ABSTRACT

Kinetic Studies of Thiosemicarbazone Inhibitors of Cruzain, a Validated Target of *Trypanosoma cruzi*

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Chagas' disease is caused by the flagellate protozoan *Trypanosoma cruzi.* As determined by the World Health Organization, effective therapeutic medications are needed for the treatment of Chagas' disease. Cruzain, a powerful cysteine protease involved in cell invasion, immune evasion, and metabolism of the *T. cruzi* parasite, is a validated target for the disease. A number of thiosemicarbazone derivatives are inhibitors of cruzain. In this study, the kinetic mechanism of a potent thiosemicarbazone inhibitor of cruzain was investigated. A microplate reader using a 96-well plate format was used to perform fluorometric assays. Progress curves provided evidence that this thiosemicarbazone compound is a time-dependent inhibitor of cruzain. This was a collaborative study between the Trawick and Pinney groups at Baylor University.

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KINETIC STUDIES OF THIOSEMICARBZONE INHIBITORS OF CRUZAIN, A VALIDATED TARGET OF *TRYPANOSOMA CRUZI*

A Thesis Submitted to the Faculty of

Baylor University

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By

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LIST OF ABBREVIATIONS

Asn	Asparagine
Cys	Cysteine
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
His	Histidine
IC_{50}	Half Maximal Inhibitory Concentration of Inhibitor that Results in 50% Inhibition of Reaction
k_{obs}	Rate Constant for Conversion of V_o to V_s
mM	Millimolar
NaOAc	Sodium Acetate Buffer
nM	Nanomolar
SDS	Sodium Dodecyl Sulfate
T. cruzi	*Trypanosoma cruzi*
V_{max}	Maximal Velocity
V_o	Initial velocity
V_i	Inhibited velocity
V_s	Steady-State Velocity
Z-FR-AMC	*N*-Carbobenzoxy-L-Phenylalanyl-Arginine Amide, Hydrochloride
µM	Micromolar

ACKNOWLEDGEMENTS

I would like to thank God for blessing me with the opportunity to do research with implications into a possible therapy for an incurable disease.

Secondly, I would like to thank Dr. Mary Lynn Trawick for her patience, care, and guidance. I do not know many thesis advisors that would stand by my side from 11am to 12am on Diadeloso in the Baylor Sciences Building to ensure that my thesis copy to my Defense Committee is well-polished. Thank you Dr. Trawick for all that you have done and will continue to do for students at Baylor University. I have truly enjoyed my time here with you.

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Chapter One

Introduction to Chagas' Disease

Epidemiology

Chagas' disease (American Tripanosomiasis) is named after Charles Chagas, a Brazilian physician who discovered this tropical disease more than one hundred years ago.[1] Chagas' disease affects more than 8 million people in Latin America and twenty five percent of the total population are at risk for contracting the disease.[2] The disease is caused by *Trypanosoma cruzi* (Figure 1). This parasite is usually transmitted via triatomine insect vectors (Figure 2). The triatomine insect vector is found in Latin America, with a higher prevalence in rural and poor areas.[2,3] The mode of transmission is as follows: the insect becomes infected after feeding on the blood of an infected host; it then transmits *T. cruzi*, located in the gut of the triatomine insect, to its next uninfected host by defecating near the bite wound; lastly, the uninfected host becomes infected by transmitting the *T. cruzi* into its blood stream through scratching the triatomine insect bite. Contraction of Chagas' Disease can also be transmitted through blood transfusions or organ transplantation of infected hosts or by consuming food contaminated by the triatomine's fecal matter.[2, 4]

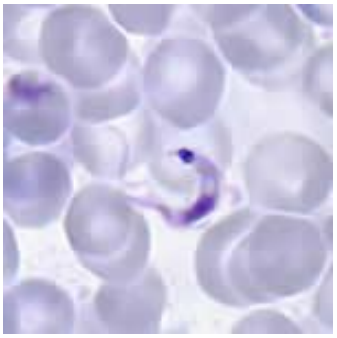


Figure 1.*T. cruzi* in blood smear[2]

Figure 2. Triatomine insect[2]

(Reproduced from http://www.dpd.cdc.gov/dpdx.)[2]

The danger of contracting Chagas' disease is not restricted to Latin America. In fact, there has been an increase in patients with Chagas' disease in non-endemic areas. The Netherlands, Spain, France, Portugal, Switzerland, Germany and the United Kingdom have all reported cases of Chagas' disease. (Figure 3) Some epidemiologists suggest that this phenomenon is due to Latin Americans immigrating into non-endemic countries.[5]Their theory suggests that the Latin American immigrants could possibly be hosts of the vector for Chagas' disease. So by emigrating from their Latin American country, they inadvertently bring the vector causing the Chagas' disease to be spread within a non-endemic area. The countries listed above all have a substantial immigrant population—more than 3 million immigrants—and as a result more than one hundred thousand cases of Chagas' disease are predicted in these countries.[6](Figure 4)

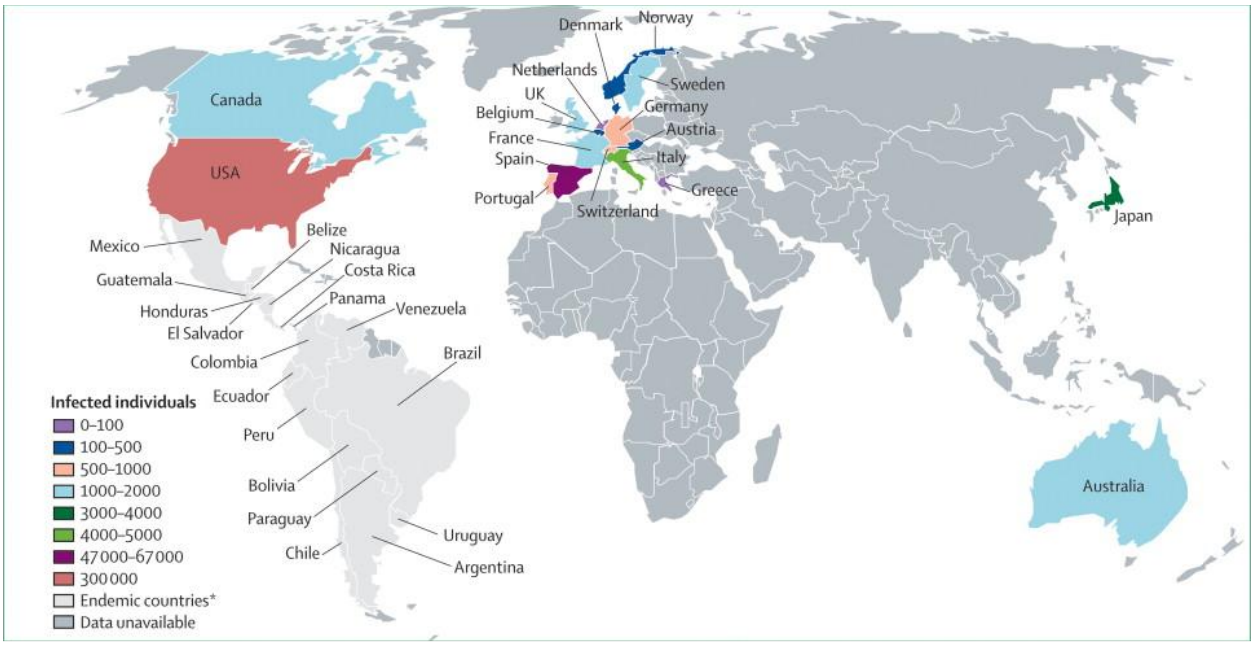


Figure 3.Estimated number of immigrants with *Trypanosoma cruzi* infection living in non-endemic countries. (Reproduced from Rassi et al., 2010[7])

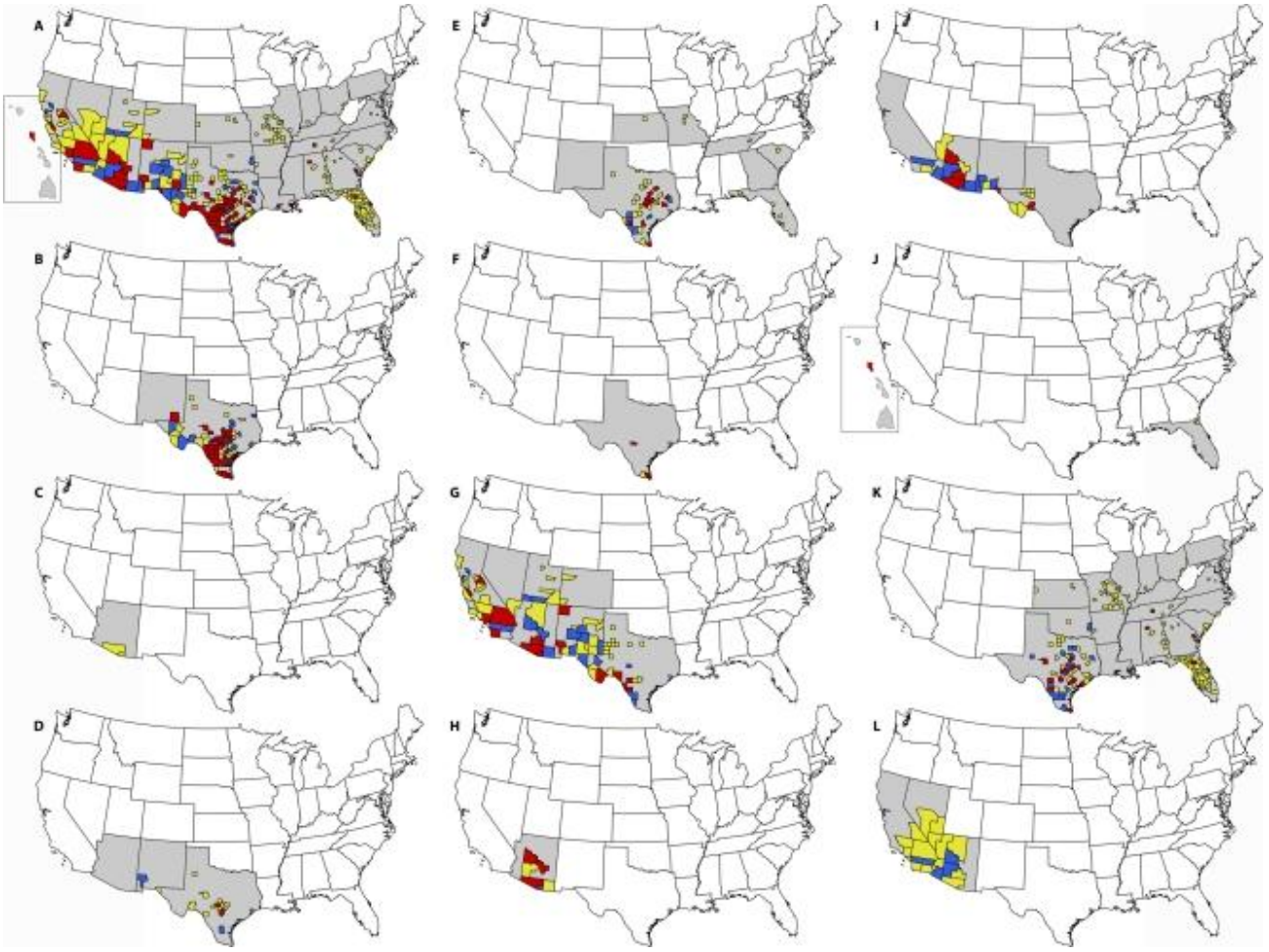


Figure 4. Triatomine species geographic distribution by state (gray areas) and county and *Trypanosoma cruzi* infection status by county in the continental United States and Hawaii. (A) All species; (B) *Triatoma gerstaeckeri; (C) T. incrassate* (D) *T. indictiva*; (E) *T. lecticularia*; (F) *T. neotomae*; (G) *T. protracta*; (H) *T. recurva*; (I) *T. rubida*; (J) *T. rubrofasciata*; (K) *T. sanguisuga*; (L) *Paratriatoma hirsuta*. Red, *T. cruzi*-positive specimens; blue, negative specimens; yellow, no testing reported. (Reproduced from Bern, 2011[6])

