; alternatively R³⁰ and R³¹

can be taken together to form an optionally substituted 4- to 7-membered ring carbocyclic or heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^z is H or OH and the dashed line is absent; or R^z is absent where the dashed line is an sp^2 bond;

R^5 and R^6 are each independently selected from the group consisting of hydrogen and C_{1-5} alkyl;

n is an integer selected from 0, 1 or 2;

R^7 and R^8 are each independently hydrogen, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} alkoxy, C_{1-5} alkylthio, halogen, or a 4-7 membered carbocyclyl or heterocyclyl; alternatively, R^7 and R^8 can be taken together to form an optionally substituted 5- to 7-membered carbocyclic or heterocyclic ring, which ring may be unsaturated or aromatic, and may be optionally substituted with between one and three substituents independently selected from the group consisting of halo, cyano, amino, hydroxy, nitro, R^4 , R^4O- , R^4S- , $R^4O(Ci-5alkylene)-$, $R^4O(C=O)-$, $R^4(C=O)-$, $R^4(C=S)-$, $R^4(C=O)O-$, $R^4O(C=O)(C=O)-$, R^4SO_2 , $NHR^{44}(C=NH)-$, $NHR^{44}SO_2-$, an $NHR^{44}(C=O)-$;

R^4 is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl)N-, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

R^{44} is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl)N-, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl, or (C_{1-5} heterocyclyl) C_{1-6} alkylene- and R^{43} is C_{1-5} alkylene, phenylene, or divalent C_{1-5} heterocyclyl;

Ar represents a monocyclic or bicyclic aryl or heteroaryl ring, optionally substituted with between 1 and 3 substituents independently selected from the group consisting of halogen, C_{1-5} alkoxy, C_{1-5} alkyl, C_{2-5} alkenyl, cyano, azido, nitro, $R^{22}R^{23}N$, $R^{24}SO_2$, $R^{24}SO$, $R^{24}OC=O$, $R^{22}R^{23}NC=O$, C_{1-5} haloalkyl, C_{1-5} haloalkoxy, C_{1-5} haloalkylthio and C_{1-5} alkylthio;

R^{22} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, phenethyl, benzyl, C_{1-5} heterocyclyl, C_{2-8} acyl, aroyl, $R^{38}OC=O$, $R^{25}R^{26}NC=O$, $R^{38}SO$, $R^{38}SO_2$, $R^{38}S$, or $R^{25}R^{26}NSO_2$;

R^{23} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl or C_{1-5} heterocyclyl; alternatively, R^{22} and R^{23} can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^{24} is C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl, or C_{1-5} heterocyclyl;

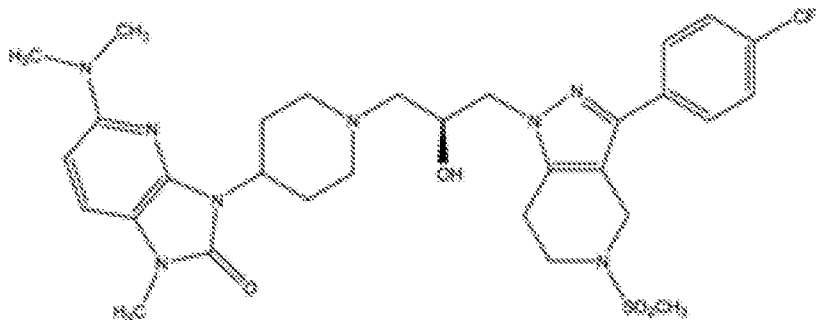
R^{25} and R^{26} independently are hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl or C_{1-5} heterocyclyl; or alternatively, R^{25} and R^{26} can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^{38} is H, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl, phenethyl or C_{1-5} heterocyclyl;

wherein each of the above hydrocarbyl or heterocarbyl groups, unless otherwise indicated, and in addition to any specified substituents, is optionally and independently substituted with between 1 and 3 substituents selected from the group consisting of methyl, halomethyl, hydroxymethyl, halo, hydroxy, amino, nitro, cyano, C_{1-5} alkyl, C_{1-5} alkoxy, $-COOH$, C_{2-6} acyl, $[di(C_{1-4} alkyl)amino]C_{2-5} alkylene$, $[di(C_{1-4} alkyl)amino]C_{2-5} alkyl-NH-CO-$, and C_{1-5} haloalkoxy; or a pharmaceutically acceptable salt, amide or ester thereof; or a stereoisomeric form thereof.

[0044] In one embodiment, the inhibitor of cathepsin S comprises the general formula $C_{31}H_{39}F_3N_8O_4S$ (free base) or $C_{31}H_{39}F_3N_8O_4S-HCl$ (HCl salt). Other salt forms include the therapeutically active non-toxic acid addition salt forms. These can conveniently be obtained by treating the base form with an appropriate acid. Appropriate acids comprise, for example, inorganic acids such as hydrohalic acids, e.g. hydrochloric or hydrobromic acid; sulfuric; nitric; phosphoric and the like acids; or organic acids such as, for example, acetic, propanoic, hydroxyacetic, lactic, pyruvic, oxalic, malonic, succinic, maleic, fumaric, malic, tartaric, citric, methanesulfonic, ethanesulfonic, benzenesulfonic, p-toluenesulfonic,

cyclamic, salicylic, p-aminosalicylic, palmoic and the like acids. The term addition salt also comprises the solvates which the disclosed compounds, as well as the salts thereof, are able to form. Such solvates are for example hydrates, alcoholates and the like. Conversely the salt form can be converted by treatment with alkali into the free base form. In another embodiment, the inhibitor of cathepsin S comprises Formula II:



Formula II.

[0045] Compounds of Formula I and II may be formulated for delivery as extended release, delayed release or slow release formulations. For example, the compound may be prepared as tablets or granules and enterically coated for delivery to the small intestine or may be formulated for gastric retention as desired. Such formularies are recognized in the art.

[0046] The selective inhibitor of cathepsin S activity and/or expression may be administered in the form of a pharmaceutical composition, as defined below.

[0047] In one embodiment, the inhibitor is administered in a therapeutically effective amount. By a "therapeutically effective amount" is meant a sufficient amount of the inhibitor of cathepsin S to treat and/or to prevent the infection of or spread of *T. cruzi* or to ameliorate symptoms of Chagas Disease and related disorders.

[0048] It will be understood that the total daily usage of the compounds and compositions of the disclosure will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient or subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition

employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient (e.g., from 1 mg to about 100 mg of the active ingredient). An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day. In general it is contemplated that a therapeutically effective dose would be from 0.001 mg/kg to 5 mg/kg body weight, more preferably from 0.01 mg/kg to 0.5 mg/kg body weight. It may be appropriate to administer the therapeutically effective dose as two, three, four or more sub-doses at appropriate intervals throughout the day. Said sub-doses may be formulated as unit dosage forms, for example, containing 0.05 mg to 250 mg, and in particular 0.5 to 50 mg of active ingredient per unit dosage form. Examples include 2 mg, 4 mg, 7 mg, 10 mg, 15 mg, 25 mg, and 35 mg dosage forms. Compounds of the disclosure may also be prepared in time-release or subcutaneous or transdermal patch formulations. Disclosed compound may also be formulated as a spray or other topical or inhalable formulations.

[0049] The disclosure also provides a pharmaceutical composition for treating and/or preventing Chagas disease or disorder or an infection by *T. cruzi*, said composition comprising an inhibitor of Cathepsin S expression and/or activity, typically a selective inhibitor of Cathepsin S expression and/or activity.

[0050] In one embodiment, the disclosure provides a pharmaceutical composition for treating and/or preventing a disease or disorder associated with *T. cruzi* infection.

[0051] In another embodiment, the disclosure provides a pharmaceutical composition for inhibiting or preventing the parasitic load of *T. cruzi* in a subject.

[0052] The inhibitor(s) of Cathepsin S may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

[0053] In the pharmaceutical compositions of the disclosure for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

[0054] In one embodiment, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

[0055] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must

be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[0056] Solutions comprising compounds of the disclosure as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0057] The inhibitor of Cathepsin S of the disclosure can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[0058] The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

[0059] Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0060] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

[0061] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

[0062] The inhibitor of Cathepsin S of the disclosure may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

[0063] In addition the compounds of the disclosure can be formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations: time release capsules; and any other form currently used.

[0064] A further embodiment relates to a pharmaceutical composition for killing *T. cruzi* in a subject comprising administering a cathepsin S inhibitor to the subject.

EXAMPLES

Example 1

[0065] **CDD database and Chagas datasets.** An analysis of the Chagas disease literature was performed resulting in the curation of over 500 molecules with associated target information. The Broad Chagas screening data were also collected and both datasets were uploaded into the CDD database (Collaborative Drug Discovery Inc. Burlingame, CA) from sdf files and mapped to custom protocols. All public datasets used in model building are available for free public read-only access and mining upon registration in the CDD database ([\[https://www.collaborativedrug.com/pages/public_access\]](https://www.collaborativedrug.com/pages/public_access)).

[0066] **Data annotation and Pathway Genome Data Base construction.** By using a combination of genetic validation from the literature, bioinformatic analyses, and available assays, a prioritized set of *T. cruzi* targets for experimental validation were identified as the binding targets of screening hits. Furthermore, SRI has developed "choke point" analyses to assess the likelihood that a particular metabolic pathway step is essential for an organism. In order to use such approaches a Pathway Genome Data Base (PGDB) for *T. cruzi* was constructed (which is referred to as "TCruCyc") using the complete genome sequence of the Dm28c strain. The Dm28c strain was chosen since it is a model organism for studying Chagas Disease and its recently assembled genome sequence is more complete than CL-Brener. This was completed by using the "Pathologic" workflow within the Pathway Tools suite. The existing workflow imports the complete genome sequence and then assigns proteins from annotated sequences. A patch to Pathologic to enable proteins to be searched by Uniprot/TrEMBL identifiers was used. This process will not

assign proteins unless they are annotated in the genome sequence, which will miss some obvious sequence-based homologies (e.g., the tubulin gene is not annotated in the Dm28c sequence). Workflows were also examined that would enable the automatic import of protein annotations from a closely related organism (e.g., CL-Brener), but ended up manually annotating a number of orphan proteins for the current dataset. The underlying genome sequence consisted of 5,287 contigs assembled into 1,378 scaffolds of 30,716,540 base pairs. Pathologic found 11,349 distinct gene products, at least 880 of which were found to be enzymes and at least 16 of which are transporters. Pathologic was able to infer 1030 enzymatic reactions and 122 pathways from these assignments as well as the existence of 806 metabolic compounds. This set was filtered to 358 molecules after removal of compounds with R- groups and small nuisance molecules. This dataset was then used to infer potential targets by comparing the Tanimoto similarity with a phenotypic screening hit.

