Probe Report

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Title: Identification of ML359 as a Small Molecule Inhibitor of Protein Disulfide Isomerase

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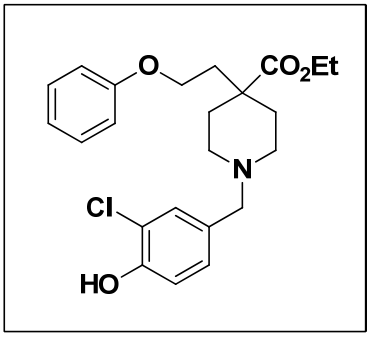
Abstract:

Arterial thrombosis causes heart attacks and strokes and is the leading cause of morbidity and mortality in the United States. However, there are only a few adequate therapies available for treating arterial thrombosis. Thus, there is a clear need for new approaches and new targets in the prevention and treatment of arterial thrombosis. Protein disulfide isomerase (PDI) is expressed on vascular cells following injury and has been shown to be a critical regulator of thrombus formation *in vivo.* Since inhibition of PDI prevents platelet accumulation and fibrin generation, it makes it a tractable target for the development of new antithrombotics. A high throughput screen (HTS) was conducted to identify potent and selective inhibitors of PDI. An insulin-based turbidometric assay was used to screen 348,505 compounds of the MLSMR library. Potential PDI inhibitors were further characterized via additional specificity and cytotoxicity assays. We report the identification and characterization of a small-molecule probe (**ML359**) shown to be a specific inhibitor of PDI. **ML359** shows no cytotoxicity in three human cell lines, and some activity in inhibiting platelet aggregation *in vitro*. **ML359** will provide more understanding of the mechanisms of PDI in platelet functions. It also could lead to the development of a new class of antithrombotics that could establish PDI as suitable drug target.

Probe Structure & Characteristics:



ML359

CID/ML No.	Targets	PDI_inhibition (uM) [SID, AID]	Anti-Target	Anti-Target_inhibition (uM) [SID, AID]	Fold Selective*
23723882 **ML359**	Protein Disulfide Isomerase (PDI)	**IC_{50}**: 250.0 nM [160843258, 652146]	**1.** Erp5 **2.** Thioredoxin **3.** Thioredoxin reductase	**1.** **IC_{50}**: >30.0 [160843258, 652270] **2.** **IC_{50}**: >30.0 [160843258, 652214] **3.** **IC_{50}**: >30.0 [160843258, 652269]	>100x

* Selectivity = Anti-target IC_{50}/Target IC_{50}

Recommendations for scientific use of the probe:

Researchers studying arterial thrombosis mediated tissue infarction in coronary artery disease, cerebrovascular disease, and peripheral vascular disease will find this probe useful to better understand the role protein disulfide isomerase (PDI) plays in arterial thrombosis. Despite the potential importance of PDI in these disease processes, potent and selective small molecule inhibitors are not presently available. The probe, **ML359** will be used to further deconvolute the functions of PDI in the regulation platelet activation, fibrin formation and thrombus formation. **ML359** will also serve as a useful probe in evaluating the pharmacology of PDI, enabling determination of which domains within enzyme are particularly susceptible to pharmacological manipulation. As this probe is more selective than the prior art molecules, the probe will be further analyzed for specific function in inhibiting PDI in biological assays and *in vivo*. Proposed future target ID experiments, including examining the crystal structure of PDI in complex with the probe will enlighten this area. The probe and additional analogs that will come from additional medicinal chemistry efforts will also be used in advanced anti-thrombotic investigations, which include *in vivo* mouse model studies. By gaining a better understanding of the functions of PDI, it is possible that a novel PDI inhibitor could pave the way towards the development of novel class of therapeutics that potentially would improve the therapeutic outcomes with arterial thrombosis-related diseases.

1 Introduction

Protein disulfide isomerase (PDI, EC 5.3.4.1) is the founding member of a large family of disulfide oxidoreductases (1). This family of enzymes catalyzes posttranslational disulfide exchange, contributing to the proper folding of newly synthesized proteins. Gene knockdown of PDI is lethal, thus preventing the use of standard genetic approaches to evaluate function and selectivity. Specific functions of the 19 PDI disulfide oxidoreductase family members have been difficult to study and the degree of redundancy between family members has been impossible to evaluate due to the lack of selective small molecule inhibitors for thiol isomerases.

In addition to its function in protein folding, PDI can also be released into the extracellular environment, where it functions in thrombus formation, cancer metastasis, and invasion of infectious organisms. PDI secreted by cells can reattach to the plasma membrane, where it functions as an extracellular oxidoreductase (2). In particular, PDI has been identified on the surface of vascular cells. PDI is secreted by stimulated platelets, binds the extracellular membrane, and functions in platelet activation (3-15). It has been shown that vascular PDI serves a critical regulatory role in the control of thrombus formation following vessel injury (6, 16). Thrombus formation is the underlying pathology in myocardial infarction and stroke, the first and third most common cause of death in the industrialized world. The critical role of PDI in thrombus formation raises the possibility that PDI could represent an important target for the inhibition of arterial thrombosis. In addition, extracellular PDI may function in modifying proteins for endocytosis or membrane fusion (17). Entry of HIV into lymphophocytes is inhibited by antibodies to PDI, bacitracin, or cell-impermeable sulfhydryl blockers (18-21). The cytotoxicity of diphtheria toxin (2, 22), the attachment and entry of Chlamydia (23, 24), and phagocytosis of *Leishmania chagasi* promastigotes (25) are blocked by inhibition of PDI. Extracellular PDI has also been implicated in gamete fusion (26) and in insulin metabolism (27). A role for PDI in cancer progression has been proposed and inhibition of PDI by irreversible inhibitors or bacitracin interferes with tumor cell growth *in vitro* and *in vivo* (28). Thus, PDI is an important target not only for biological probes designed to study thiol isomerase function, but also for several life-threatening pathological processes.