The residents of the United States are not immune to Chagas' disease infection.[7]The Pan American Health Organization and World Health Organization estimates that approximately 89 to 700 thousand Latin Americans infected with the *T. cruzi* parasite migrated to the United States from 1981 to 2005.[4] Recently, *T. cruzi*has been detected inpatient casesin the southern United States of Texas, California, Arizona, Florida, Georgia, Tennessee, Alabama and Louisiana.[6] For these reasons, the Center for Disease Control (CDC) considers Chagas disease as one of the Neglected Parasitic Infections, a group of five parasitic diseases that have been targeted for public health action.[2]

Transmission and Life Cycle of T. cruzi

The transmission and life cycle of *T. cruzi* consists of two stages: the triatomine insect stage and the human stage.[6] For the sake of this discussion, only the human stage as the second stage of the *T. cruzi* transmission and life cycle will be considered; although, other animal hosts are possible. Thetransmissionand life cycle begins within the triatomine insect. The triatomine insect ingests infected blood from an infected human host. In the infected blood, *T. cruzi* exists as a trypomastigote, the first morphological stage of *T. cruzi.*The ingested trypomastigotes differentiate and multiply into epimastigotes while in the midgut of the triatomine insect. Next, the epimastigotedifferentiate into metacyclic trypomastigotes in the hindgut and are ready for transmission into a host. The metacyclic trypomastigote enters the blood stream of a

human host after the host scratches his/her bite wound and inadvertently places the triatomine's fecal matter into the blood stream.[6]While in the human host, metacyclic trypomastigotes infect various cells near the bite wound. Within these local cells, the metacylic trypomastigotes become amastigotes. Amastigotes are capable of rapid binary fission. The amastigotes carry out substantial binary fission and then become trypomastigotes once again. This causes cell lysis and the trypomastigotes enter the host's blood stream. Once in the blood stream, the trypomastigotes can infect other non-localized cells and transform into amastigotes. Thus, causing a massive scale infection of *T. cruzi*. The infected host can become bitten by another non-vector triatomine insect, causing the transmission cycle of *T. cruzi* to restart.[6] Figure 5 illustrates the life cycle of *T. cruzi*.

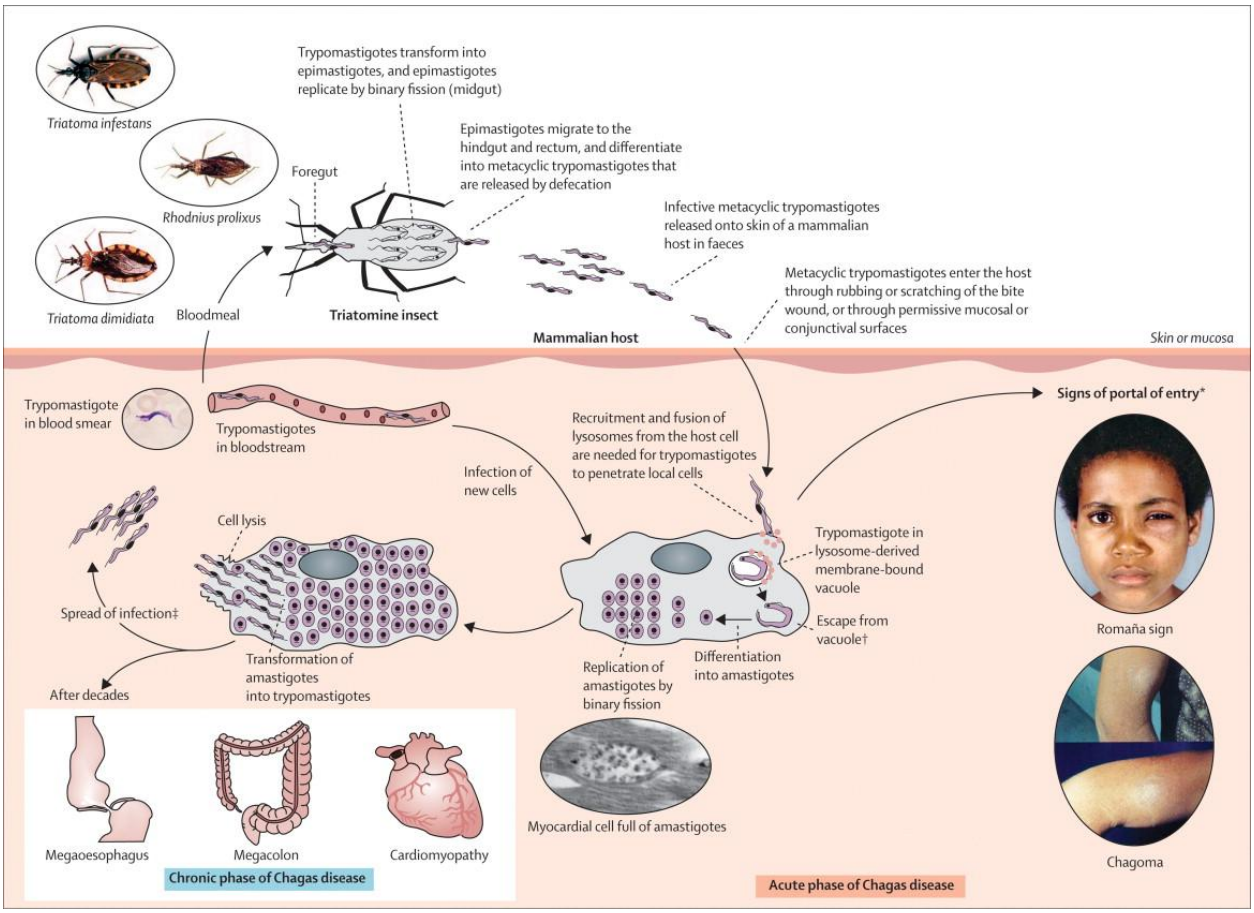


Figure 5. Vector-borne transmission and life cycle of *Trypanosoma cruzi* (Reproduced from Rassi et al., 2010[7])

Other modes of transmission not directing involving this life cycle have still been linked to transmission of *T.* cruzi.[8] These alternative modes of transmission include: blood transfusion, organ transplants, contaminated food and placental infection to newborns.[9-11]

Clinical Stages and Symptoms

Chagas' disease can be divided into two distinct clinical stages: acute (asymptomatic and symptomatic) and chronic.[2, 12]

The asymptomatic acute stage, indeterminate acute stage, is difficult to detect due to its lack of symptoms and the unknown presence of the parasite within the blood stream. This presents a great danger to infected individuals. Treatment of the less severe asymptomatic stage would save the lives of many infected individuals because it would keep the *T. cruzi* from infecting more than one tissue site due to blood stream infiltration.[2,6]

The symptomatic acute phase determinate acute phase occurs shortly after the *T. cruzi* enters the host via the triatomine insect's feces. On average, the phase persists for the first few weeks or months after the initial infection. The most characteristic symptom of Chagas' disease occurs during the acute phase. This symptom is Romana's sign (Figure 5). Romana's sign is a severe swelling of the right eyelid, due to the triatomine insect bite or accidental transmission of the insect's feces into the eye. Other mild symptoms may occur during the acute phase. These can include fever, fatigue, body aches, headache, rash, loss of appetite, diarrhea, and vomiting. Upon physical examination, the patient can show splenomegaly (enlarged spleen), hepatomegaly (enlarged liver), swollen lymph nodes, and chagomas (local swelling) due to the propagation of the amastigote form of *T. cruzi* in these tissues. These symptoms do not

normally contribute to death of the infected individual. However, if the organism has infected the heart muscle (myocarditis) or the brain (meningoencephalitis), death will result: myocarditis being the more likely case.[2, 6]

Although the acute phase's symptoms usually regress, *T. cruzi* continues to invade other tissues in its metacyclic trypomastigote form. Once the metacylic trypomastigote form infects cells it rapidly propagates in its amastigote form and causes cell lysis. This causes an inflammatory response in certain tissues. This gives rise to the chronic phase of Chagas' disease. In this phase, approximately 20-40% of infected individuals are at risk of developing life-threatening heart and/or digestive disorders while the other 60-80% remain asymptomatic. During the symptomatic chronic stage, the heart, digestive system and the nervous system are most often sites of tissue infection. Severe heart problems like arrhythmias and bradiarrhythimias can result due to the destruction of heart tissue. Infection of the digestive tract results in severe weight loss, swallowing difficulties and malnutrition due tomegacolon (enlarged colon) and megesophagus (enlarged esophagus). Dementia, neuritis, confusion, chronic encephalopathy and sensory and motor nerve malfunctions occur as a result of brain tissue infection. It should be noted that the most common tissue infected is cardiac muscle.[2,6]

Methods of Detection

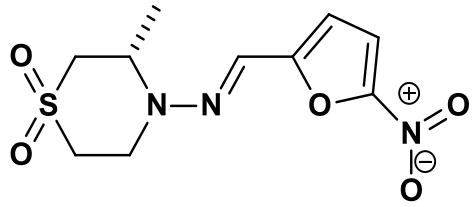
Below are the most important detection systems presently available:

1. Electrocardiograms are taken in order to detect abnormal arrhythmias, tachycardia, heart block and ventricular aneurysms.[7]
2. Parasitic staining techniques, Giemsa or Wright tests in particular, are able to detect the presence of *T. cruzi* within a variety of human fluids and tissues.[13]
3. Cell culture, xenodiagnoses and other methods of parasite isolation can be employed although these methods are cumbersome and costly. This is because these experiments require the inoculation of healthy mammals with infected blood.[14]
4. Indirect Immunofluorescence Assay (IFA), Enzyme-Linked immunosorbent assay (ELISA), hemagglutination, radioimmunoprecipitation and other serological tests are the most popular and accurate methods of detection and diagnosis of Chagas' disease. These tests are not 100% accurate due to false positives occurring with parasites from the Leishmania family.[15, 16]
5. Polymerase chain reaction (PCR) and immunoblotting techniques are useful techniques when attempting to detect the parasite.[17]
6. ABBOTT ESA [Trypanomsoma *cruzi* (E. *coli*, Recombinant) Antigen] are used an in vitro enzyme strip assay for the qualitative detection of antibodies against T. *cruzi*.[48]

Treatment

Presently, there is no cure for the chronic phase of Chagas' disease. There are many anti-parasitic treatments for those with acute Chagas' disease and reoccurring Chagas' disease.[18]The most used of these anti-parasitic treatments are nifurtimox and benznidazole (Figure 6). Nifurtimox is a nitrofuran derivative compound while benznidazole is a nitroimidazole derivative compound. Both compounds are used chemotherapeutically in order to rid the infected individual of the parasite during the acute phase of Chagas' disease.[18, 19]They have proven to be effective at shortening the acute phase and decreasing mortality by approximately 60%. Although both compounds are effective therapeutic agents, they cause a great deal of side effects—that appear to increase in severity with age— due to high toxicity. In addition, benznidazole and nitroimidazole are ineffective against the chronic stage of Chagas' disease. [20,21]The drugs' toxicity levels result in dizziness or vertigo, headache, vomiting, nausea, polyneuropathy (nerve disease), weight loss and anorexia.[2]Due to the sideeffects of these compounds they are administered only under close medical supervision.

