Probe Report
6-(3,5-difluorophenylamino)-9-ethyl-9H-purine-2-carbonitrile

Title: Promiscuous and Specific Inhibitors of Cruzain

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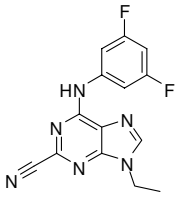
Screening Center Name & PI: NIH Chemical Genomics Center, Christopher Austin
Chemistry Center Name & PI: NIH Chemical Genomics Center, Christopher Austin
Assay Submitter & Institution: Shoichet, Brian, University of California, San Francisco
PubChem Summary Bioassay Identifier (AID): 1476, 1478

Probe Structure & Characteristics:

NCGC00181918



CID**	Target Name[‡]	IC50/EC50 (nM) [SID, AID][†]	Anti-target Name(s)[‡]	IC50/EC50 (µM) [SID, AID][†]	Selectivity*	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID][§]
44143088	**Cruzain - qHTS**	**100 nM**	Papain	300 nM	3:1	**Cruzain – Secondary Cuvette Assay: 0.2 nM**
	Rhodesain	**< 6 nM**				
	TbCatB	**28000 nM**				

‡Short descriptive name of target or pathway (similar for antitarget, if applicable).
†IC50/EC50 value in nM along with the PubChem SID and AID where this value can be found.
§For secondary assay provide the following information: Name, IC50/EC50 [SID, AID]
***Selectivity = antitarget IC50/target IC50
**Provide the PubChem CID for the probe.
***See complete protease profile provided at the end of this report in Appendix B.**

Recommendations for the scientific use of this probe:

This probe exhibits potent near-stoichiometric inhibition of Cruzain and should be useful as both a potent effector of the enzyme *in vitro* and as a starting point for development of anti-trypanosomal agents. The majority of current advanced cruzain inhibitors act via irreversible covalent inhibition, such as the probe detailed within. Our probe acts via *reversible covalent inhibition*, and to the best of our knowledge, we provide the first co-crystallization of nitrile-based inhibitor to cruzain. In addition to the chemotype presented here, a different probes series (2-oxo-1,2-diphenylethyl 2-(cyclohexanecarboxamido) acetate) has been developed for this target; that probe acts as a non-covalent reversible inhibitor and is featured in the complementary report.

1. Scientific Rationale for Project

The goals of the project are at least threefold. First, we aim to discover and develop novel cruzain inhibitors to ultimately aid in the development of antitrypanosomal agents. Second, using the cruzain enzymatic assay as a model screening system of an enzyme carrying a reactive cysteine catalytic residue, we attempt to profile the MLSMR collection with respect to several sources of false-positive or promiscuous types of inhibition: compound autofluorescence, colloidal aggregation, and reactive compounds. Third, to improve the chances for discovering novel competitive cruzain inhibitors and to compare the two techniques, we perform a virtual screen of the collection and characterize the top docking hits using the same cruzain enzymatic assay.

Cruzain is a key cysteine protease in *Trypanosoma cruzi (T. cruzi)*; it is essential for the parasite survival and replication, and has been validated as a drug target for this organism. *Trypanosoma cruzi*, a protozoan parasite, is the causative agent of Chagas disease. This is a major neglected tropical disease affecting over 16 million people, primarily in Central and South America. Discovering novel inhibitors of cruzain and determining their enzyme-bound structures is expected to provide a platform for drug-discovery efforts against this resistance target.

In search for cruzain inhibitors in the MLSMR, we miniaturized and implemented a kinetic fluorogenic assay for cruzain activity. Additionally, each compound was tested at a minimum of 7 concentrations following our previously-reported quantitative high-throughput screening (qHTS) paradigm. Further, to profile the MLSMR for promiscuous inhibitors acting via colloidal aggregation, we compared the hits derived from screens performed in the absence and presence of detergent (0.01% Triton X-100): compounds that inhibited only in the absence of detergent were considered likely promiscuous aggregators. The utilization of kinetic read and the application of detergent-sensitivity screening allowed us to evaluate the effects of the three major modes of compounds interference: aggregation, autofluorescence, and reactivity. Lastly, we compared qHTS hits with the outcome of an independently-performed docking experiment of the MLSMR collection against the cruzain active site.

To measure the enzymatic activity of cruzain, we utilized its model fluorogenic substrate Z-Phe-Arg-AMC. Upon cruzain-catalyzed hydrolysis, a highly fluorescent 7-amino-4-methylcoumarin reporter is released, whose fluorescence was measured at 340 nm excitation and 450 nm emission. Purified cruzain was supplied by the labs of

Prof. James McKerrow and Brian Shoichet, UC San Francisco.

Project Description

PubChem AID	Type	Target	Conc. Range	Samples Tested
1476	Confirmation	Cruzain (-) detergent	1nM – 57uM	197,861
1478	Confirmation	Cruzain (+) detergent	1nM – 57uM	197,861
2158	Confirmation	Cruzain	1nM – 57uM	
2161	Counterscreen	Papain	1pM – 50uM	

a. Assay implementation and screening: **Kinetic Fluorogenic qHTS Assay for inhibitors of cruzain, detergent-free screen [AID: 1476; Primary DR]**

Assay Description

Three µL of reagents were dispensed into 1536-well Greiner black solid-bottom assay plate. Compounds and controls (23 nL) were transferred via Kalypsys PinTool equipped with 1536-pin array (10 nL slotted pins, V&P Scientific, Palo Alto, CA). The plate was incubated for 15 min at room temperature, and then a 1 µL aliquot of 8 µM substrate solution was added to start the reaction. The plate was transferred to ViewLux high-throughput CCD imager (Perkin-Elmer, Waltham, MA), where kinetic measurements (4 reads, one read every 30 seconds) of the AMC fluorescence were acquired using standard 340 nm excitation and 450 nm emission filter sets. During dispense, reagent bottles were kept submerged into 4°C recirculating chiller bath, and all liquid lines were covered with aluminum foil to minimize fluorophore degradation. All screening operations were performed on a fully integrated robotic system (Kalypsys Inc, San Diego, CA) as described elsewhere. Plates containing DMSO only (instead of compound solutions) were included approximately every 50 plates throughout the screen to monitor any systematic trend in the assay signal associated with reagent dispenser variation or decrease in enzyme specific activity.