[0067] Building and validating dual-event machine learning models with novel bioactivity and cytotoxicity data. Laplacian-corrected Bayesian classifier models were developed with bioactivity and cytotoxicity data to create dual-event models using Discovery Studio versions 3.5 and 4.1 (Biovia, San Diego, CA). This was applied to the Broad Chagas dose response data (AID 2044) using the EC₅₀ data, where values less than 1 μ M are classed as actives and were used for the single event models. Further revision of the actives using the cytotoxicity data when a greater than 10 fold difference with cytotoxicity was observed and these compounds were considered active. The models were all generated using the following molecular descriptors: molecular function class fingerprints of maximum diameter 6 (FCFP_6), AlogP, molecular weight, number of rotatable bonds, number of rings, number of aromatic rings, number of hydrogen bond acceptors, number of hydrogen bond donors, and molecular fractional polar surface area which were all calculated from input sdf files.

[0068] The resulting single- and dual-event datasets were validated using leave-one-out cross-validation, 5 fold validation and by leaving out 50% of the data and rebuilding the model 100 times using

a custom protocol to generate the receiver operator curve area under the curve (ROC AUC), concordance, specificity and selectivity.

[0069] These models were used to score the following drug libraries; Selleck Chemicals (Houston, TX) natural product library (139 molecules), GSK kinase library (367 molecules), Malaria box (400 molecules), Microsource (Gaylordsville, CT) Spectrum (2320 molecules), CDD FDA drugs (2690 molecules), Prestwick Chemical (Illkirch, France) library (1280 molecules) and Traditional Chinese Medicine components (373 molecules). The top scoring molecules with the dual event model were selected and purchased from (eMolecules, La Jolla, CA) and then 97 underwent primary *in vitro* screening.

[0070] Primary *In vitro* screening. Mouse myoblast cell line C2C12 (ATCC #CRL-1772) was cultivated in Dulbecco's Modified Eagle's Medium containing 4.5 g/l glucose (DMEM), supplemented with 5% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. *T. cruzi* CA-I/72 trypomastigotes were obtained from C2C12 infected-culture supernatants after 4-7 days of infection. Cultures were maintained at 37°C with 5% CO₂. For the infection assay to assess anti-parasitic activity of the compounds, 500 C2C12 cells were seeded in 384-well plate in 40 µl of DMEM media per well. Compounds were added at 10 mM in 50 nl per well using a Biomek FX (Beckman Coulter) for a final 10 µM concentration in 50 µl total volume, and 2,500 parasites were added in 10 µl per well. The plate was incubated for 72 hours at 37°C with 5% CO₂. After the incubation, the plate was fixed with the addition of 50 µl of 8% paraformaldehyde solution, followed by two successive washing steps using PBS. Finally, a staining solution containing 0.5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) was added to each well of the plate and incubated for at least 4 hours prior to reading. Images were acquired by an IN Cell Analyzer 2000 (GE Healthcare) and analyzed by IN Cell Analyzer Developer 1.6 software. The size parameters used to segment host and parasite organelles were 125 µm² for host nucleus, and 1-2 µm² for parasite nucleus/kinetoplast. Numbers of host cells and intracellular amastigotes were determined based on host cell and parasite nucleus quantification, providing a measure of growth inhibition during the first 72 h of post-infection treatment compared to untreated

controls. The anti-parasitic results were expressed in terms of relative activity normalized based on the average infection ratio (number of infected cells/total number of cells) of negative controls (0.1% DMSO, 0% activity) and positive controls (50 μ M of benznidazole, EC₁₀₀, 100% activity). The host cell viability was assessed based on the total number of cells divided by the average number of cells from untreated controls (0.1% DMSO), being <0.5 considered a cytotoxic compound. This assay was performed in duplicate.

[0071] Hit selection and secondary screening (dose-response assay).

The hit selection criteria: >50% activity at 10 μ M and >0.5 host cell viability in the primary screening. To determine the potency of the hit compounds, a dose-response assay was performed. EC₅₀ values of compounds were determined applying the same assay used in the primary screening. For this, an intermediate plate (384-well plate) was prepared by serial diluting each hit compound (10 mM, 5 mM, 2.5mM, 1.125 mM, 0.625 mM, 0.312 mM, 0.156 mM, 78 μ M, 36 μ M, 18 μ M) in 100 % DMSO. Then, 50 nl of each sample were diluted in 50 μ l media (DMEM H-21) and added to the experimental plate followed by incubation at 37°C with 5% CO₂ for 72 h. Cells were then fixed with 4% paraformaldehyde, and rinsed with a solution of 150 mM NaCl, 100 mM NH₄Cl, 0.1% Triton X-100 and 0.1% NaN₃. After that, they were treated for 4 h with 0.2 μ g/ml of the DNA fluorescent dye, DAPI (4,6-diamidino-2-phenylindole), diluted in the same solution. Plates were kept at ambient temperature until image acquisition and analysis were performed as described in the primary screening.

[0072] In vivo studies. To assess *in vivo* efficacy of test

compounds, a 4-day mouse model of infection by transgenic *T.cruzi* Brazil luc strain expressing firefly luciferase was used. Six-week-old female Balb/c mice (average weight 20g) were obtained from Simonsen Labs (Gilroy, CA). All animal protocols were approved and carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee from UCSD (Protocol S14187). Mice were housed at a maximum of 5 per cage and kept in a specific-pathogen free (SPF) room at 20 to 24°C under a 12-h light/12-h dark cycle and provided with sterilized water and chow ad libitum. To infect the mice, trypomastigotes of *T. cruzi* Brazil luc

strain were harvested from culture supernatant and injected intraperitoneally, 10^5 trypomastigotes per mouse. Two control groups included untreated mice, which received a vehicle (20% Kolliphor HS 15, a.k.a. Solutol), and the positive control groups, which received 50 mg/kg benznidazole, all via oral gavage (p.o.), twice a day (b.i.d). Starting on day 3 the infected mice were treated with test compounds at 50 mg/kg administered in 20% Kolliphor, i.p., b.i.d., for four consecutive days. At day 7 post-infection, the luminescent signal from infected mice was read upon injection of D-luciferin. The absolute numbers of measured photons/s/cm² were averaged between all five mice in each group and compared directly with compound-treated mice and the control groups. The efficacy percentage was calculated based on relative luminescence signal reduction compared to the controls.

[0073] Statistics. Two tailed paired Student *t* test was used to assess statistical significance between luminescence values from vehicle-treated and compound-treated groups at day 7 post-infection; values are statistically significant when $p \leq 0.05$.

[0074] Bayesian Models. Using either dose response data alone or the combination of dose response and cytotoxicity (dual activity) resulted in statistically comparable models. Both had one ROC values greater than 0.8 left out (Table 1).

Table 1. Leave-out cross validation data for Bayesian Models

Model	Best cutoff	Leave-one out ROC	5-fold cross validation ROC	5-fold cross validation sensitivity (%)	5-fold cross validation specificity (%)	5-fold cross validation concordance (%)
Dose response (1853 actives, 2203 inactives)	-0.676	0.81	0.78	77	89	84
Dose response and cytotoxicity (1698 actives, 2363 inactives)	-0.337	0.82	0.80	80	88	84

[0075] The use of FCFP₆ fingerprints enabled the good features important for activity to be visualized in the dose response data alone model (Figure 2) which included tertiary amines, piperidines and aromatic fragments containing basic nitrogen functionality while those features that were negatively related to activity included cyclic hydrazines prone to tautomerization as well as a number of electron-poor chlorinated aromatic systems (Figure 3). Similarly for

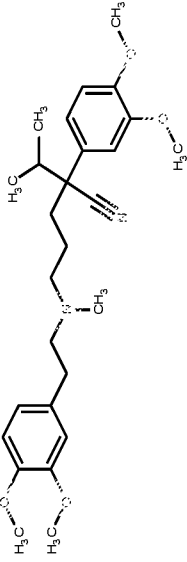
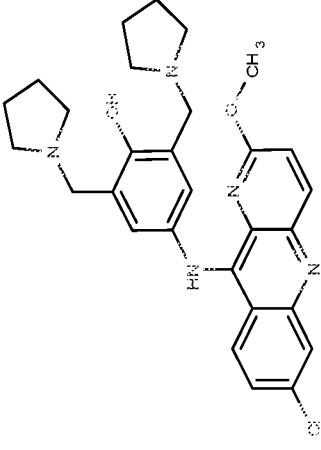
the dual activity the good features were tertiary amines, piperidines and aromatic fragments containing basic nitrogen functionality (Figure 4) and the bad features were again a number of cyclic hydrazines prone to tautomerization and a number of electron-poor chlorinated aromatic systems (Figure 5) providing very clear trends and perhaps a rationale for why the enrichments are so dramatic for these systems. Upon 5 fold cross validation the ROC was greater than 78% for both models and sensitivity, specificity and concordance values were comparable and greater than 77% (Table 1). The more exhaustive leave out 50% x 100 fold for the dual activity model resulted in an external ROC of 0.79 and while concordance and specificity was greater than 73%, sensitivity declined to 66% (Table 2).

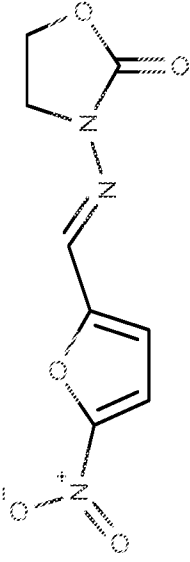
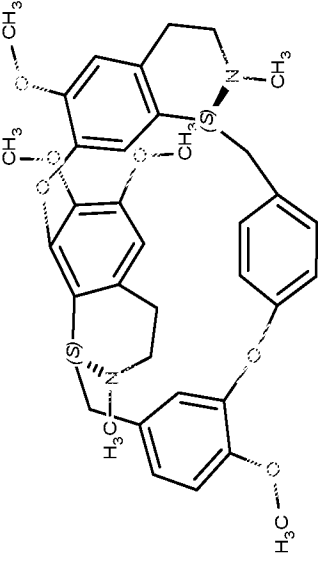
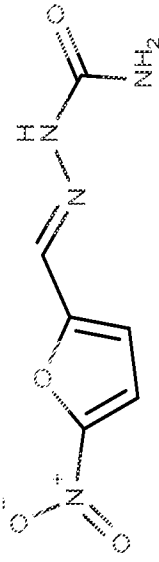
Table 2. Leave-out 50% x 100 fold for Chagas dose response and cytotoxicity Bayesian model.