Despite its potential importance in disease, potent, selective, and reversible small molecule inhibitors of PDI with suitable stability and physicochemical characteristics are not presently available. Three types of inhibitors constitute the vast majority of antagonists that have

previously been used for studying PDI in cellular systems. These include sulfhydryl blockers, antibodies, and the macrocyclic dodecapeptide antibiotic, bacitracin.

1. Sulfhydryl blockers are irreversible, non-specific, and demonstrate IC_{50}s of 100-500 uM against PDI (2). Poor selectivity and potency limits their use as probes to evaluate PDI function. A series of propynoic acid carbamoyl methyl amides identified on the basis of their ability to inhibit growth of ovarian cancer cells was recently described (28). These compounds have improved potency over previous sulfydryl blockers. However, these are irreversible inhibitors that react with the active site of PDI and would likely not be selective among thiol isomerases, which share a common active site motif.

2. Antibodies to PDI have been developed. However, they cannot be used to study the function of intracellular PDI. Antibodies have limitations as antithrombotics owing to their long half-lives, as they are not easily reversed in the setting of hemorrhage. In addition, even antibodies widely used in the field suffer from lack of selectivity in biological assays. For example, RL90 is a widely used monoclonal antibody directed against PDI. It has recently been shown, however, that RL90 also inhibits ERp57 activity, complicating interpretation of previous data (29).

3. Bacitracin (structure shown in **Figure 6**) is a widely used inhibitor of PDI (2). It is a 1423 Da cyclic dodecapeptide that blocks PDI activity. However, it is non-selective, inhibiting many thiol isomerases, and not potent, with an IC_{50} of approximately 150-200 uM (2, 30, 31). Furthermore, bacitracin is extracted from *Bacillus licheniformis* and the product available through commercial suppliers is a mixture of >30 different substances, including a subtilisin-type protease that cleaves PDI (32). As a result of its impurity and protease contamination, investigators in the PDI field have advised caution in the interpretation of results from studies using commercially available bacitracin (32).

More recently, juniferdin (structure shown in **Figure 6**) was identified as an inhibitor of PDI in a high throughput screen performed with the goal of identifying PDI inhibitors to block HIV transmission (33). Since juniferdin was cytotoxic in a cell-based assay (33), the investigators synthesized juniferdin epoxide, which was nearly as potent and did not show activity in their cytotoxicity assay. In this report, we demonstrate the limitations of juniferdin epoxide and identify **ML359** as a superior probe for studying the biology of PDI.

2 Materials and Methods

See subsections for a detailed description of the materials and methods used for each assay.

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 Primary assay - Turbidometric HTS to detect the inhibition of insulin aggregation in the presence of PDI (AID 588391, 602350, 624274, 624310, 652146, 652199)

To identify inhibitors of PDI, an assay was developed to analyze PDI-mediated aggregation of insulin. The assay measured the aggregation of insulin chains by monitoring absorbance at 650nm. When PDI is inhibited, there is a decrease in aggregation, and therefore a decrease in absorbance signal. Recombinant human PDI was expressed and purified from *E. coli*. (16), mixed with bovine insulin (Sigma), and added to assay plates. For the primary HTS, 1536-well assay ready plates (ARPs) were prepared prior to adding the assay components. ARPs were prepared with 7.5 nL of 10 mM compounds for a final screening concentration of 10 uM. For retests performed at Broad, the assay was performed in 1536-well and 384-well plates. For 384-well plates, compounds were added by pin transfer in a volume of 100 nL into an assay volume of 30 uL. The final concentration of compounds per well started at 20uM and were titrated 1:3. Following the addition of PDI, insulin, and compounds, DTT was added to initiate the reaction. Following a 90-minute incubation, plates were read for absorbance at 650 nm. The primary HTS data were analyzed using Genedata Screener Assay Analyzer. All values were normalized against the neutral control (DMSO) and positive control (100 uM Rutin Hydrate, CID 45479757) wells. The average of the two replicates was used to determine activity and to choose compounds for retests. For dose studies, the percent activity for each replicate, for each concentration was determined, and the concentration response curves were generated using Genedata Screener Condoseo.

For the reconfirmation assays performed in D. Kennedy's laboratory, the assay was performed in 384-well plates, at a volume of 30 uL. To each well, the final concentration of the constituents were 50 ug/mL purified human PDI, 0.4 mM insulin, and 2 mM EDTA in 100 mM potassium phosphate, pH 7.4, at 25°C. The reaction was read initially and again after 15 minutes. The raw

data was normalized by the following formula $OD[_{15min}] - OD[_{0min}]$ Enzyme inhibition was subsequently calculated with the following formula: PDI activity (%) = $(OD[_{compound + PDI + DTT}] - OD[_{DTT}])/(OD[_{PDI + DTT}] - OD[_{DTT}]) \times 100\%$.