Table 1: Cruzain qHTS assay protocol for 1536-well plate format

Cruzain qHTS Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3 µL	Enzyme: 3 nM Cruzain final
2	Compound	23 nL	Compound library
3	Control	23 nL	Control plate
4	Time	12 min	RT Incubation
5	Reagent	1 µL	Substrate : 2 µM final Z-FR-AMC
6	Detector	EX 340 / EM 450450	ViewLux fluorescence intensity kinetic read
Step	**Notes**		
1	Black solid plates, 1 tip dispense, Dispenser 2 head B, 80 µL pre-dispense, 1L glass bottle (+4℃), no enzyme in col 3-4; Triton X-100 concentration: 0.00005%		
2	Pintool transfer of compounds to assay plate (col 5-48)		
3	Pintool transfer of control inhibitor to col 2 of all assay plates. Two-fold, 16 pt dilution in duplicate.		
4	RT incubation in auxiliary hotel		
5	8 tip dispense, Dispenser 1 head C, 80 µL pre-dispense, 1 L glass bottle RT.		
6	ViewLux program 10, emission filter wheel A, Excitation DUG 11 / Emission 450/20; 4 kinetic reads every 30 sec		

b. Assay implementation and screening: **Kinetic Fluorogenic qHTS Assay for inhibitors of cruzain, detergent-present screen [AID: 1478; Primary DR]**

Assay Description

Three µL of reagents were dispensed into 1536-well Greiner black solid-bottom assay plate. Compounds and controls (23 nL) were transferred via Kalypsys PinTool equipped with 1536-pin array (10 nL slotted pins, V&P Scientific, Palo Alto, CA). The plate was incubated for 15 min at room temperature, and then a 1 µL aliquot of 8 µM substrate solution was added to start the reaction. The plate was transferred to ViewLux high-throughput CCD imager (Perkin-Elmer, Waltham, MA) where kinetic measurements (4 reads, one read every 30 seconds) of the AMC fluorescence were acquired using standard 340 nm excitation and 450 nm emission filter sets. During dispense, reagent bottles were kept submerged into 4°C recirculating chiller bath and all liquid lines were covered with aluminum foil to minimize fluorophore degradation. All screening operations were performed on a fully integrated

robotic system (Kalypsys Inc, San Diego, CA) as described elsewhere. Plates containing DMSO only (instead of compound solutions) were included approximately every 50 plates throughout the screen to monitor any systematic trend in the assay signal associated with reagent dispenser variation, or any decrease in enzyme specific activity.

Table 2: Detergent-present Cruzain qHTS assay protocol for 1536-well plate format

Cruzain qHTS Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3 µL	Enzyme: 1.5 nM final
2	Compound	23 nL	Compound library
3	Control	23 nL	Control plate
4	Time	12 min	RT Incubation
5	Reagent	1 □L	Substrate : 2 µM final Z-FR-AMC
6	Detector	EX 340 / EM 450450	ViewLux fluorescence intensity kinetic read

Step	Notes
1	Black solid plates, 1 tip dispense, Dispenser 2 head B, 80 µL pre-dispense, 1L glass bottle (+4°C), no enzyme in col 3-4; Triton X-100 concentration: 0.01%
2	Pintool transfer of compounds to assay plate (col 5-48)
3	Pintool transfer of control inhibitor to col 2 of all assay plates. Two-fold, 16 pt dilution in duplicate.
4	RT incubation in auxiliary hotel
5	8 tip dispense, Dispenser 1 head C, 80 µL pre-dispense, 1 L glass bottle RT.
6	ViewLux program 10, emission filter wheel A, Excitation DUG 11 / Emission 450/20; 4 kinetic reads every 30 sec

Center Summary of Results

To measure the enzymatic activity of cruzain, we utilized its model fluorogenic substrate Z-Phe-Arg-AMC, which is converted to a highly fluorescent 7-amino-4-methylcoumarin reporter upon cruzain-catalyzed hydrolysis. Assay optimization was performed directly in 1,536-well format at a final reaction volume of 4 µL. For the detergent-present screen, Triton X-100 was used at 0.01%, and the cruzain concentration selected for screening was 1.5 nM. During the optimization of the detergent-free assay, low stability and high variability in the specific activity of cruzain was noted, likely due to protein denaturation promoted by the large surface-to-volume ratio in the 1,536-well polystyrene assay plates. In order to stabilize the enzyme's performance, its final concentration was raised to 3 nM and a trace of Triton

X-100 (final concentration of 0.00005%) was included in the detergent-free assay; we note that a similar adjustment step was needed for the AmpC beta-lactamase aggregation screen described earlier. At the conditions selected - 1.5 nM or 3 nM final cruzain concentration and 2 μM final substrate concentration (the latter chosen to match previously reported conditions and being close to the Km value for this substrate) - the signal evolution was robust and low substrate conversion could be conveniently monitored over the course of 1 minute, making the assay highly sensitive to cruzain inhibitors. All assay components were tested and found stable for at least 24 hours when formulated as stock solutions at their working concentrations, in both the detergent-present and the detergent-free buffers (data not shown). Such demonstrated stability permitted the implementation of an unattended overnight screening operation.