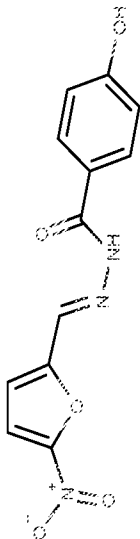
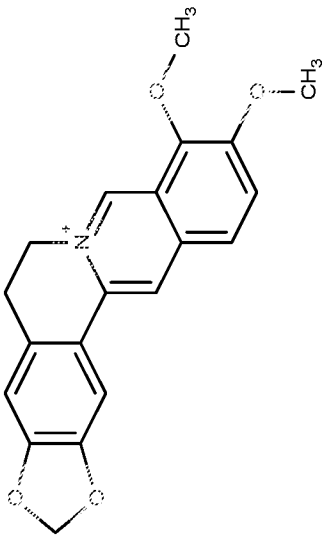
External ROC	Internal ROC	Concordance (%)	Specificity (%)	Sensitivity (%)
0.79 ± 0.01	0.80 ± 0.01	73.48 ± 1.05	79.08 ± 3.73	65.68 ± 3.89

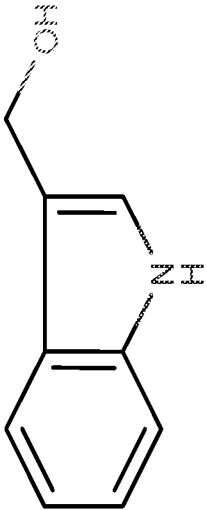
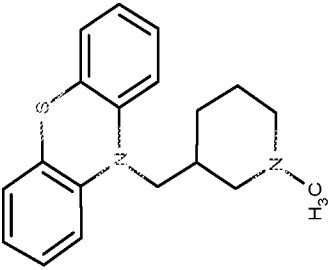
[0076] *In vitro* Screening. Molecules with the highest Bayesian score in the dual event model were selected by an experienced medicinal chemist and purchased. Ninety seven molecules were tested and 11 were found to have EC₅₀ values less than 10μM (see, Table 3).

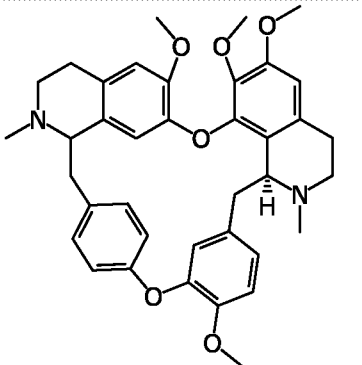
[0077] Table 3. Primary and dose response results

Molecule Name	Structure	Synonyms	Infection Ratio	EC50 (uM)	Hill slope
SC-0005695		(±)-Verapamil hydrochloride, SC-0011762	0.019458858, 0.016758591	0.0383	1.67
SC-0011799		29781612, Pyronaridine	0, 0	0.225	2.03

SC-0011742		511176, Furazolidone	7.712766E-03, 8.769277E-03	0.257	2.81
SC-0011496		501337, SC- 0011777, Tetrandine	0, 0	0.508	1.95
SC-0011754		511054	0.012269939, 0.010091743	0.775	1

SC-0011833		511499	4.020101E-03, 7.333683E-03	1.04	1.34
SC-0011790		739223	0.018524756, 0.017738359	1.68	0.743
SC-0011752		2735992	0.028985507, 0.011235955	1.69	6.04

SC-0011772		539712	0.012753502, 6.218333E-03	2.44	2.95
SC-0011803		876406	0.027158313, 0.038105727	2.72	6.86
SC-0011796		515815	7.481297E-03, 8.14901E-03	4.94	2.18

	501337, SC-0011777, Tetrandrine	0, 0	0.508	1.57	1.95	1.3	43.6
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[0080] In vivo testing. To assess *in vivo* efficacy of test compounds, a 4-day treatment mouse model of infection by transgenic *T. cruzi* Brazil luc strain³⁵ expressing firefly luciferase was used, which enabled the activity in the mouse to be visually measured. All compounds were dosed at 50mg/kg bid. Benznidazole was used as a positive control and showed 100% efficacy alongside furazolidone (Figure 1, Table 4). Hydroxymethylnitrofurazone is a prodrug of nitrofurazone (which had *in vitro* activity) and is an additional known active compound against Chagas Disease, with an efficacy of 78.5%. The prodrug form was chosen to reduce the toxicity of nitrofurazone in the mouse model. Pyronaridine showed 85.2% efficacy while verapamil showed 55.1% and tetrandrine 43.6%, respectively. Apart from tetrandrine, these are statistically significant (Figure 1, Table 4).

[0081] Target prediction. Using several available datasets and resources the potential target/s of pyronaridine were investigated. A similarity search was performed in the Chagas Disease dataset composed of literature data and targets which was curated in this study. The molecules with the highest Tanimoto similarity in CDD were *T. cruzi* GAPDH inhibitors. The metabolites created from the *T. cruzi* pathway model created in this study were also searched. The most similar molecule being S-adenosyl 3-(methylthio)propylamine with a Tanimoto similarity of 0.67 using the MDL Keys in Discovery Studio (Biovia, San Diego, CA). This would point to polyamine biosynthesis. A further approach was to query the ChEMBL database from within the MMDS mobile app. This retrieved several analogs similar to the antimalarial quinacrine, suggesting trypanothione disulfide reductase as a possible target. Quinacrine has also been shown to be a Topoisomerase VI inhibitor elsewhere.

[0082] The computational drug discovery work in tuberculosis was made possible by the existence of datasets with genetic validation of essential genes *in vivo*. The work profited from the existence of the tier one TBCyc metabolic pathway database, the natural divergence of prokaryotic *M. tuberculosis* genome from the genome of the eukaryotic human host, and the availability of a well-annotated *M. tuberculosis* genome. In contrast, *T. cruzi*, the eukaryotic parasite that causes Chagas Disease, and several other eukaryotic human pathogens including the parasites that cause malaria, human African trypanosomiasis, and leishmaniasis, have larger genomes, higher similarity to human enzymes and biological pathways, and have less well annotated genomes. Investment in high throughput screening efforts has resulted in the release of screening data and hit lists for several of these eukaryotic pathogens. It was hypothesized that for pathogens, such as *T. cruzi*, with fewer sources of available data to support bioinformatics approaches to target identification, a reverse approach can be used as compared to the work in tuberculosis. More specifically, it begins with interesting phenotypic screening hits and apply cheminformatic and bioinformatic approaches to map those hits onto potential targets. As a preliminary step in this direction public data was used to build computational models.

[0083] The CDD Public database now includes structural and biological activity data for over 300,000 molecules from the Broad Institute compounds that have been screened against *T. cruzi*. In addition over 500 compounds and their known targets and over 740 compounds from DNDi based around the fungicide fenarimol have been curated. In this study, a subset of the Broad HTS screening data was used to build Bayesian machine learning models to classify compounds as likely actives against *T. cruzi in vitro*. These models were then used to virtually screen several libraries of compounds including drugs and drug-like compounds, to identify compounds with potential activity that may have not been tested yet. Some of these compounds were purchased and tested *in vitro* and then several more tested *in vivo*. Historically, for a diversity-based library undergoing HTS, it is expected a range of 1 to 2% of hits based on observed activity (usually >50% antiparasitic activity at 10 μ M and no signs of

cytotoxicity at this concentration) will be observed. Applying the current method, 11/97 (11%) hits were identified and confirmed with $EC_{50} < 10 \mu M$.

[0084] Out of these hits derived from searching 8 relatively small libraries of compounds, several of the compounds were known actives against *T. cruzi*. Verapamil was previously shown as active in the Broad dataset with an $EC_{50} < 0.1 \mu M$, and has a well-known effect in reducing acute mortality in mice and cardiomyopathy if treated early in infection. It should be noted that others have retested some of the active HTS hits from the Broad *T. cruzi* screen and found higher IC_{50} values. For example the IC_{50} for verapamil in one study was $>50 \mu M$. Pyronaridine is an antimalarial and P-glycoprotein inhibitor and was shown to have an $EC_{50} < 0.587 \mu M$ in the Broad dose response dataset, which is comparable to this study ($EC_{50} 0.225 \mu M$).

Apparently both of these compounds were retrieved as various salt forms from the vendor databases and were initially not considered to be in the training sets. Pyronaridine, was overlooked following the published initial screening and so these compounds were pursued further *in vivo*. Furazolidone is used as a *H. pylori* treatment and has known *in vivo* activity against *T. cruzi* and was not in the dose response training set (but is in the larger Broad screening dataset of over 300,000 compounds), so can be considered a true 'prediction'. Tetrandrine is a P-glycoprotein inhibitor that has been tested in malaria in combination with chloroquine. This molecule was not in the training dataset but was in the larger Broad HTS screening dataset to identify inhibitors of replication as an 'inactive', so the ability to identify a previous false negative as an active prediction is an interesting observation, although this compound does not appear to have statistically significant efficacy *in vivo*. The known *T. cruzi* active compound Nitrofurantoin (nitrofurazone) was also not in the model training set or the Broad dataset, but was predicted as 'active' *in vitro* (experimentally confirmed $EC_{50} 0.77 \mu M$ and $CC_{50} > 10 \mu M$), and its prodrug form hydroxymethylnitrofurazone was used as an internal control (while benznidazole was a positive control) in the *in vivo* experiments. These results illustrate that the dose response and cytotoxicity machine learning model based on *T. cruzi* replication HTS data used

in this case, could retrieve known active compounds useful for Chagas Disease. While the Broad screen and the assay used in this study are similar in that they are both cell-based, they each use different cell lines for *T. cruzi* culture and different readouts. The Broad screen used the Tulahuen genetically modified to express Beta-galactosidase which is biased towards finding CYP51 inhibitors, while these experiments used the CA-I/72 strain with an image-based readout. There are no apparent publications describing pyronaridine being tested in the mouse model for Chagas Disease and the observation of 85.2% efficacy (higher than nitrofurantoin) suggests this molecule is therefore worthy of further study (Figure 1). In particular, the identification of the likely target or targets for this molecule would be very important. Using various informatics resources the methods attempted to predict these in this study. The prior work on *Mtb* resulted in many datasets relating to small molecules and their targets in the bacteria, which in turn lead to the development of the TB Mobile app which contains Bayesian models that can be used for target prediction.

