Probe Report

Title: Identification of Small-Molecule Inhibitors of *Trypansoma cruzi* Infection-Probe 3

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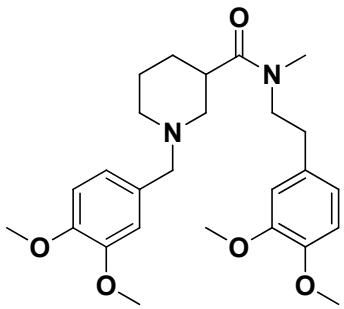
Chemistry Center Name and PI: Broad Institute Probe Development Center, Stuart Schreiber
Assay Submitter and Institution: Ana Rodriguez, New York University
PubChem Summary Bioassay Identifier (AID): 1885

Abstract:
Chagas disease is a tropical disease caused by the parasite *Trypanosoma cruzi (T. cruzi)*, which is endemic to Central and South America. Approximately 13 million people are infected with the parasite, and 25-30% of infected patients suffer from irreversible damage to the heart and digestive tract resulting in disability and death within 20 years of infection. Few treatments are available with limited effectiveness only against the early (acute) stages of disease, significant toxicity, and widespread drug resistance. We report the outcome of a high-throughput chemical library screen to identify novel, nontoxic, small-molecule inhibitors of *T. cruzi,* which will aid the development of more potent and selective therapies for both the acute and chronic stages of Chagas disease. Of the 303,224-screened compounds, 35 compounds were chosen based on their selectivity, potency, and chemical tractability. Of those, 27 dry powder-validated compounds were retested in the primary screen, secondary assays, and an orthogonal screen. Three scaffolds were prioritized to identify potential probes. One of these compounds (CID3238551/ML158) displayed greater than 100-fold selective inhibition of *T. cruzi confirmed* in immunofluorescent imaging assays and is inactive against host cells at the highest tested dose. This new probe should be very useful in future cell-based investigations and *in vivo* studies of *T. cruzi* inhibition.

Probe Structure and Characteristics:



ML158

Compound Summary in PubChem

IUPAC Chemical Name	*N*-[2-(3,4-dimethoxyphenyl)ethyl]-1-[(3,4-dimethoxyphenyl)methyl]-*N*-methylpiperidine-3-carboxamide
PubChem CID	3238551
Molecular Weight	456.57444 g/mol
Molecular Formula	C26H36N2O5
XLogP	3.6
H-Bond Donor	0
H-Bond Acceptor	6
Rotatable Bond Count	10
Exact Mass	456.262422
Topological Polar Surface Area	60.5
Solubility (PBS, µM)	>500

CID/MLS No.	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target	IC_{50}/EC_{50} (µM) [SID, AID]	Fold Selective*	Secondary Assay(s) [SID, AID]
3238551/ ML158	*Trypansoma cruzi* replication	109 [SID 87219036, AID 2630]	NIH/3T3 Cell Toxicity	Inactive [SID 87219036, AID 2586]	109 nM vs inactive	Immunofluorescence Static [SID 87219036, AID 2630]

*Selectivity = anti-target IC_{50}/target IC_{50}.

Recommendations for scientific use of the probe:

The goal of this project is to identify a small-molecule inhibitor of the protist *Trypanosoma cruzi (T. cruzi)*, the causative parasite of Chagas disease. Although a few treatments for Chagas disease are available, these treatments are limited because they are only effective against the early (acute) stages of disease and have significant toxicity to the patient (1,2,3). Novel antitrypanosomal agents will aid in developing a drug treatment for both the acute and chronic stages of Chagas disease. This probe (CID3238551/ML158) is expected to inhibit the replication of *T. cruzi* without having effects on the viability of the mammalian host cell.

The probe (CID3238551/ML158) described in this report inhibits the replication of *T. cruzi* with an IC_{50} of 109 nM and is inactive toward mammalian cells, NIH/3T3 up to concentrations of 19,500 nM. In immunofluorescent imaging assays, the compound inhibited *T. cruzi* amastigote replication within host cells. Since amastigote morphology was maintained, this probe (CID3238551/ MLS002725691) is categorized as a trypanostatic (static) compound.

This probe (CID3238551/ML158) provides a valuable tool that will allow microbiologists investigating Chagas disease to identify new targets in *T. cruzi,* which may potentially lead to better drugs. Furthermore, the Assay Provider, Dr. Ana Rodriguez and her colleagues, will continue their own studies with this probe (CID3238551/ML158) in a mouse model of Chagas disease.

1 Introduction

Scientific Rationale

Chagas disease is a tropical parasitic disease caused by the parasite *Trypanosoma cruzi (T. cruzi)*. Approximately 13 million people are infected with the parasite, which is endemic to Central and South America and results in 14,000 deaths per year (2, 3). Twenty-five to thirty percent of infected patients suffer from irreversible damage to the heart and digestive tract resulting in a high incidence of disability and death within 20 years of infection (1, 2). Chagas disease is the leading cause of heart failure in the region.

In **Figure 1**, the drugs currently used to treat Chagas disease are: benznidazole **A** (Rochagan®, Radanil® Hoffman Roche) and nifurtimox **B** (Lampit®, Bayer), whose anti-*T. cruzi* activities were discovered more than 30 years ago. Both of these drugs are effective only in the acute phase of infection, rather than the key chronic stage. They often do not completely eliminate the parasite despite long-term administration, while exhibiting unacceptable side effects (such as nausea, vomiting, weight loss, neurological effects, and signs of testicular and ovarian injury). Both treatments are actually nonspecific for *T. cruzi;* in addition, resistance to these drugs is becoming more widespread. Thus, there is a clear need for more potent and selective therapies.

Figure 1. Current Treatments for Chagas Disease

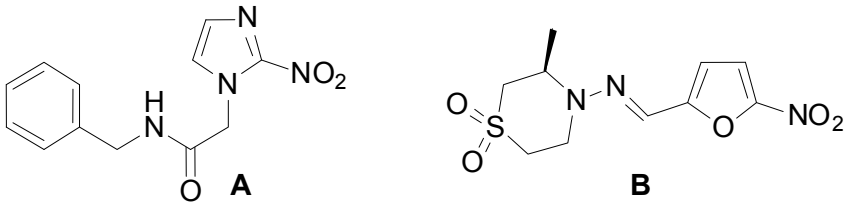
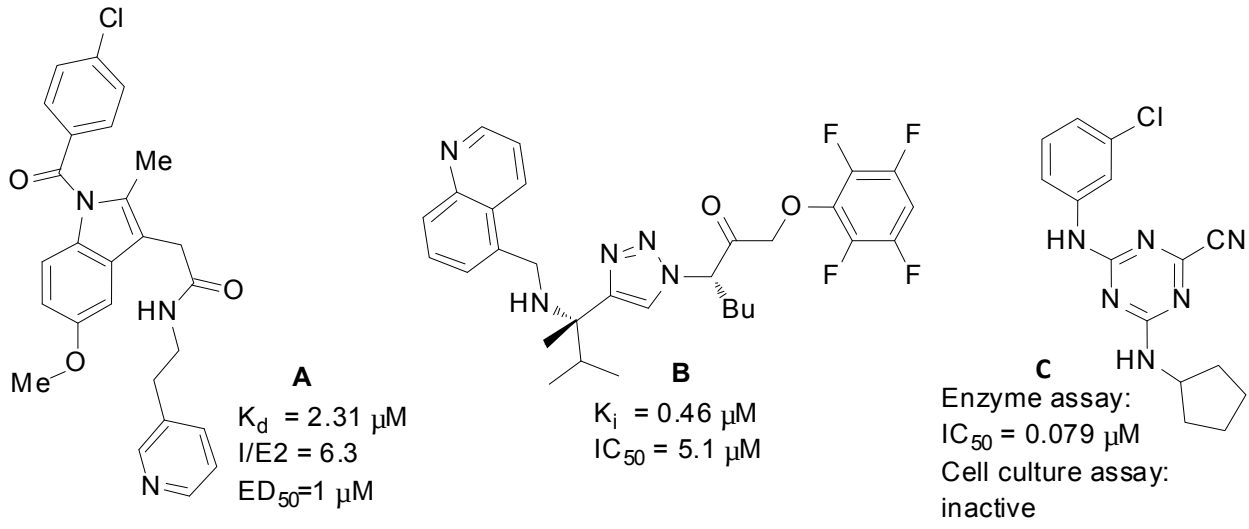


Figure 1. Benznidazole (Rochagen®, Radanil®, Hoffman-La Roche) (*A*); Nifurtimox (Lampit® ,Bayer) (*B*)

Several recent reports have revealed promising new lead compounds that target *T. cruzi*, including inhibitors of the cysteine protease cruzain and inhibitors of sterol demethylase (4). However, while the protease inhibitors (see **Figures 2A**, **2B**, and **2C**) exhibit excellent activity in biochemical assays (0.079 μM to 2 μM), they are inactive or demonstrate high micromolar activity in parasite inhibition in cell culture (1 μM to inactive) (4, 5, 6). Thus, there is a continued

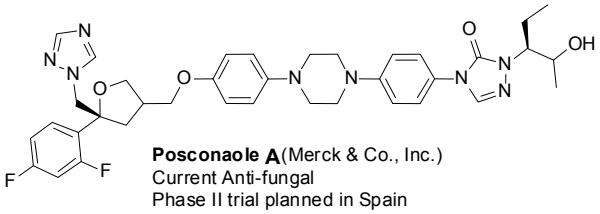
unmet need to identify scaffolds that are potent in cell culture against *T. cruzi* and nontoxic to the mammalian host cell. Our approach overcomes this problem of lack of cellular activity by employing a phenotypic screen measuring parasite replication in a host cell. Counterscreening in the mammalian host filters out toxic compounds.

Figure 2. Recent Inhibitors of *T. cruzi* Cysteine Proteases

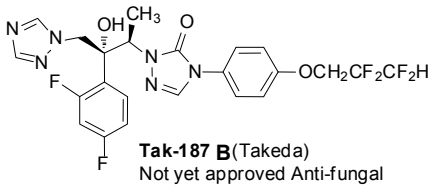


There are currently four compounds in clinical trials for Chagas disease (see **Figure 3**). Benznidazole (see **Figure 1A**) initially developed for treatment of acute Chagas disease is in Phase III trials to expand its use to include the treatment of chronic Chagas cardiomyopathy.
Three antifungal drugs from Merck (see **Figure 3A**), Takeda (see **Figure 3B**), and Eisai (structure not disclosed) are in Phase I or II trials for expanding their use to include treatment of Chagas disease. Two heart arrhythmia treatments (see **Figure 3C** and **Figure 3D**) are the most frequent treatments for the chronic stage heart issues associated with Chagas disease and have shown anti-parasitic activity in some patients. The only novel compound currently in the pipeline is a peptide mimic from the Sandler Center for Drug Discovery (see **Figure 3E**) which is in preparation for a Phase I safety trial. Since all of these compounds are nonspecific for *T. cruzi*, the side effects may be problematic as is the case for benzidazole in its current use. Therefore, a selective, nontoxic inhibitor of *T. cruzi* replication could be of significant use to the scientific community.

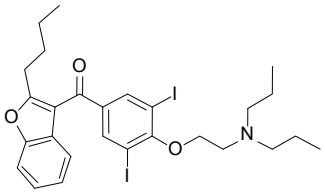
Figure 3. Compounds in Clinical Trials for Chagas Disease



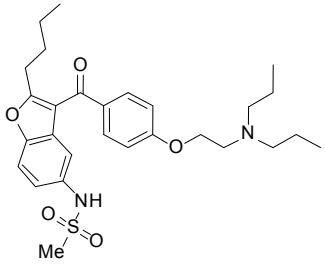
Posconaole A (Merck & Co., Inc.)
Current Anti-fungal
Phase II trial planned in Spain



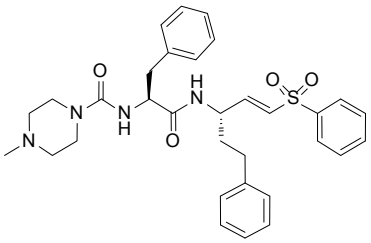
Tak-187 B (Takeda)
Not yet approved Anti-fungal
Phase I completed



Amiodarone C (Off patent)
Heart Arrhythima treatment
Frequently used anti-arrhythima drug
in Chagas patients; anecdotal anti-parasitic
activity in individual patients



Dronedarone D (Sanofi-Aventis)
Heart Arrhythima treatment
Frequently used anti-arrhythima drug
in Chagas patients; anecdotal anti-parasitic
activity in individual patients



K777 E (Sandler Center for Drug Discovery)
Not yet approved
In preparation for Phase I safety trial

This project aims to identify novel antitrypanosomal agents for the treatment of Chagas disease. One key design of the project is to target the parasites co-cultured with host cells and to demonstrate little to no toxicity to the host cell. This screening campaign has identified a probe, *N*-[2-(3,4-dimethoxyphenyl)ethyl]-1-[(3,4-dimethoxyphenyl)methyl]-*N*-methylpiperidine-3-carboxamide (CID3238551/ML158), which has an IC_{50} of 109 nM and has no toxicity towards NIH/3T3 cells at the highest concentration tested (i.e., 19.5 μM).

2 Materials and Methods

The methods in this section were either performed as described in Bettiol et al. (i.e., immunofluorescence) (7) or modified for high throughput screening (co-culture and host cell toxicity).

Materials and Reagents

T175 culture flasks with vented caps were obtained from BD Falcon, and hyperflasks were obtained from Corning (Corning, NY; Catalog no.10024). Disposable sterile filter units (500 ml or 1 L; pore size, 0.20 μm were obtained from Nalgene (Catalog no. 566-0020). Dulbecco's modified Eagle's medium (DMEM) with Phenol Red, high glucose, with L-glutamine and sodium

pyruvate was obtained from Cellgro (Mediatech Inc, Manassas, VA; Catalog no. 10-013-CM). Penicillin-streptomycin-L-glutamine (PSG, Catalog no. 10378-016), FBS-heat inactivated fetal bovine serum (FBS, Catalog no.16140-089), and 0.25% Trypsin-EDTA 1X (Catalog no. 25200-072) were purchased from Gibco-Invitrogen. Sterile horse serum, from donor herd (if appearance of epimastigotes) was obtained from Sigma (Catalog no. H1270). Sterile, Ca++/ Mg++-free Phosphate Buffered Saline (PBS) 1X was prepared in house.

Nonidet P-40 (NP40, now called Igepal CA 360) was obtained from Fluka (Sigma-Aldrich, St. Louis, MO; Catalog no. 56741) and Gal-Screen® Buffer B was obtained from AB Biosciences (Allston, MA; Catalog no.T1031).

Cell Lines

- The following cell lines were used in this study: LLC-MK2 cells (rhesus monkey kidney epithelial cell line) and NIH/3T3 cells (mouse embryonic fibroblastic cell line) were initially obtained from the Assay Provider, then from ATCC. *T. cruzi* expressing β-galactosidase (*T. cruzi* -β-gal: Tulahuen strain, clone C4; was obtained from the Assay Provider with derivation as described in Buckner et al. (8).
- Alexa Fluor 488 goat anti-rabbit IgG secondary antibody was from Molecular Probes®, Invitrogen (Carlsbad, CA).
- Polyclonal rabbit anti-*T. cruzi* was a gift from Dr. B. Burleigh, Harvard School of Public Health, Boston, MA).

2.1 Assays

A summary listing of completed assays and corresponding PubChem AID numbers is provided in **Appendix A** (Table A1). Refer to **Appendix B** for the detailed assay protocols.

2.1.1 *T. cruzi* Inhibition Assay (AID Nos.1885, 2044, 2294, 2630)

For cell propagation: 90% DMEM, Phenol Red, 10% FBS, and 1% PSG were mixed and filtered through a 0.2 micron membrane. The cells were kept at 4°C, and then warmed up to 37°C in a water bath before use.

For *T. cruzi* culture and assays: 98% DMEM, Phenol Red, 2% FBS, and 1% PSG were mixed and filtered through a 0.2 micron membrane. The cells were kept at 4°C, and then warmed up to 37°C in a water bath before use.

Solutions: Gal-Screen + 0.05% NP40. Using a Gal-Screen base kit, Buffer B (Catalog no. T2361) was mixed with 1:25 substrate (Catalog no. T2359) with a 1:400 dilution of 20% NP40.

NIH/3T3 Cell Culture. NIH/3T3 cells were cultivated in DMEM supplemented with 10% FBS and 1% PSG in T175 in 50 ml total of medium.

LLC-MK2 Cell Culture. LLC-MK2 cells were cultivated in DMEM supplemented with 10% FBS and 1% PSG in T175 flasks in 50 ml total of medium. Cells were usually passaged twice a week at 1:4 to 1:8 ratios.