A



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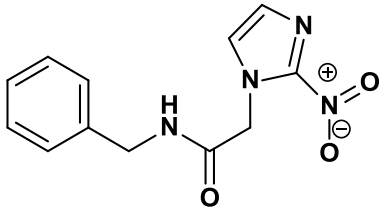


Figure 6. Chemical Structures of A. Nifurtimox B. Benznidazole[18,19]

Introduction to Cruzain

Cruzain, the Papain-like Cysteine Protease

Cruzain is considered a papain-like protease. Proteases are enzymes that catalytically cleave the bonds of the polypeptide chains via a hydrolysis reaction.[25] Proteases come in two forms: endopeptidases and exopeptidases. An endopeptidase cleaves the amide bond within the polypeptide chain while the exopeptidase cleaves the amide bond at the N or C-terminus of the polypeptide. Proteases can be furthered divided into five classes based on the mechanism of peptide hydrolysis: serine, cysteine, aspartic, threonine, and metallo-proteases.[24]Theresearch of this thesis will focus on cruzain, a cysteine protease.It is a parasitic hydrolase protease because it is the main enzyme involved in the parasite infection. Cruzain activity was first correctly identified in 1977 by Itow and colleagues.[22]This gave rise to Rangel's research which reported the first purified form of cruzain a few years later.[22]

Cruzain's structure and mode of catalysis also classifies it as a papain-like cysteine protease (Figure 7). This is because papain-like cysteine proteases possess a common conserved active site of cysteine (Cys25), histidine (His159), and asparagine (Asn175) residues.[4]This specific structure of the active site gives rise to certain properties of the enzyme. For example, the cysteine residue thiol is near the His 159

imidazolium cation and participates in nucleophilic attack of the substrate as the thiolate anion.

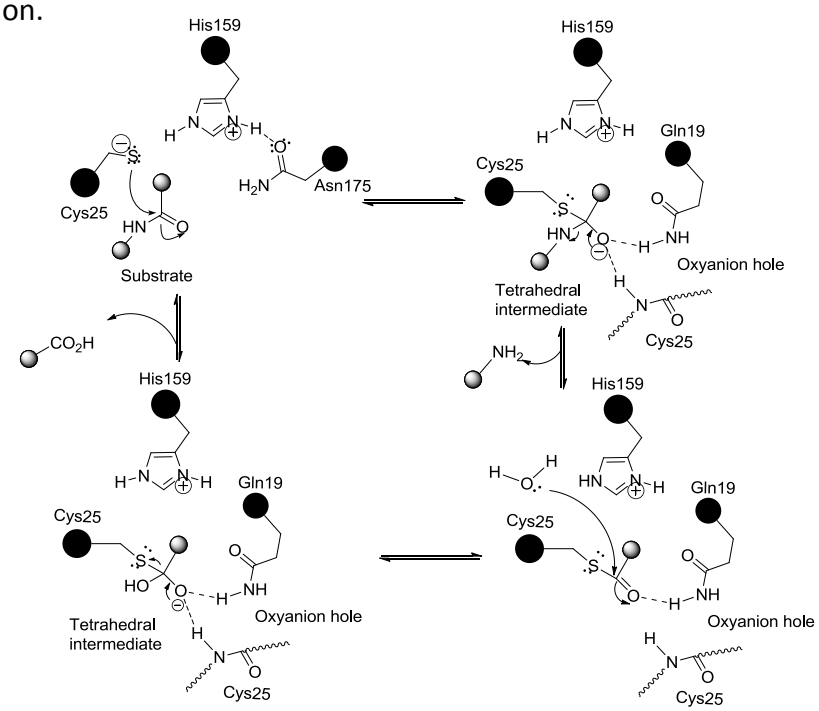


Figure 7: Proposed Mechanism of cruzain[38]

Cruzipain's Role in Chagas Disease

Cruzipain is the primary cysteine protease involved in cell invasion and immune evasion of *T. cruzi*. Cruzain is the recombinant form of cruzipain. Cruzain does not retain the C-terminal tail of cruzipain. This difference doesn't interfere with the mechanistic properties of the enzyme. Therefore, their mechanisms of action are, for experimental purposes, identical.

The immune evasion role of cruzipain has been recently confirmed through mice model studies. In these studies, infected mice immunized against cruzipain showed

strong responses against muscle and/or cardiac myosin and a clear enlargement of their spleens. These strong responses manifested as autoimmune reactions against both skeletal muscle and tissue damage.[27, 28]

Cell remodeling, activation of bradykinin receptors, mediation of apoptotic processes, invasion of host cells, protein processing or degradation of proteins to generate nutrients are all roles of cruzipain.[29, 30, 31]

Cruzipain plays an integral role in the lifecycle of *T. cruzi*.

Cruzain's Substrate Specificity

Fluorogenic synthetic substrates were tested with a cathepsin-L like cysteine protease.[32]Five different fluorogenic substrates with a Z-XR-AMC—X where X represents an amino acid residue at the P2 position. The peptide from P2 to P2' are correspond to the enzyme sub site: S1, S2, etc. (Figure 8).These explorations were carried out under a wide range of pH values: 3-8. The results indicated that cruzain prefers substrates with hydrophobic residues at the P2 position over hydrophilic residues. Although, cruzain still retains the ability to catalyze cleavage at hydrophilic residues.[33]

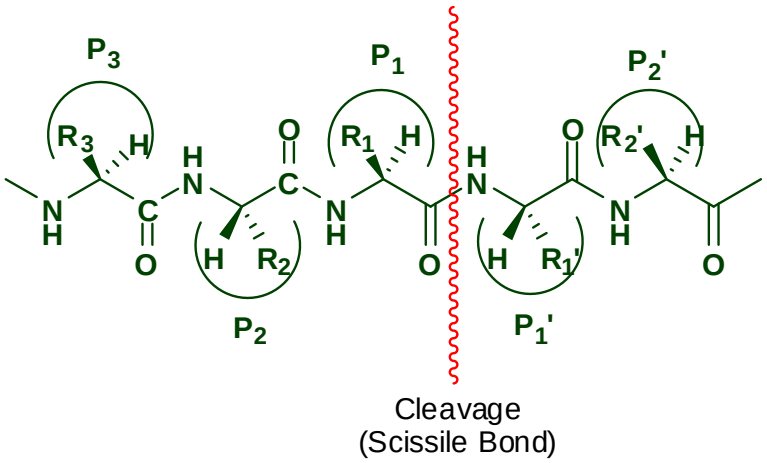


Figure 8.Nomenclature for peptide substrate of a cysteine protease

(Reproduced with permission from Dr. Gustavo Chavarria)[51]

Structure of Cruzipain and Cruzain

Cruzipain is expressed as a preproenzyme of 467 amino acid residues which is divided up into four well defined portions: the mature portion (215 residues), the propeptide (104 residues), a signal peptide (19 residues) and a carboxy-terminal domain (129 residues).[23]In addition, all papain-like cysteine proteases have a signal peptide, assists the protease in translocation, and a propeptide, responsible for acting as a scaffold for protein folding, a chaperone for transport.[4]The propeptide region functions to protect unnecessary hydrolytic activity of the enzyme. The mature portion is a monomer with little known function. Lastly, the carboxyl terminal remains as the characteristic feature of the enzyme cruzain. Currently, no other enzyme has this

carboxyl terminal.[23]Figure 9 shows the complete amino acid sequence divided up into the four regions.

1 MSGWARALLLAAVLVVMACLPAATASLHAEETLTSQFAEFKQKHGRVYESAAEEAFRLS
61 VFRENLFLARLHAAANPHATFGVTPFSDLTREEFRSRYHNGAAHFAAAQERARVPVKVEV
121 VGAPAAVDWRARGAVTAVKDQGQCGSCWAFSAIGNVECQWFLAGHPLTNLSEQMLVSCDK
241 PQDEAQIAAWLAVNGPVAVAVDASSWMTYTGGVMTSCVSEQLDHGVLLVGYNDSAAVPYW
301 IIKNSWTTQWGEEGYIRIAKGSNQCLVKEEASSAVVGGPGPTPEPTTTTTTSAPGPSPSY
361 FVQMSCTDAACIVGCENVTLPTGQCLLTTSGVSAIVTCGAETLTEEVFLTSTHCSGPSVR
421 SSVPLNKCNRLLRGSVEFFCGSSSSGRLADVDRQRRHQPYHSRHRRL

Figure 9.Amino Acid Sequence of Preprocruzain. Legend: Green: signal peptide; blue: propeptide; red: mature cruzain; purple: C-terminal.[23]

As Figure 9 indicates, alanine, glycine, asparagine, and valine are the three most abundant residues within the protease cruzain. Hydrophobic residues (A, F, I, L, M, P, V and W) make up forty percent of cruzain's amino acid sequence. In addition, hydrophilic uncharged residues make up 38 percent of cruzain's amino acid sequence.[23]Table 1 is also helpful in understanding the amino acid composition of cruzain.

While cruzipain has not been crystallized, cruzain, the recombinant form of cruzipain with the C-terminal tail deleted, A model of cruzain from it X-ray crystal structure is shown in Figure 10.[39,42]

AA	#	%	AA	#	%	AA	#	%
Ala	25	11.6	Ile	9	4.2	Arg	3	1.4
Cys	8	3.7	Lys	5	2.3	Ser	19	8.8
Asp	9	4.2	Leu	11	5.1	Thr	14	6.5
Glu	13	6.1	Met	4	1.9	Val	21	9.8
Phe	3	1.4	Asn	10	4.7	Trp	9	4.2
Gly	23	10.7	Pro	8	3.7	Tyr	7	3.3
His	4	1.9	Gln	10	4.7	Total	215	100

Table 1. Amino Acid Composition of Mature Cruzain[42]

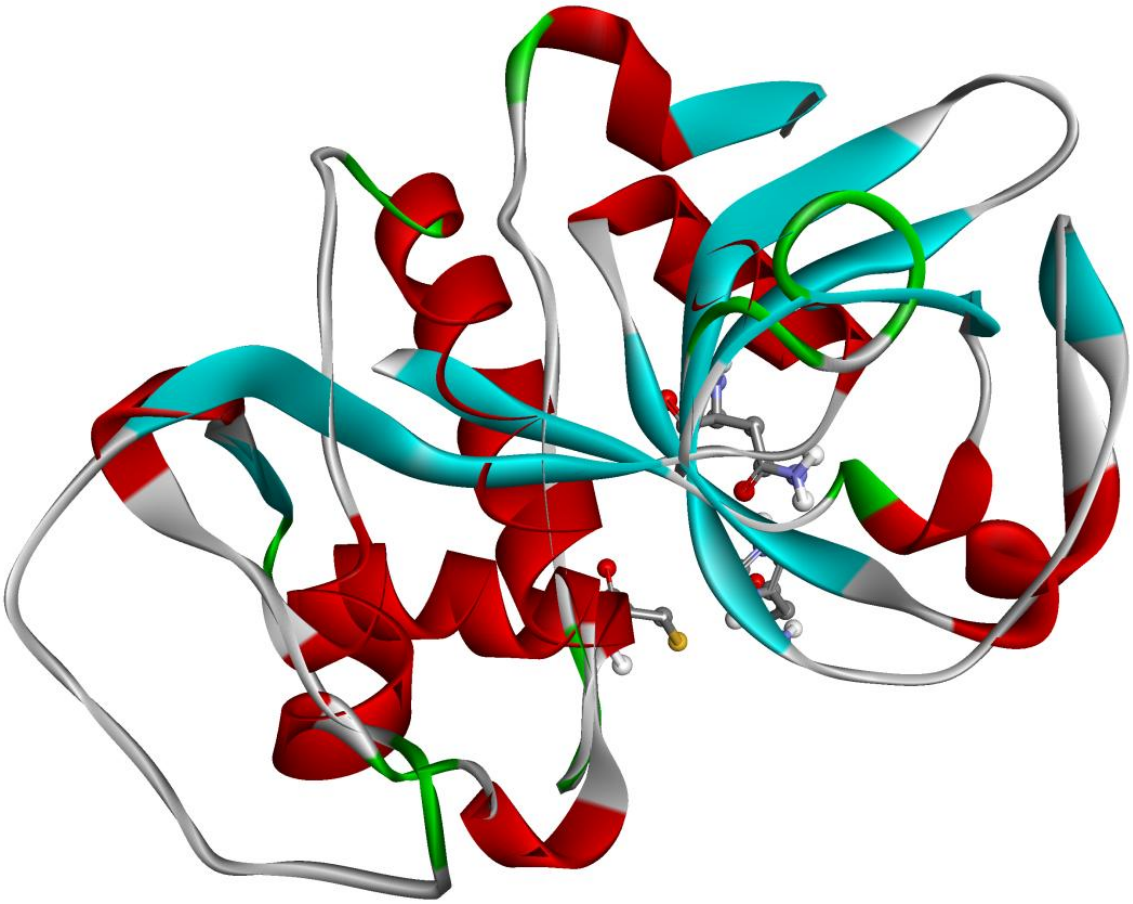


Figure 10.Model of Cruzain, from the PDB: 1AIM[42, 39]

Crystal Structure of Cruzain

McGrath and colleagues reported the first crystal structure of cruzain in 1995.[34]The crystal structures reveal that cruzain is composed of one polypeptide chain of 215 amino acid residues and divided into two distinct domains: L and R. The L domain's secondary structure is mostly composed of α-helices while the R domain's secondary structure is mostly composed of antiparallel β-pleated sheets. The active site is located at the interface of the two domains.