[0085] The disclosure highlights how the *in vivo* transgenic *T. cruzi* Brazil luc strain expressing firefly luciferase data can be stored in the software (Figure 3). In the process of this study *T. cruzi* data was curated, constructed a Pathway Genome Data Base for *T. cruzi*, developed multiple Bayesian machine learning models, tested molecules *in vitro* and *in vivo* as well as proposed potential targets for one of the *in vivo* active compounds. In the process pyronaridine was identified as having promising *in vivo* activity in the mouse model of Chagas Disease.

Example 2

[0086] Experiments will be performed to confirm *in vivo* efficacy of cathepsin S in *Trypanosoma cruzi* mouse model of infection. Using *T. cruzi* parasites transfected with the luciferase gene, a rapid 4-day dosing mouse model is employed to compare cathepsin S inhibitor to benznidazole and untreated control group, utilizing *In vivo* Imaging System (IVIS - Perkin Elmer) to quantify and confirm anti-parasitic activity.

[0087] The target for cathepsin S inhibitors in *Trypanosoma cruzi* is the major protease, cruzain (cruzipain). This is a Clan CA

(papain family) cysteine protease homologous in catalytic mechanism and structure to cathepsin S. In fact, there has been a "proof of principle" protease inhibitor, K777, which has demonstrated, in rodent and dog models of infection, a capacity to cure disease and prevent cardiac manifestations at doses which are orally available and safe. However, K777 is a Michael-acceptor vinylsulfone, resulting in irreversible inhibition of the enzyme target. A noncovalent inhibitor, such as either of the two available NCATS compounds (RWJ-445380 and SAR114137) would be preferable. Notably, K777 was originally synthesized as a cathepsin S inhibitor, but that program was abandoned due to lack of efficacy in human disease models. Extensive evaluation of this inhibitor in rodents, dogs, and primates has confirmed acceptable PK parameters and safety for a small molecule cathepsin S inhibitors.

[0088] The target for cathepsin S inhibitors in *Trypanosoma cruzi* is the major cysteine protease, cruzain (aka cruzipain). This target has been validated chemically by showing that a number of active site directed cysteine protease inhibitors, varying in scaffold and inhibitory mechanisms result in killing of *T. cruzi* parasites *in vitro*, as well as in a mouse model of infection. A screening *in vivo* assay, utilizing luciferase-transfected parasites is available as an initial evaluation tool. In addition, a mouse models of Chagas Disease which lead to cardiac failure have been optimized utilizing the clinical isolate strain CAI-729, representing a chronic model. Finally, a selective active-site probe of the target cruzain is available. This allows covalent modification of the active site, both *in vitro* and *in vivo*, providing validation of the cysteine protease target by the industry-provided cathepsin S inhibitors. With these tools and models already in place, rapid assessment can be performed whether or not either of the two cathepsin S inhibitors are effective in clearing *T. cruzi* infection, and if they target the expected parasite enzyme.

[0089] The project approach to validating either of the two cathepsin S inhibitors as potential drugs for the treatment of Chagas Disease will have three phases. In the first phase, these two inhibitors will be directly tested in mouse models of *T. cruzi*

infection, utilizing both the 4-days treatment with luciferase-transfected parasites (rapid activity assessment), and the 20-days treatment with the CA-I/72 clinical isolate parasite strain to assess parasitological cure. If efficacy in the animal model is equal to or greater than benznidazole, either of these compounds can progress to clinical studies. However, even if partial activity is noted, there remains the option of testing derivatives of these compounds.

[0090] Previous studies have indicated that these cathepsin S inhibitors are reasonably safe in the human host. SAR114137 has been dosed for as long as 14 days. RWJ-445380 was dosed as long as 13 weeks. This provides confidence that a 20-day course of therapy, as recommended for Chagas Disease, could be achieved without significant side effects.

[0091] Initial dose response curves as described above, were performed with RWJ-445380 (Formula II, above). The dose response curve demonstrated an IC₅₀ of 1.7 μ M (see, e.g., Figure 6).

[0092] *In vivo* pharmacokinetics in the mouse for the compound of Formula II dosed at 2 mg/mouse (100 mg/kg) are provided in Table 5.

[0093] Table 5: *In vivo* pharmacokinetics (Mouse)

Time (Hour)	Plasma Concentration (ng/mL)					
	2 mg/Mouse PO			2 mg/Mouse IP		
Dose	Mouse 1	Mouse 2	Avg ^a	Mouse 3	Mouse 4	Avg ^a
	BLOQ	BLOQ		BLOQ	BLOQ	
1	1913.6	992.3	1453.6	3344.8	4536.2	4940.5
2	2731.7	1589.7	2160.7	3092.8	3628.3	4360.6
4	1212.4	1122.3	1167.3	3632.2	2624.1	3128.2
8	898.8	687.6	892.3	1689.3	1344.6	1515.1
24	67.6	123.7	95.7	27.6	6.1	16.8

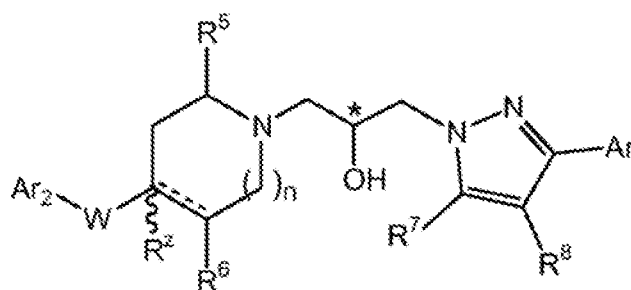
[0094] Using *T. cruzi* CA-I/72 clinical isolate a 20-day mouse dosing study is performed, comparing to benznidazole-treated group as a reference drug control, an untreated control group and a non-infected control group. Parasitological "cure" will be determined based on quantitative PCR from blood sample right after the treatment ends, and also include a follow-up re-test after immunosuppression to detect any "latent" parasites 60 days post-infection.

[0095] In collaboration with colleagues in South America. *T. cruzi*-infected patients will be recruited by *T. cruzi* serology (Ortho), and positive blood PCR.

[0096] Carry out Phase 2a clinical trial using model of posaconazole and ravuzonazole trials 20-day dosing of cathepsin S inhibitors orally, with reevaluation of PCR and serology positivity at one month and six months. Expected Result: Compared to benznidazole, cathepsin S inhibitors should result in negative PCR in treated patients at both one month and six months.

WHAT IS CLAIMED IS:

1. A method of treating a *T. cruzi* infection comprising contacting a subject with the infection with an agent that inhibits a cathepsin S or a homolog having cysteine protease activity.
2. The method of claim 1, wherein the agent comprises a compound of Formula I:



Formula I

wherein

Ar₂ is a monocyclic or bicyclic ring system, unsaturated, saturated or aromatic, optionally fused, optionally including between 1 and 5 heteroatom ring moieties independently selected from the group consisting of O, S, N, SO₂ and C=O; wherein said Ar₂ ring system is optionally substituted with between 1 and 4 substituents;

W represents O, S, NR²⁷, C=O, (C=O)NH, NH(C=O)₁CHR²⁸, or a covalent bond;

R²⁷ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, naphthyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R²⁹OC=O, R³⁰R³¹NC=O, R²⁹SO, R²⁹S, R²⁹SO₂ or R³⁰R³¹NSO₂; or alternatively, R²⁷ and part of Ar₂ can be taken together to form an optionally substituted 5- to 6-membered heterocyclic ring with optionally 1 to 3 additional heteroatom moieties in the ring selected from O, NR⁹, NR¹⁰, N, SO₂, C=O and S; which ring may be saturated, unsaturated or aromatic; wherein R⁹ and R¹⁰ are independently selected from the group consisting of H, C₁₋₃ alkyl, and -CH₂CO₂(C₁₋₄alkyl);

R²⁸ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, hydroxy, phenyl, benzyl, C₁₋₅ heterocyclyl, R²⁹O, R³⁰R³¹NC=O, R²⁹S, R²⁹SO, R²⁹SO₂ or R³⁰R³¹NSO₂;

R²⁹ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl;

R³⁰ and R³¹ are each independently selected from the group consisting of hydrogen, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl,

phenethyl, naphthyl, and C₁₋₅ heteroaryl; alternatively R³⁰ and R³¹ can be taken together to form an optionally substituted 4- to 7-membered ring carbocyclic or heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R² is H or OH and the dashed line is absent; or R² is absent where the dashed line is an sp² bond;

R⁵ and R⁶ are each independently selected from the group consisting of hydrogen and C₁₋₅ alkyl;

n is an integer selected from 0, 1 or 2;

R⁷ and R⁸ are each independently hydrogen, C₁₋₅ alkyl, C₂₋₅ alkenyl, C₁₋₅ alkoxy, C₁₋₅ alkylthio, halogen, or a 4-7 membered carbocyclyl or heterocyclyl; alternatively, R⁷ and R⁸ can be taken together to form an optionally substituted 5- to 7-membered carbocyclic or heterocyclic ring, which ring may be unsaturated or aromatic, and may be optionally substituted with between one and three substituents independently selected from the group consisting of halo, cyano, amino, hydroxy, nitro, R⁴, R⁴O-, R⁴S-, R⁴O(Ci-₅alkylene)-, R⁴O(C=O)-, R⁴(C=O)-, R⁴(C=S)-, R⁴(C=O)O-, R⁴O(C=O)(C=O)-, R⁴SO₂, NHR⁴⁴(C=NH)-, NHR⁴⁴SO₂-, an NHR⁴⁴(C=O)-;

R⁴ is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, C₁₋₅ heterocyclyl, (C₁₋₅ heterocyclyl) C₁₋₆ alkylene, phenyl, benzyl, phenethyl, NH₂, mono- or di(C₁₋₆ alkyl)N-, (C₁₋₆ alkoxy)carbonyl- or R⁴²OR⁴³-; wherein R⁴² is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, phenyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, or (C₁₋₅ heterocyclyl) C₁₋₆ alkylene- and R⁴³ is C₁₋₅ alkylene, phenylene, or divalent C₁₋₅ heterocyclyl;