The screens of the 197,861-compound collection were completed within a period of two separate workweeks. During the first week, approximately 60% of the 1,107 library 1,536-well plates were screened in the detergent-free assay first; this was immediately followed by a screen of the same set of library plates in the detergent-present assay. The remaining 40% of the library was screened in the same manner during the second week. The screening of each compound against the two assays in close succession minimized the possibility for sample-age related differences in results. Overall, two sets of 1,107 1,536-well assay plates were run under the detergent-free and detergent-present conditions, respectively, leading to the generation of 197,861 concentration responses consisting of at least seven points per compound per assay, and corresponding to a total of approximately 1.5 million samples tested per detergent condition.

The Z' screening factors associated with each plate and each screening condition remained high and stable throughout the two screens (Figure 1): the average Z' for the detergent-free screen was 0.78, while the corresponding average for the detergent-present screen was 0.93. Of the two screens comprising a total of 2,214 plates, only a group of six plates failed and was re-screened immediately using the same batches of enzyme and substrate. As a further quality control measure, we included a concentration response of the known vinyl sulfone cruzain inhibitor K1177721, 26 added as a 16-point dilution series in duplicate between 5.7 μM and 0.175 nM into the second column of every assay plate. The shape and quality of the concentration response remained consistent throughout both screens (Figure 3, green data points) with the associated minimum significant ratios (defined by Eastwood et al (6)) of 2.5 and 1.4 for the detergent-free and detergent-present conditions, respectively, further indicating stable runs.

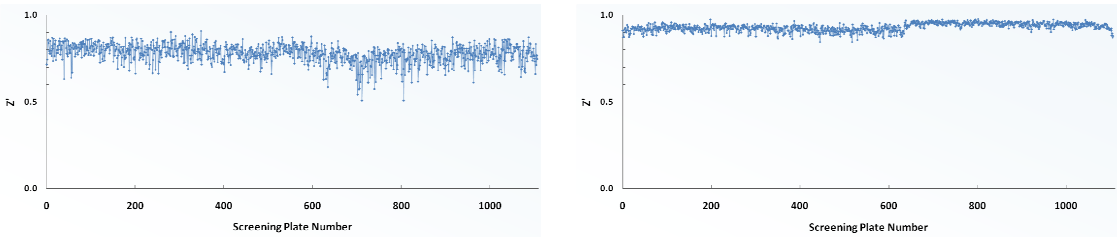
The cumulative effect of all library compounds on the cruzain activity at each screening condition is shown in Fig. 1C. On the plots, concentration responses were color-coded and positionally sorted based on activity with inactive samples (flat concentration responses) represented by the black dots, activators in red, and inhibitors in blue; green points in the very front of the 3D plots represented the 1,107 duplicate responses of the intra-plate control titration of K11777. Similar to our AmpC beta-lactamase profiling, the outcomes from the detergent-free versus detergent-present screens were strikingly different: the detergent-free screen yield over 15 times more hits than its detergent-present counterpart. Also as noted previously, we observed a large number (~12,000) of apparent activators in the detergent-free screen. Of these samples, almost all turned completely inactive upon inclusion of detergent. Over 85% of the activators were associated with partial or

single-point top concentration responses, indicating that the condition-dependent activation was being observed only at the highest compound concentrations where complicating phenomena such as transient precipitation, light scatter, and compound aggregate-assisted enzyme stabilization have been known to lead to false positive effects. We did not consider those compounds further.

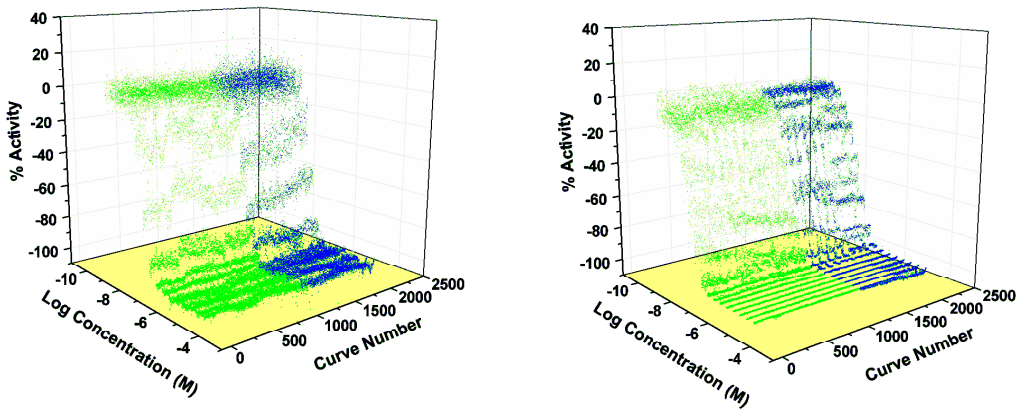
The detergent-insensitive actives were clustered based on chemical similarity and minimum common scaffolds were derived. A set of representative members of the top-ranked clusters were acquired for follow-up testing.

Figure 1. Cruzain dual-qHTS Screening Performance (Z' trend (A) and intraplate control titrations (B)) and Cumulative Compound Activity.