Cruzain's structure shares similarities and differences with that of papain. Figure 11 show the structure of cruzain. Figure 12 goes on to show a model of cruzain with a thiosemicarbazone inhibitor. Lastly, Figure 13 illustrates the active site of cruzain at high magnification. At this magnification, the Cys thiolate group can be seen in very close proximity to the thiocarbonyl carbon of thiosemicarbazone inhibitor. A major difference between papain and cruzain is their loops and turns. In addition, a unique feature of cruzain is the presence of cysteine residue at position 36 (Cys36). There is no current data that indicates any other cysteine protease with cysteine located at position 36.[23]

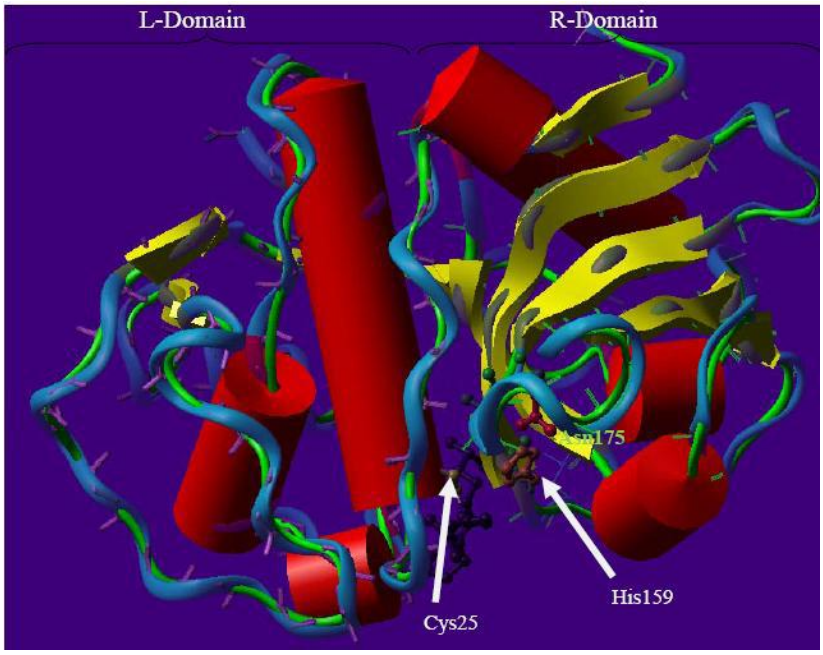


Figure 11.Structure of cruzipain with labeled catalytic groups.Red cylinders (α-helices); Yellow sheats (β-sheets). (Reproduced from Chen[38])

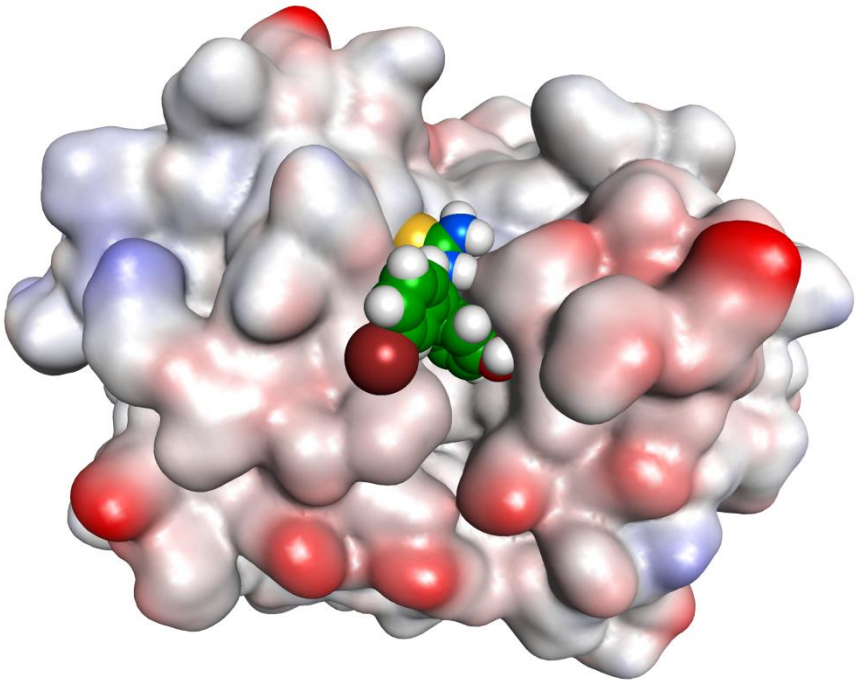


Figure 12. Cruzain with thiosemicarbazone inhibitor (Reproduced from Johnson[39])

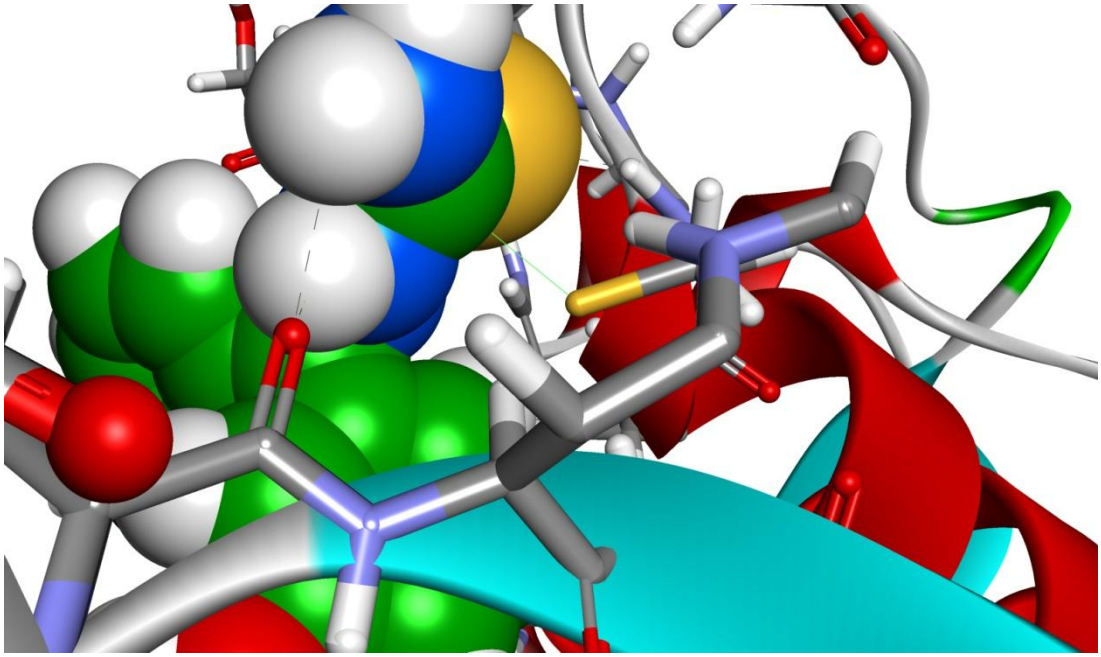


Figure 13.Cruzain with inhibitor. The cys25 thiolate group forms a transient covalent bond with the thiocarbonyl carbon of thiosemicarbazone inhibitor (Reproduced from Johnson[39])

Introduction to Cruzain Inhibitors

Chagas' disease has no effective therapeutic treatment for the chronic stage and a highly toxic treatment for the acute phase. This presents an urgent need for an effective therapy. Cruzain, a validated target of *T. cruzi*, inhibition can potentially provide an effective therapy. In this section, both natural and synthetic inhibitors will be covered.

The Importance of Cruzain Inhibition

Inhibition studies of cruzain in cell models indicated prevention and reduction of infection. Inhibitory studies also demonstrate the ability of cruzain inhibitors to rescue mice from the acute phase of lethal experimental *T. cruzi* infection.[20]

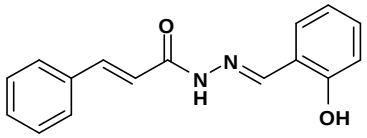
Natural Inhibitors of Cruzain

One of *T. cruzi's* proteins, chagasin, acts as a natural inhibitor to papain-like cysteine proteases. Studies verify chagasin as a validated tight-binding inhibitor of cruzipain. The two proteins have intracellular pathways that converge within reservosomes, endocytic organelles of *T. cruzi*, allowing the inhibition of cruzipain activity.[35]

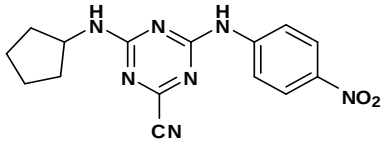
Cystatins are natural inhibitors mammalian cysteine proteases of cruzain. Endogenous cystatins are notable to effectively inhibit *T. cruzi* infection.[36]

Synthetic Inhibitors of Cruzain

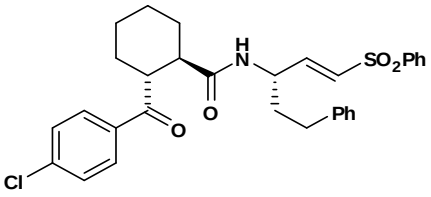
A number of cruzain inhibitors have been reported in the literature (Figure 14). Nonpeptidic thiosemicarbazones were first reported as cruzain inhibitors by Du and colleagues in 2002. Du and colleaguesreported on 100 thiosemicarbazones as potential inhibitors of cruzain. A number of these thiosemicarbazones showed IC_{50} values in the nanomolar range. 3'-ethylmonobromopropiophenone thiosemicarbazone was a potent inhibitor of cruzain (Figure 15).[32]Inspired by Du and colleagues, Rogelio Siles in the laboratory of Professor Kevin G. Pinney successfully synthesized cruzain inhibitors, the most active are listed in Tables 2 and 3.[40]



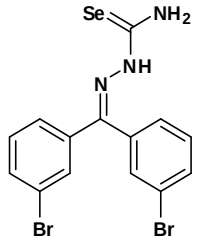
Cinnamic *N*-acylhydrazone (IC_{50}: 52 μM)



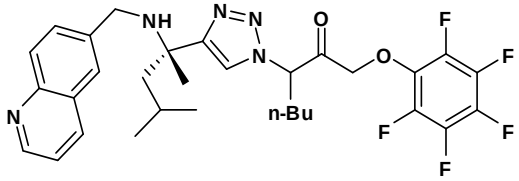
Nitro-substituted triazine (IC_{50}: 1 nM)



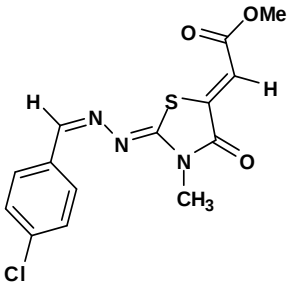
Vinylsulfone Derivative (IC_{50}: 50 nM)