R⁴⁴ is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, C₁₋₅ heterocyclyl, (C₁₋₅ heterocyclyl) C₁₋₆ alkylene, phenyl, benzyl, phenethyl, NH₂, mono- or di(C₁₋₆ alkyl)N-, (C₁₋₆ alkoxy)carbonyl- or R⁴²OR⁴³-; wherein R⁴² is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, phenyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, or (C₁₋₅ heterocyclyl) C₁₋₆ alkylene- and R⁴³ is C₁₋₅ alkylene, phenylene, or divalent C₁₋₅ heterocyclyl;

Ar represents a monocyclic or bicyclic aryl or heteroaryl ring, optionally substituted with between 1 and 3 substituents independently selected from the group consisting of halogen, C₁₋₅ alkoxy, C₁₋₅ alkyl, C₂₋₅ alkenyl, cyano, azido, nitro, R²²R²³N, R²⁴SO₂, R²⁴SO, R²⁴OC=O, R²²R²³NC=O, C₁₋₅ haloalkyl, C₁₋₅ haloalkoxy, C₁₋₅ haloalkylthio and C₁₋₅ alkylthio;

R²² is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, phenethyl, benzyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R³⁸OC=O, R²⁵R²⁶NC=O, R³⁸SO, R³⁸SO₂, R³⁸S, or R²⁵R²⁶NSO₂;

R²³ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; alternatively, R²² and R²³ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R²⁴ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl, or C₁₋₅ heterocyclyl;

R²⁵ and R²⁶ independently are hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; or alternatively, R²⁵ and R²⁶ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

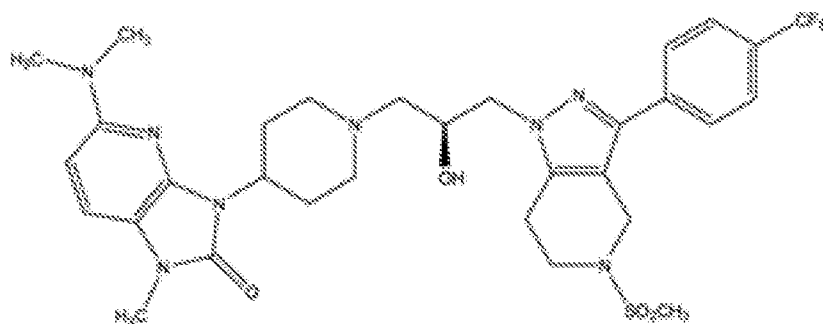
R³⁸ is H, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl, phenethyl or C₁₋₅heterocyclyl;

wherein each of the above hydrocarbyl or heterocarbyl groups, unless otherwise indicated, and in addition to any specified substituents, is optionally and independently substituted with between 1 and 3 substituents selected from the group consisting of methyl, halomethyl, hydroxymethyl, halo, hydroxy, amino, nitro, cyano, C₁₋₅ alkyl, C₁₋₅ alkoxy, -COOH, C₂₋₆ acyl, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkylene, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkyl-NH-CO-, and C₁₋₅ haloalkoxy;

or a pharmaceutically acceptable salt, amide or ester thereof; or a stereoisomeric form thereof.

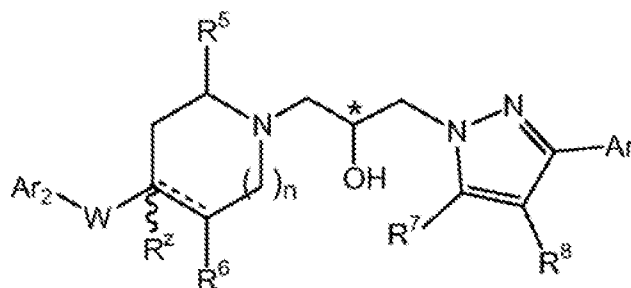
3. The method of claim 1, wherein the agent comprises a compound having the general formula C₃₁H₃₉F₃N₈O₄S or salt thereof.

4. The method of claim 1, wherein the agent comprises a compound of Formula II:



Formula II.

5. The method of claim 1, wherein the homology having cysteine protease activity comprises cruzipain.
6. The method of claim 1, wherein the agent inhibits cruzipain activity.
7. A method of treating Chagas Disease comprising contacting a subject with Chagas Disease with an agent that inhibits a cathepsin S or a homolog having cysteine protease activity.
8. The method of claim 7, wherein the agent comprises a compound of Formula I:



Formula I

wherein

Ar₂ is a monocyclic or bicyclic ring system, unsaturated, saturated or aromatic, optionally fused, optionally including between 1 and 5 heteroatom ring moieties independently selected from the group consisting of O, S, N, SO₂ and C=O; wherein said Ar₂ ring system is optionally substituted with between 1 and 4 substituents;

W represents O, S, NR²⁷, C=O, (C=O)NH, NH(C=O)₁CHR²⁸, or a covalent bond;

R^{27} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, naphthyl, benzyl, phenethyl, C_{1-5} heterocyclyl, C_{2-8} acyl, aroyl, $R^{29}OC=O$, $R^{30}R^{31}NC=O$, $R^{29}SO$, $R^{29}S$, $R^{29}SO_2$ or $R^{30}R^{31}NSO_2$; or alternatively, R^{27} and part of Ar_2 can be taken together to form an optionally substituted 5- to 6-membered heterocyclic ring with optionally 1 to 3 additional heteroatom moieties in the ring selected from O, NR^9 , NR^{10} , N, SO_2 , C=O and S; which ring may be saturated, unsaturated or aromatic; wherein R^9 and R^{10} are independently selected from the group consisting of H, C_{1-3} alkyl, and $-CH_2CO_2(C_{1-4}alkyl)$;

R^{28} is hydrogen, C_{1-5} alkyl, C_{3-5} alkenyl, hydroxy, phenyl, benzyl, C_{1-5} heterocyclyl, $R^{29}O$, $R^{30}R^{31}NC=O$, $R^{29}S$, $R^{29}SO$, $R^{29}SO_2$ or $R^{30}R^{31}NSO_2$;

R^{29} is C_{1-5} alkyl, C_{3-5} alkenyl, phenyl, benzyl or C_{1-5} heterocyclyl;

R^{30} and R^{31} are each independently selected from the group consisting of hydrogen, $C_{1-5}alkyl$, $C_{3-5}alkenyl$, phenyl, benzyl, phenethyl, naphthyl, and C_{1-5} heteroaryl; alternatively R^{30} and R^{31} can be taken together to form an optionally substituted 4- to 7-membered ring carbocyclic or heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R^Z is H or OH and the dashed line is absent; or R^Z is absent where the dashed line is an sp^2 bond;

R^5 and R^6 are each independently selected from the group consisting of hydrogen and C_{1-5} alkyl;

n is an integer selected from 0, 1 or 2;

R^7 and R^8 are each independently hydrogen, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} alkoxy, C_{1-5} alkylthio, halogen, or a 4-7 membered carbocyclyl or heterocyclyl; alternatively, R^7 and R^8 can be taken together to form an optionally substituted 5- to 7-membered carbocyclic or heterocyclic ring, which ring may be unsaturated or aromatic, and may be optionally substituted with between one and three substituents independently selected from the group consisting of halo, cyano, amino, hydroxy, nitro, R^4 , R^4O- , R^4S- , $R^4O(Ci-5alkylene)-$, $R^4O(C=O)-$, $R^4(C=O)-$, $R^4(C=S)-$, $R^4(C=O)O-$, $R^4O(C=O)(C=O)-$, R^4SO_2 , $NHR^{44}(C=NH)-$, $NHR^{44}SO_2-$, an $NHR^{44}(C=O)-$;

R^4 is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{1-5} heterocyclyl, (C_{1-5} heterocyclyl) C_{1-6} alkylene, phenyl, benzyl, phenethyl, NH_2 , mono- or di(C_{1-6} alkyl)N-, (C_{1-6} alkoxy)carbonyl- or $R^{42}OR^{43}-$; wherein R^{42} is H, C_{1-5} alkyl, C_{2-5} alkenyl, phenyl, benzyl, phenethyl, C_{1-5} heterocyclyl,

or (C₁₋₅ heterocyclyl) C₁₋₆ alkylene- and R⁴³ is C₁₋₅ alkylene, phenylene, or divalent C₁₋₅ heterocyclyl;

R⁴⁴ is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, C₁₋₅ heterocyclyl, (C₁₋₅ heterocyclyl) C₁₋₆ alkylene, phenyl, benzyl, phenethyl, NH₂, mono- or di(C₁₋₆ alkyl)N-, (C₁₋₆ alkoxy)carbonyl- or R⁴²OR⁴³-; wherein R⁴² is H, C₁₋₅ alkyl, C₂₋₅ alkenyl, phenyl, benzyl, phenethyl, C₁₋₅ heterocyclyl, or (C₁₋₅ heterocyclyl)C₁₋₆ alkylene- and R⁴³ is C₁₋₅ alkylene, phenylene, or divalent C₁₋₅ heterocyclyl;

Ar represents a monocyclic or bicyclic aryl or heteroaryl ring, optionally substituted with between 1 and 3 substituents independently selected from the group consisting of halogen, C₁₋₅ alkoxy, C₁₋₅ alkyl, C₂₋₅ alkenyl, cyano, azido, nitro, R²²R²³N, R²⁴SO₂, R²⁴SO, R²⁴OC=O, R²²R²³NC=O, C₁₋₅ haloalkyl, C₁₋₅ haloalkoxy, C₁₋₅ haloalkylthio and C₁₋₅ alkylthio;

R²² is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, phenethyl, benzyl, C₁₋₅ heterocyclyl, C₂₋₈ acyl, aroyl, R³⁸OC=O, R²⁵R²⁶NC=O, R³⁸SO, R³⁸SO₂, R³⁸S, or R²⁵R²⁶NSO₂;

R²³ is hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; alternatively, R²² and R²³ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

R²⁴ is C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl, or C₁₋₅ heterocyclyl;

R²⁵ and R²⁶ independently are hydrogen, C₁₋₅ alkyl, C₃₋₅ alkenyl, phenyl, benzyl or C₁₋₅ heterocyclyl; or alternatively, R²⁵ and R²⁶ can be taken together to form an optionally substituted 4- to 7-membered heterocyclic ring, which ring may be saturated, unsaturated or aromatic;