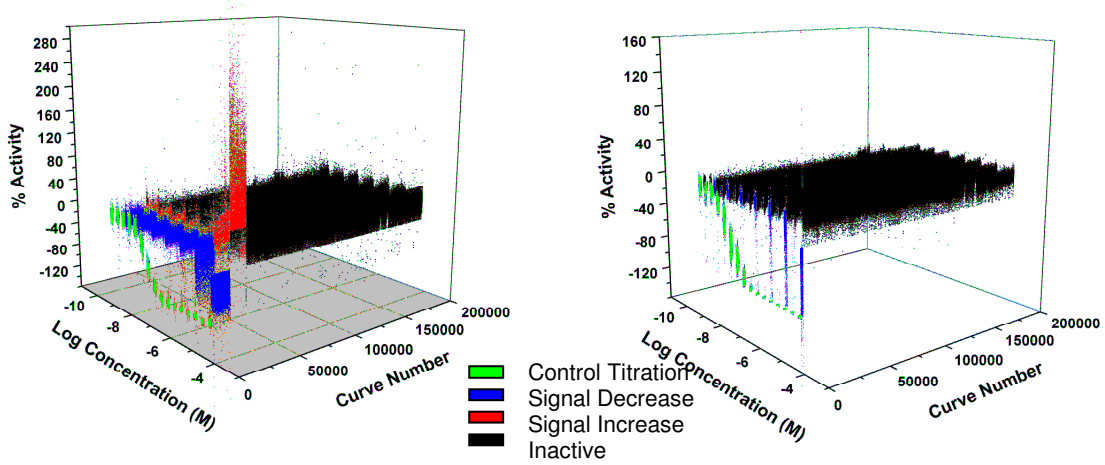
A) Plate Z' Trends



B) Intra-plate Control



C) Library Activity Profile



c. Assay implementation and screening: **Confirmation Concentration-Response Assay for cruzain inhibition [AID: 2158; Confirmatory DR]**

Assay Description
Select samples active in the primary screen were obtained in DMSO solution from the MLSMR and/or as powders from compound vendors to confirm activity in the original assay.

Assay Protocol
The assay protocol and reagents are identical to the cruzain primary qHTS assay described above.

Center Summary of Results
Prioritized compounds had a high confirmation rate.

d. Assay implementation and screening: **qHTS Counterscreen Assay for Papain inhibition [AID: 2161; DR]**

Assay Description

Although high selectivity was not a stated prerequisite for probe nomination, we wished to evaluate the selectivity of our top small molecules against related proteases. Papain, another cysteine protease known to utilize the same Z-FR-AMC fluorogenic substrate, was selected as a convenient profiling target. The fluorogenic assay used for cruzain was utilized here as well, with the exception of the reducing agent, which was 5 mM cystein here instead of DTT.

Assay Protocol

Table 3: Papain assay protocol for 1,536-well plate format

Cruzain qHTS Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3 µL	Enzyme: 12 nM final
2	Compound	23 nL	Compound dilution
3	Control	23 nL	Control plate
4	Time	12 min	RT Incubation
5	Reagent	1 µL	Substrate : 2 µM final Z-FR-AMC
6	Detector	EX 340 / EM 450450	ViewLux fluorescence intensity kinetic read

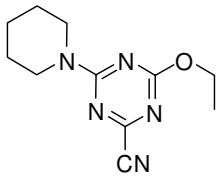
Step	Notes
1	Black solid plates, 1 tip dispense, Dispenser 2 head B, 80 µL pre-dispense, 1L glass bottle (+4°C), no enzyme in col 3-4; Triton X-100 concentration: 0.01%
2	Pintool transfer of compounds to assay plate
3	Pintool transfer of control inhibitor to col 2 of all assay plates. Two-fold, 16 pt dilution in duplicate.
4	RT incubation
5	Dispense of 4x substrate.
6	ViewLux program 10, emission filter wheel A, Excitation DUG 11 / Emission 450/20; 4 kinetic reads every 30 sec

Center Summary of Results

The papain profiling assay was miniaturized to a final 4 µL volume in 1536-well format. Consistently high signal-to-background and Z' score were noted.

Probe Optimization

Lead Compounds from Primary Screen:



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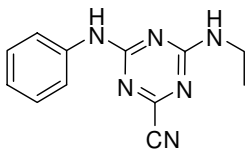

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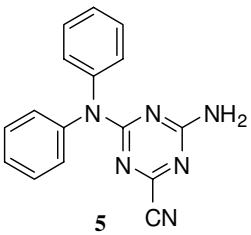
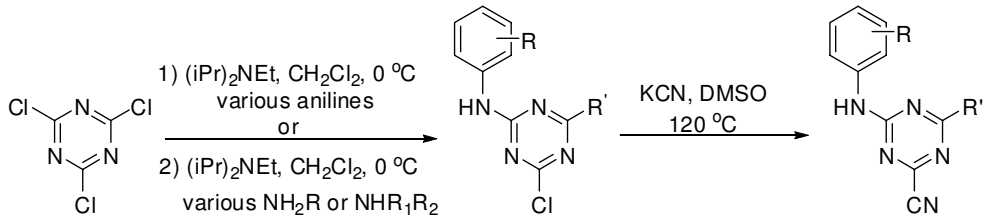


5

SAR Plan:

The top actives from qHTS, compounds **3**, **4**, and **5** (CIDs 729558, 778602, 712283 respectively) all showed comparable *in vitro* potency against cruzain in the low μM range. Since neither computer docking nor a crystal structure was available at the outset of the project, modifications were based on standard methods of functionality scan through library synthesis. Triazine nitriles are known reversible covalent inhibitors of various cysteine proteases with inhibition occurring through nucleophilic attack of the active site cysteine on the nitrile moiety. As such, this functional group was kept constant while changes to both the aniline and amino substituent's were attempted.