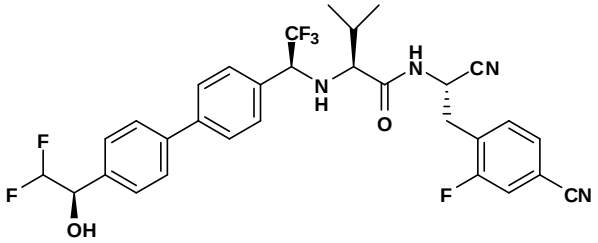
Selenosemicarbazone
(99% inhibition, [I]: 10 μM)



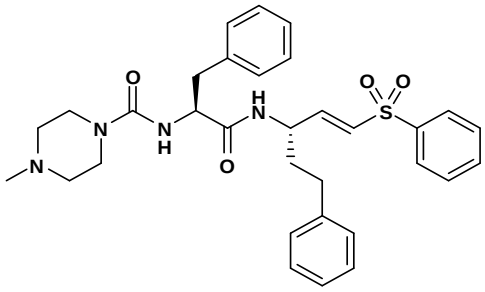
Tetrafluorophenoxymethyl (K_I: 100 nM)



Thiazolidinones (75% inhibition, [I]: 100 μM)



Amino nitrile derivative (IC_{50}: 0.4 nM)



K777 (vinyl sulfone)

Figure 14. Selected cruzain inhibitors reported between 2009 and 2012[43-47]

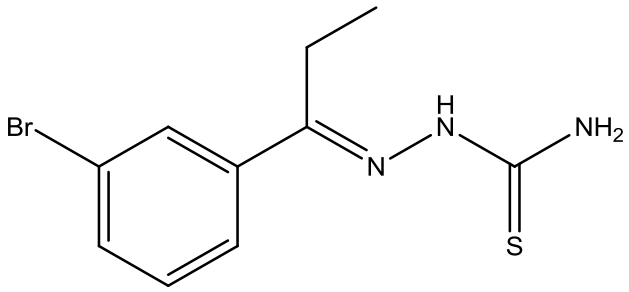
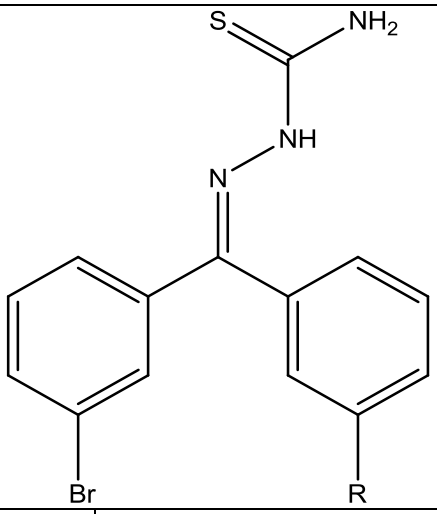
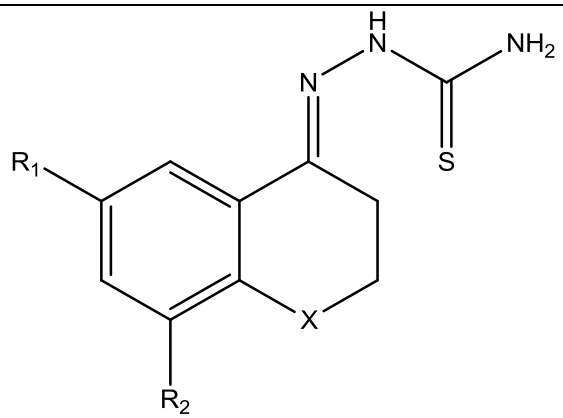


Figure 15. 3’-bromopropiophenone[32]



Compound #	R	IC_{50} nM
13	H	80
14	Br	24

Table 2.Active benzophenone inhibitors of cruzain[40]



Compound #	R_1	R_2	X	IC_{50} nM
21	Br	H	CH_2	17
22	Br	H	O	110
23	Br	H	SO_2	210

Table 3.Active thiosemicarbazone inhibitors of cruzain[40]

Statement of Purpose

Chagas' disease affects an estimated 8-11 million people infected with *Trypanosoma* cruzi (the flagellate protozoan) worldwide. Until recently, Chagas' disease was once confined to the Americas, principally in Latin America. Currently, there is no effective treatment for Chagas' disease in the chronic stage, and the medications that do exist for the acute stage tend to be highly toxic. There is an urgent need for effective therapeutic agents for the treatment of Chagas' disease. Research in cultured cells and mouse models have shown that cruzipain, a protease that is essential for the parasite's survival and a member of the papain family of cysteine proteases, is a validated target for potential therapeutic drugs.

In this study, a series of thiosemicarbazone derivativeswere tested against cruzain, a recombinant form of the target protease cruzipain. This thiosemicarbazone series was first synthesized in the laboratory of Professor Kevin G. Pinney's group as cathepsin-L inhibitors as a collaborative project between the Trawick and Pinney groups. Since cruzain is a cathepsin-L like enzyme, we hypothesized that these thiosemicarbazone derivates of cathepsin-L should also be potent candidates as cruzain inhibitors.

The strategy of this study was to use 10 μM as the initial concentration for testing.Compounds that demonstrated greater than 50% inhibition in the cruzain enzyme assay, were further evaluated to determine their IC_{50} values (lowest concentration of inhibitor that achieve 50% inhibition of enzyme) for cruzain inhibition. The enzyme assay was a fluorometric one using a 96-well plate format and a fluorescence microplate reader.

Advanced kinetic studies were used to examine the mechanism of the potent cruzain inhibitor.

CHAPTER TWO

Experimental Procedure for the Biological Evaluation of Potential Inhibitors of cruzain

These studies are part of a partnership between the Trawick and Pinney laboratory groups in the Department of Chemistry and Biochemistry at Baylor University. All thiosemicarbazone compounds were synthesized in the laboratory of Professor Kevin G. Pinney.

Chemical Sources and Equipment

Preparation of recombinant cruzain was carried out by Dr. Wara Milenka Arispe Angulo at Baylor University.[37] Z-Phe-Arg-Aminomethylcoumarin (Z-FR-AMC) and Brij 35 were purchased from Sigma. EMD Biosciences and Fisher Scientific supplied sodium acetate (NaoAc) and dithiothreitol (DTT). Dimethyl sulfoxide (DMSO) was purchased from Acros Chemicals, and Omnipur provided the ethylenediaminetetraacetic acid disodium salt (EDTA). A Thermo Fluoroskan Ascent FL microplate reader and black 96 well Corning 3686 assay microplates were utilized for the cruzain assays. Graphpad 5.0 software was used to apply non-linear regression analysis of the data for each experiment. Distilled water (DI) was used throughout all experimentation. A Mettler Toledo AX microbalance with an accuracy of 0.01mg was used to weigh various compounds.

130mM NaOAc buffer, pH 5.5

Due to the extreme sensitivity of enzymes to pH levels the experiment required sodium acetate buffer. The acetate buffer, pH of 5.5, was prepared by adding 9.053 grams of sodium acetate (82.03 g/mol) into a 1 L volumetric flask and adding 1125 µl of glacial acetic acid. Next, pure water was added into the volumetric flask until the final volume of the solution was 1 L, giving a final concentration of 130mM. To obtain the final pH of the buffer, sodium hydroxide (NaOH) and acetic acid ($C_2H_4O_2$) were added as necessary.

80mM DTT

The 80mM DTT solution was prepared each day of an experiment in order to ensure that it would remain an active reducing agent. A solid sample of DTT was weighed and dispensed into a 50mL conical vial. Next, the sodium acetate (130mM) buffer pH 5.5 was added to the conical vial in a ratio of 1 mL for every 12.34mg of DTT. The ratio gave the DTT/sodium acetate buffer solution a final concentration of 80mM. Mechanical homogenization through the use of a vortex was required for the entire solid DTT sample to dissolve in the sodium acetate buffer. The 80mM DTT solution was stored on ice until the time of experimentation.

40mM EDTA

EDTA is required in protein assays to ensure chelation of metal ions that might otherwise interfere with the protein activity. EDTA disodium salt (372.2 g/mol) was weighed and pure water was added in a ratio of 1mL of pure water for every 14.89 mg of EDTA. The result of this ratio was a solution of 40mM concentration. Mechanical homogenization through the use of a vortex was then employed. The solution was stored on ice until the time of experimentation.

Assay buffer

130mM NaOAc buffer (pH 5.5), DTT (80mM), EDTA (40mM), and Brij 35 were added according to Table 4 for a final concentration of 130mM NaOAc buffer, 3.25 DTT, 1.3mM DTT, and 0.01% Brij. Mechanical homogenization was employed and the solution was then stored on ice until the time of experimentation (Table 4).

			Exact		To make		EDTA	DTT	BRIJ	NaOAc
Columns							40	80	30	195
2	16	samples	1600	uL	2400	uL	78.00	97.50	0.80	2223.70
4	32	samples	3200	uL	4800	uL	156.00	195.00	1.60	4447.40
6	48	samples	4800	uL	7200	uL	234.00	292.50	2.40	6671.10
8	64	samples	6400	uL	9600	uL	312.00	390.00	3.20	8894.80
10	80	samples	8000	uL	12000	uL	390.00	487.50	4.00	11118.50
12	96	samples	9600	uL	14400	uL	468.00	585.00	4.80	13342.20

Table 4. Assay Buffer Solutions

Thiosemicarbazone Inhibitors

The synthetic thiosemicarbazones utilized in this study were synthesized by the Pinney research group at Baylor University. 20 mM stock solutions of these compounds were made by dissolving roughly 1 mg of compound in the necessary volume of 100% DMSO. Mechanical homogenization was employed in order to completely dissolve the dry compound in each stock solution.

To determine IC_{50} value, seven 1:10 dilutions of the selected synthetic inhibitors were created using each stock solution of inhibitor and pure DMSO. The final concentrations of inhibitor in 35% DMSO ranged between 10µM to 0.01nM (Table 5).

The advanced kinetic studies protein assay had different inhibitor dilutions. Compound **15**was tested using dilutions in the following range: 1 µM≤ [Compound **15**] ≤ 1 nM. (Table 5)

				DMSO	35	%	
Final	units	Stock	units	Solution		DMSO	Water
10	uM	200	uM	A	10	25	65
5	uM	100	uM	A	5	30	65
1	uM	20	uM	B	10	25	65
100	nM	2	uM	C	10	25	65
50	nM	1	uM	C	5	30	65
10	nM	0.2	uM	D	10	25	65
1	nM	20	nM	E	10	25	65
0.5	nM	10	nM	E	5	30	65
0.1	nM	2	nM	F	10	25	65
0.05	nM	1	nM	F	5	30	65
0.01	nM	0.2	nM	G	10	25	65
0	nM	0	nM	G	0	35	65

Table 5. Inhibitor Solutions for Inhibition Studies

Substrate Solution

A *benzyloxycarbonyl-Lphenylalaginyl-L-argininyl-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC)* stock solution (29.94 mM) was prepared by dissolving 19.43 mg of Z-Phe-Arg-AMC (78.13 g/mol) in 1mL DMSO.

Both the preliminary 10 µM screening protein assay and secondary protein assay required a fixed concentration of the Z-Phe-Arg-AMC substrate. 100% DMSO and pure water were added into a conical vial wrapped in aluminum foil according to Table 6. The conical vial was wrapped in aluminum foil to ensure that the light-sensitive substrate would not react. The stock solution was micropipetted into theprepared assay buffer for a final concentration of 150µM.