2.1.2 Secondary assay – Cytotoxicity in HEK293 cells (AID 652119)

HEK293 cells were treated with compounds for 48 hours, and then measured for cell viability using the CellTiter-Glo Assay (Promega), a luciferase-based reagent that measures cellular ATP levels. The compounds were tested at concentrations ranging from 26 uM to 0.004 uM, to determine IC_{50} values. Compounds that were inactive ($IC_{50} \geq 30$ uM) in this assay were considered for probe development. Data were normalized against the neutral control wells (DMSO) and positive control wells (20 uM Staurosporine, CID 44259) in Genedata Screener Assay Analyzer. Curves were generated with Genedata Screener Condoseo.

2.1.3 Secondary assay – Cytotoxicity in HepG2 cells (AID 652118)

This assay was performed in conjunction with assay 2.1.2. The same procedures were used with the exception of cell type.

2.1.4 Secondary assay – Cytotoxicity in HeLa cells (AID 652117)

This assay was performed in conjunction with assay 2.1.2. The same procedures were used with the exception of cell type.

2.1.5 Secondary assay – Selectively screen to determine compound activity against ERp5 (AID 624320, 652270)

Reductase activity was assayed measuring the ERp5-catalyzed reduction of insulin in the presence of DTT. The reaction results in the aggregation of insulin chains, which can be measured by absorbance at 650 nM. For the assay performed at Broad, ERp5 and insulin were combined in an assay buffer (100 mM Potassium Phosphate, pH 7.0; 2 mM EDTA) at 26.5 ug/mL and 142 uM, respectively. The ErP5/insulin mixture was added to 384-well plates with a liquid handler, at 30 uL. Compounds arrayed at dose were added via pin transfer (CyBiWell, Cybio) in 100 nL. Bacitracin (CID 3083711), used as a positive control, was added to designated positive control wells, at a final concentration of 1 mM. Following compound addition, the reaction was catalyzed with the addition of 2 uL of 5.6 mM DTT to a final concentration of 350 uM. The reaction was incubated for 120 minutes at room temperature. After the incubation, the plates were read for absorbance at 650 nm. The data was analyzed using Genedata Screener Assay Analyzer where the percent activity for each replicate, at each

concentration was determined, and the concentration response curves were generated using Genedata Screener Condoseo.

For the assay performed in D. Kennedy's laboratory, the reactions were performed in a 384-well plate in 30 uL, in the presence of 100 ug/mL purified ERp5 (16), 0.4 mM insulin, and 2 mM EDTA in 100 mM potassium phosphate, pH 7.4, at 30°C. DTT was added to initiate the reaction. Absorbance at 650 nm was read at time = 0 and again after 20 minutes, with a Spectra Max M3 (Molecular Devices). The data were normalized by subtracting the earlier time points from the later. Enzyme inhibition was subsequently calculated with the following formula: ERP5 activity (%) = $(OD[_{compound + ERP5 + DTT}] - OD[_{DTT}])/(OD[_{ERP5 + DTT}] - OD[_{DTT}]) \times 100\%$.

2.1.6 Secondary assay – Selectively screen to determine compound activity against Thioredoxin Reductase (AID 652269)

The disulfide reductase activity of thioredoxin-1/ thioredoxin reductase/NADPH (TRN) system was assessed by the turbidometric insulin reduction assay (based on (34)). Reactions were carried out in assay buffer consisting of 100 mM sodium phosphate, 2 mM EDTA (pH 7.4). Compounds were prepared as a stock solution (1 mM) in 50% DMSO/50% H_2O. A solution of 50% DMSO/50% H_2O was prepared as control buffer.

Thioredoxin reductase (140 nM) was pre-incubated with compound (at concentrations of 0.005, 0.015, 0.046, 0.137, 0.411, 1.23, 3.7, 11.1 and 33.33 uM) or control buffer for 30 minutes at room temperature. NADPH is then added at a concentration of 0.12 mM, followed by thioredoxin at 0.83 uM, and insulin at 100 uM in a buffer containing 100 mM sodium phosphate and 2 mM EDTA. The total reaction volume was 100 uL. The precipitation of insulin B chains is monitored as at OD 650 nm in a 96-well plate. The percent (%) inhibition of insulin reduction is expressed as (1-OD max of thioredoxin reductase in the presence of compound/OD max of thioredoxin in the presence of buffer alone) x 100, Insulin reduction by thioredoxin/ DTT was used as a positive control to gauge positive activity.

2.1.7 Secondary assay – Selectively screen to determine compound activity against Thioredoxin (AID 652214)

Reductase activity was assayed by measuring the thioredoxin–catalyzed reduction of insulin in the presence of DTT. The thioredoxin (TRX) assay was performed in a 384-well plate at 30 uL, in the presence of 100 ug/mL purified human thioredoxin, 0.4 mM insulin, and 2 mM EDTA in 100 mM potassium phosphate, pH 7.4, at 25°C. DTT was added to initiate the reaction.