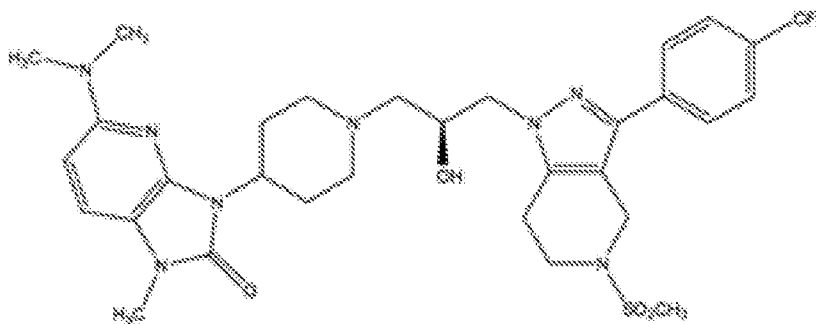
R³⁸ is H, C₁₋₅alkyl, C₃₋₅alkenyl, phenyl, benzyl, phenethyl or C₁₋₅heterocyclyl;

wherein each of the above hydrocarbyl or heterocarbyl groups, unless otherwise indicated, and in addition to any specified substituents, is optionally and independently substituted with between 1 and 3 substituents selected from the group consisting of methyl, halomethyl, hydroxymethyl, halo, hydroxy, amino, nitro, cyano, C₁₋₅ alkyl, C₁₋₅ alkoxy, -COOH, C₂₋₆ acyl, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkylene, [di(C₁₋₄ alkyl)amino]C₂₋₅ alkyl-NH-CO-, and C₁₋₅ haloalkoxy;

or a pharmaceutically acceptable salt, amide or ester thereof;
or a stereoisomeric form thereof.

9. The method of claim 7, wherein the agent comprises a compound having the general formula $C_{31}H_{39}F_3N_8O_4S$ or salt thereof.

10. The method of claim 7, wherein the agent comprises a compound of Formula II:



Formula II.

11. The method of claim 7, wherein the homology having cysteine protease activity comprises cruzipain.

12. The method of claim 7, wherein the agent inhibits cruzipain activity.

13. A combination therapy comprising an agent that inhibits cathepsin S, and at least one of benznidazole and/or nifurtimox.

14. A pharmaceutical composition comprising an agent that inhibits cathepsin S and an agent selected from benznidazole, nifurtimox and a combination thereof.

15. The pharmaceutical composition of claim 14, wherein the agent that inhibits cathepsin S comprises an agent of Formula I and/or Formula II.

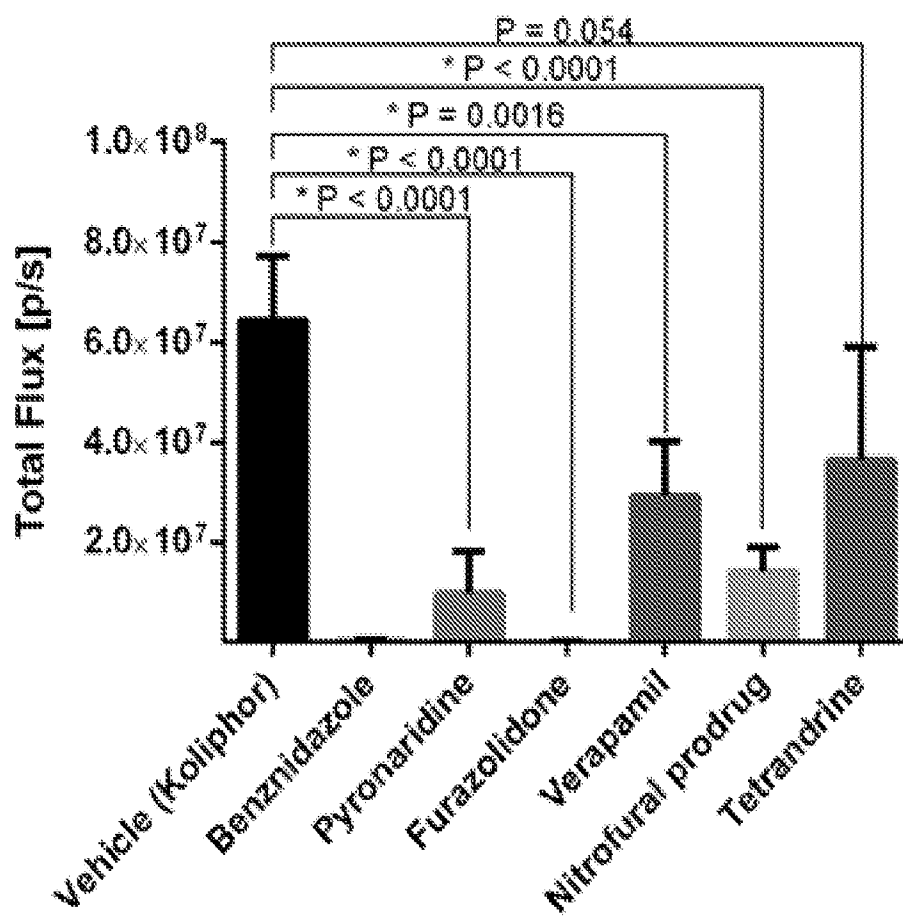
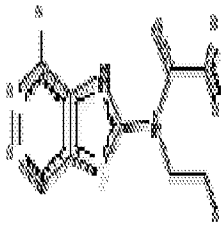
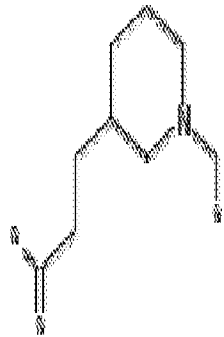
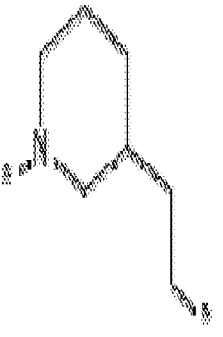
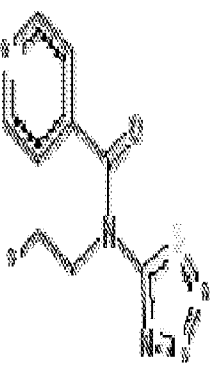
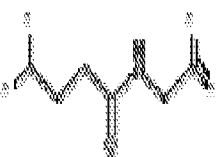
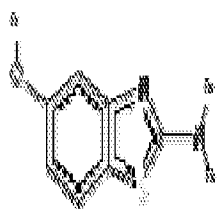
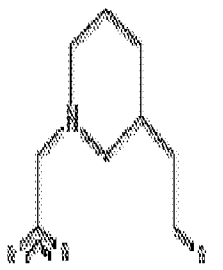
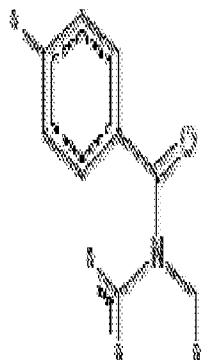
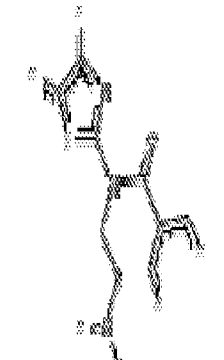


FIGURE 1

Figure 2. Broad Chagas T. Cruzi dose response: good features from FCFP_6

 G1: 1151340232 56 out of 57 good Bayesian Score: 0.754	 G2: -1031377555 27 out of 27 good Bayesian Score: 0.750	 G3: -553669137 27 out of 27 good Bayesian Score: 0.750	 G4: 284237667 48 out of 49 good Bayesian Score: 0.748	 G5: 193414534 25 out of 25 good Bayesian Score: 0.747
 G6: -551668701 25 out of 25 good Bayesian Score: 0.747	 G7: -1214801979 25 out of 25 good Bayesian Score: 0.747	 G8: 84111596 23 out of 23 good Bayesian Score: 0.743	 G9: -1799269324 38 out of 39 good Bayesian Score: 0.737	 G10: 776700060 19 out of 19 good Bayesian Score: 0.734