Parasite Culture: *Tcruzi* β-gal (Tc). *T. cruzi* -β-gal were cultivated in DMEM supplemented with 2% FBS and 1% PSG in T175 flasks with vented caps (important to avoid spills!) in 50 ml total of medium.

2.1.2 Growth Inhibition Assay for HTS (384-well plates)

The medium was warmed up with 2% FBS/DMEM. The parasites were harvested in 50-ml tubes, and spun for 10 minutes at 2200 rpm. Approximately 15 ml of media was aspirated, and the samples were incubated for 3-5 hours. The NIH/3T3 cells were trypsinized (refer to cell culture protocol). When the NIH/3T3 cells were detached, the cells were harvested in DMEM, 2% FBS, and 1% PSG, then counted using the Nexcelom Cellometer. The cells were diluted to 166,667 cells/ml, and then added to a flask and plated 5,000 cells/ 30 µL per well using a standard cassette multiwell drop Combi. The cells were incubated for 3 hours, and then *T. cruzi* cells were counted, diluted to 0.250 million cells/ml, and transferred to a 2-liter flask. Then, 50 nL compounds/DMSO were pinned to each well with NIH/3T3 cells. Next, 20 µL/well of parasites (5000 *T. cruzi*) were added with a standard cassette multiwell drop Combi on slow speed, and incubated for 4 days (or a minimum of 90 hours). Gal-Screen was prepared with 0.05% NP40, 30 µL per well were dispensed in a 384-well plate, incubated for 60 minutes, and the luminescence was read using Envision (Perkin-Elmer) at 0.1 sec/well.

2.1.3 Cell Toxicity Assay: NIH/3T3 Cells (AID Nos. 2010, 2586, 493247)

For the cell toxicity assay with NIH/3T3 cells, the same materials as for *T. cruzi* co-culture assay were used. NIH/3T3 cells were cultivated in DMEM supplemented with 10% FBS and 1% PSG in T175 in 50 ml total of medium.

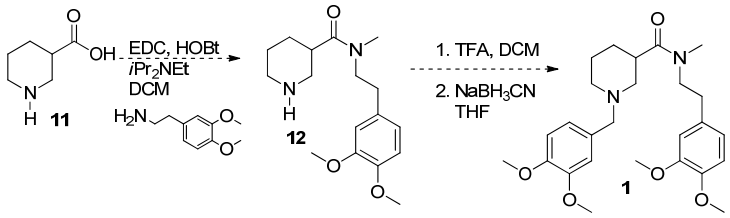
2.1.4 Intracellular *T. cruzi* Immunofluorescence Assay (AID No. 2632)

Fifty thousand NIH/3T3 cells were seeded on sterile glass coverslips in 12-well plates and allowed to adhere overnight. Five million *T. cruzi* parasites were added (Mechanism of inhibition 100:1) and allowed to infect for 2 hours in DMEM+2% FBS and PSG. Parasites were rinsed out 3X with PBS, and compounds were added at 10X their IC_{50} (as determined in AID 2044 and AID 2294). Infected cells were further incubated for 4 days and fixed for 15 minutes with 4% paraformaldehyde.

Fixed cells on coverslips were rinsed with PBS and permeabilized for 15 minutes in PBS with 0.1% Triton X-100. After blocking for 20 minutes in PBS with 10% goat serum, 1% bovine serum albumin (BSA), 100 mM glycine, and 0.05% sodium azide, cells were incubated for 1 hour at room temperature with a polyclonal rabbit anti-*T. cruzi* at 1:2000 dilution. After rinsing, an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody was added for 1 hour at a 1:800 dilution. DNA was stained with DAPI, and coverslips were mounted with anti-fade mounting media. Images were taken using an inverted Olympus IX70 microscope with a 60X oil objective.

2.2 Probe Chemical Characterization

Scheme 1. Synthesis of Probe (CID3238551/ML158)



The probe (CID3238551/ML158) **1** was purchased from ChemDiv (ID 5979-0184); however, the probe could be synthesized starting from nipecotic acid in three steps as shown in **Scheme 1**. The above route was used to synthesize several analogs. Amide coupling of nipecotic acid **11** with 3,4-dimethoxyphenethyl amine would result in the amide **12**. Reductive amination of **12** with 3,4-dimethoxybenzaldehyde would provide probe **1** (CID3238551/ML158). The probe (CID3238551/ML158) was determined to have a solubility of >500 µM in PBS at room temperature.

The probe (CID3238551/ML158) and five analogs were submitted to the SMR collection (MLS002725691, MLS002725685, MLS002725682, MLS002725692, MLS002725694).

The ^{1}H NMR (300 MHz, DMSO-d6) spectra and LC-MS chromatograms of the probe (CID3238551/ML158) and analogs (CID16187238, CID16191661, CID44629407, CID44629409, CID44629410, CID44629413, and CID 16190091) are provided in **Appendix C**.

2.3 Probe Preparation

Not applicable. The probe (CID3238551/ML158) was purchased from ChemDiv (ID 5979-0184).

3 Results

Probe Attributes:

- Inhibits extracellular parasite invasion into host cell or inhibits intracellular parasitic replication.
- Non-toxic to host cells (100-fold selectivity towards parasite vs. host cells).

3.1 Summary of Screening Results

Figure 4 displays the critical path for probe development.

A high-throughput screen of 303,224 compounds (AID 1885) was performed in duplicate in the recombinant Tulahuen strain of *T. cruzi* stably expressing beta-galactosidase reporter co-cultured with host cell, mouse fibroblast NIH/3T3 (8). The signal was normalized to neutral (DMSO) controls, and a 55% inhibition cutoff at a screening concentration of 3.75 μM average was used to define a hit. Next, 4,394 hits were identified as inhibitors of *T. cruzi* replication when co-cultured with host cells and, of these, 4,063 were available as cherry picks. The cherry-picked compounds were retested in dose in the primary assay using co-cultured *T. cruzi* strain with host cells to confirm their inhibitory activity (AIDs 2044, 2294). From the 4,063 compounds retested at dose concentrations, 4,016 compounds (99%) re-confirmed in this assay. In parallel, these 4,063 compounds were included in toxicity assays against host cell NIH/3T3 (AID 2010) to determine if these compounds were cytotoxic to mammalian cells and, thus, false positives. This secondary screen identified 1,011 compounds that reduced viability of NIH/3T3 cells; therefore, these compounds were excluded as viable hits.

After completing retests and a secondary assay at dose from DMSO stocks, 3,005 compounds were clustered into groups with 70% similarity. The 25 clusters with at least five members were

given to a team of chemists; 35 representative compounds, at least one from each cluster, were prioritized based on molecular weight, presumed solubility, and lack of toxic or reactive functionality. Of the 35 compounds selected, 27 authentic dry powders were obtained. From this set of dry powder compounds, a piperidine series was prioritized. Additional analogs were ordered and tested in the primary and secondary toxicity assay. The results are reported in **Table 1**, **Table 2**, and **Table 3**. Also, these compounds were tested in an additional immunofluorescence assay to determine the mode of action of the compounds (AID 2632).

Intracellular *T. cruzi* was treated with the probe (CID3238551/ML158). The compound inhibited replication of *T. cruzi* within the mammalian host cell but did not lyse *T. cruzi*. Therefore, the compound was classified as a 'static' inhibitor. Related analogs were also tested (AIDs 2630, 2586, 2632).

Figure 4. Critical Path for Probe Development

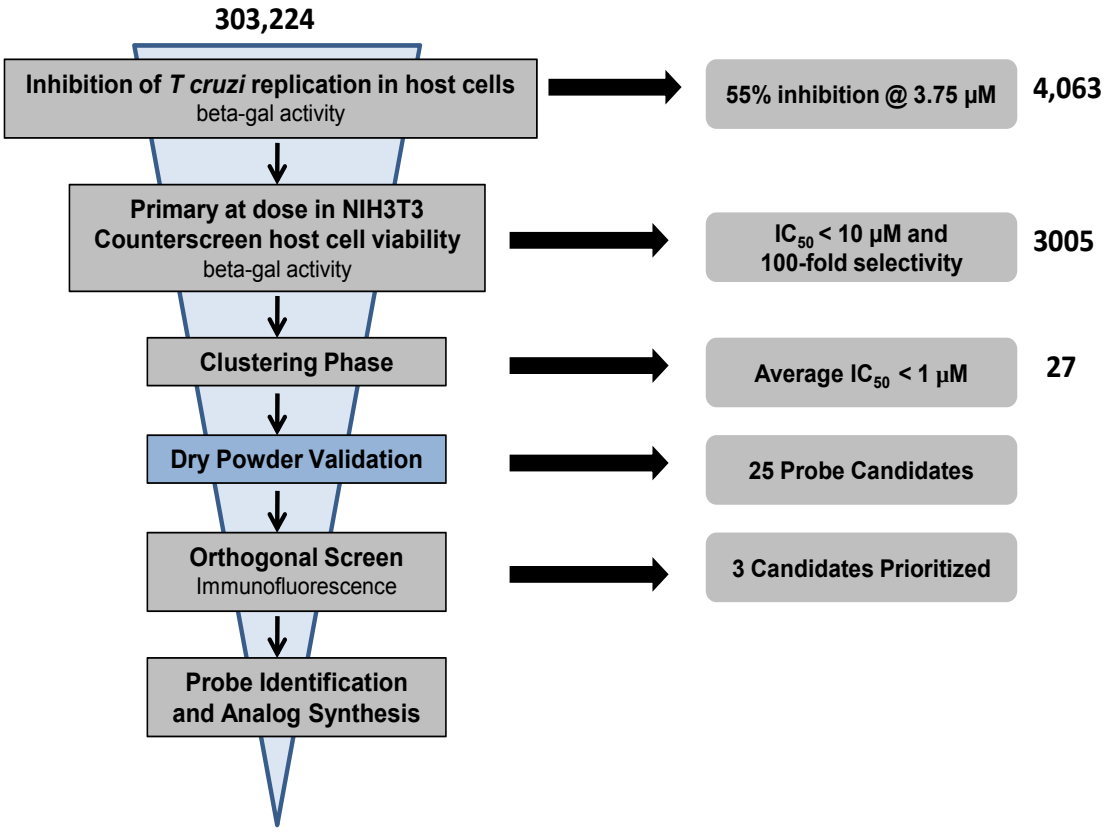


Figure 4. In the primary screen, 303,224 compounds were screened. Of these, 4,063 compounds were available for evaluation in the primary screen retest at dose and secondary assay. Of the 3,005 that passed this filter, 27 dry powder compounds were selected to re-test in the primary screen at dose, secondary assays, and were then carried through to the orthogonal assay. From the orthogonal screen, three scaffolds were prioritized for medicinal chemistry efforts to identify potential probes

3.2 Dose Response Curves for Probe

Figure 5 displays dose response curves for the probe.

The probe (CID3238551/ML158) met the defined probe criteria by displaying greater than 100-fold selective inhibition of *T. cruzi* versus NIH/3T3 (109 nM versus inactive). Furthermore, the inhibition of *T. cruzi* replication was confirmed in immunofluorescent imaging assays (AID 2632). The compound inhibited replication as compared to DMSO control conditions, and the compound did not lyse the cells. The probe (CID3238551/ML158) has acceptable solubility and stability in aqueous conditions.

Figure 5. Dose-dependent Activities of Probe (CID3238551/ML158) in Target and Counterscreen Assays

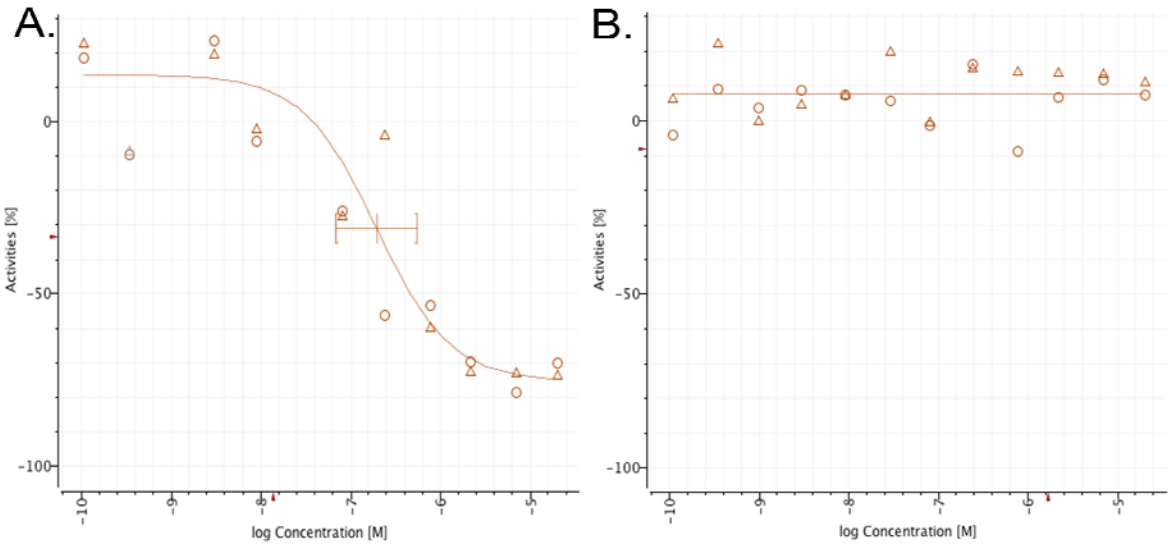


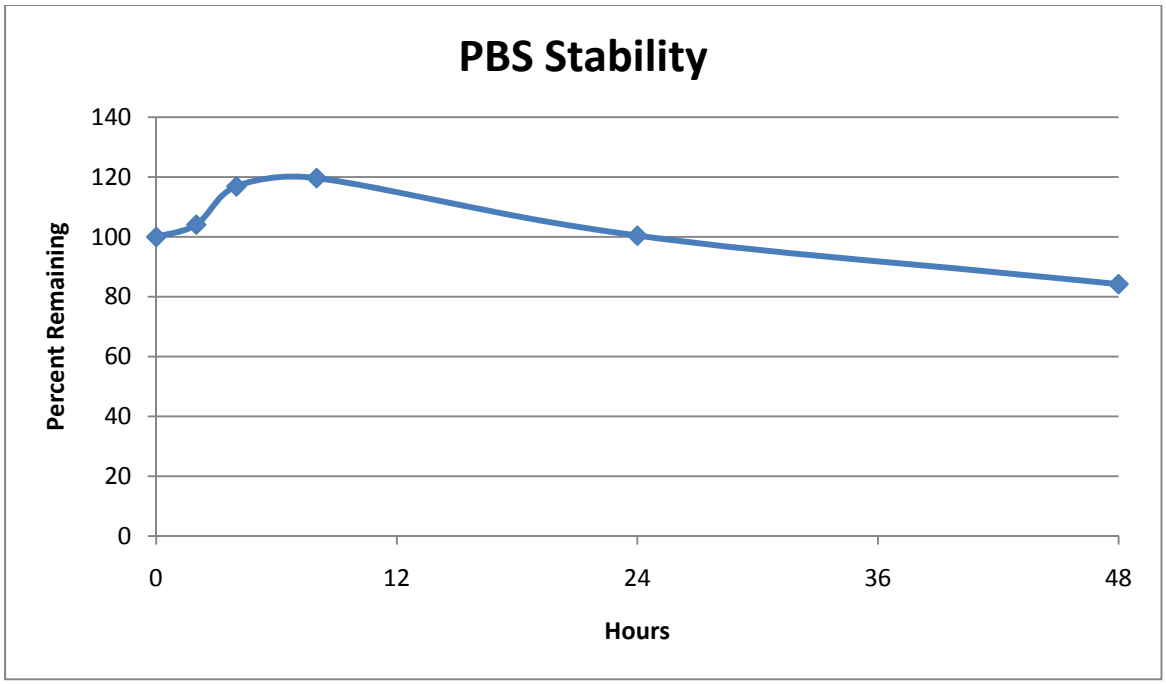
Figure 5. Primary screen-from dry powders (IC_{50} 109 nM) (AID 2630) (*A*); NIH3T3 (inactive) Toxicity (AID 2586) (*B*).

3.3 Scaffold/Moiety Chemical Liabilities

A search of PubChem for the probe (CID3238551/ML158) revealed that the probe has been tested in 359 BioAssays and was confirmed as active only in our assays. Therefore, the compound is not promiscuous. The compound has a drug-like structure with no obvious chemical liabilities that would be a concern.