Absorbance at 650 nm was read at time = 0 and again after 15 minutes, on a Spectra Max M3 (Molecular Devices). The raw data were normalized by subtracting the earlier time points from the later. Enzyme inhibition was subsequently calculated with the following formula: TRX activity (%) = $(OD[_{compound + ERP5 + DTT}] - OD[_{DTT}])/(OD[_{ERP5 + DTT}] - OD[_{DTT}]) \times 100\%$.

2.1.8 Secondary assay – Selectively screen to determine compound activity against ERp57 (AID 652266)

ERp57 reductase activity was assayed by measuring the reduction of insulin in the presence of DTT. The ERp57 assay was performed in a 384-well plate at 30 uL, in the presence of 100 ug/mL purified human ERp57 (AbCam, Cambridge MA.), 0.4 mM insulin, and 2 mM EDTA in 100 mM potassium phosphate, pH 7.4, at 30°C. DTT was added to initiate the reaction. Absorbance at 650 nm was read at time = 0 and again after 20 minutes, with a Spectra Max M3 (Molecular Devices). The raw data were normalized by subtracting the earlier time points from the later. Enzyme inhibition was subsequently calculated with the following formula: ERp57 activity (%) = $(OD[_{compound + ERP57 + DTT}] - OD[_{DTT}])/(OD[_{ERP57 + DTT}] - OD[_{DTT}]) \times 100\%$.

2.1.9 Secondary assay – Determination of platelet aggregation activity (AID 652249)

Platelet Rich Plasma (PRP) obtained from individual donors was treated with 10 U/mL hirudin for 20 minutes at 37°C. Acid Citrate Dextrose (ACD) was added to 15% of total volume. PGE-1 was added to a final concentration of 0.15 uM. PRP was spun at 1000g x 10 minutes and the supernatant was discarded. The platelet pellet was resuspended in Modified HEPES-Tyrodes buffer pH 7.3. The 1X washed platelets were incubated for 20 minutes at 37°C until they were in a quiescent state. The above washing procedure was then repeated. The 2X washed platelets were allowed to rest for 20 minutes at 37°C. Platelets were then counted and the concentration of platelets in suspension was adjusted to 200,000 platelets/uL. The 2X washed platelets were treated with compounds at 30 uM, and were incubated for 30 minutes at 37°C. Following incubation, platelets were added to a cuvette and stirred at 37°C in a Chrono-Log Platelet Aggregometer. In order to initiate aggregation, a concentration of SFLLRN, the thrombin receptor activating peptide, sufficient to elicit 60-70% light transmittance was added to the cuvette. Therefore, a range of 2-3 uM SFLLRN was used in order to accommodate small differences in sensitivity to SFLLRN between individual donors. Prior to reading, the aggregometer was scaled so that unstimulated, washed platelets are set to 0% light transmittance, and buffer alone with no platelets is set to 100% light transmittance. The light transmittance of each sample was recorded as a measure of aggregation. Data were recorded

as a single point representing maximum aggregation in the presence of compound and were compared to samples exposed to vehicle (DMSO) alone.

2.1.10 Secondary assay – Determination of platelet aggregation reversibility (AID 652274)

Platelet Rich Plasma (PRP) obtained from individual donors was treated with 10 U/mL hirudin for 20 minutes at 37°C. Acid Citrate Dextrose (ACD) was added to 15% of total volume. PGE-1 was added to a final concentration of 0.15 uM. PRP was spun at 1000g x 10 minutes and the supernatant was discarded. The platelet pellet was resuspended in Modified HEPES-Tyrodes buffer pH 7.3. The 1X washed platelets were incubated for 20 minutes at 37°C until they were in a quiescent state. The above washing procedure was then repeated. The 2X washed platelets were allowed to rest for 10 minutes at 37°C. After the resting, 0.1 mM $CaCl_2$ was then added, and the platelets were further rested for another 10 minutes. Platelets were then counted and the concentration of platelets in suspension was adjusted to 200,000 platelets/uL. The 2X washed platelets were treated with compounds at 30 uM, and were incubated for 30 minutes at 37°C. Following incubation, platelets were added to a cuvette and stirred at 37°C in a Chrono-Log Platelet Aggregometer. In order to initiate aggregation, a concentration of SFLLRN sufficient to elicit 60-70% light transmittance was added to the cuvette. Therefore, a range of 2-3 uM SFLLRN was used in order to accommodate small differences in sensitivity to SFLLRN between individual donors. Prior to reading, the aggregometer was scaled so that unstimulated, washed platelets are set to 0% light transmittance, and buffer alone with no platelets is set to 100% light transmittance. An initial read of light transmittance was recorded as a measure of aggregation. Data were recorded as a single point representing maximum aggregation in the presence of compound and were compared to samples exposed to vehicle (DMSO) alone. Following the initial read, the platelets were washed as before, and then resuspended with the modified HEPES-Tyrodes buffer to the original volume. The 3X washed platelets were then allowed to rest for 10 minutes at 37°C. After resting, 0.1 mM $CaCl_2$ was then added and the platelets were further rested for another 10 minutes. Samples were then returned to a cuvette for a second reading to measure for reversibility.