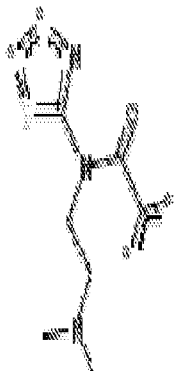
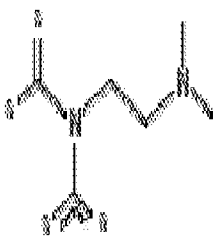
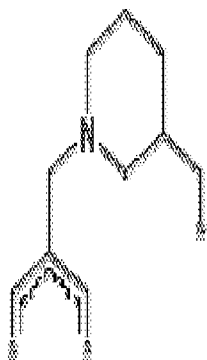
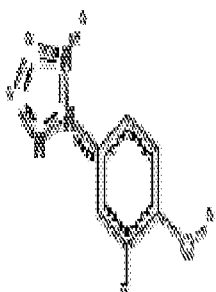
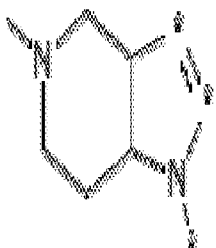
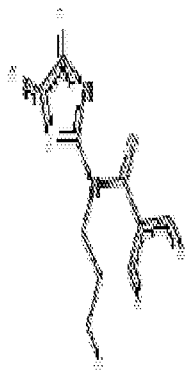
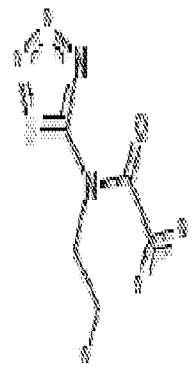
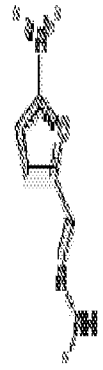
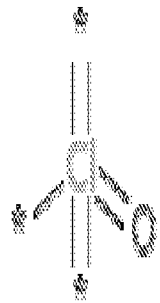
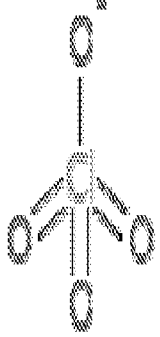
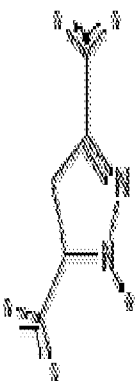
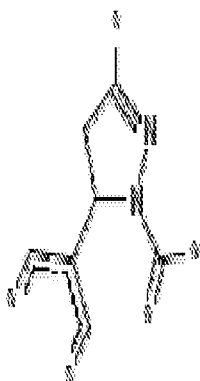
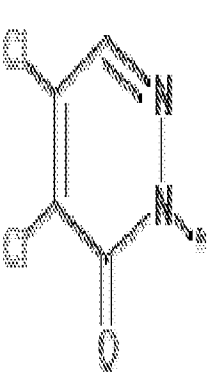
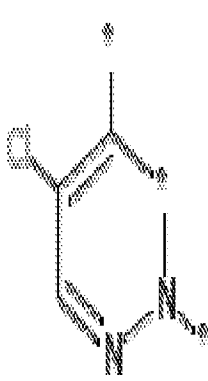
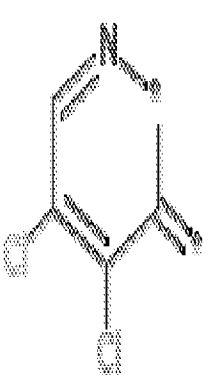
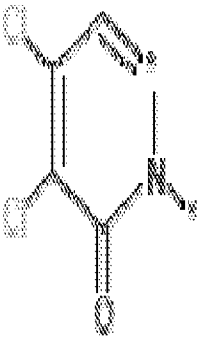
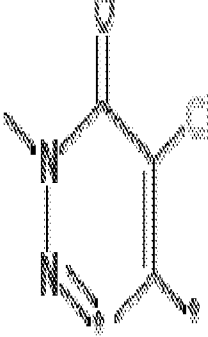
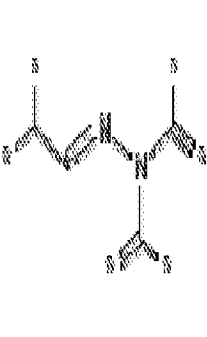
 G11: 1566208234 19 out of 19 good Bayesian Score: 0.734	 G12: -356513920 19 out of 19 good Bayesian Score: 0.734	 G13: -1541471493 73 out of 77 good Bayesian Score: 0.724	 G14: -301872638 16 out of 16 good Bayesian Score: 0.724	 G15: 675763288 16 out of 16 good Bayesian Score: 0.724
 G16: 774560441 30 out of 31 good Bayesian Score: 0.724	 G17: -609539162 58 out of 61 good Bayesian Score: 0.724	 G18: -1808404930 56 out of 59 good Bayesian Score: 0.721	 G19: 1873107836 15 out of 15 good Bayesian Score: 0.720	 G20: -6220344 15 out of 15 good Bayesian Score: 0.720

FIGURE 2 (cont.)

Figure 3. Broad Chagas T Cruzi dose response: bad features from FCFP_6

 B1: 453075031 0 out of 23 good Bayesian Score: -2.435	 B2: -975093520 0 out of 19 good Bayesian Score: -2.262	 B3: -1782262991 0 out of 18 good Bayesian Score: -2.214	 B4: 1485918631 0 out of 18 good Bayesian Score: -2.214	 B5: -1110304493 0 out of 18 good Bayesian Score: -2.214
 B6: -1116653357 0 out of 18 good Bayesian Score: -2.214	 B7: 357154323 0 out of 18 good Bayesian Score: -2.214	 B8: 1585170049 0 out of 18 good Bayesian Score: -2.214	 B9: 1249923161 0 out of 16 good Bayesian Score: -2.109	 B10: 1108944866 0 out of 16 good Bayesian Score: -2.109

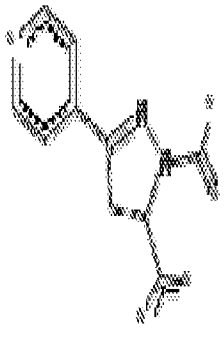
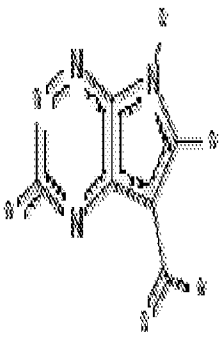
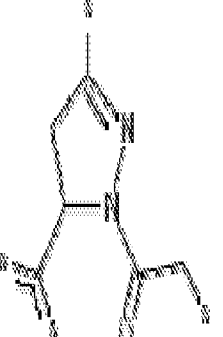
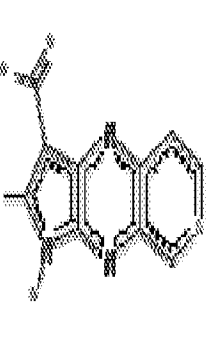
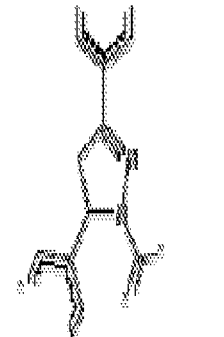
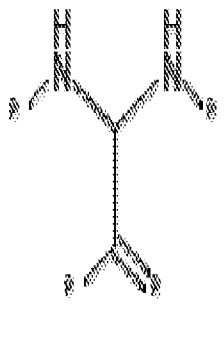
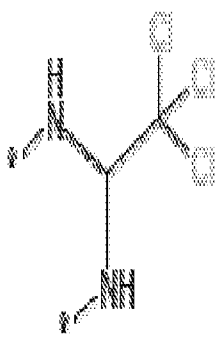
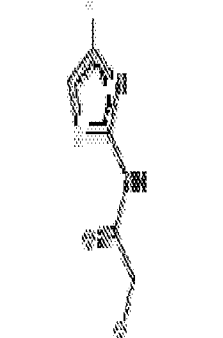
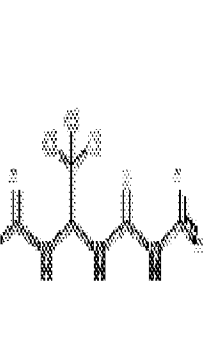
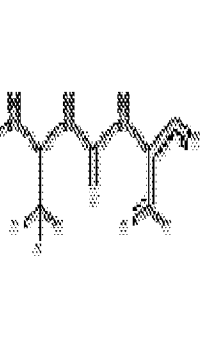
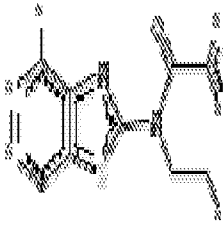
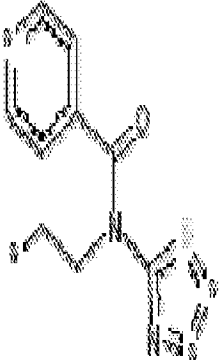
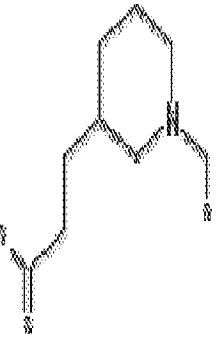
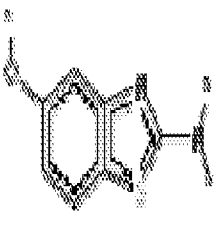
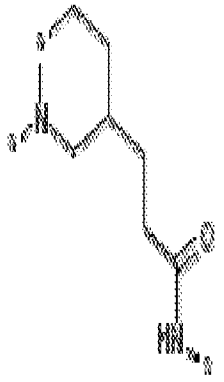
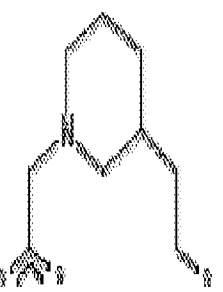
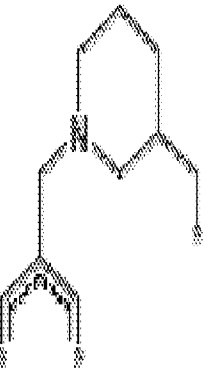
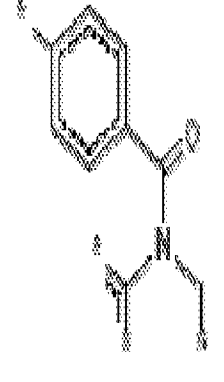
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 B16: -109786778 0 out of 13 good Bayesian Score: -1.929	 B17: 32983469 0 out of 12 good Bayesian Score: -1.861	 B18: -144598603 0 out of 12 good Bayesian Score: -1.861	 B19: 632156824 0 out of 12 good Bayesian Score: -1.861	 B20: -2082572339 0 out of 12 good Bayesian Score: -1.861

FIGURE 3 (cont.)

Figure 4. Broad Chagas T Cruzi dose response and cytotox: good features from FCFP_6

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 G6: 193414534 25 out of 25 good Bayesian Score: 0.825	 G7: -551668701 25 out of 25 good Bayesian Score: 0.825	 G8: 84111596 23 out of 23 good Bayesian Score: 0.821	 G9: -1541471493 74 out of 77 good Bayesian Score: 0.821	 G10: -1799269324 38 out of 39 good Bayesian Score: 0.818