							Substrate 2.50%		
			Exact		To make		Stock	DMSO	Water
Columns								2.5	
2	16	samples	320	uL	480	uL	2.405	9.595	468.000
4	32	samples	640	uL	960	uL	4.810	19.190	936.000
6	48	samples	960	uL	1440	uL	7.214	28.786	1404.000
8	64	samples	1280	uL	1920	uL	9.619	38.381	1872.000
10	80	samples	1600	uL	2400	uL	12.024	47.976	2340.000
12	96	samples	1920	uL	2880	uL	14.429	57.571	2808.000

Table 6. Substrate Solutions for Inhibition Studies

For the advanced kinetics study, different concentrations of substrate were made by diluting the stock solution (Table 7).

			To make		Stock	DMSO	Water
Concentration						2.5	
0.5µM	48	samples	7220	uL	1.203	198.8	7020
1µM	32	samples	4332	uL	1.443	118.6	4212
5µM	16	samples	1440	uL	2.400	33.60	1404

Table 7. Substrate Solutions for Inhibition Studies

Enzyme Solution

Pure water, 130mM NaOAc buffer (pH 5.5), 80 mM DTT, 40mM EDTA, and Brij 35 were micropipetted into a glass flask according to Table 6. Then, the glass flask was sealed with parafilm and stored on ice until it was time to run the assay. Depending on the number of wells utilized in the assay, the appropriate amount—as determined by Table 8—of cruzain was added to the enzyme solution just before the assay. This was the last step before the assay was carried out.

Cruzain assays

The 10-100 µL multichannel micropipette was used to load all of the samples into the 96 well microplate. First, 100 µL of assay buffer and 10 µL of inhibitor solution were added to the appropriate wells in the microplate. With the appropriate amount of cruzain stock being added to the enzyme solution (Table 8), 70 µL of the enzyme

solution was added to the microplate. The enzyme-inhibitor mixture was incubated for 5 minutes at 25˚C. Following the 5 minute preincubation, 20 µL of substrate were quickly added to each well for a final concentration of 1 mM EDTA, 2.5 mM DTT, 0.01% Brij, 100 mM NaOAc buffer, 2% DMSO, 0.1001 nM cruzain, and 15 µM substrate in the cruzain assay. The reaction (Figure 15)at λ_x=355 nm and λ_m=460nm for 50 minutes. GraphPad 5.0 was used to fit the data into equation 1 and to perform a nonlinear regression to determine the IC_{50} values. The final IC_{50} value was the average of at least two experiments. Only those synthetic inhibitors secondarily screened were subject to more than one experimental IC_{50} value.

Throughout the advanced kinetic protein assay each trial progressed as follows: 100 µl of assay buffer was added; then 10 µl of the inhibitor and 20 µl of Z-Phe-Arg-AMC; lastly, 70µl of cruzain stock solution was added and the assay was carried out without preincubation. The data were restricted by means of determining when the substrate was 10% consumed. This was carried out by taking the transformed fluorescence unit (TFU) from control values at time zero and subtracting it from the total concentration of substrate present. The transformed value was determined using Equation 1. The sum was then taken and multiplied by 0.1. This value was added to the transformed florescence unit from control values at time zero (Equation 2).

			Exact		To make		EDTA	DTT	BRIJ	Cruzain	NaOAc	WATER
Columns							40	80	30	1110	130	0
1	8	samples	560	uL	840	uL	21.00	26.25	0.28	0.22	646.15	146.10
2	16	samples	1120	uL	1680	uL	42.00	52.50	0.56	0.43	1292.31	292.20
3	24	samples	1680	uL	2520	uL	63.00	78.75	0.84	0.65	1938.46	438.30
4	32	samples	2240	uL	3360	uL	84.00	105.00	1.12	0.87	2584.62	584.40
5	40	samples	2800	uL	4200	uL	105.00	131.25	1.40	1.08	3230.77	730.50
6	48	samples	3360	uL	5040	uL	126.00	157.50	1.68	1.30	3876.92	876.60
7	56	samples	3920	uL	5880	uL	147.00	183.75	1.96	1.52	4523.08	1022.70
8	64	samples	4480	uL	6720	uL	168.00	210.00	2.24	1.73	5169.23	1168.80
9	72	samples	5040	uL	7560	uL	189.00	236.25	2.52	1.95	5815.38	1314.90
10	80	samples	5600	uL	8400	uL	210.00	262.50	2.80	2.16	6461.54	1461.00
11	88	samples	6160	uL	9240	uL	231.00	288.75	3.08	2.38	7107.69	1607.10
12	96	samples	6720	uL	10080	uL	252.00	315.00	3.36	2.60	7753.85	1753.20

Table 8. Enzyme Solutions

CHAPTER THREE

Results and Discussion

Inhibition Studies with SyntheticThiosemicarbazones

A series of thiosemicarbazone derivate inhibitors was synthesized in Professor Kevin. G. Pinney's laboratory as cathepsin-L inhibitors , as a collaborative project between the Trawick and Pinney groups. Since cruzain is a cathepsin-L like enzyme, we hypothesized that compounds from this series of thiosemicarbazone derivates could also be potent cruzain inhibitors. The strategy to evaluate these compounds was to use 10 µM as the initial concentration of potential inhibitors. Only the compounds that showed more than 50 % inhibition at the fixed concentration of10 µM were further evaluated to determine their IC_{50} values, concentration of inhibitor that resulted in 50% inhibition of enzyme.

Initially, aminomethylcoumarin (AMC) curves were created to determine the relationship between relative fluorescence units (RFU) and AMC. As expected, there was a linear correlation between RFU and AMC. This allowed us to measure the AMC product produced due to the fluorescence of AMC produced by cleavage of Z-FR-AMC. In order to validate my technique, I determined theIC_{50}value of compound **14** that had been previously reported by Dr. Gustavo Chavarria under the same assay conditions.

inhibition assay, many assays were successfully run in order to duplicate previously validated IC_{50} values of compound **14**, first validated by Dr. Gustavo Chavarria.

The IC_{50} values for this library were determined under final experimental conditions (100 mM sodium acetate pH 5.5, 1 mM EDTA, 2.5 mM DTT, 0.01 % brij 35, 0.1 nM cruzain, Inhibitor, 2% DMSO and 15 μM Z-Phe-Arg-AMC). The reaction (Figure 16) was carried out after a five minute pre-incubation time using a fluorescence microplate reader (Thermo Fluoroskan Ascent Fl) and a 96 well plate. The microplate reader measured the reaction at λ_x=355 nm and λ_m=460 nm for 50 minutes. GraphPad 5.0 was used to fit the data into Equation 1 and to perform a nonlinear regression analysis to determine the IC_{50} values.

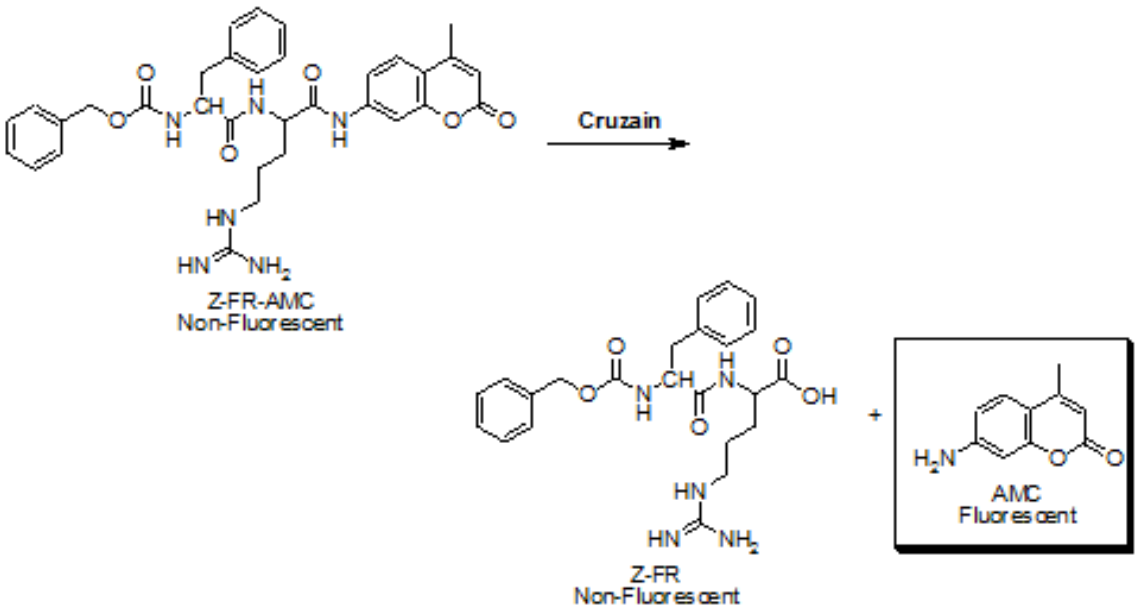


Figure 16. Fluorogenic Assay Reaction[39]

$$\frac{v_i}{v_0} = \frac{Bottom + (Top - Bottom)}{1 + 10^{(\log(IC_{50} + x) Hill\ slope)}}$$

Equation 1

$$10\%\ Substrate\ Consumed = \left(0.1\left([AMC] - [AMC]_{Transformed}\right)\right) + [AMC]_{Transformed}$$

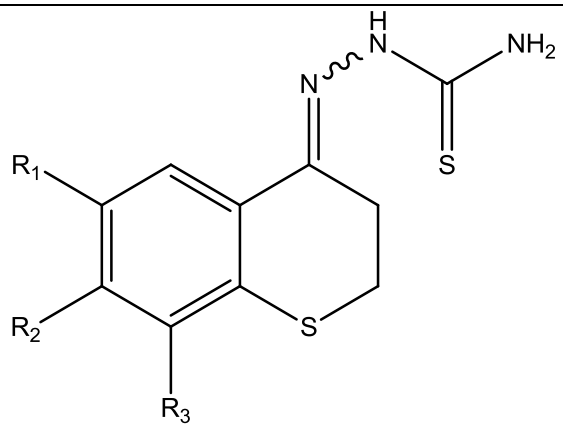
Equation 2

The IC_{50} values for the 15 compounds evaluated in this study are shown in Tables 9-11. It should be noted that the bond between –N and –NH on the thisemicarbazone moiety is represented by a curved arrow because of the possibility of isomerization at this position.[41] This is important to note because it could affect the inhibitor's docking to the enzyme active site.

Table 9 illustrates a collection of thiochromanone thiosemicarbazones. Compound **1,2**, and **6** show no substantial inhibition (IC_{50}>10,000) against cathepsin-L cruzain or cathepsin L.[41] Compound **3** has two halide substituents (-F and –Br, respectively) in the R1 and R3 positions. The IC_{50} value against cruzain was determined to be 45.3nM while it is almost tenfold larger against cathepsin-L (IC_{50} value = 434 nM).[41] This is a promising lead because the compound **3** is much less active toward cathepsin-L, a human enzyme. Compound **5** has a trifluoro-methyl group ($–CF_3$) at the R_1 position. The IC_{50} value against cruzain was 101 nM while it was higher against cathepsin-L (IC_{50} = 284 nM).[41] Compound **7** presents quite interesting data. The IC_{50} value against cruzain (> 10,000nM) is substantially higher than the corresponding cathepsin-L IC_{50} value (68 nM).[41] Lastly, Compound **8** has two halide substituents (-F) at the R_1 and R_2 positions. The cruzain IC_{50} value is 180 nM while it is more active against the cathepsin-L (IC_{50} value = 46 nM).[41] These values are quite close and suggest a similar inhibition activity in both proteases.