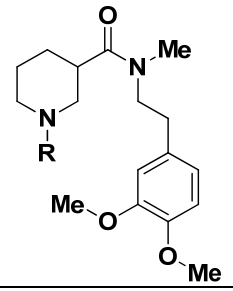
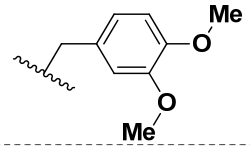
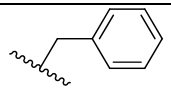
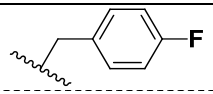
The stability of the probe (CID3238551/ML158) in PBS was monitored over 48 hours, and the data is presented in **Figure 6**. After 24 hours, 100% of the compound was remaining. The amount of compound decreased to 84.2% between 24 to 48 hours. Recovery with acetonitrile was done at each time point to account for any precipitation and showed that no precipitation occurred.

Figure 6. PBS Stability of the Probe (CID3238551ML158)



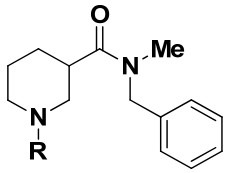
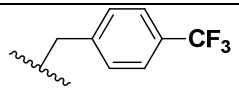
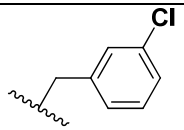
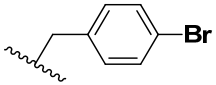
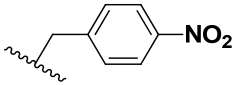
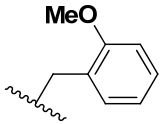
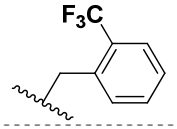
3.4 SAR Tables

Table 1. SAR Analysis of Dimethoxy-phenethyl Amide Series

SAR Analysis for *T. cruzi*						Potency (μM) mean ± S.E.M. (n=replicates)				Target to Antitarget Fold Selectivity
Entry	**CID**	**SID**	**Broad No.**	*****	**R**	**n**	***T. cruzi* inhibition**	**n**	**Toxicity**	
1	3238551	87219048	BRD-A98248982	P		3	0.109 ±6.5	3	inactive	>500
	Solubility: 105.4 μM					Purity: 94%				
2	3377343	99351065	BRD-A54545770	P		3	1.4 ±0.8	3	47.4	34
	Solubility: ND					Purity: >95%				
3	46897906	99351063	BRD-A72455176	P		3	4.33 ±1.76	3	64.7 ±21.5	15
	Solubility: ND					Purity: >95%				

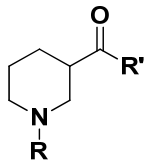
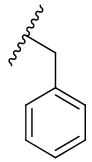
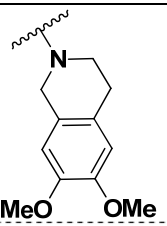
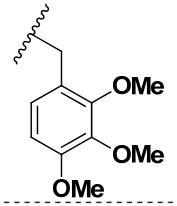
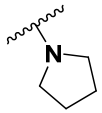
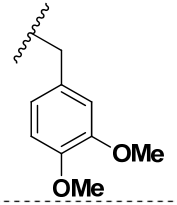
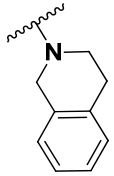
ND= Not determined; * P = Purchased

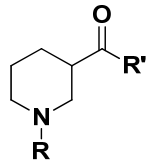
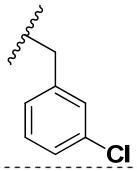
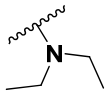
Table 2. SAR Analysis of the Benzyl Amide Series

SAR Analysis for *T. cruzi*						Potency (μM) mean ± S.E.M. (n=replicates)				Target to Antitarget Fold Selectivity
Entry	**CID**	**SID**	**Broad No.**	*****	**R**	**n**	***T. cruzi* inhibition**	**n**	**Toxicity**	
1	46912157	99376496	BRD-A84438227	S		3	0.008 ±0.005	3	61.6 ±29.5	>500
	Solubility: ND					Purity: 81%				
2	3500831	87219049	BRD-A83402799	P		3	0.570 ±0.247	3	70.3 ±67.5	123
	Solubility: >500 μM					Purity: >95%				
3	16470923	99376495	BRD-A30944476	S		3	3.01 ±1.12	3	74.3 ±18.5	25
	Solubility: ND					Purity: >95%				
4	46912177	99376497	BRD-A51613646	S		3	7.63 ±5.05	3	51.0 ±14.9	6.7
	Solubility: ND					Purity: >95%				
5	3237704	87556793	BRD-A74871599	P		3	10.8 ±4.45	3	77.3 ±58.4	7.2
	Solubility: ND					Purity: >95%				
6	87556794	2930928	BRD-A16581344	P		3	15.9 ±4.48	3	63.3 ±37.6	7.2
	Solubility: >500 μM					Purity: >95%				

ND= Not determined; * P = Purchased; S = Synthesized

Table 3. SAR Analysis for *T. cruzi*

SAR Analysis for *T. cruzi*							Potency (μM) mean ± S.E.M. (n=replicates)				Target to Antitarget Fold Selectivity
Entry	**CID**	**SID**	**Broad No.**	*****	**R**	**R'**	**n**	***T. cruzi* inhibition**	**n**	**Toxicity**	
1	46897916	99351067	BRD-A04542699	P			3	0.763 ±0.0413	3	52.7 ±19.9	69
	Solubility: ND						Purity: 91%				
2	4207918	87556801	BRD-A99518825	P			3	1.01 ±0.655	3	inactive	>500
	Solubility: >500 μM						Purity: 93%				
3	99351072	3422467	BRD-A36791463	P			3	3.54 ±2.12	3	41.7 ±6.96	12
	Solubility: 388.9 μM						Purity: 88%				

SAR Analysis for *T. cruzi*							Potency (μM) mean ± S.E.M. (n=replicates)				Target to Antitarget Fold Selectivity
Entry	**CID**	**SID**	**Broad No.**	*****	**R**	**R'**	**n**	***T. cruzi* inhibition**	**n**	**Toxicity**	
4	87556795	2838441	BRD-A98050686	S			3	9.91 ±3.67	3	164 ±53.1	17
	Solubility: >500 μM						Purity: 85%				

ND= Not determined; * P = Purchased; S = Synthesized

Screening results for the primary assay are described in the above tables. The most potent IC_{50} of inhibition without toxicity was measured with compound 1 (CID3238551/ML158), which has been designated as the probe for this series. As can be seen in **Table 1**, substitutions at the 3 and 4 positions of the benzyl amine substituent seem to be tolerable, while substitution at the 2 position results in a significant decrease in activity.

The substitution preference is reinforced by the results shown in **Table 2**. The 4-trifluoromethyl substituted compound (CID46912157) was the most potent compound at 8 nM; however, it showed a significant increase in toxicity over the probe. The 2-trifluoromethyl substituted compound (CID87556794) was one of the least potent compounds tested at 15.9 μM; however, it had similar toxicity.

Replacement of the amide with other amides resulted in a decrease in activity; however, tying the amide back (CID46897916) resulted in similar activity (0.765 μM) when compared to the benzyl substituted compound from the dimethoxyphenethyl series (CID3377343) (1.4 μM). Several other combinations tested with various amides and benzyl substituents showed a range of activity.

Overall, compounds containing an aromatic amide and a benzylic amine substituent substituted at the 3- or 4-position resulted in the highest activities. The probe compound provided the best combination of potency without toxicity and that is why it was selected. All the compounds in this series showed excellent solubility (greater than 100 μM; therefore, this probe (CID3238551/ML158) will serve as a useful probe for cell biology research.

See **Appendix C** for ^{1}H NMR spectra and LC-MS chromatograms of the probe (CID3238551/ML158) and analogs (CID5113379, CID46897906, CID3377343, CID46912157, CID3500831, CID16470923, CID16470922, CID46912177, CID3237704, CID2930928, CID46897916, CID4207918, CID3422467, CID2838441).

Figure 7 shows the activity of the series of analogs in *T. cruzi* inhibition assays.

Figure 7. Activity of the Series Analogs in *T. cruzi* Inhibition Assays (AID 2630)

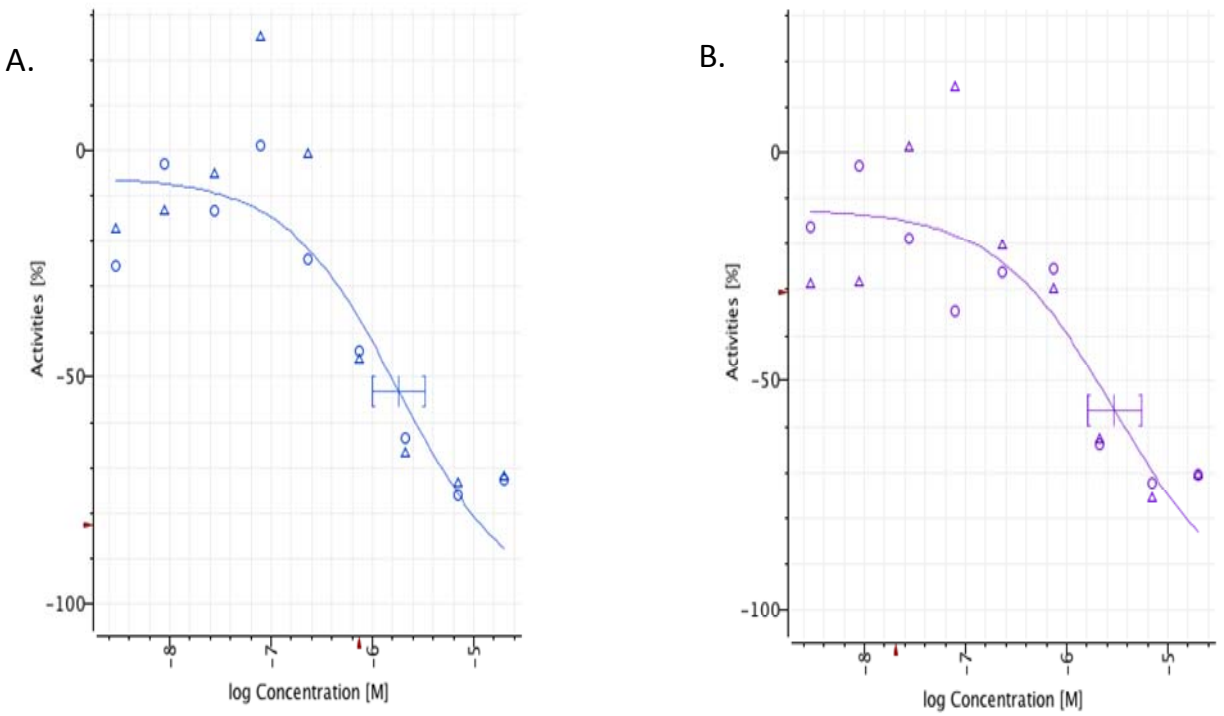


Figure 7. Representative curves of analogs tested in the primary screen at dose. CID3377343(*A*); CID46897906(*B*); CID46912157(*C*);CID3500831(*D*);CID16470922(*E*);CID46912177(F);CID3237704(*G*);CID87556794(*H*); CID46897916(*I*);CID4207918(*J*);CID99351072(*K*); CID87556795(*L*)

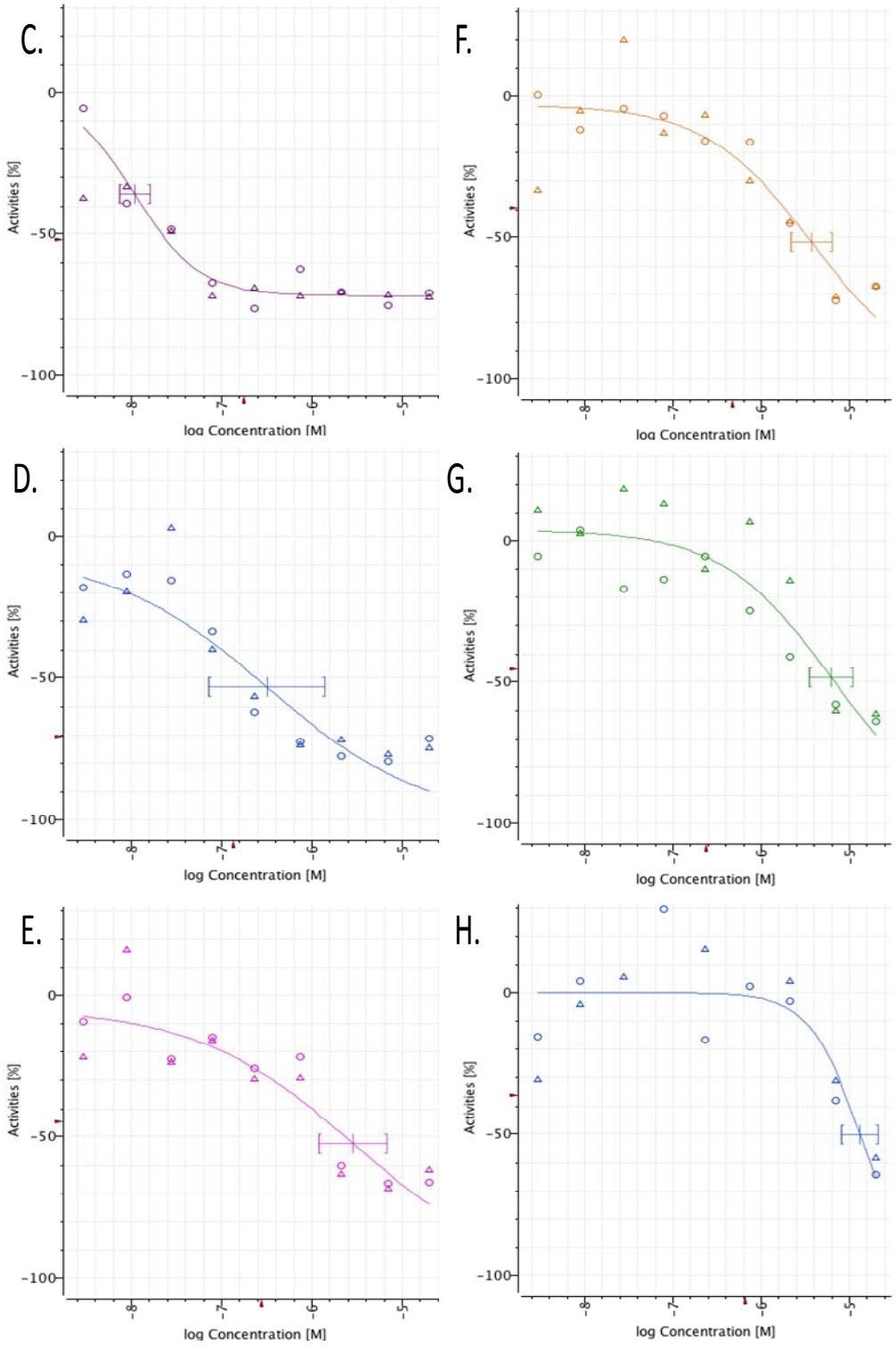


Figure 7 (cont'd). Representative curves of analogs tested in the primary screen at dose. CID3377343(*A*); CID46897906(*B*);CID46912157(*C*);CID3500831(*D*);CID16470922(*E*);CID46912177(F);CID3237704(*G*); CID87556794(*H*);CID46897916(*I*);CID4207918(*J*);CID99351072 (*K*); CID87556795 (*L*)