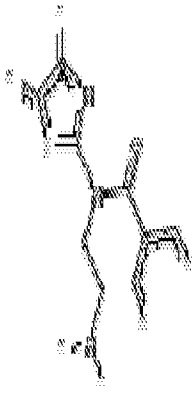
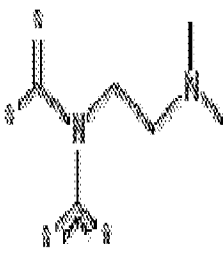
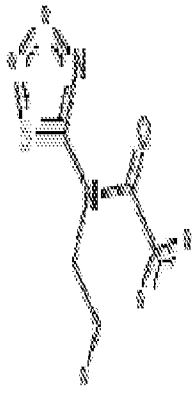
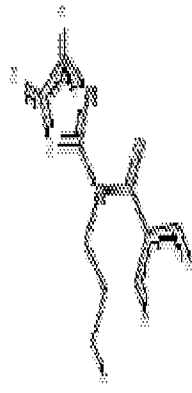
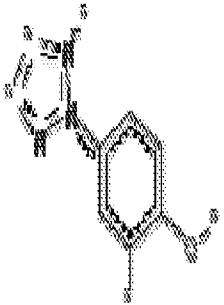
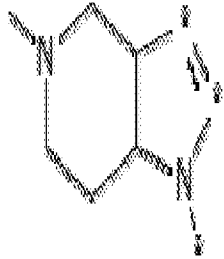
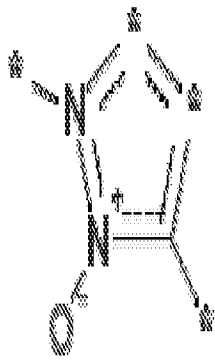
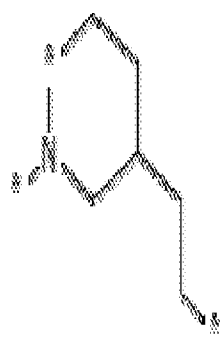
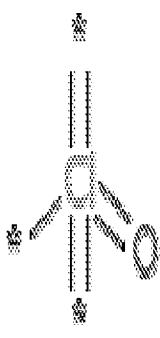
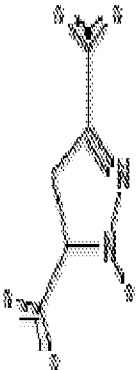
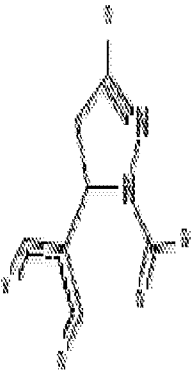
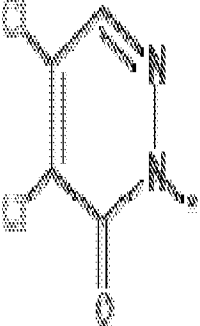
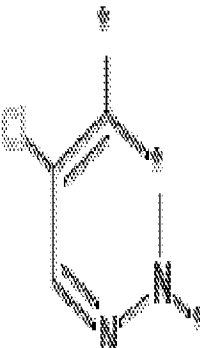
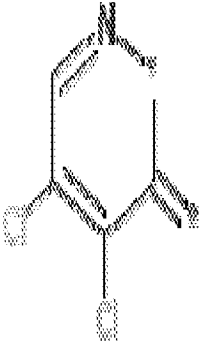
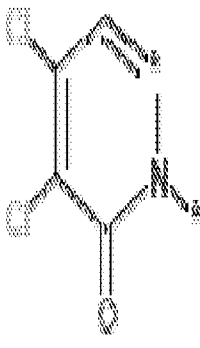
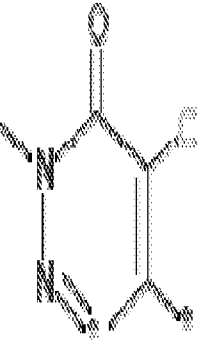
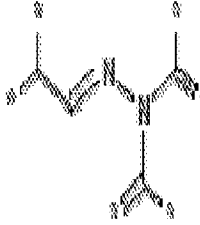
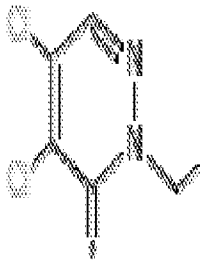
 G11: 1566208234 19 out of 19 good Bayesian Score: 0.810	 G12: 776700060 19 out of 19 good Bayesian Score: 0.810	 G13: -356513920 19 out of 19 good Bayesian Score: 0.810	 G14: -609539162 58 out of 61 good Bayesian Score: 0.806	 G15: 774560441 30 out of 31 good Bayesian Score: 0.803
 G16: -301872638 16 out of 16 good Bayesian Score: 0.798	 G17: 675763288 16 out of 16 good Bayesian Score: 0.798	 G18: -1715686892 28 out of 29 good Bayesian Score: 0.798	 G19: 1422550102 27 out of 28 good Bayesian Score: 0.795	 G20: 1873107836 15 out of 15 good Bayesian Score: 0.794

FIGURE 4 (cont.)

Figure 5. Broad Chagas T Cruzi dose response and cytotox: bad features from FCFP_6

 B1: 453075031 0 out of 23 good Bayesian Score: -2.357	 B2: -975093520 0 out of 19 good Bayesian Score: -2.186	 B3: 1474313868 0 out of 19 good Bayesian Score: -2.186	 B4: -1782262991 0 out of 18 good Bayesian Score: -2.138	 B5: 1485918631 0 out of 18 good Bayesian Score: -2.138
 B6: -1110304493 0 out of 18 good Bayesian Score: -2.138	 B7: -1116653357 0 out of 18 good Bayesian Score: -2.138	 B8: 357154323 0 out of 18 good Bayesian Score: -2.138	 B9: 1585170049 0 out of 18 good Bayesian Score: -2.138	 B10: 1108944866 0 out of 16 good Bayesian Score: -2.035

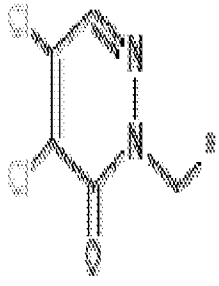
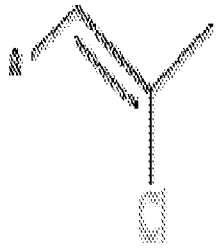
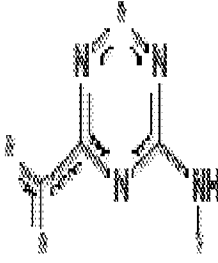
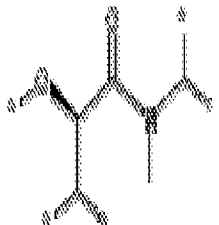
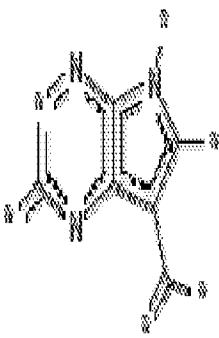
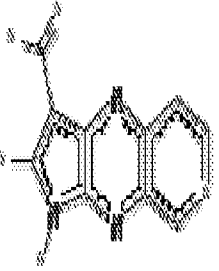
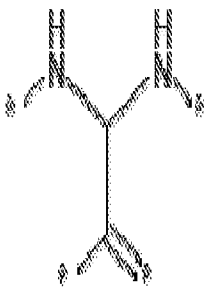
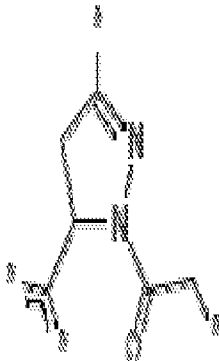
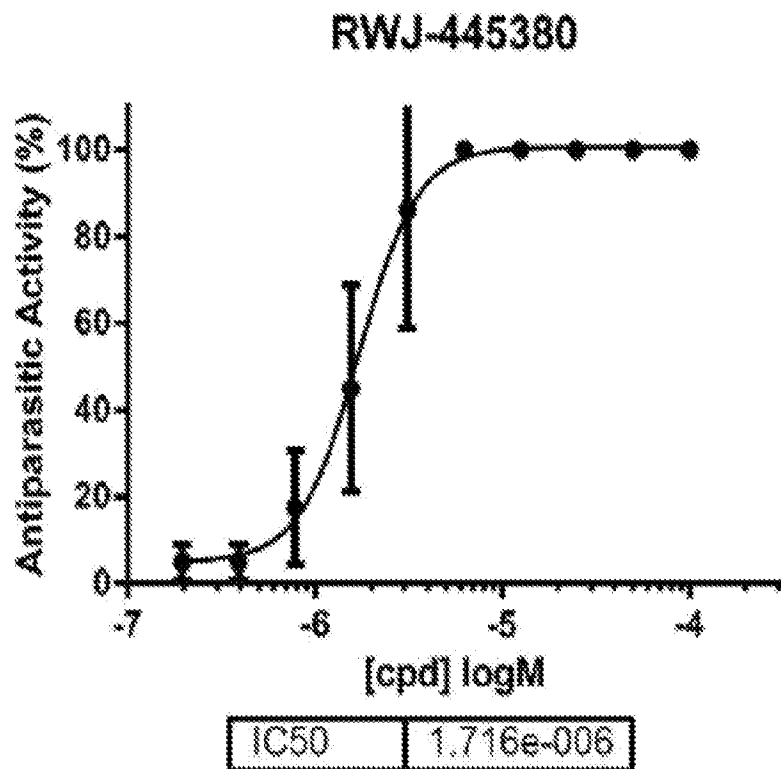
 B11: 1249923161 0 out of 16 good Bayesian Score: -2.035	 B12: 501530171 1 out of 34 good Bayesian Score: -2.024	 B13: -1775135141 0 out of 15 good Bayesian Score: -1.979	 B14: -36630029 0 out of 14 good Bayesian Score: -1.920	 B15: -950109695 0 out of 14 good Bayesian Score: -1.920
 B16: 1796123205 0 out of 13 good Bayesian Score: -1.857	 B17: 1559056674 0 out of 13 good Bayesian Score: -1.857	 B18: -109786778 0 out of 13 good Bayesian Score: -1.857	 B19: 76171628 0 out of 13 good Bayesian Score: -1.857	 B20: 433286102 0 out of 13 good Bayesian Score: -1.857

FIGURE 5 (cont.)



1.716 μ M=1,161ng/ml

FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/28510

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/445; A61K 31/415; A61P 33/00 (2016.01)

CPC - C07D471/04; C07D401/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

CPC: C07D471/04; C07D401/14

IPC(8): A61K 31/445; A61K 31/415; A61P 33/00 (2016.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/320; 514/326; 514/406 (See Search Words Below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATBASE: Full-text = AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO

Google: Scholar/Patents: Cathepsin S inhibitor trypanosoma cruzi, T.cruzi cruzipain cysteine protease homolog benzimidazole
combination therapy chagas tetrahydropyrazol[4,3-c]pyridin-1-yl piperidine imidazo[4,5-d]pyridin-2-one trifluoromethylphenyl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2009/075778 A2 (The Regents of the University of California) 18 June 2009 (18.06.2009) para [0004];[0008];[0086];[0088]-[0091];[0094];[0095]	1, 5-7, 11-12 ----- 2-4, 8-10
X ---- Y	US 2012/0101053 A1 (BLACK et al.) 26 April 2012 (26.04.2012) para [0029];[0032];[0044];[0045];[0050]	13, 14 ----- 15
Y	US 2002/0055497 A1 (BUTLER et al.) 09 May 2002 (09.05.2002) para [0069];[0070]; [0260];[0261];[0532], Example 31	2-4, 8-10, 15

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 June 2016

Date of mailing of the international search report

26 JUL 2016

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