Synthesis of triazine analogues:

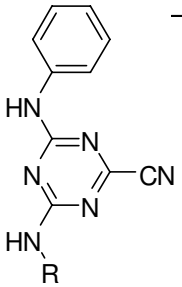


R' = various amines

Equimolar amounts of either alkyl/cycloalkyl-amines or anilines (depending on modification strategy) were added to a solution of cyanuric chloride and Hunig's base in dichloromethane at 0 °C. Depending on the starting material used in the first step, either anilines or amines were added using the procedure described above. Finally, the nitrile was then installed using KCN in DMSO at 120 °C for 10 minutes. Importantly, careful monitoring of the reaction was required as prolonged reaction times led to decomposition products.

<u>SAR of selected analogues</u>

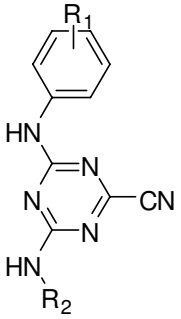
Alkyl moiety modifications-Round 1



Compound	R	AC_{50} (μM)[a]
4	Ethyl	0.71
6	Cyclopentyl	0.71
7	Propyl	0.79
8	Isopropyl	0.89
9	Methylcyclopropyl	0.89
10	Isobutyl	0.89
11	Butyl	0.89
12	4-hydroxybutyl	0.89
13	Methyl	1.26
14	Cyclohexyl	1.41
15	2-hydroxyethyl	1.58

AC50 values determined at NCGC

Aniline moiety modifications-Round 2



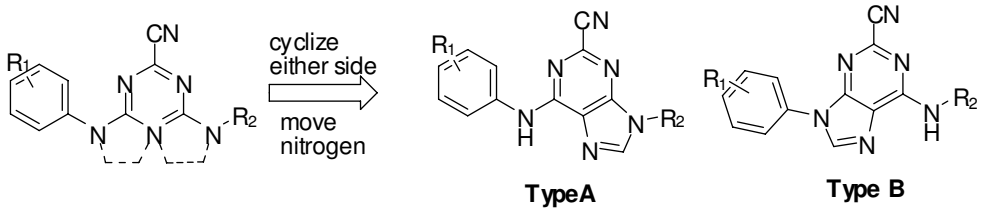
Compound	R_1	R_2	AC_{50} (μM)
16	3-nitro	cyclopentyl	0.063
17	3,5-difluoro	cyclopentyl	0.063
18	3-fluoro	cyclopentyl	0.079
19	3-chloro	cyclopentyl	0.079
20	3-bromo	cyclopentyl	0.1
21	4-fluoro	cyclopentyl	0.126
22	3-methyl	cyclopentyl	0.126
23	3,5-dichloro	cyclopentyl	0.398
24	4-bromo	cyclopentyl	1.122
25	3-phenyl	cyclopentyl	2.239
26	3-trifluoromethyl	cyclopentyl	inactive
27	3,5-difluoro	2,2-difluoroethyl	0.025

AC50 values determined at NCGC

Summary of Important SAR

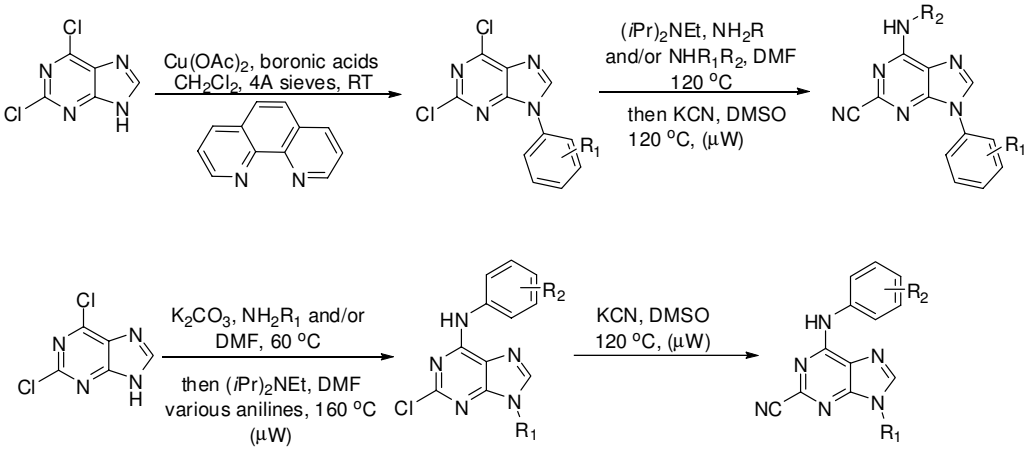
- There is tolerance for various amino groups, not much movement in potency.
- Electron-withdrawing groups on the aniline improve potency, while larger hydrophobic moieties are detrimental.
- 3,5-difluoro aniline provides the most potent analogue, yet interestingly the 3,5-dichloro is much less active presumably due to unfavorable steric interactions. (*vide supra*)

Scaffold hop: From Triazine to Purine Nitrile



Upon completion of the triazine nitrile chemotype, we sought to explore purine-based inhibitors, as we hoped the increased rigidity and hydrophobicity of these molecules would provide an increase in potency. Using the lessons learned from our previous series, we where able to synthesized a more targeted library. However, while some literature precedence exists for the use of purine nitriles against cysteine proteases, they have not been reported for the inhibition of Cruzain. Thus, our initial plan was to determine what the optimal regiochemistry of both the aniline group and alkyl group

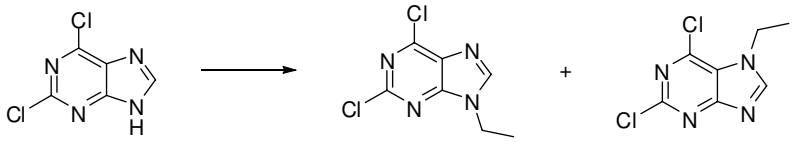
Synthesis of Purine Nitrile analogues:



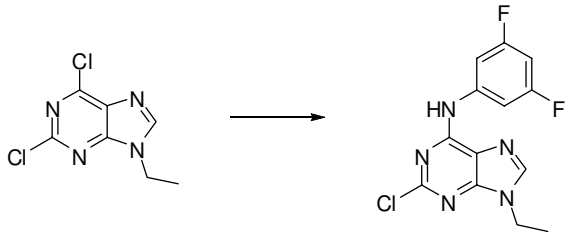
Starting from 2,6-dichloropurine, the 9-position was alkylated using various alkyl halides in the presence of K_2CO_3 in DMF at 60 °C. Two regioisomers were obtained, providing alkylation primarily at N9. The ratio of the product distribution was dependent on the nature of the alkyl group, and the two isomers were easily separable by column chromatography. Displacement of the 6-position chlorine was accomplished by heating various anilines in DMF at 160 °C in the presence of Hunig's base. Finally, the nitrile was installed at the 2-position using KCN in DMSO at 120 °C. Of note, performing both of these transformations in the microwave greatly accelerated the reaction.

Analogues of Type B required a modified first step. The aromatic ring was first installed at the 9-position using a Buchwald-Hartwig-like copper catalyzed cross coupling between the aromatic amine and desired boronic acids. Alkyl amines could then be installed at the 6-position in the presence of Hunig's base in DMF at elevated temperature (120 °C). These analogues were also completed by displacing the final 2-Cl with KCN in DMSO at 120 °C in the microwave.

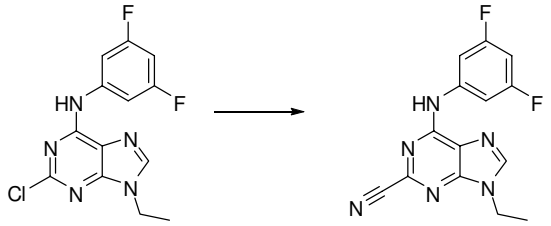
General procedures for the synthesis of probe molecule and analogues:



Example procedure for the formation of *2,6-dichloro-9-alkyl-9H-purines:*
To a solution of 2,6-dichloro-9H-purine (2 g, 10.58 mmol) in acetone (45 mL) was added sodium carbonate (2.25 g, 21.2 mmol). The reaction vessel was equipped with a reflux condenser, and the mixture was heated to reflux for 20 minutes. After that time, iodoethane (0.86 mL, 10.6 mmol) was added in one portion, and the reaction mixture was allowed to stir for 5 h. Upon completion, the reaction mixture was concentrated under reduced pressure and directly purified on silica column. Gradient elution with ethyl acetate (2→40%) in hexanes provided regioisomers 2,6-dichloro-9-ethyl-9H-purine and 2,6-dichloro-7-ethyl-7H-purine as pale yellow solids: yield (1.5 g, 6.9 mmol and 0.4 g, 1.8 mmol, respectively).



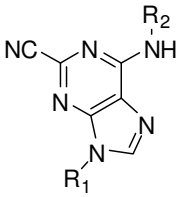
Example procedure for the formation of *2-chloro-N-phenyl-9-ethyl-9H-purin-6-amines*: To a solution of 2,6-dichloro-9-ethyl-9H-purine (0.5 g, 2.3 mmol) in DMF (2 mL) was added 3,5-difluoroaniline (0.29 g, 2.30 mmol) and Hunig's base (0.40 mL, 2.30 mmol). The reaction mixture was sealed in a microwave tube (2-5 mL) and heated to 110 °C for 30 min in a Biotage Initator® microwave. Upon completion, the reaction mixture was diluted with ethyl acetate and washed several times with 3N lithium chloride solution. The organic layer was separated, dried on magnesium sulfate, filtered, concentrated under reduced pressure and directly purified on silica column. Gradient elution with ethyl acetate (5→50%) in hexanes provided 2-chloro-N-(3-chlorophenyl)-9-ethyl-9H-purin-6-amine as a pale yellow solid: yield (0.64 g, 2.1 mmol, 90 %).



Example procedure for the formation of *6-phenylamino-9-ethyl-9H-purine-2-carbonitriles:*
To a solution of 2-chloro-N-(3,5-difluorophenyl)-9-ethyl-9H-purin-6-amine (50 mg, 0.16 mmol) in DMSO (1 mL) was added KCN (11 mg, 0.16 mmol). The reaction mixture was sealed in a microwave tube (0.5-2 mL) and heated in a Biotage Initiator® microwave to 140 °C for 1 h. Upon completion, the reaction mixture was dilute with ethyl acetate and washed several times with saturated sodium chloride solution. The organic layer was separated, dried on magnesium sulfate, filtered, concentrated under reduced pressure and directly purified on silica column. Gradient elution with ethyl acetate (10→70%) in hexanes provided the product as a colorless solid: yield (45 mg, 0.15 mmol, 93 %);

<u>SAR of selected analogues</u>

In vitro potency of Purine Nitrile Analogues



Compound	R_1	R_2	AC_{50} (μM)
28	Ethyl	3,5-difluorophenyl	0.010
29	2,2-difluoroethyl	3,5-difluorophenyl	0.013
30	Cyclopentyl	3,5-difluorophenyl	0.018
31	Ethyl	3-chlorophenyl	0.018
32	Cyclopentyl	3-chlorophenyl	0.040
33	3,5-difluorophenyl	2,2-difluoroethyl	0.050
34	3-chlorophenyl	Ethyl	0.063
35	3-chlorophenyl	2,2-difluoroethyl	0.071
36	3,5-difluorophenyl	Ethyl	0.251
37	3,5-difluorophenyl	Cyclopentyl	0.316