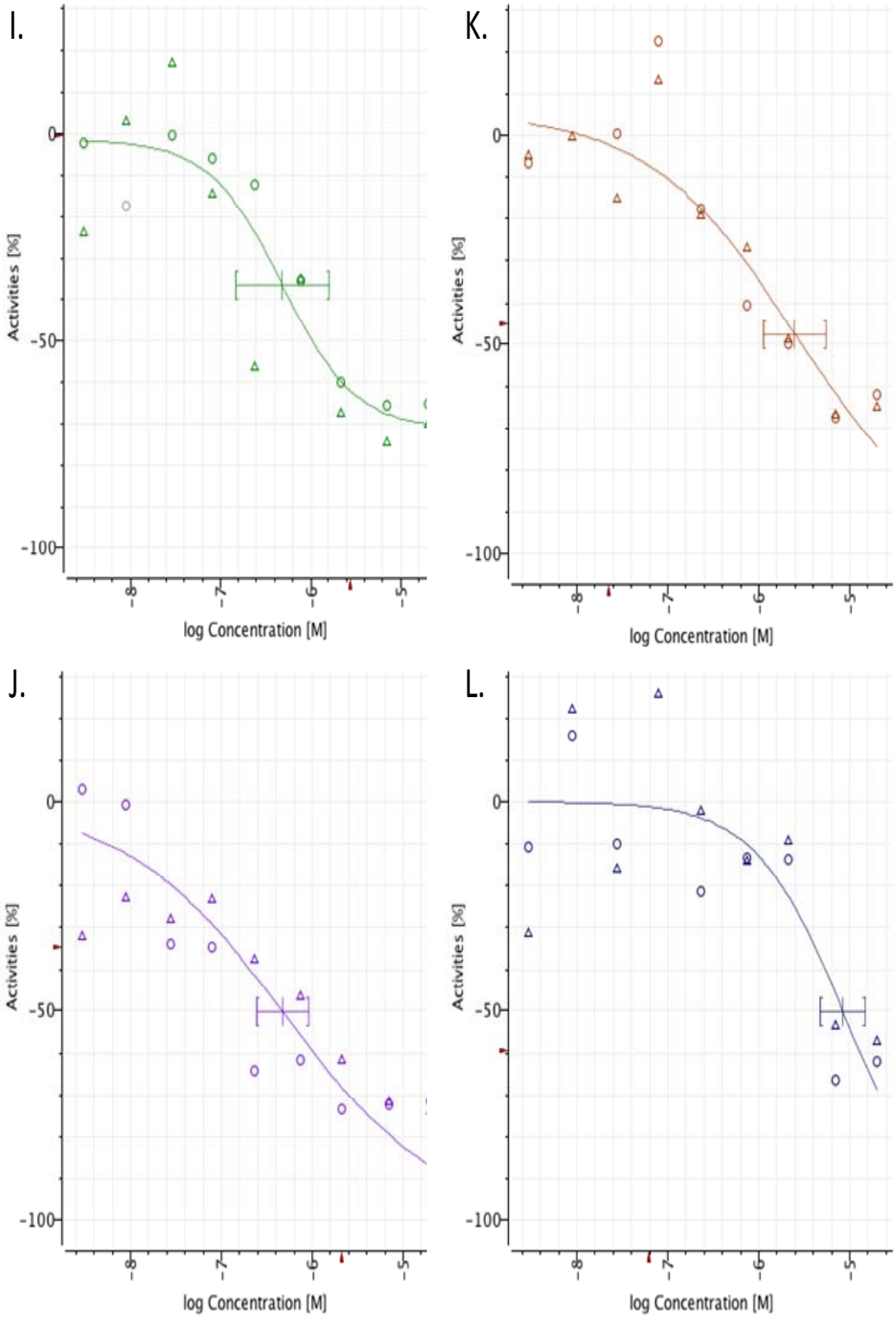


Figure 7 (cont'd). Representative curves of analogs tested in the primary screen at dose. CID3377343(*A*); CID46897906(*B*);CID46912157(*C*);CID3500831(*D*);CID16470922(*E*);CID46912177(F);CID3237704(*G*); CID87556794(*H*);CID46897916(*I*);CID4207918(*J*);CID99351072(*K*);CID87556795(*L*)

3.5 Cellular Activity

As the primary assay is a phenotypic, cell-based assay, the low nanomolar (nM) activity of the probe (CID3238551/ML158) as described above by definition indicates excellent permeability and good physical properties.

3.6 Profiling Assays

Not applicable.

4 Discussion

4.1 Comparison to Existing Art and Feature/Benefits of the New Probe

The current state of the art for probe molecules in cell culture assays against *T. cruzi* is a potency of about 1 μM (6), whereas our new probe (CID3238551/ML158) is much more potent at 109 nM, a 10-fold improvement. Also, the current treatments for Chagas disease are toxic to host cells. The probe (CID3238551/ML158) is inactive against host cells at the highest tested dose (19.5 μM), a difference of greater than 500-fold compared with its activity against *T. cruzi*. These features should make this probe (CID3238551/ML158) very useful as a probe molecule in cell assays and potentially facilitate future *in vivo* studies.

Investigation into relevant prior art entailed searching the following databases: SciFinder, Patent Lens, PubChem, and PubMed. The search terms applied and hit statistics are provided in **Table 4.** Abstracts were obtained for all references returned and were analyzed for relevance to the current project. The searches were performed on and are current as of February 16, 2011.

Table 4. Search Strings and Databases Employed in the Prior Art Search

Search String	Database	Hits Found
“t. cruzi inhibition”	Sci Finder	726
“ t. cruzi inhibition + small molecule”	Sci Finder	10
“ t. cruzi inhibition ”	Patent Lens	6,714
“ t. cruzi inhibition + small molecule ”	Patent Lens	5,525
“ t. cruzi inhibition ”	PubChem Bioassay	668
“ t. cruzi inhibition ”	PubMed	461
“ t. cruzi inhibition + small molecule ”	PubMed	1

4.2 Mechanism of Action Studies

In order to elucidate how this probe (CID3238551/ML158) works, an immunofluorescent assay was employed to determine its mode of action and the extent of inhibition of *T. cruzi* replication in the host cell. To quantitate relative inhibition of parasite replication, 50 infected cells were selected for analysis for the probe (CID3238551/ML158) and each analog. The number of infected cells with more than four amastigotes (where the parasite has invaded and is actively replicating) was divided by the total number of infected cells. As observed by immunofluorescence, all compounds in this series inhibited *T. cruzi* replication compared with control, but the parasite maintained defined amastigote bodies. Therefore, the mode of action was determined as static. No diffuse staining in the cytosol of host NIH/3T3 cells was observed in any case, suggesting no lysis of the amastigotes as described in Bettiol et al. (7). Therefore, none of the compounds were categorized as lytic.

Additional experiments could be developed to identify the actual molecular target of this probe. A phenotypic cell-based screening approach is extremely powerful for identifying novel compounds of interest when the exact mechanisms of parasite growth are not fully understood. At the Broad Institute, we have developed a robust, scalable method for confident identification of the protein targets of small molecules in their cellular context called stable isotope labeling by amino acids in cell culture (SILAC). SILAC-based target identification technology overcomes prior difficulties with affinity-based target identification methods. This technology is routinely

applied at the Broad Institute to identify targets of a variety of small molecules with drug-like properties, including kinase inhibitors, immunophilin modulators, and others.

Another method for target identification would be to develop a *T. cruzi* strain resistant to the probe (CID3238551/ML158). In brief, *T. cruzi* could be co-cultured with the probe and compound-resistant parasites would be identified after culturing through many passages. A genomic approach would then be undertaken to identify the gene(s) that differed in the resistant strain versus the wild type. The identification of common mutated genes across multiple experiments could narrow the list of possible targets. Biochemical and biophysical approaches could then be applied for further characterize and optimize the probe (CID3238551/ML158) for *in vivo* testing.

4.3 Planned Future Studies

The probe (CID3238551/ML158) will be tested for its inhibition of related parasites, *Leishmani* major and *Leishmania amazonensis*, with the aim of developing a treatment for *Leishmania* infections.

The probe (CID3238551/ML158) and certain analogs are also being tested in a mouse model of Chagas disease using fluorescently tagged *T. cruzi*. Compounds are being dosed intravenously and the extent of *T. cruzi* replication is assessed by relative fluorescence in the infected area. These studies are ongoing at the University of Georgia by Drs. A. Rodriguez and R. Tarleton.

5 References

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Appendix A: Assay Summary Table

Table A1. Summary of Completed Assays and AIDs

PubChem AID No.	Type	Target	Concentration Range (µM)	Samples Tested
1885	Primary	*T. cruzi* co-culture	Average 3.75 µM	303,224
2044	Primary	*T. cruzi* co-culture	100 nM-6.0 µM	4063
2294	Primary	*T. cruzi* co-culture	4.0 nM-1.0 µM	319
2630	Primary	*T. cruzi* co-culture	0.11 nM-19.5 µM	42
2396	Primary	*T. cruzi* co-culture	0.11 nM-6.8 µM	27
493197	Primary	*T. cruzi* co-culture	3 nM-19.5 µM	46
2010	Secondary	NIH3T3 Toxicity	100 nM-6.0 µM	4,063
2586	Secondary	NIH3T3 Toxicity	0.11 nM-19.5 µM	27
493247	Secondary	NIH3T3 Toxicity	3 nM-19.5 µM	46
2632	Orthogonal	Inhibition of *T. cruzi* replication	Single dose; 10x IC_{50} as defined in AID 2204 and 2294	27
1968	Summary	NA	NA	NA

NA= Not applicable

Appendix B: Detailed Assay Protocols

T cruzi Inhibition Assay Protocol(AID Nos.1885, 2044, 2294, 2630)

Cell Propagation

1) 90% DMEM+Phenol red, 10% FBS, 1% PSG.
2) Mix and filter through 0.2 microns membrane.
3) Keep at 4°C. Warm up to 37°C in water bath before use.

***T cruzi* Culture and Assays**

1) 98% DMEM+Phenol red, 2% FBS, 1% PSG.
2) Mix and filter through 0.2 microns membrane.
3) Keep at 4°C. Warm up to 37°C in water bath before use.

Cell Culture Protocols

NIH/3T3 Cell Culture

1) Aspirate medium.
2) Rinse cells with 10 ml PBS/plate.
3) Aspirate PBS.
4) Add 5 ml of pre-warmed trypsin-EDTA, swirl the dish to make sure the trypsin covers all the cells.
5) Place the dishes back in the incubator for 5 min.
6) Check that the cells are detaching.
7) Add 20 ml of medium, pipette up and down to detach all the cells.
8) Dilute into 2% FBS/DMEM for assay or 10% FBS/DMEM for propagation.

LLC-MK2 Cell Culture

1) Aspirate medium.
2) Rinse cells with 10 ml PBS/plate.
3) Aspirate PBS.
4) Add 5 ml of pre-warmed trypsin-EDTA, swirl the dish to make sure the trypsin covers all the cells.
5) Place the dishes back in the incubator for 5 min.
6) Check that the cells are detaching.
7) Add 20 ml of medium, pipette up and down to detach all the cells.
8) Dilute into 2% FBS/DMEM for assay or 10% FBS/DMEM for propagation.

T. cruzi β-gal (Tc) Parasite Culture

1) The day before infection (or at least 2-3 hours before), plate 3 million LLC-MK2 cells/T175 in DMEM+10% FBS.
2) Either thaw *T. cruzi* in from liquid nitrogen stock into 50 ml 2% FBS/DMEM or remove the media from a propagating LLC-MK2 co-culture; in other words, harvesting the medium containing the free *T. cruzi* in 50-ml Falcon tubes.
3) Spin 10 min at 2200 rpm.
4) Aspirate the supernatant until 15 ml is left.
5) Place back the tubes in the incubator delicately (so as not to disturb the pellet).
6) Incubate minimum 3 hours at 37°C.
7) Take supernatant to fresh tube.
8) To count: Mix 75 ml *T. cruzi* with 25 ml 16% PFA, mix, put 75 ml on Nexcelom cellometer cassette.

9) Wait for 2-5 min to let the *T. cruzi* settle; use the *T. cruzi* method saved on the M10 machine, press display image, focus, and count.
10) Aspirate LLC-MK2 media (used 10% for plating) and replace with 2% FBS/DMEM.
11) Plate 17-35 million on *T. cruzi* on fresh LLC-MK2.
12) Change medium after 2 days (Use 2% FBS/DMEM).
13) Harvest *T. cruzi* trypomastigotes on Day 6 and Day 7.

Growth Inhibition Assay Protocol for HTS (384-well plates)

1) Warm up medium 2% FBS/DMEM.
2) Harvest parasites in 50-ml tubes (1 flask per tube).
3) Spin 10 min at 2200 rpm.
4) Aspirate media approximately 15 ml and place them on a rack in the incubator to let trypomastigotes swim out of the pellet for 3-5 hours.
5) In the meantime, trypsinize NIH/3T3 cells as described in cell culture protocol.
6) When NIH/3T3 are detached, harvest them in DMEM- 2% FBS and 1% PSG and count using the NIH3T3 program using the Nexcelom Cellometer.
7) Dilute cells to 166,667 cells /ml and add to flask.
8) Plate 5,000 cells/30 µL per well using standard cassette multiwell drop Combi, adding at a fast speed.
9) Put back in incubator for 3 hours to allow cells to attach.
10) Count *T. cruzi* as described in the parasite culture protocol.
11) Dilute to 0.250 million/ml and transfer to a 2-liter flask.
12) Add to a 2- liter flask, stirring.
13) Pin 50 nL compounds/DMSO to each well with NIH/3T3s.
14) Add shortly after 20 µL per well of parasites (5000 *T. cruzi*) with standard cassette multiwell drop Combi on slow speed.
15) Incubate for 4 days (or minimum 90 hours).
16) Prepare Gal-Screen with 0.05% NP40.
17) Add 30 µL per well of 384-well plate.
18) Incubate for 60 minutes.
19) Read using the ultrasensitive luminescence program on the Envision (Perkin-Elmer, 0.1 sec/well).

Cell Toxicity Assay Protocol: NIH/3T3 Cells (AID Nos. 2010, 2586, 493247)

1) Warm up medium 2% FBS/DMEM.
2) Trypsinize NIH/3T3 cells as described in cell culture protocol.
3) When NIH3T3 cells are detached, harvest them in DMEM- 2% FBS and 1% PSG and count using the NIH3T3 program using Cellometer Auto T4 (Nexcelom Biosciences).
4) Dilute cells to 166,667 cells /ml and add to flask.
5) Plate 5,000 cells/50 µL per well using a Thermo MultiDrop Combi liquid dispenser and a sterilized standard sized dispensing cassette adding at a fast speed in a tissue culture hood.
6) Put back in incubator for 3 hours to allow cells to attach.
7) Pin 50 nL compounds/DMSO to each well.
8) Incubate for 4 days (or minimum 90 hours).
9) On day of substrate addition, prepare CellTiter-Glo.

CellTiter-Glo® Protocol

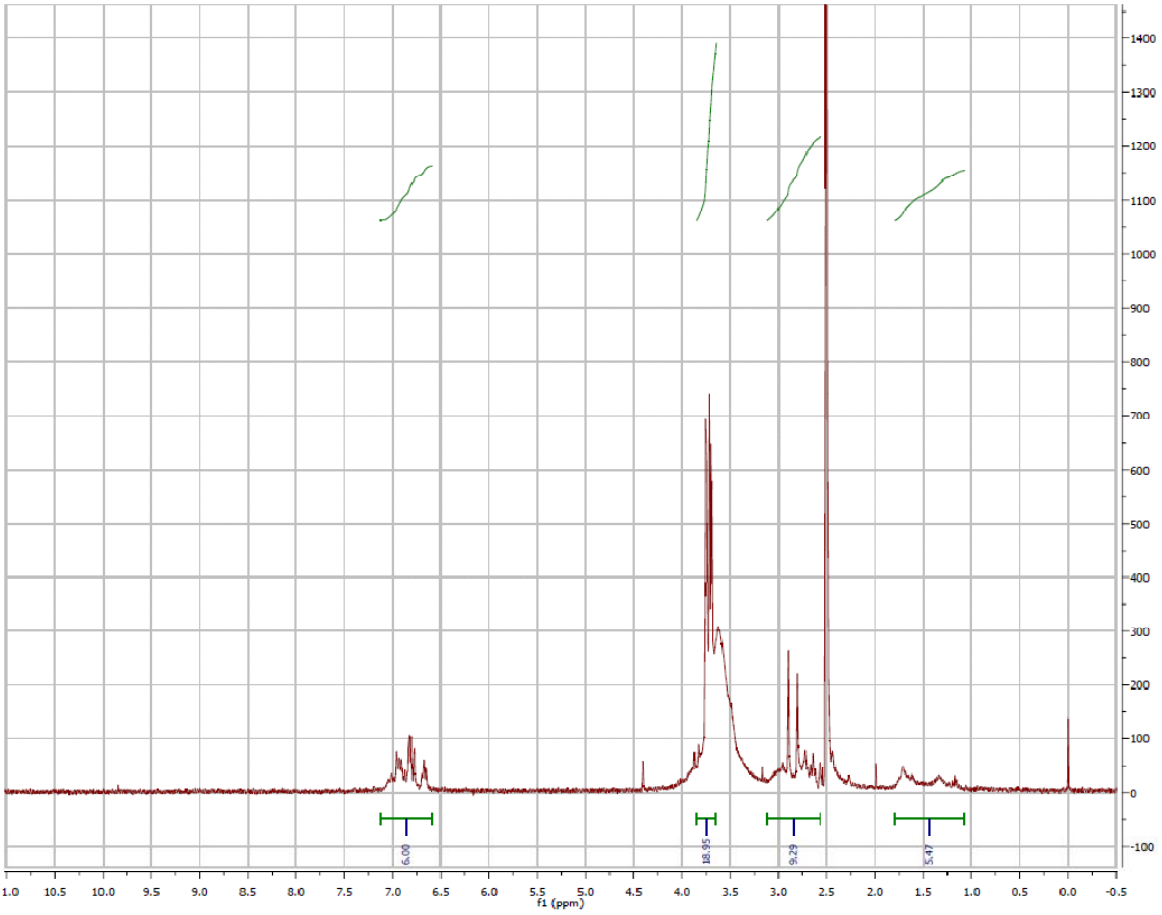
1) Thaw the CellTiter-Glo Buffer, and equilibrate to room temperature prior to use. For convenience the CellTiter-Glo Buffer may be thawed and stored at room temperature for up to 48 hours prior to use.
2) Transfer the appropriate volume of CellTiter-Glo Buffer into the amber bottle containing CellTiter-Glo Substrate to reconstitute the lyophilized enzyme/substrate mixture.
3) Mix by gently vortexing, swirling, or by inverting the contents to obtain a homogeneous solution.
4) Dilute solution 1:3 with PBS.
5) Take out plates from incubator and incubate at r.t. for 30 minutes.
6) Add 30 µL/well using a Thermo MultiDrop Combi liquid dispenser and a sterilized standard sized dispensing cassette adding at a fast speed.
7) Allow the plate to incubate at room temperature for 10 minutes.
8) Read using the ultrasensistive luminescence program on the Envision (Perkin Elmer, 0.1 sec/well).