2.2 Probe Chemical Characterization

The probe (**ML359**) was synthesized as described in Section 2.3. **ML359** was subsequently analyzed by ^{1}H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry. The NMR and mass spectroscopy data are consistent with the structure of the probe, and isolated purity was determined to be greater than 95% by UPLC. The reaction schemes, complete synthetic protocol and the relevant spectral data are provided in **Appendix C** and **Appendix E**. The physical properties of the probe **ML359** are summarized in **Table 1**. The solubility of **ML359** was determined to be 62.7 uM in PBS with 1% (v/v) DMSO (PBS; pH 7.4, 23°C).

Table 1. Summary of Probe Properties Computed from Structure **ML359**

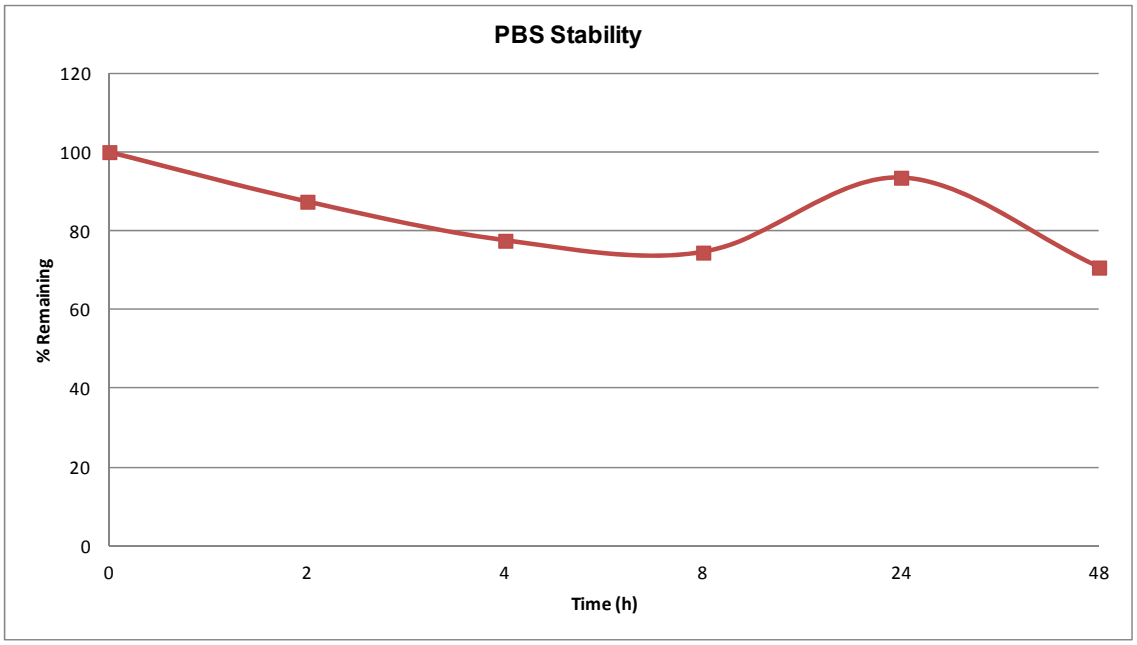
Description	ML359
IUPAC chemical name*	ethyl 1-(3-chloro-4-hydroxybenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate
PubChem CID	23723882
Molecular Formula	C23H28ClNO4
Molecular Weight (g/mol)	417.92572
Exact Mass (amu)*	417.17069
ClogP #	4.462
Topological Polar Surface Area#	59 Å^2
H-Bond Donors#	1
H-Bond Acceptors#	5
Rotatable Bond Count#	9

* Generated or calculated with ChemBioDraw Ultra 12.0. #Calculated with StarDrop 5.2

The stability of the probe **ML359** was measured in the presence of PBS pH 7.4 with 1% DMSO. **ML359** was added (in triplicate at 1 uM) on six separate plates and allowed to equilibrate at room temperature for 48 hours. At each time point (0, 2, 4, 8, 24, and 48 hours), one plate was