AC50 values determined at NCGC

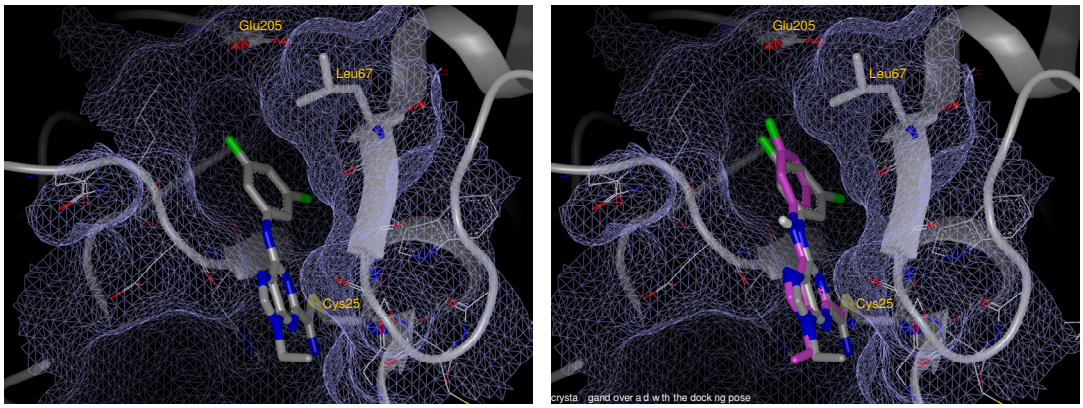
In vitro and in vivo potency of Purine Nitrile Analogues

Compound	cruzain IC_{50} (μM)[a]	rhodesain IC_{50} (μM)[a]	TbCatB IC_{50} (μM)[a]	Tbb IC_{50} (μM)[b]
3	0.3	0.5	>1	**>100**
4	>10	>10	>1	**na**
5	0.07	>10	>1	**>100**
16	0.001	0.008	>1	81
17	0.004	0.005	>1	5
18	0.01	0.02	>1	79
19	0.002	0.006	>1	5
20	0.002	0.006	>1	5
21	0.03	0.04	>1	>100
22	0.03	0.05	6	20
23	0.01	0.03	>10	22
24	0.06	0.1	>10	>100
25	0.05	0.3	>10	21
26	0.04	0.7	>10	21
27	0.001	<0.006	1	2
28	*0.0002*	*<0.006*	*28*	*9*
31	0.0004	<0.01	41	7
32	0.0003	<0.006	26	53

IC50 values determined at UCSF

Note: UCSF IC50 values are left-shifted compared to NCGC IC50 values due to improved assay sensitivity.

Co-crystallization of Probe Molecule with Cruzain-docking pose also shown:



Left-Co-crystallization of probe molecule with Cruzain. Right- docking pose (purple) overlaid with co-crystal structure (grey).

Summary of Important SAR

- Lessons learned from triazine SAR translated well to the purine nitrile chemotype. 3,5-difluoro analogue still provides the most potent compound.
- Analogues of type B (i.e. the regiochemistry of the aniline and amino moieties are flipped) are much less active.
- Potency of top active is ~200 pM, yet these compounds lack appreciable activity against either *T. brucei* or *T. cruzi* parasites presumably due to cell permeability/solubility.

- Co-crystallization of the probe molecule with cruzain seems to indicate that the N9 substituent is exposed to solvent and could be utilized to improve pharmacokinetic properties (i.e. solubility and cell permeability).

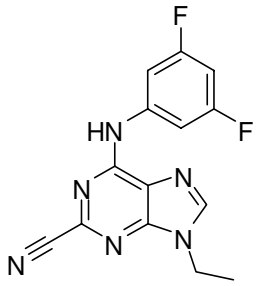
- Co-crystallization also helps explain the observation that larger/hydrophobic groups off the aniline are unfavorable. The S2 binding pocket is fairly narrow and can not accommodate larger groups.

3. **Probe**

e. Chemical name of probe compound

6-(3,5-difluorophenylamino)-9-ethyl-9H-purine-2-carbonitrile

f. Probe chemical structure including stereochemistry if known



g. Structural Verification Information of probe SID

^{1}H NMR (DMSO-d6) δ 1.46 (t, J = 7.3 Hz, 3H), 4.30 (q, J = 7.3 Hz, 2H), 6.94 (tt, J = 9.3 and 2.4 Hz, 1H), 7.63 - 7.83 (m, 2H), 8.67 (s, 1H), and 10.79 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 15.49, 98.74, 103.82, 104.11, 117.33, 122.34, 135.68, 141.91, 142.05, 145.31, 149.58, 151.78, 161.61, and 164.02; HRMS (ESI) *m/z* 301.1008 $(M+H)^+$ ($C_{14}H_{10}F_2N_6$ requires 301.1006).

LC/MS (Agilent system) Retention time t_1 (short) = 3.71 min. and t_2 (long) = 6.09 min.
Column: 3 x 75 mm Luna C18, 3 micron
Run time: 4.5 min (short); 8.5 min (long)
Gradient: 4 % to 100 %
Mobile phase: Acetonitrile (0.025 % TFA), water (0.05 % TFA).
Flow rate: 0.8 to 1.0 mL
Temperature: 50 ^{o}C
UV wavelength: 220 nm, 254 nm

h. PubChem CID (corresponding to the SID): 44143088

i. If available from a vendor, please provide details.