Intracellular *T. cruzi* Immunofluorescence Assay Protocol (AID No. 2632)

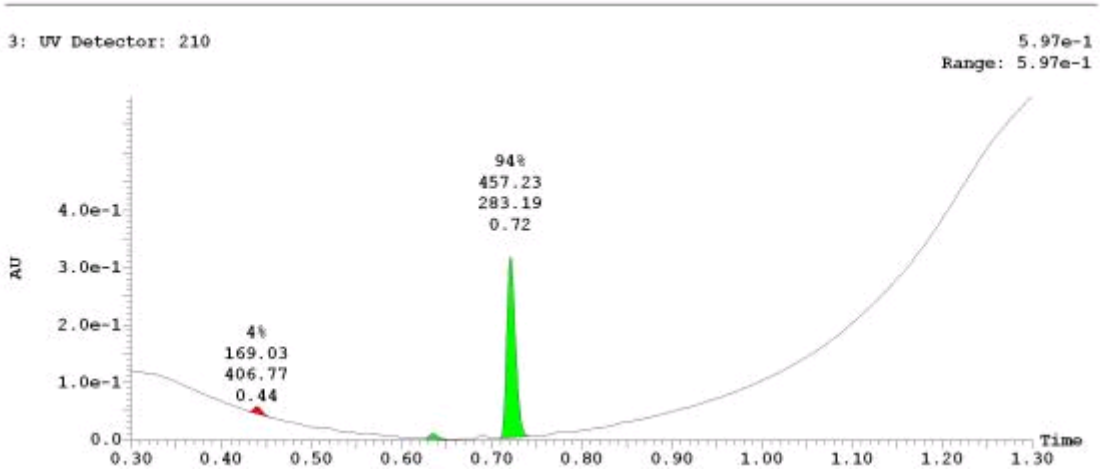
1) Seed NIH/3T3 cells on sterile glass coverslips in 12-well plates and allow cells to adhere overnight.
2) Add *T. cruzi* parasites (MOI 100:1) and allow to infect for 2 h in DMEM+2% FBS and PSG.
3) Rinse parasites out 3 times with PBS, and add compounds at 10X their IC_{50} (as determined in AID 2044 and AID 2294).
4) Incubate infected cells for 4 days and fix for 15 min with 4% paraformaldehyde.
5) Rinse fixed cells on coverslips with PBS and permeabilize for 15 min in PBS with 0.1% Triton X-100.
6) Block for 20 min in PBS with 10% goat serum, 1% bovine serum albumin (BSA), 100 mM glycine, and 0.05% sodium azide.
7) Incubate cells for 1 h at room temperature with a polyclonal rabbit anti-*T cruzi* at 1:2000 dilution.
8) Rinse,then add an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes, Invitrogen) for 1 h at a 1:800 dilution.
9) Stain DNA with DAPI, and mount coverslips with anti-fade mounting media.
10) Take images using an inverted Olympus IX70 microscope with a 60X oil objective.

Appendix C: NMR and LC Data of Probe and Analogs

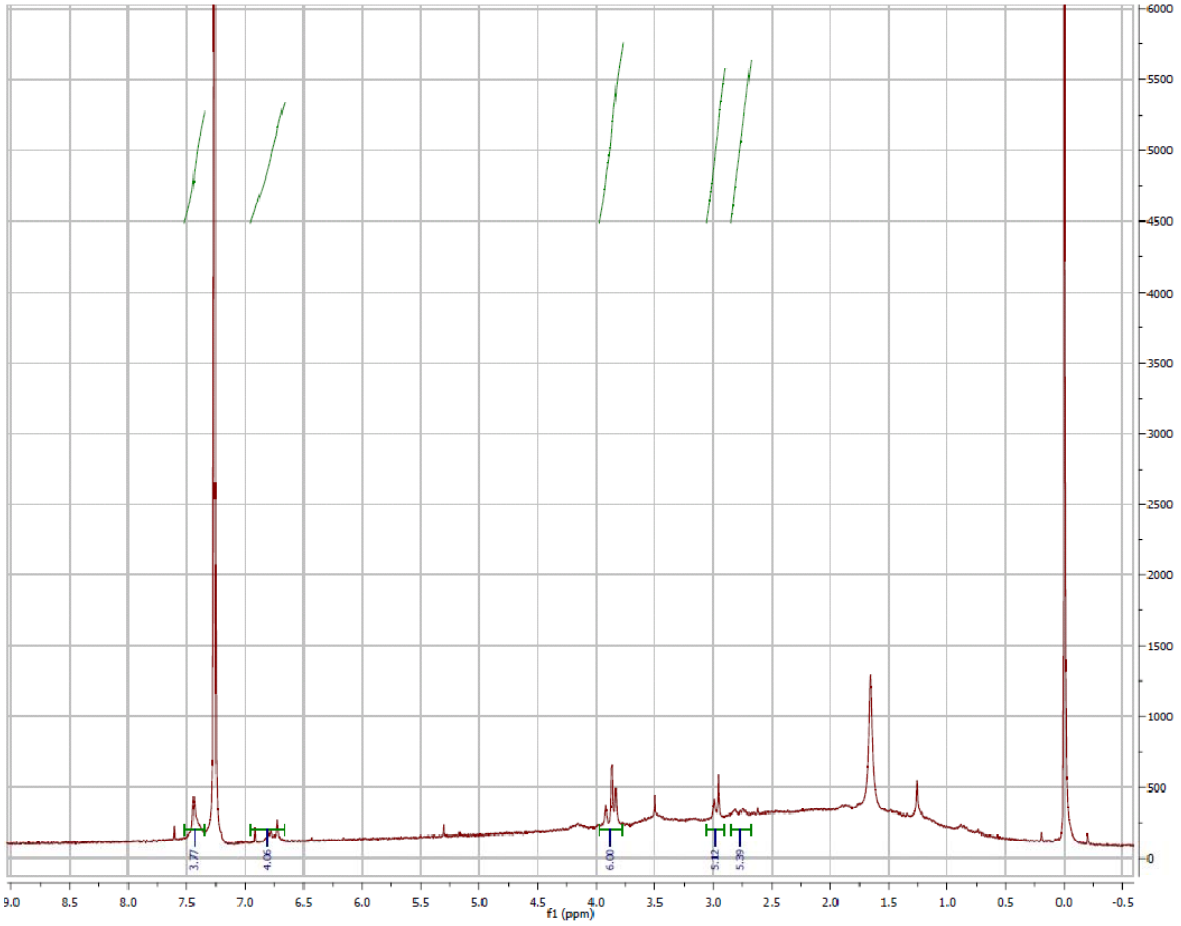
^{1}H NMR (300 MHz, DMSO-d6) Spectrum of the Probe (CID3238551/ML158)



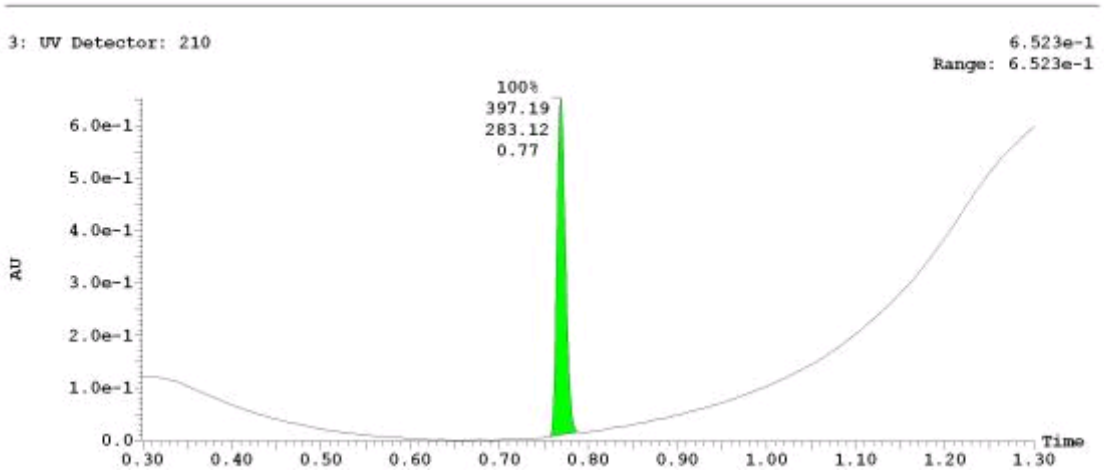
LC-MS Chromatogram of the Probe (CID3238551/ML158)



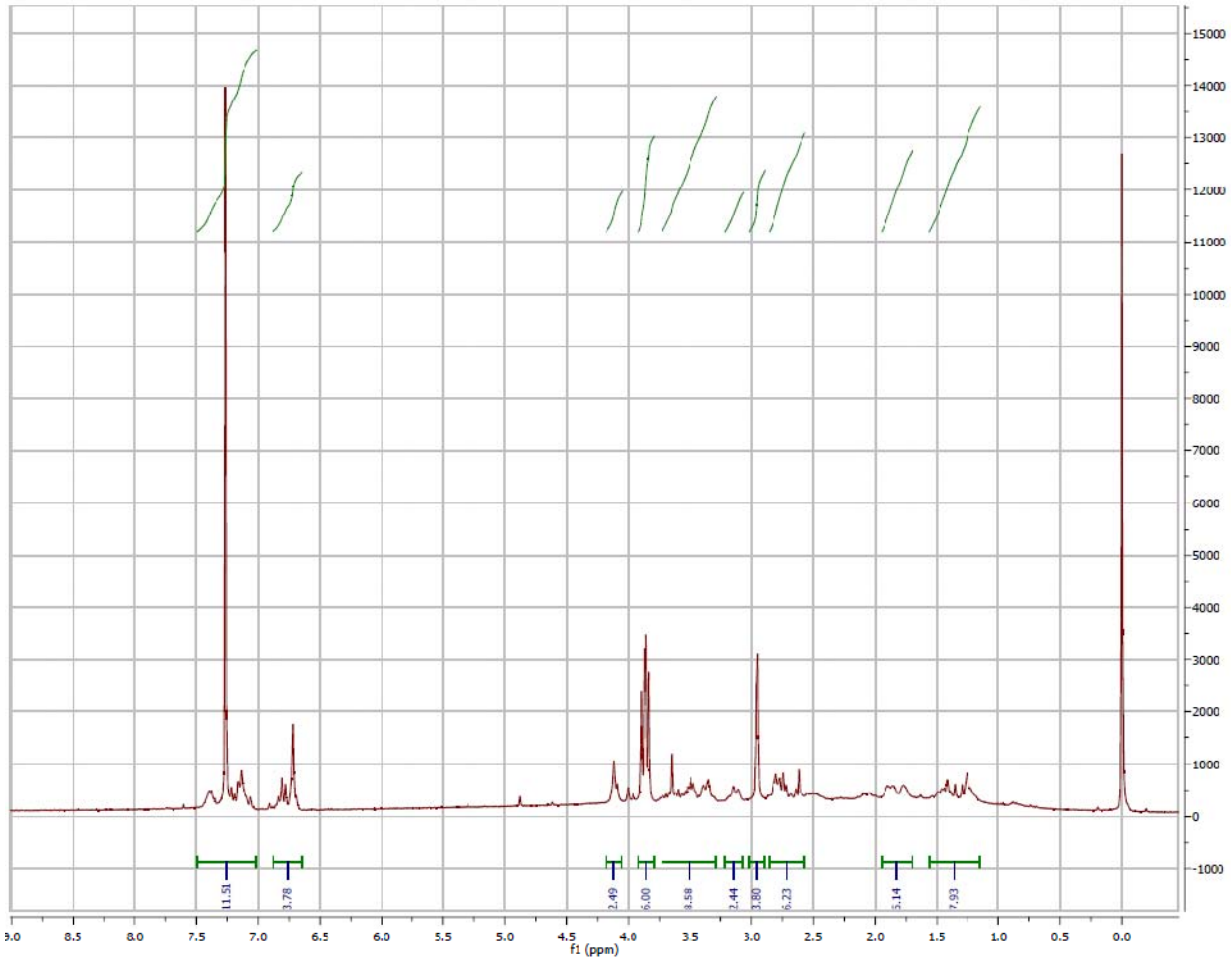
^{1}H NMR ($CDCl_3$) Spectrum of Analog CID3377343



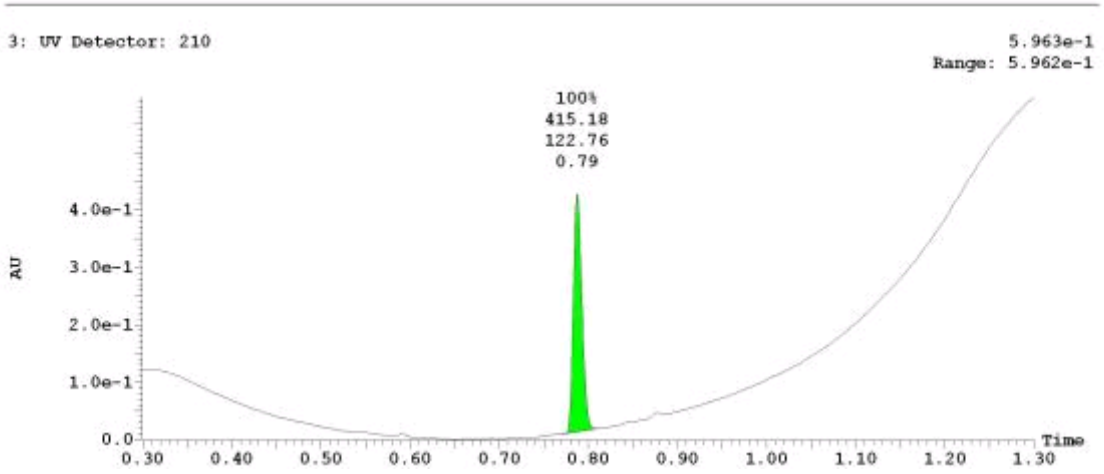
LC-MS Chromatogram for Analog CID3377343



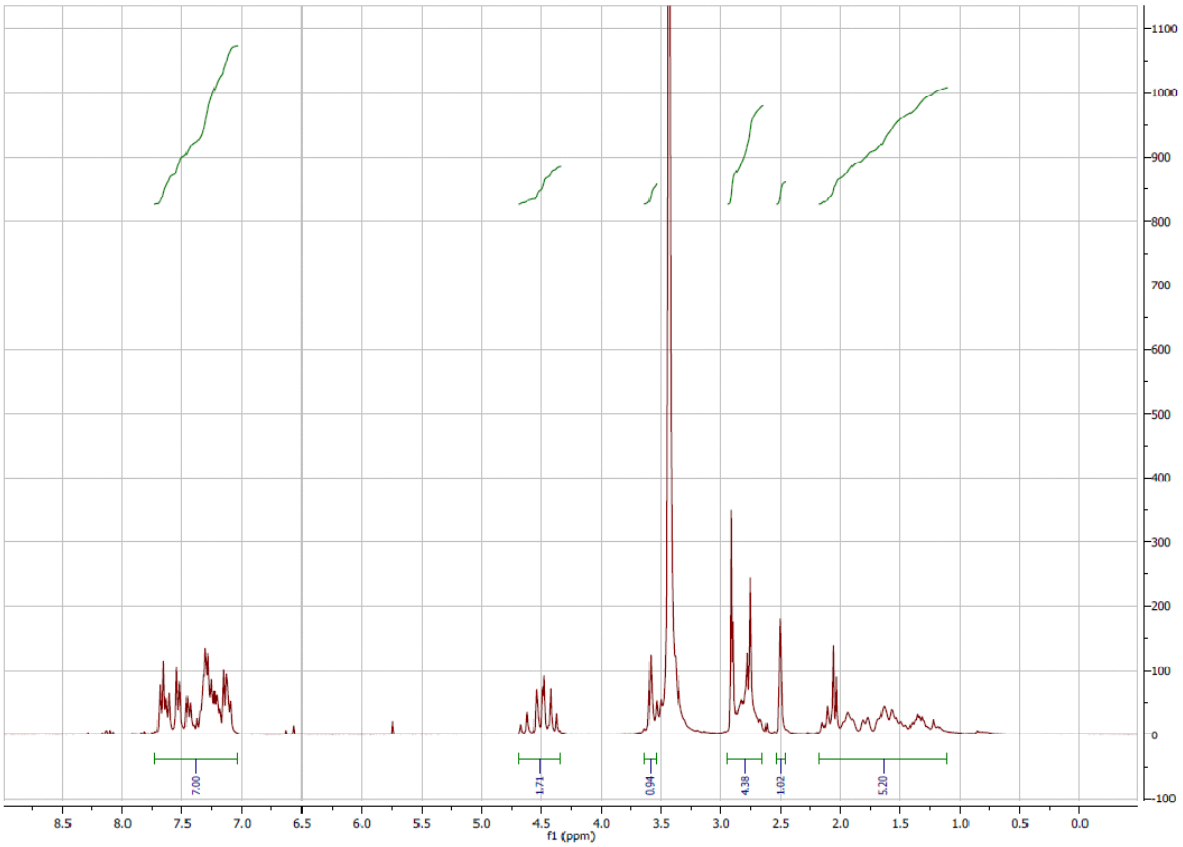
^{1}H NMR ($CDCl_3$) Spectrum of Analog CID46897906