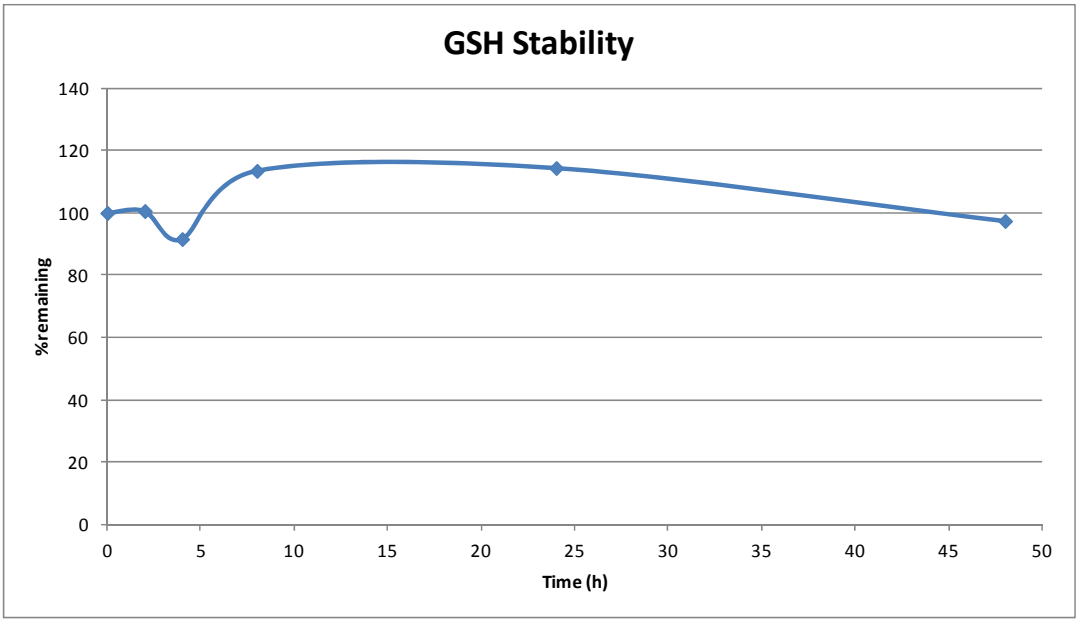
removed and an aliquot was taken out from each well and analyzed by UPLC-MS (see **Appendix D** for details). After 48 h, more than 70% of **ML359** remained present (**Figure 1**).

Figure 1. Stability of the Probe (**ML359**, CID23723882) in PBS Buffer (pH 7.4, 23°C)



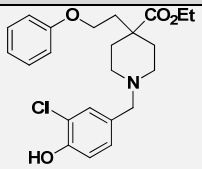
The stability of the probe was also determined in the presence of 50 uM glutathione (in PBS pH 7.4 with 1% DMSO). **ML359** was added (in duplicate at 1 uM) on six separate plates and allowed to equilibrate at room temperature for 48 hours. At each time point (0, 2, 4, 8, 24, and 48 hours), one plate was taken out and an aliquot was removed from each well and analyzed by UPLC-MS (**Appendix D**). **ML359** is stable since more than 95% remained present (**Figure 2**).

Figure 2. Stability of the probe (**ML359**, CID23723882) in presence of glutathione



ML359 showed that it was 98.7% bound in human plasma and 96.5% bound in mouse plasma (**Table 2**). The probe **ML359** is stable in human plasma (>99% remaining after a 5 h incubation period at 37°C) but unstable in murine plasma (entry 1; **Table 2**). Analogues designed to improve the murine plasma stability of the series are discussed in Section 3.4 (SAR discussion section). The probe (**ML359**) was found to be unstable to both mouse and human liver microsomes (**Table 2**). Microsomal stability data of other analogues of **ML359** are also discussed in SAR discussion section (Section 3.4.2) in detail. Experimental procedures for all analytical assays are provided in **Appendix D**.

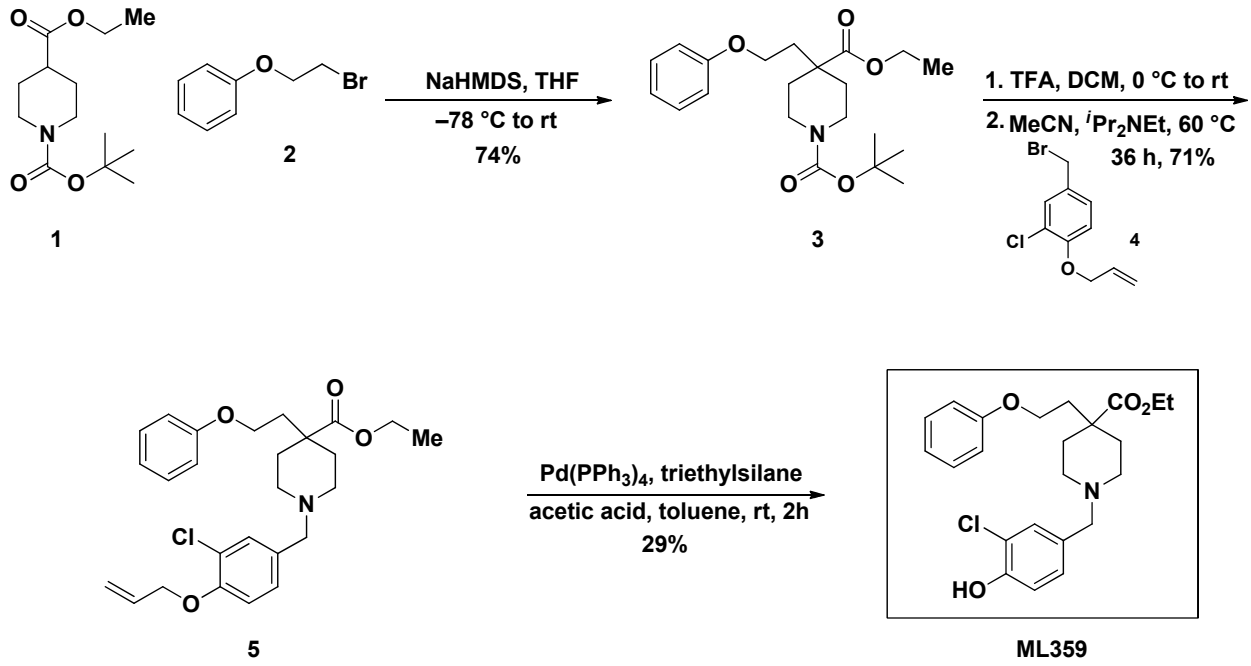
Table 2. Plasma Protein Binding, Plasma Stability and Microsomal Stability data of **ML359**