This probe is not available commercially. See (j) below for information on availability.

j. Provide MLS# that verifies the submission of probe molecules and five related samples that were submitted to the SMR collection.

Scale-up of probe molecule and 5 related analogues are in progress. Samples will be submitted to SMR immediately after completion.

k. Describe mode of action for biological activity of probe

This probe displays potent (~200 pM) reversible covalent inhibition of both cruzain and rhodesian (two homologous cysteine proteases) via nucleophilic attack of the active site cysteine moiety onto the nitrile group on the probe molecule.

l. Detailed Synthetic pathway for making probe

See above

m. Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.)

Compound is soluble at 10 mM in DMSO. The compound is not fluorescent with blue excitation wavelengths (~340 nm). Solubility in buffer has not been determined.

n. Calculated probe properties

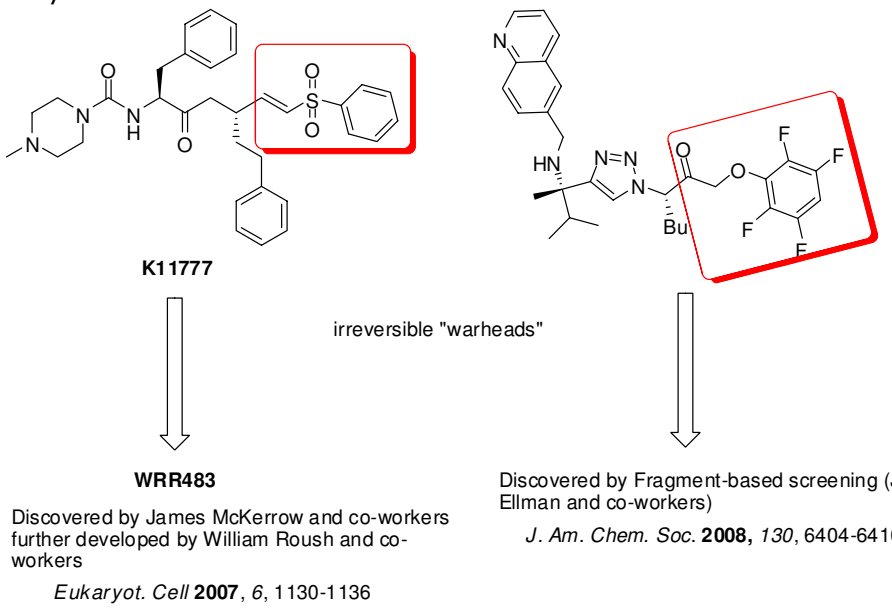
PubChem CID	44143088
Molecular Weight	300.27
Molecular Formula	$C_{14}H_{10}F_2N_6$
ALogP	2.74
H-Bond Donor	1
H-Bond Acceptor	5
Rotatable Bond Count	4
Exact Mass	300.09
Topological Polar Surface Area	79.42
Heavy Atom Count	22

Appendices

a. (1) comparative data on probe (**see above**) (2) similar compound structures/SAR (**see above**)

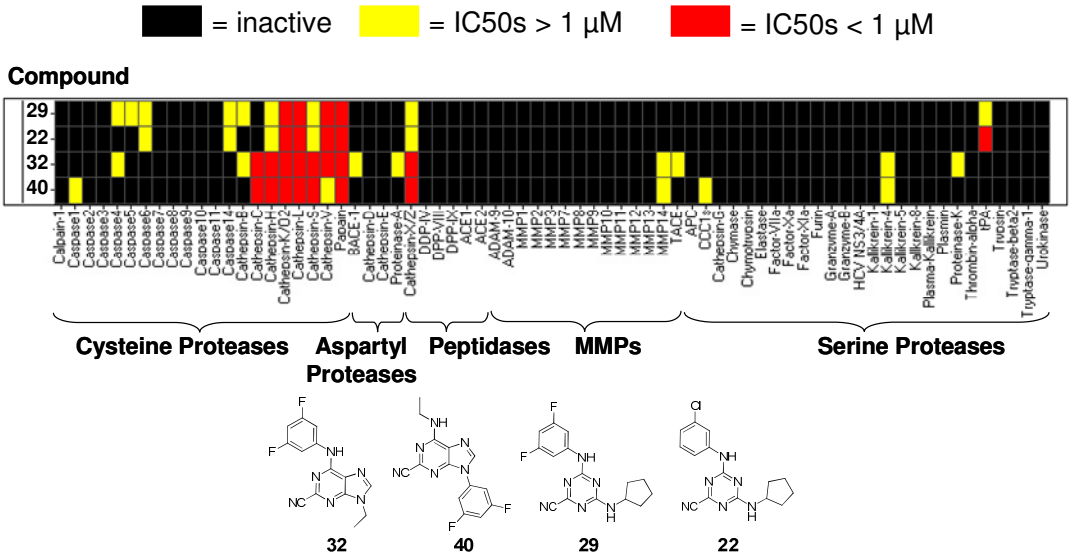
(3) Current state of the art.

The majority of current advanced cruzain inhibitors act via *irreversible* covalent inhibition such as the two compounds shown below. Our probe acts via *reversible* covalent inhibition and to the best of our knowledge we provide the first co-crystallization of nitrile-based inhibitor to cruzain.



b. Comparative data showing probe specificity for target

Selectivity towards other cysteine proteases:



Data from Reaction Biology Corp.

Note: these compounds preferentially hit the cathepsin proteases, however cathepsins are located in the lysosomes of cells whereas parasites are located

in the more accessible cytoplasm. Additionally, as Chagas is an acute ailment, off-target activity is less worrisome. Chronic administration of any treatment should not be required.

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