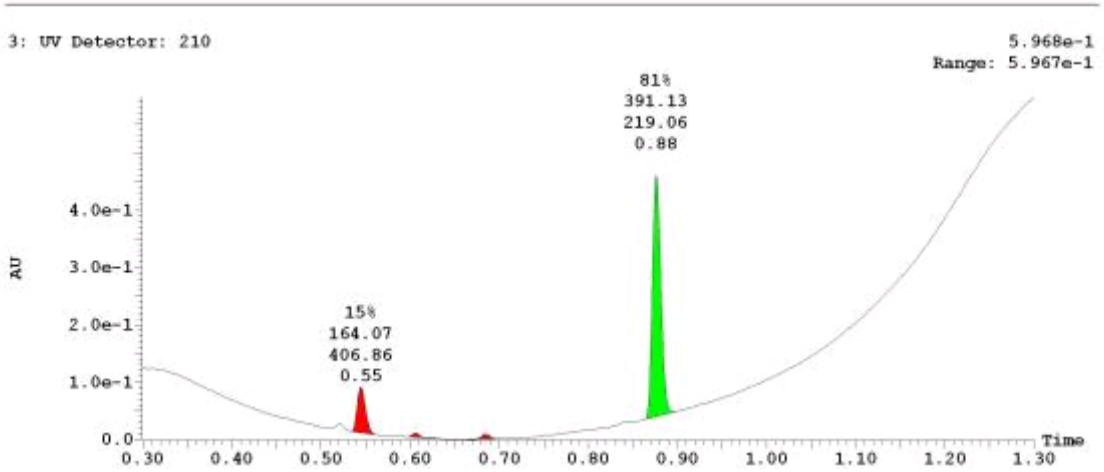
LC-MS Chromatogram of Analog CID 46897906



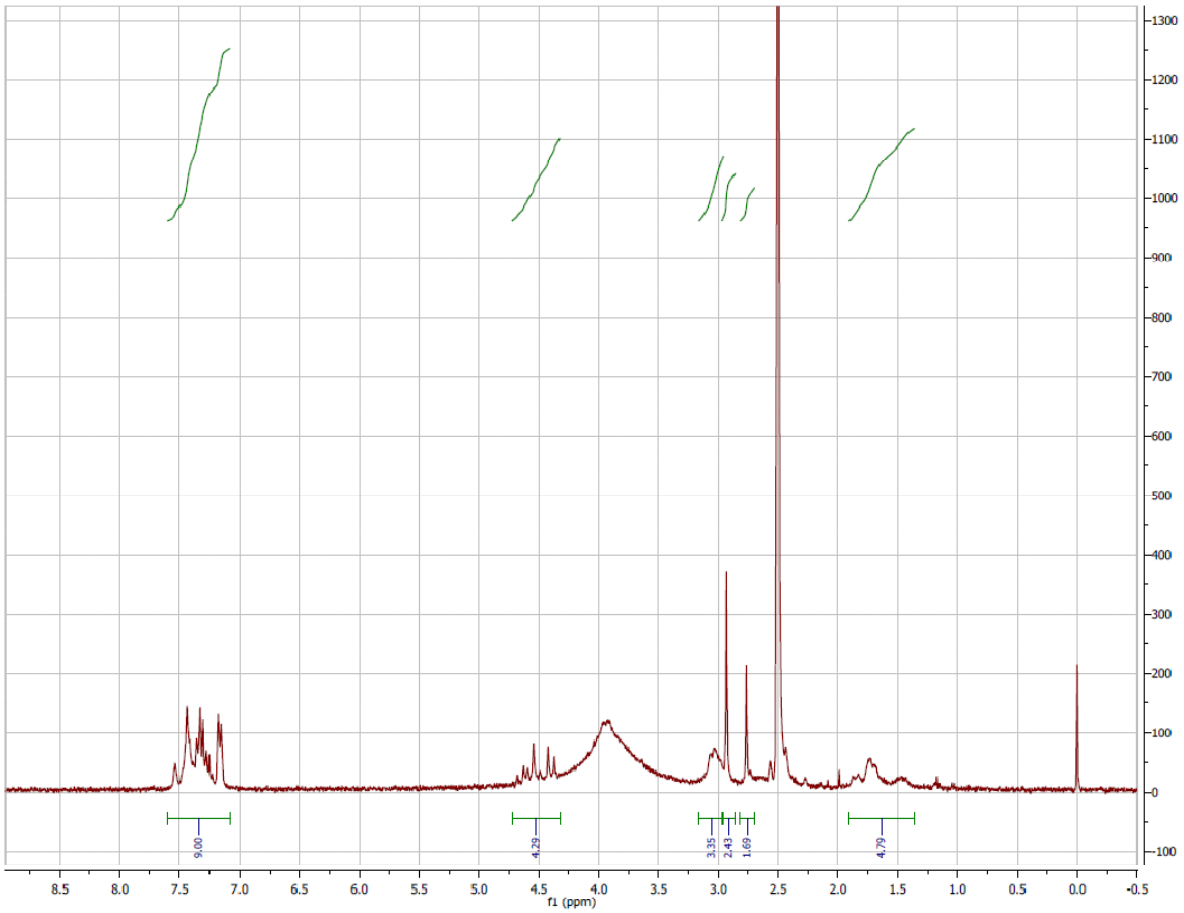
^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID 46912157



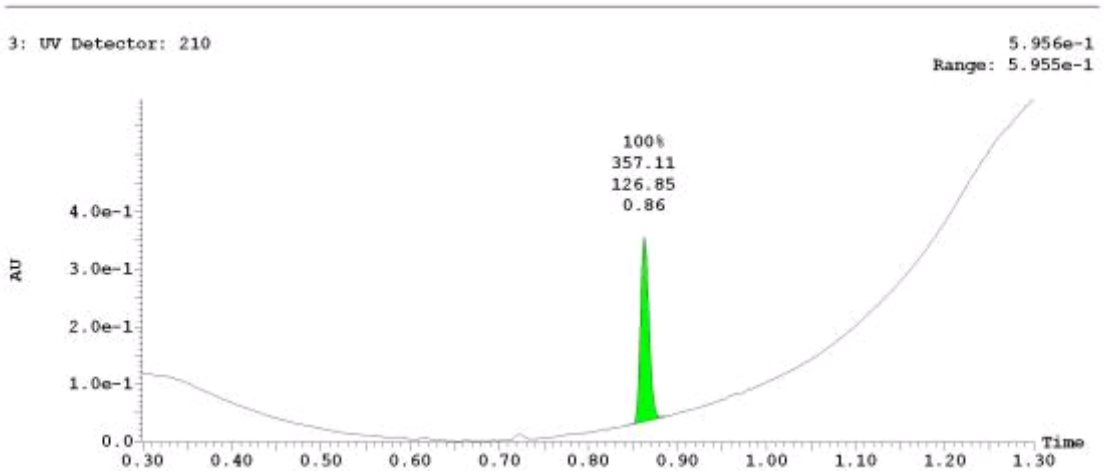
LC-MS Chromatogram for Analog CID 46912157



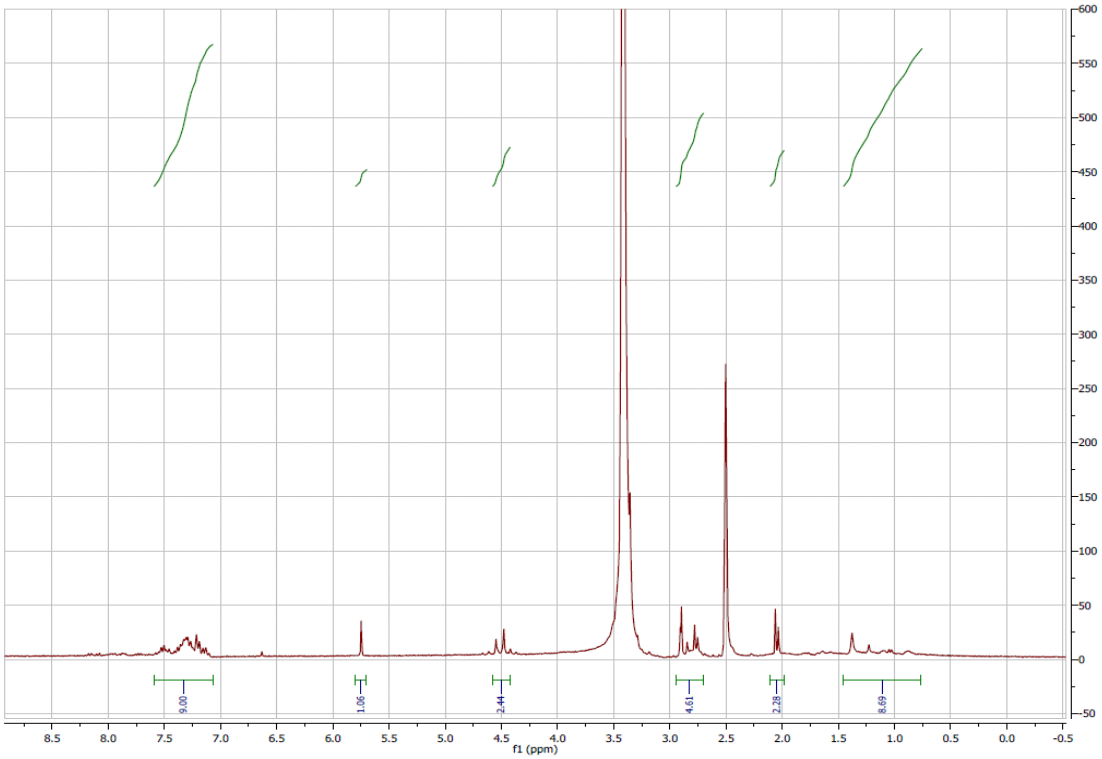
^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID3500831



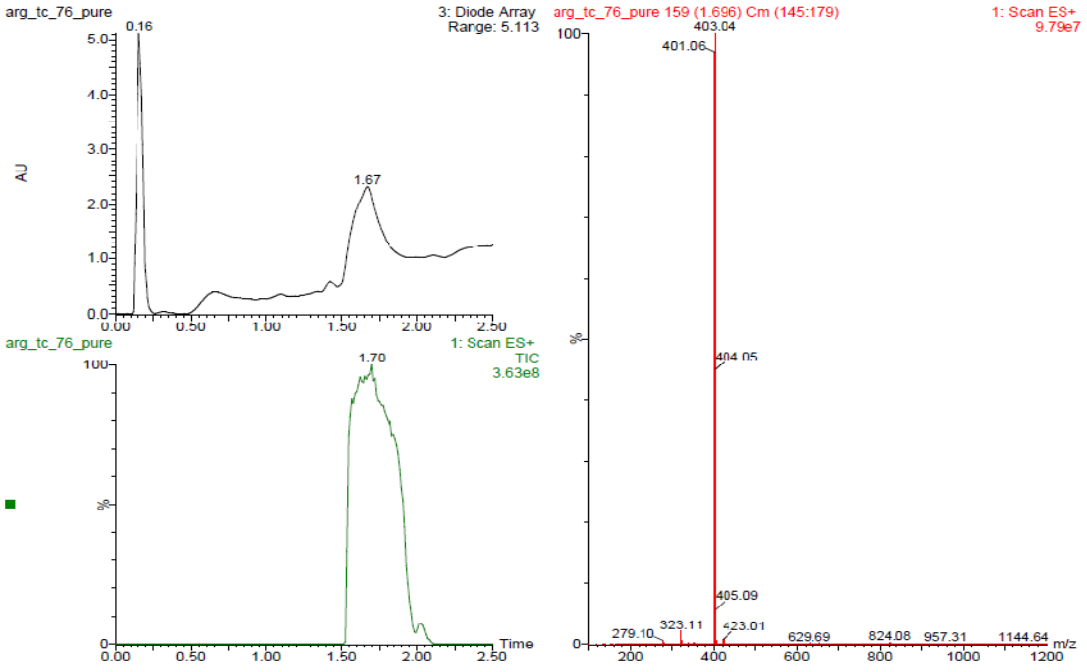
LC-MS Chromatogram for Analog CID3500831



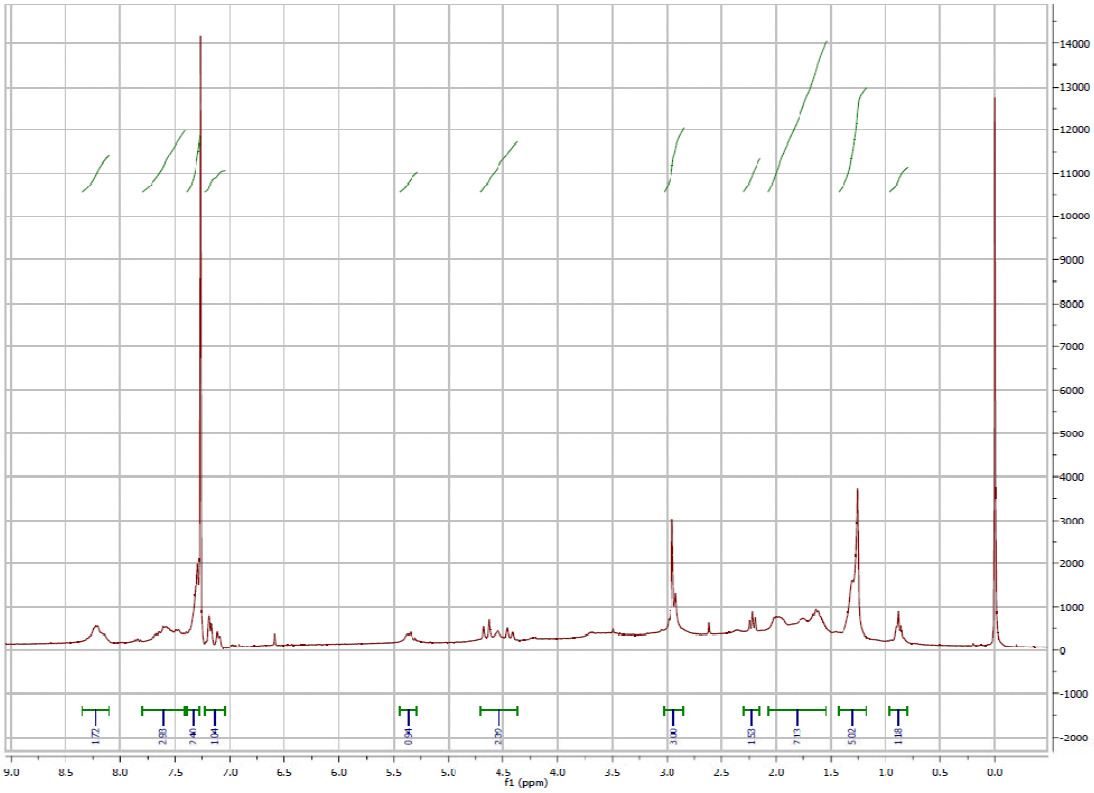
^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID16470923