Entry No.	CID	Structure	Plasma stability (% remaining after 5 h)		Plasma protein binding (% bound)		*Microsomal Stability (% remaining at 1 h)*	
			Human	Murine	Human	Murine	Human	Murine
1 (hit)	23723882 **ML359**		100	5.3	98.7	96.5	1.0	1.2

2.3 Probe Preparation

Probe **ML359** was synthesized starting from 1-*tert*-butyl 4-ethyl piperidine-1,4-dicarboxylate (**1**) in four-step as outlined below in **Scheme 1.** Deprotonation of 1-*tert*-butyl 4-ethyl piperidine-1,4-dicarboxylate (**1**) in presence of sodium hexamethyldisilazide in tetrahydrofuran followed by alkylation with 2-bromoethoxybenzene (**2**) yielded 1-*tert*-butyl 4-ethyl 4-(2-phenoxyethyl)piperidine-1,4-dicarboxylate (**3**). Removal of the Boc-functionality with TFA followed by alkylation of the secondary amine with 1-(allyloxy)-4-(bromomethyl)-2-chlorobenzene **(4)** resulted in **5**. Palladium (0) mediated allyl deprotection of the phenolic alkoxy group yielded probe **ML359**. Full experimental details, analytical characterization and additional synthetic protocol for the synthesis of **4** are provided in **Appendix C** and **Appendix E.**

Scheme 1. Synthesis of the Probe **ML359**



3 Results

Probe attributes:

- Inhibits insulin aggregation in the presence of PDI with IC_{50} < 0.25 uM
- Shows selectivity of >100x against other thiol isomerases (ERp5, thioredoxin reductase, thioredoxin, ERp57)
- Non-toxic to HeLa, HEK293, and Hep G2 cells with IC_{50} >30 uM

This project utilized an insulin-based turbidometric assay to screen the MLSMR collection of compounds to identify inhibitors to protein disulfide isomerase (PDI). In the presence of DTT, PDI catalyzes the aggregation of insulin chains, which can be measured by absorbance at 650 nm. The assay was miniaturized for high throughput screening, which allowed for the examination of 348,505 substances in duplicate from the MLSMR collection.

Analysis from the primary screen identified 181 active and 358 inconclusive compounds. From these compounds, a list of approximately 400 compounds was assembled for re-evaluation in a concentration-dependent manner. The list included those compounds from the screen that were chemically tractable, along with additional analogs to provide some SAR insight. The retest at dose verified the activity of 111 compounds that show at least a 30% decrease in absorbance, in a dose-dependent manner. Upon a more stringent examination and approaching the probe criteria, there were 12 of the 111 compounds that demonstrated an IC_{50} < 1 uM.

The 12 active compounds, along with the two prior art compounds were selected for further examination as dry powders. Based on the performance of these powders in the primary assay and selectivity assays, 2 distinct scaffolds (CID 23723882 and CID 1043221) were prioritized for further development. The two lead compounds were analyzed in a series of secondary assays that included specificity assays comparing activity in other thiol isomerases, cytotoxicity testing, and mode of action studies looking at platelet aggregation inhibition. From the 2 compounds, the piperdine compound, CID 23723882 demonstrated better potency against PDI with an observed IC_{50} of 0.25 uM **(Figure 4)**, showed no toxicity in a panel of mammalian cells, and demonstrated >300-fold selectivity towards PDI over other thiol isomerases **(Section 3.4)** and therefore was declared a probe, **ML359**.

3.1 Summary of Screening Results

As discussed above, 348,505 compounds of the Molecular Libraries and Small Molecules Repository (MLSMR) collection were tested at a single concentration (10 uM), in duplicate for their ability to inhibit the aggregation of insulin in the presence of protein disulfide isomerase (PDI). From the screen, 181 compounds were considered as actives representing a hit rate of 0.05%.

A list of compounds was assembled for retesting in a dose-response format, which was composed of the most chemically tractable compounds from the compounds determined to be active and inconclusive from the primary screen, along with additional analogs. From the retests, only 12 compounds were to be procured as dry powders for additional testing. After purity analysis and structural analysis by NMR spectroscopy, these compounds were screened as outlined in the critical path for probe development in **Figure 3**. The analysis of the dry powder compounds showed the emergence of a scaffold (**ML359**) that was a potent inhibitor to PDI **(Figure 4)** that showed specificity to PDI over other thiol isomerases, and was not toxic to three human cell lines. A number of analogs were synthesized, based on this lead compound, and were tested in the assays outlined in **Figure 3.** The results of the SAR investigation are detailed in **Tables 3, 4, and 5** in **Section 3.4.**