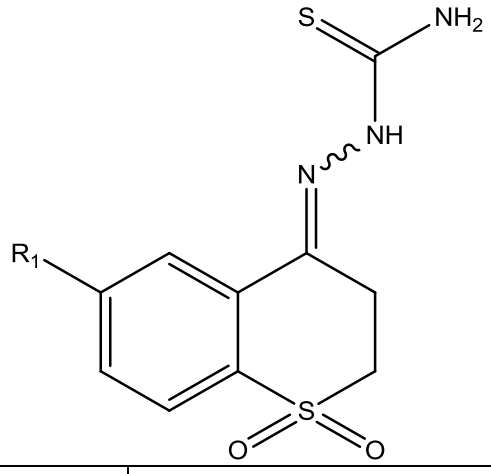
Table 10 illustrates thiosemicarbazone derivatives with a sulfone moiety. Both compounds **9** and **10** while compound **9,** with a nitro substituent, has an IC_{50} value of 112 nM against cathepsin L.[41] Compound **11** has a bromine substitution at the R_1 position. The IC_{50} value against cruzain is 247 nM while the IC_{50} value against cathepsin-L is 574 nM.[41]These results suggest similar inhibition activity against both proteases.

Table 11 shows a collection of different thiosemicarbazone compounds. Compound **12** has an ether methoxy substitution at its R_1 position. This compound showed little inhibition against both either cruzain or cathepsin-L.[41] Compound **13** has a fuzed benzene ring to the thiochromanone group. This compound is of particular interest because it shows excellent inhibition of cruzain (IC_{50} value=32.1 nM) and very little inhibition of cathepsin-L (IC_{50} value>10,000 nM).[41] This exceptional result suggests that this compound can be used to inhibit cruzain to an appreciable degree without affecting cathepsin-L.Compound **14** contains two bromine monosubstituted benzene rings while compound **15** has three halides substituted to three different benzene rings. These two compounds gave the greatest activity: IC_{50} values of 5.1 ± 0.5 nM and 2.2 ± 0.2 nM.Compound**14**also produces great inhibition of cathepsin-L.



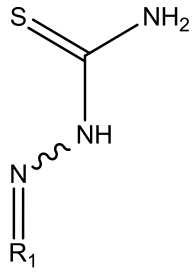
Compound	R_1	R_2	R_3	IC_{50}(nM)
1[41]	F	H	F	>10,000
2[41]	F	H	Br	>10,000
3[41]	Br	H	F	45.26 ± 2.405
4[41]	CH_2CH_3	H	H	>10,000
5[41]	CF_3	H	H	100.8 ± 6.105
6[41]	H	H	OCF_3	>10,000
7[41]	NO_2	H	H	>10,000
8[41]	F	F	H	180 ± 25.40

Table 9. IC_{50} values against cruzain for thiochromanone semicarbazone derivatives[50]



COMPOUND	R_1	IC_{50}(nM)
9[41]	NO_2	>10,000
10[41]	OH	>10,000
11[41]	Br	246.9 ± 15.10

Table 10. IC_{50} values against cruzain for sulfone thiosemicarbazones derivatives[50]



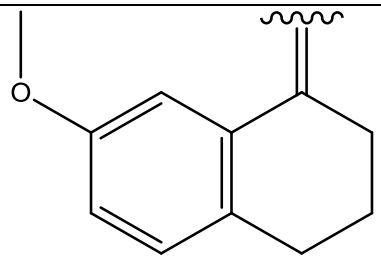
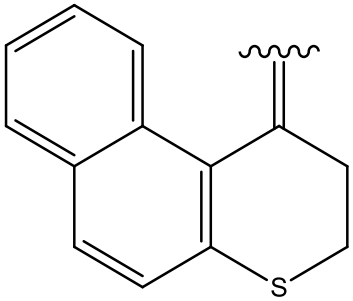
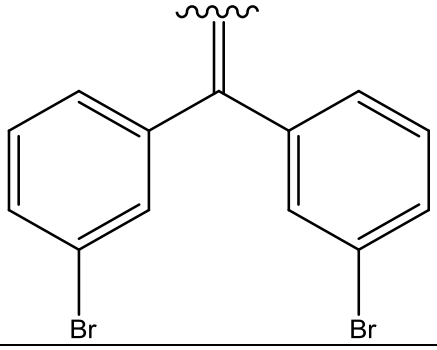
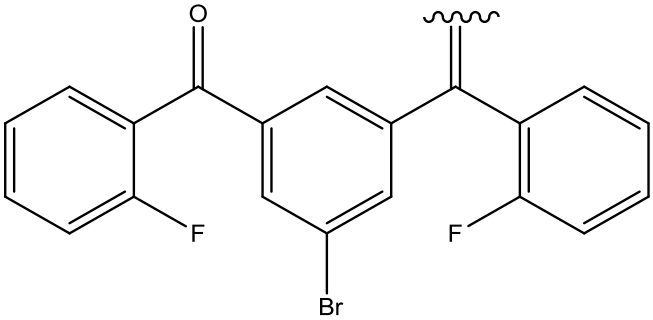
COMPOUND	R_1	IC_{50}(nM)
12[50]		>10,000
13[41]		32.14 ± 3.779
14[40]		6.135 ±0.501
15[52]		2.615 ± 0.207

Table 11. IC_{50} values against cruzain for thiosemicarbazone with ether, phenyl fused rings, and two bromo-substituted benzene rings.[41, 50]

Advanced Kinetic Studies

The reaction progress curves for the cruzain catalyzed reaction with Z-FR-AMC as substrate in the presence of varying concentrations (0-10 µM) of compound **15** are shown in Figure 17. Reactions were initiated by the addition of enzyme with no preincubation with compound **15**. Release of the fluorescent product AMC was followed as a function of time.The progress curves can be described by Equation 3. In this equation, P is the concentration of product (µM), v_i and v_s are the initial and steady-state velocities (µM/s) respectively, t is the time in seconds and k_{obs} (s^{-1}) is the rate constant for conversion of the initial velocity,v_i, to the steady state velocity v_s. These three values help to understand the progression of the reaction. The average and standard error of both k_{obs} and v_i were included in the experimental results because the experiments were done in triplicates for each substrate concentration (5µM, 1 µM, and 0.5µM) (Figures 17 and 18). An examination of Figure 17 demonstrates that higher concentrations of compound **15** are needed to achieve the same level of inhibition with increasing concentration of substrate. These results indicate that both compound **15** are competing for the enzyme active site.