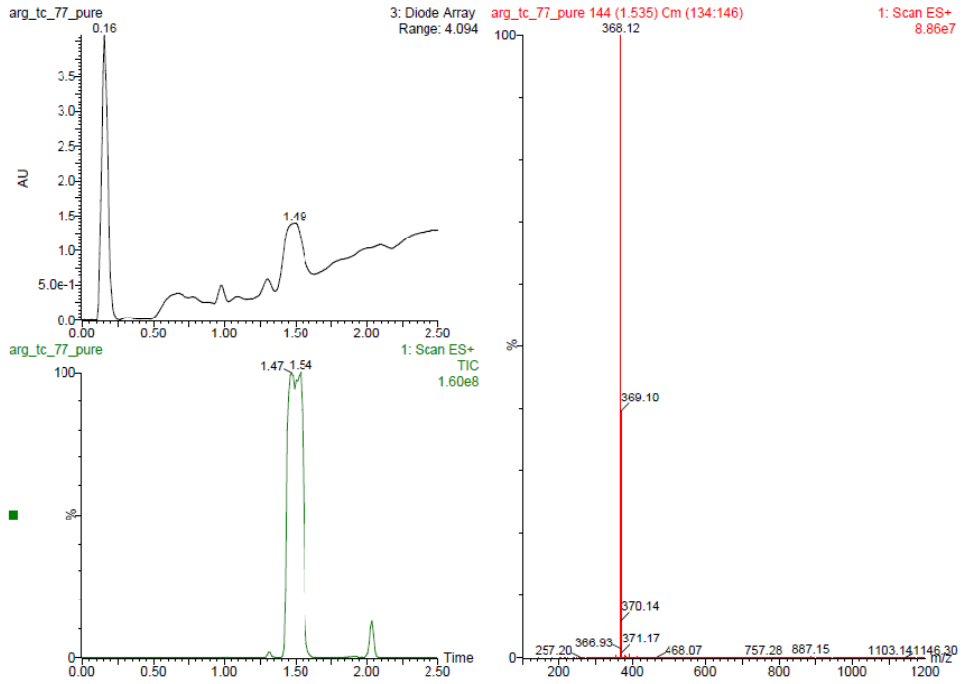
LC-MS Chromatogram for Analog CID16470923



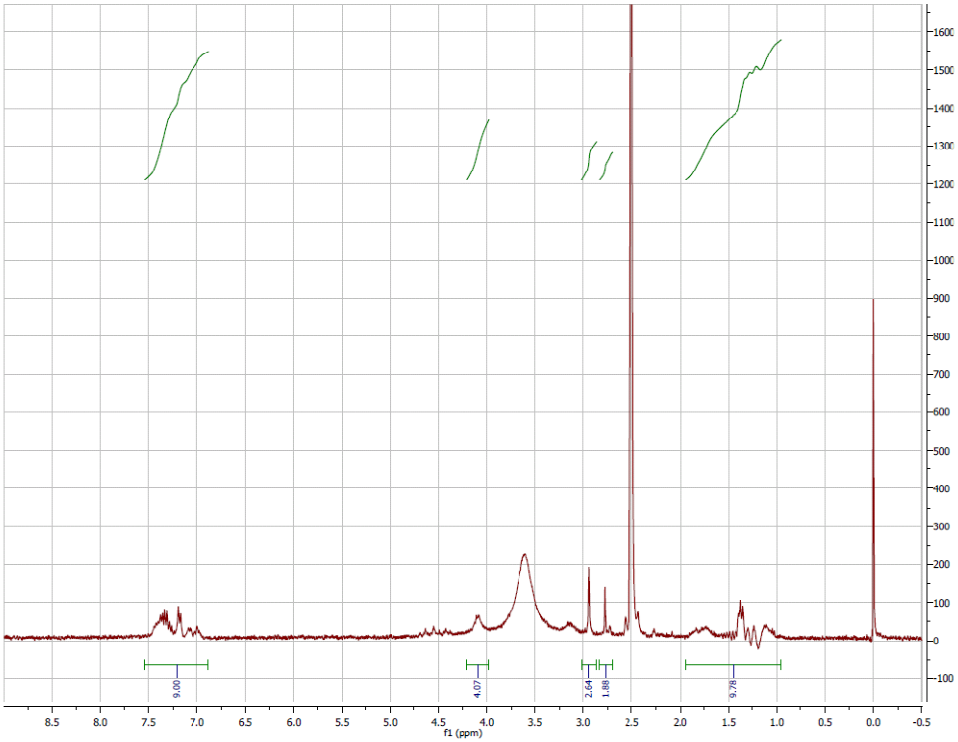
^{1}H NMR ($CDCl_3$) Spectrum for CID46912177



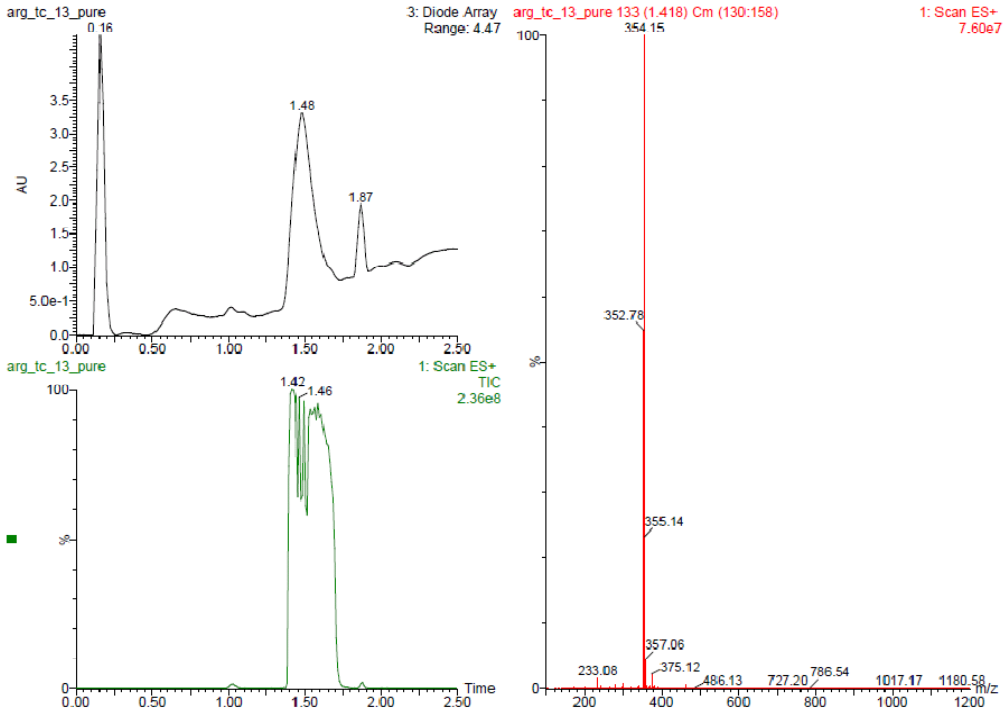
LC-MS Chromatograms for Analog CID46912177



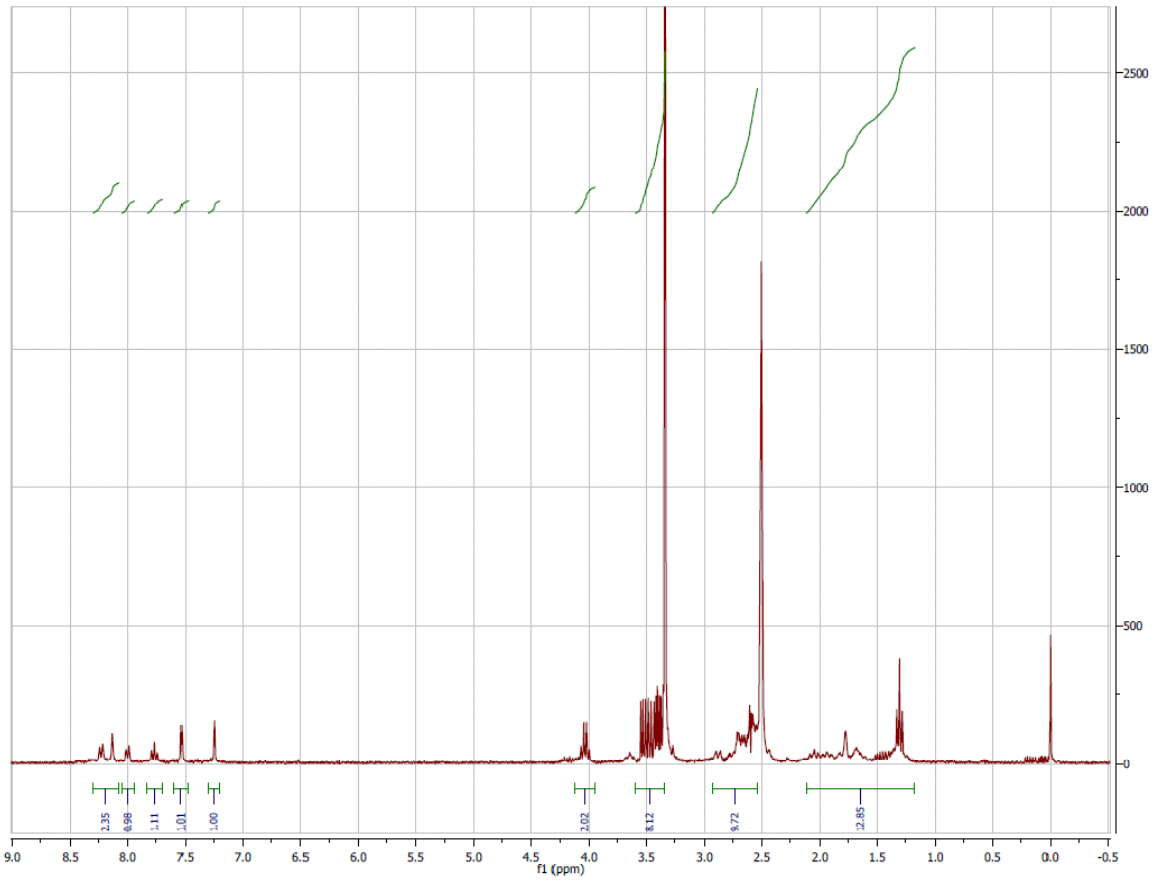
^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID 3237704



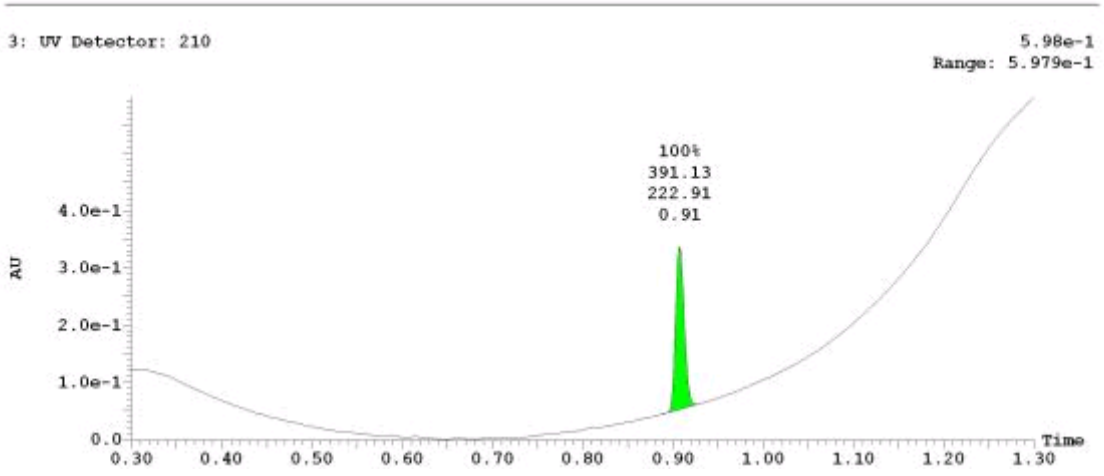
LC-MS Chromatograms for Analog CID3237704



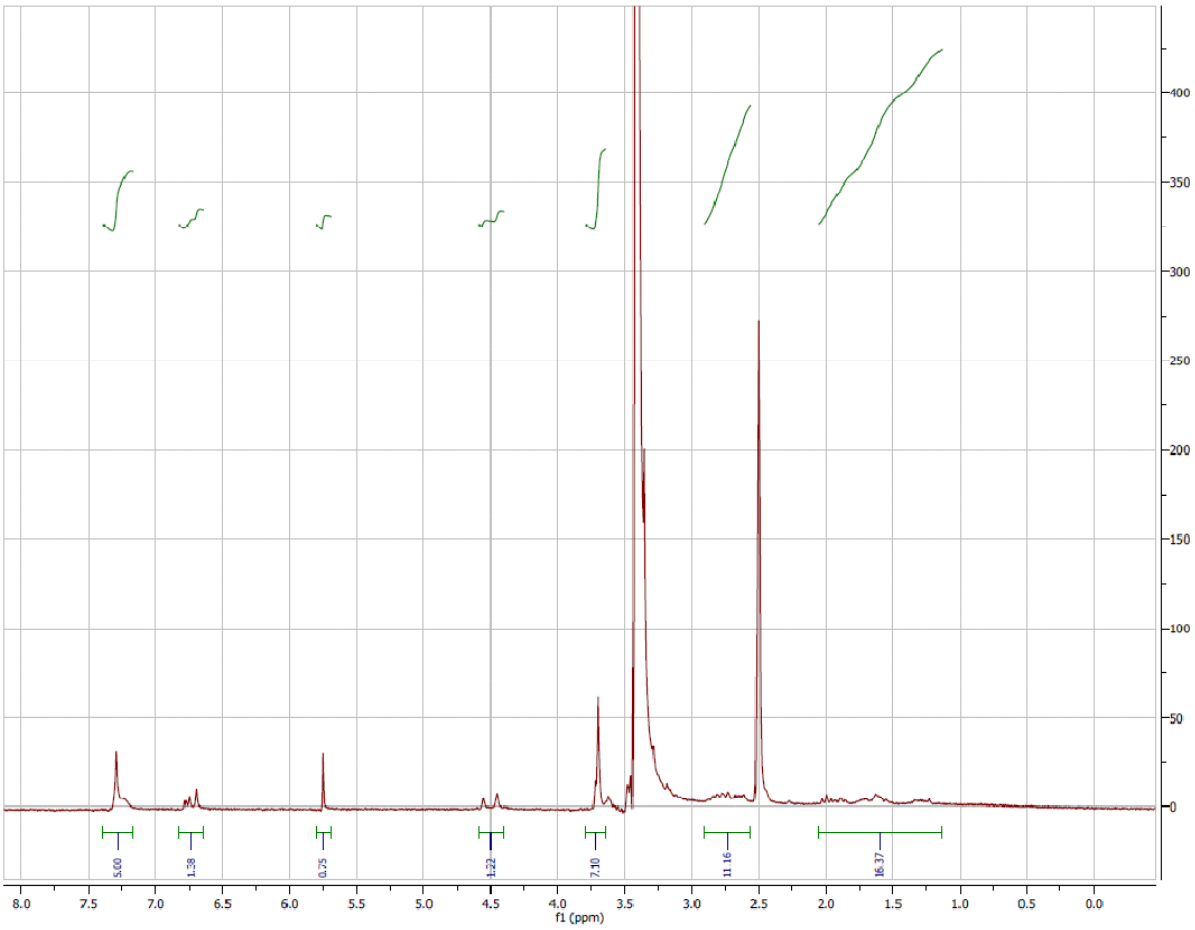
^{1}H NMR Spectrum (300 MHz, DMSO-d6) for Analog CID87556794



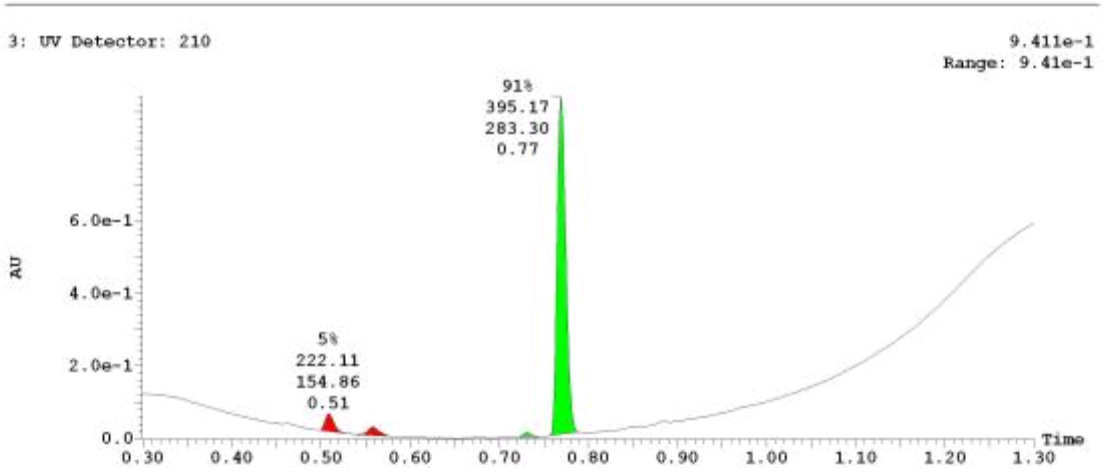
LC-MS Chromatogram for Analog CID87556794



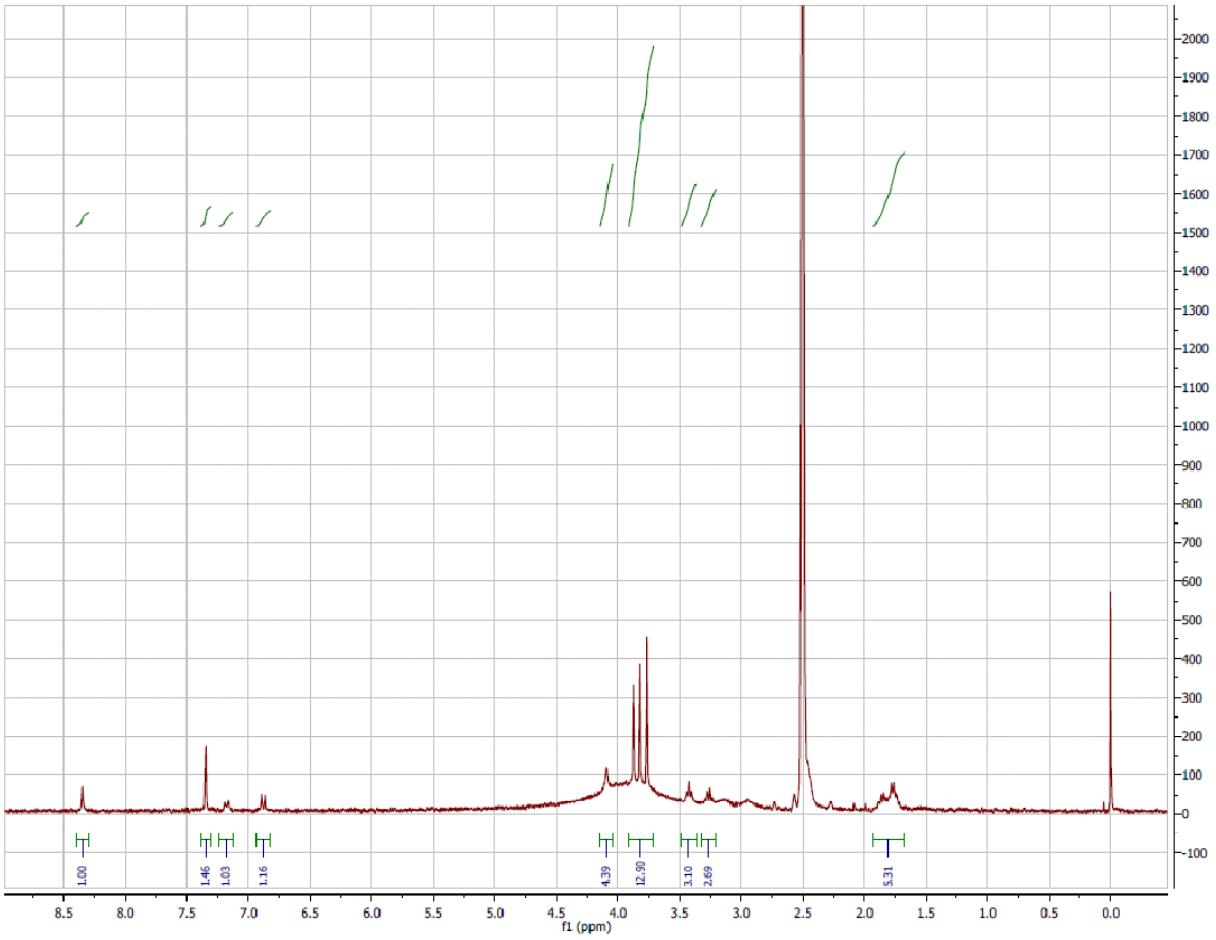
[1]H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID46897916



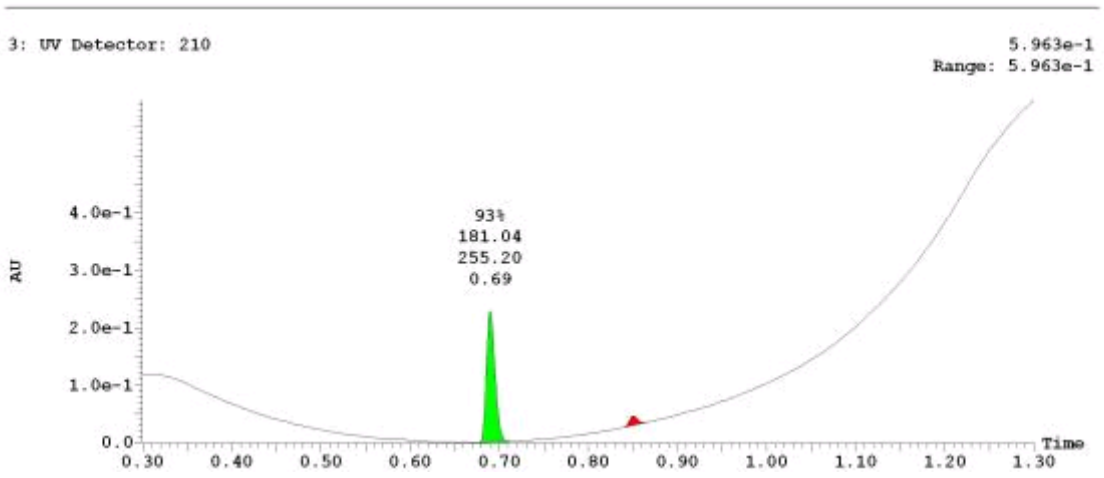
LC-MS Chromatogram for Analog CID46897916



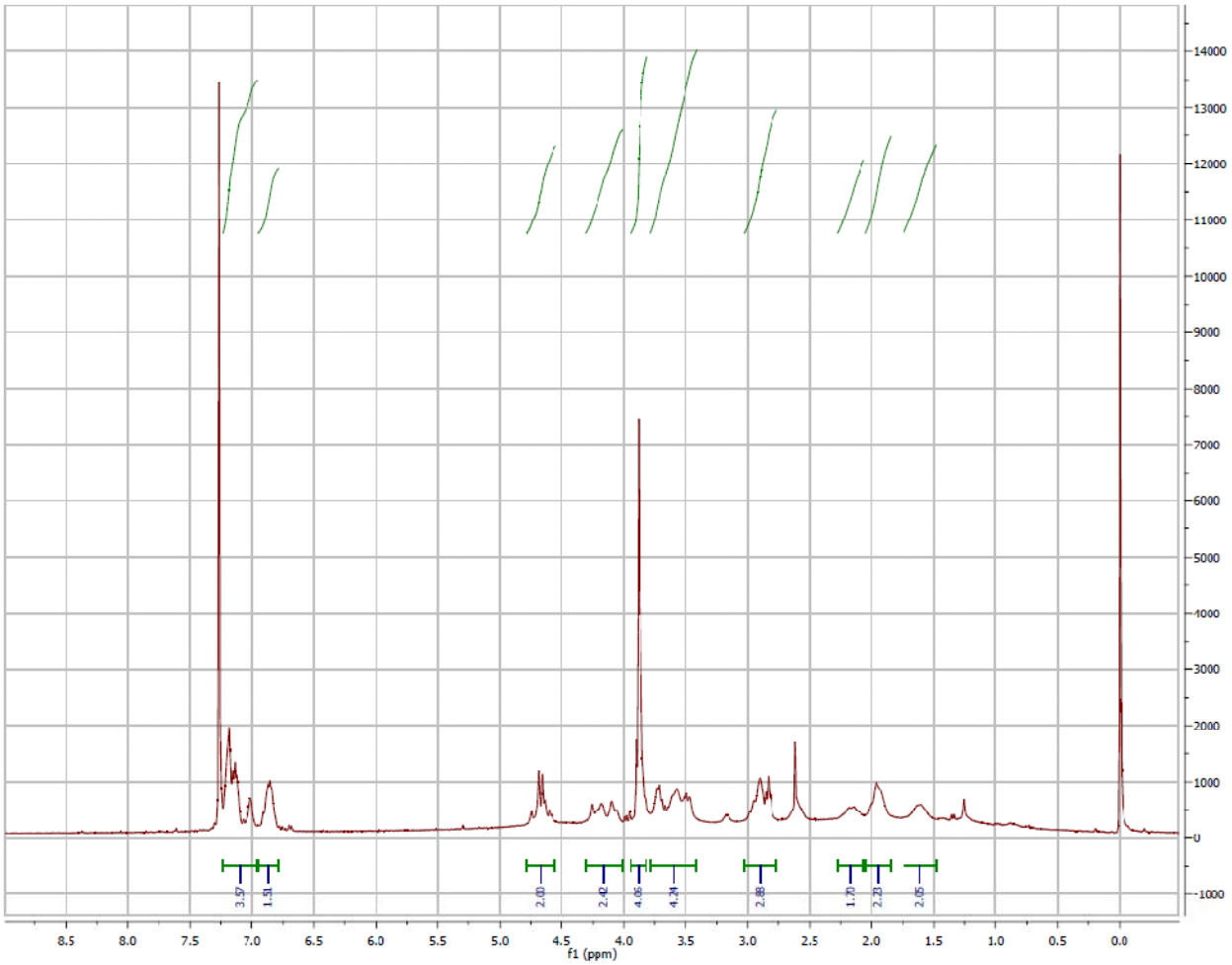
^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID4207918



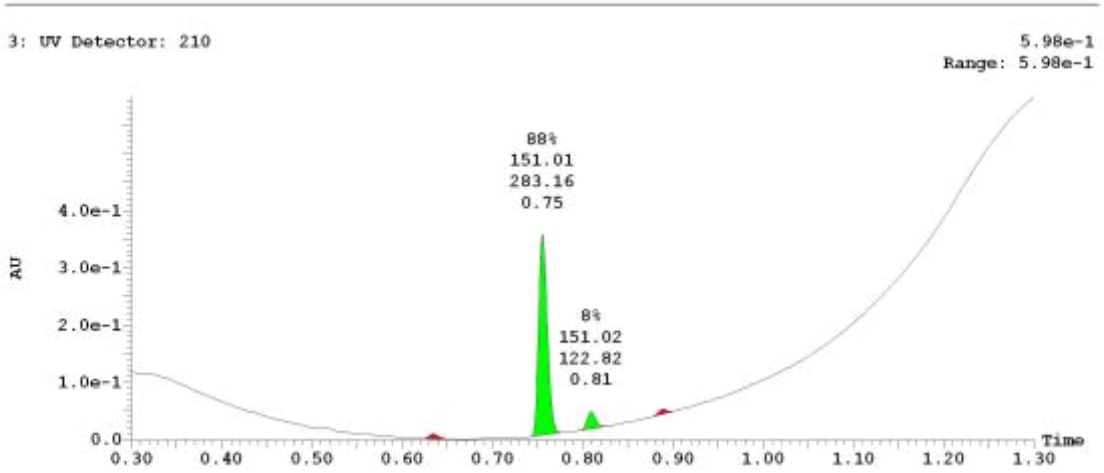
LC-MS Chromatogram for Analog CID4207918



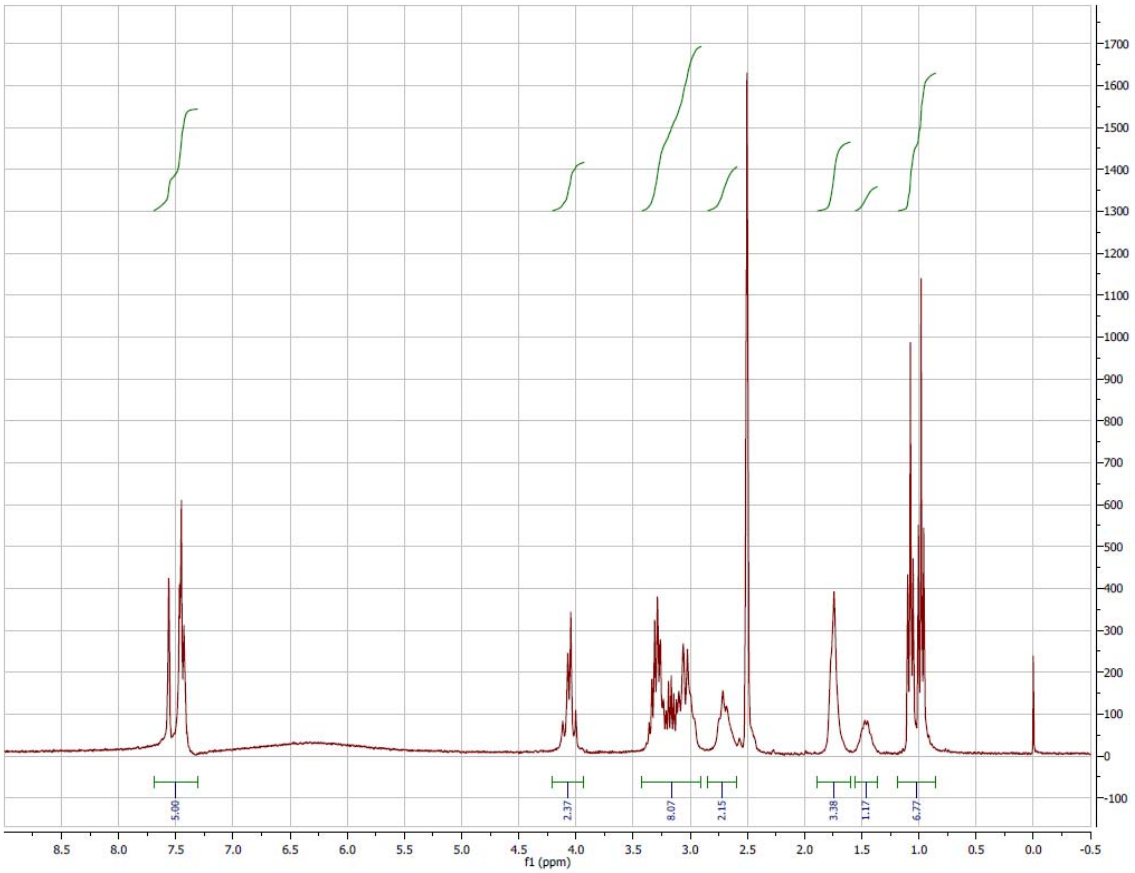
^{1}H NMR ($CDCl_3$) Spectrum for Analog CID99351072



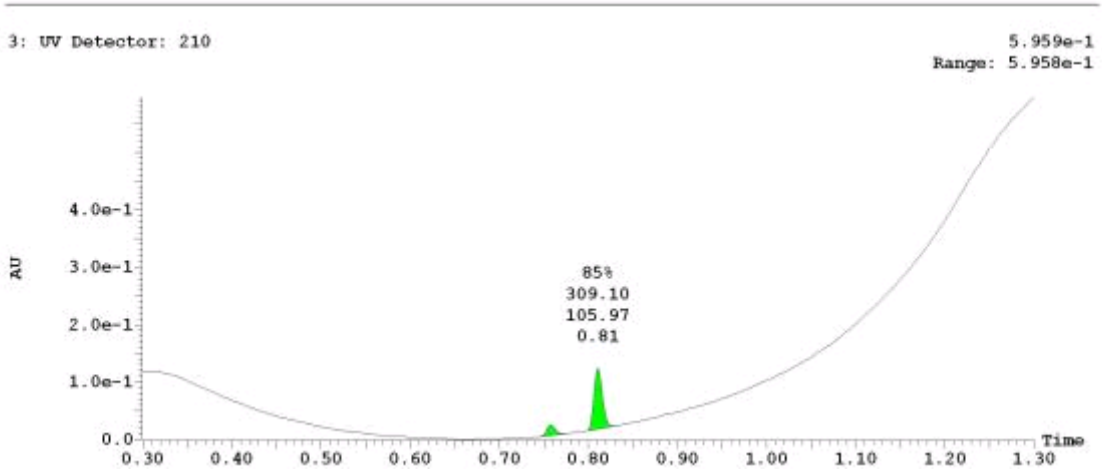
LC-MS Chromatogram for Analog CID99351072



^{1}H NMR (300 MHz, DMSO-d6) Spectrum for Analog CID87556795



LC-MS Chromatogram for Analog CID87556795



Appendix D: Compounds Submitted to BioFocus

Table A2. Probe and Analog Information

BRD	SID	CID	P/A	MLSID	ML
BRD-A98248982-001-04-9	87219048	3238551	P	MLS002725691	ML158
BRD-A99518825-001-01-1	87556801	4207918	A	MLS002725685	NA
BRD-A83402799-001-01-8	87219049	3500831	A	MLS002725682	NA
BRD-A74871599-001-01-7	87556793	3237704	A	MLS002725692	NA
BRD-A37656721-001-01-9	87556802	44629413	A	MLS002725684	NA

NA- Not applicable; P = probe; A = analog