$$[P] = \frac{(v_o - v_s)}{k_{obs}}(1 - e^{-k_{obs}t}) + v_s t$$

Equation 3

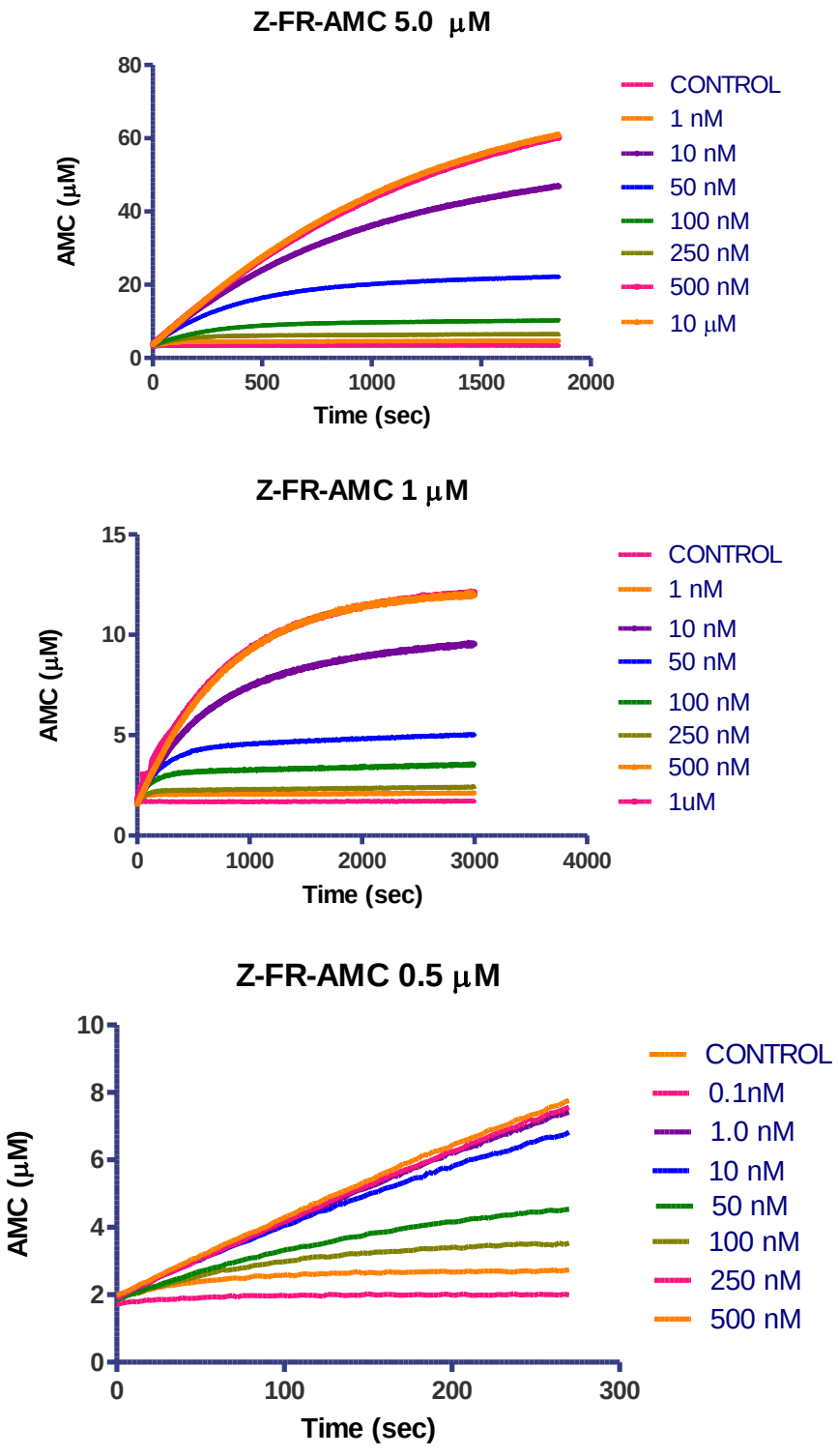


Figure 17 Progress curves at 5.0 µM, 1.0 µM, and 0.5 µM

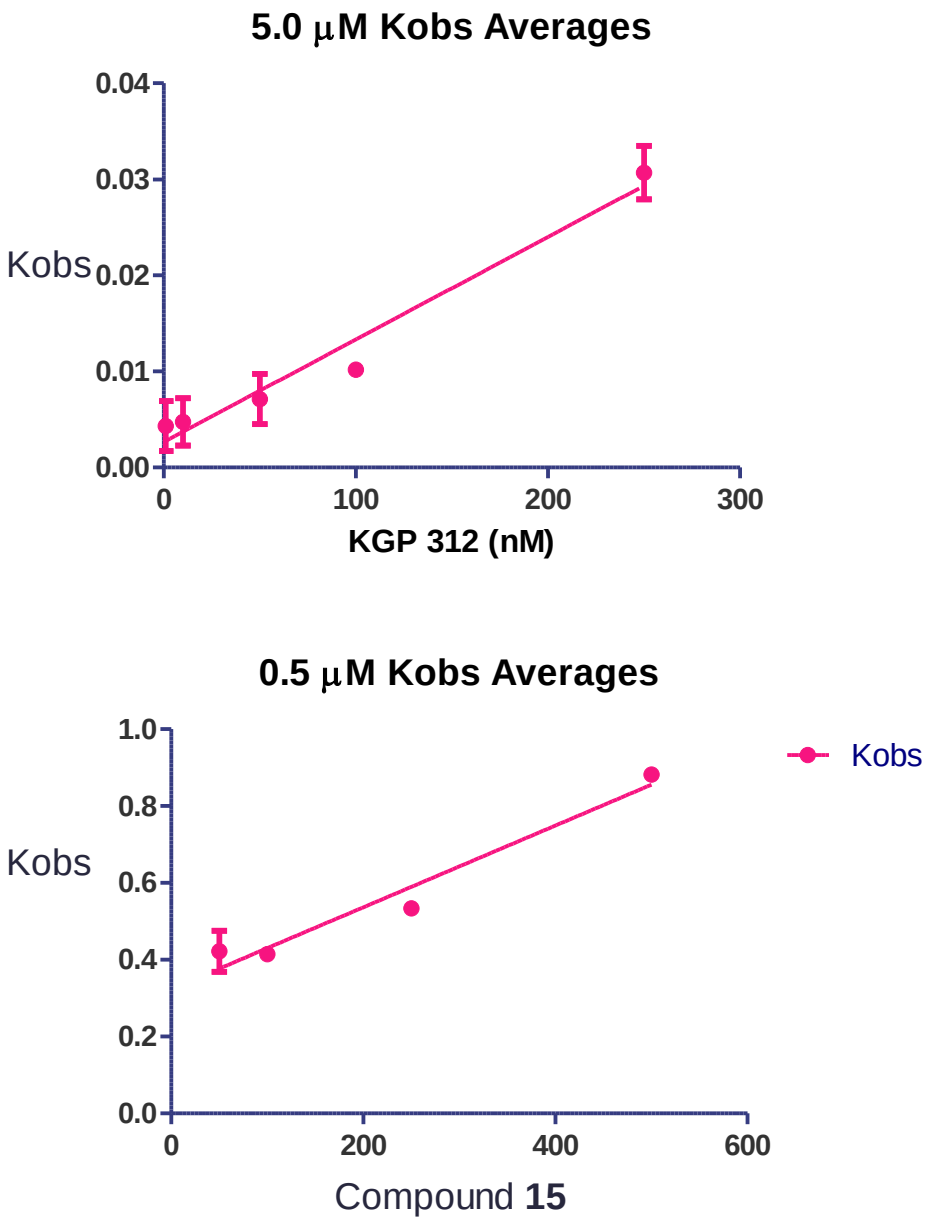


Figure 18. K_{OBS} 5.0 µM and 0.5 µM

The increase in inhibition as function of time indicates that compound **15** is a time-dependent inhibitor of cruzain. The progress curves and k_{obs} graphs clearly demonstrate that compound **15** is not a classical reversible, competitive inhibitor.

CHAPTER FOUR

Conclusions and Further Studies

Inhibition Studies with Synthetic Inhibitors

In addition to the compound **14**, five out of fifteen synthetic thiosemicarbazones were found to be active (IC_{50}< 500nM), confirming our original compounds. The results of testing this group of 15 synthetic compoundsdemonstrate that thee synthetic inhibitors with thiosemicarbazone moieties, originally synthesized as cathepsin-L inhibitors, are potent inhibitors of cruzain. The IC_{50} values of the most active compounds are as follows: compound **3** (45.3 ± 2.4 nM), compound **5** (101 ± 6.1nM), compound **8** (180 ± 25 nM), compound **11** (247± 15 nM), compound **13** (32.1 ± 3.8 nM), and compound **15** (2.6 ± 0.2 nM). It is hopeful that these compounds will be used to inhibit cruzain in the protozoan, *T. cruzi*, which is responsible for Chagas' disease. In order to evaluate these compounds as therapeutic agents, further studies were carried out.

Advanced Kinetic Studies

The advanced kinetic study, with compound **15**, demonstrates a time-dependent, non-classical competitive inhibition mechanism. The 5 µM Z-FR-AMC progress curves showed 50% inhibition at an inhibitor concentration of 250 nM as compared to the control value at 250 seconds. The 1 µM Z-FR-AMC progresscurve

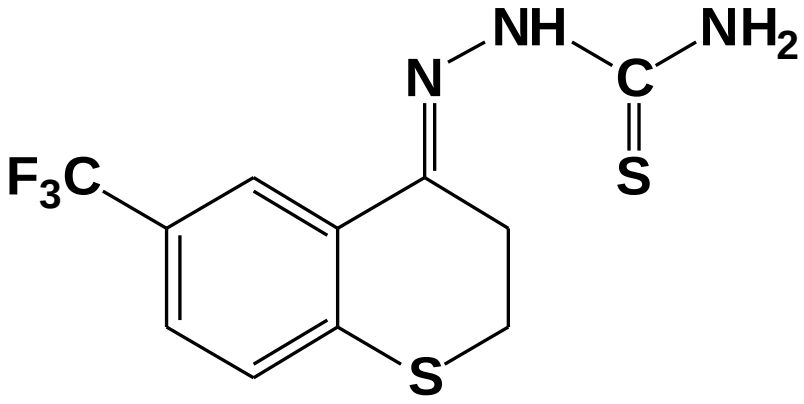
shows 50% inhibition at an inhibitor concentration of 50 nM as compared to the control value at 500 seconds. And the 0.5 μM Z-FR-AMC progression curve shows 50% inhibition at an inhibitor concentration of 10 nM as compared to the control value at250 seconds. This trend clearly indicates that the inhibitor competes with the substrate for the active site.The next step in this project would be to carry out reversibility studies.

Compound **15** appears to be a promising candidate for further studies as a therapeutic agent for the treatment of Chagas' disease.

APPENDIX

Cruzain IC_{50} Determination Data and Plots

KGP225
SJL-II-087



IC-50 (nM)
106.90
94.69

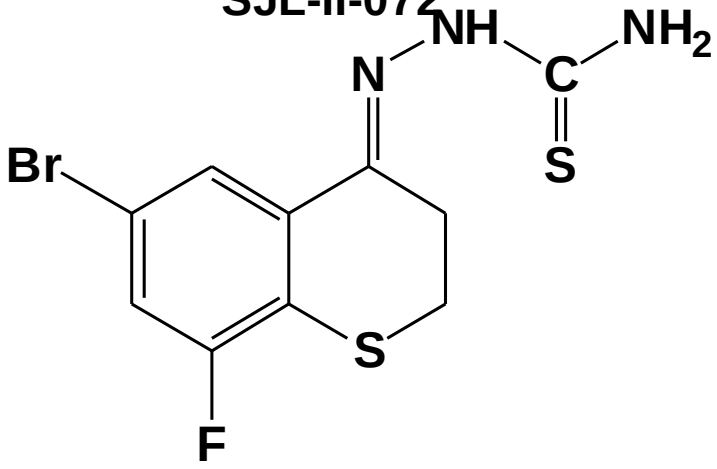
	IC-50 (nM)
Number of values	2
Mean	100.8
Std. Deviation	8.634
Std. Error	6.105
Sum	201.6

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

KGP198
SJL-II-072



IC-50 (nM)
47.66
42.85

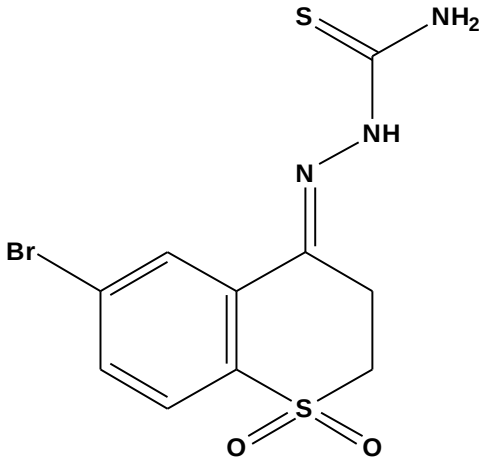
	IC-50 (nM)
Number of values	2
Mean	45.26
Std. Deviation	3.401
Std. Error	2.405
Sum	90.51

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

KGP88
LMJ-I-045



IC-50 (nM)
252.30
282.70
209.30
243.40

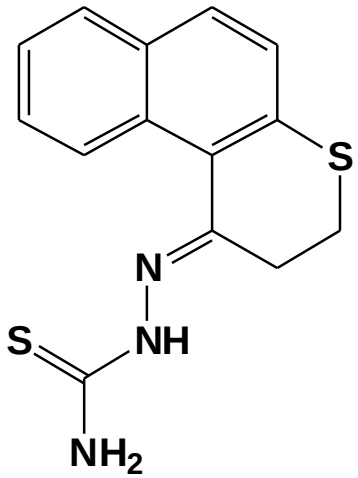
	IC-50 (nM)
Number of values	4
Mean	246.9
Std. Deviation	30.20
Std. Error	15.10
Sum	987.7

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

LMJ-I-037



IC-50 (nM)
27.70
29.07
39.66

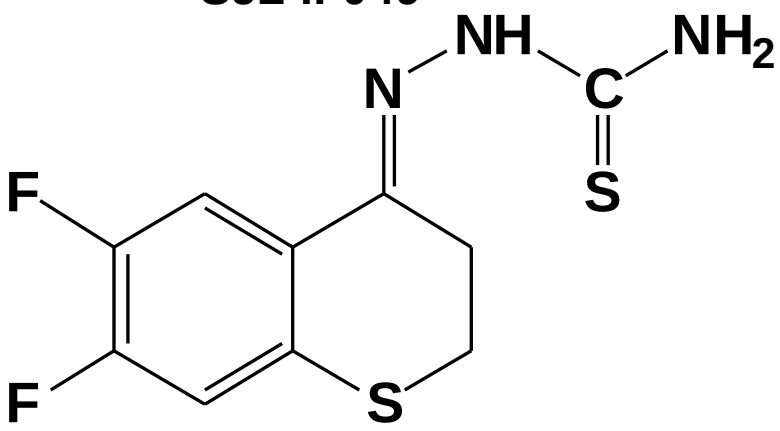
	IC-50 (nM)
Number of values	3
Mean	32.14
Std. Deviation	6.546
Std. Error	3.779
Sum	96.43

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

KGP181
SJL-II-049



IC-50 (nM)
154.60
205.40

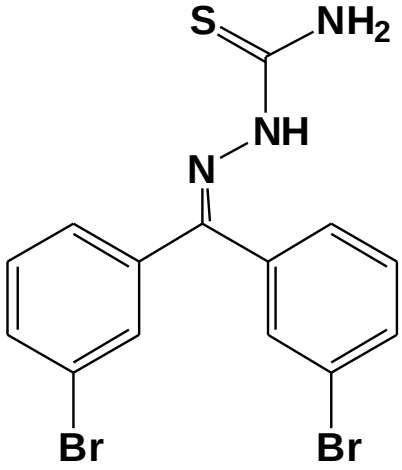
	IC-50 (nM)
Number of values	2
Mean	180.0
Std. Deviation	35.92
Std. Error	25.40
Sum	360.0

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

LMJ-I-006
KGP-068



IC-50 (nM)
5.167
5.986
4.251

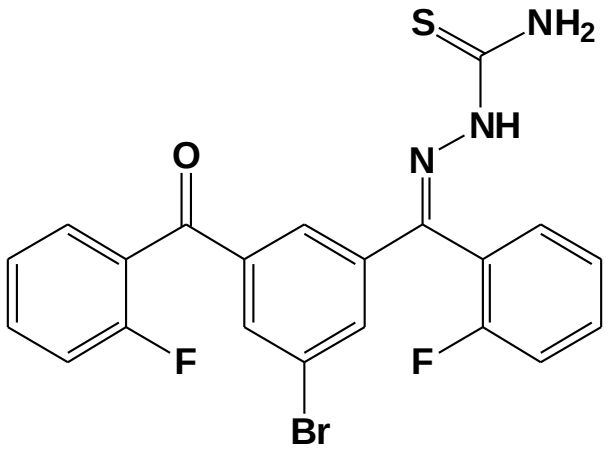
	IC-50 (nM)
Number of values	3
Mean	5.135
Std. Deviation	0.8680
Std. Error	0.5011
Sum	15.40

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

ENP-1-151
KGP312



IC-50 (nM)
2.966
2.632
2.248

	IC-50 (nM)
Number of values	3
Mean	2.615
Std. Deviation	0.3593
Std. Error	0.2074
Sum	7.846

PREINCUBATION TIME: 5 MIN

FINAL CONDITIONS

	EDTA	DTT	BRIJ	NaOAc	DMSO	Cruzain	SUBS
	mM	mM	%	mm	%	nM	uM
FC	1	2.5	0.01	100	2	0.1001	15

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