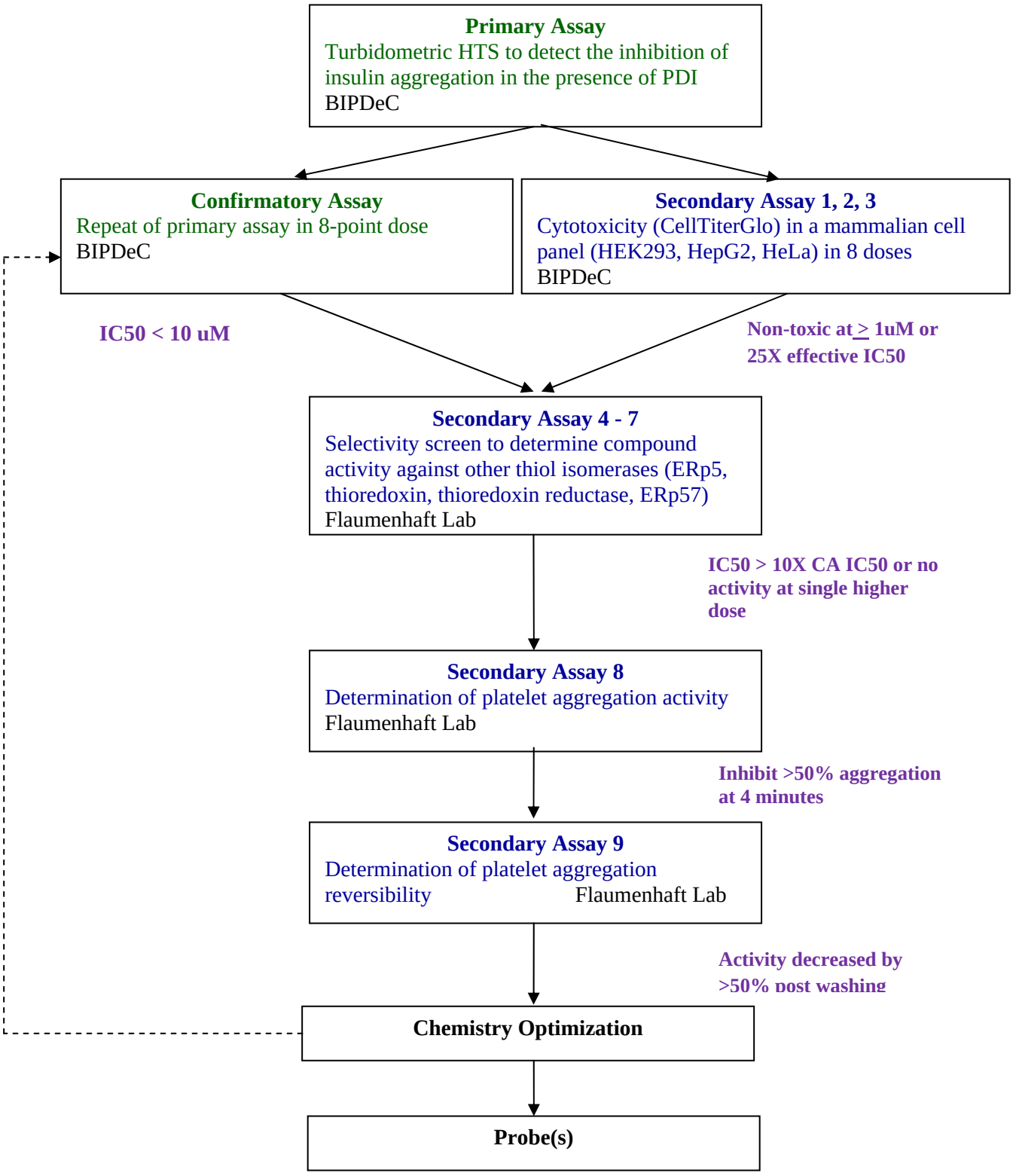


Figure 3. Critical Path for Probe Development

3.2 Representative Dose Response Curve for Probe ML359

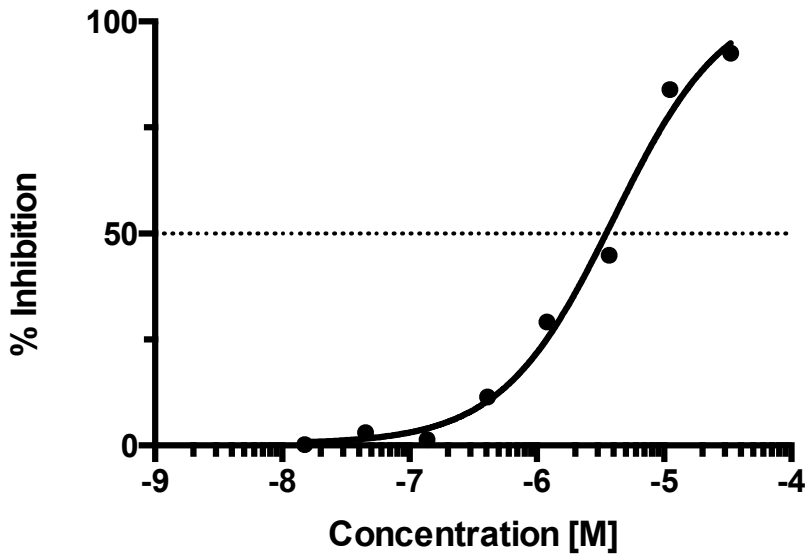


Figure 4. Dose-dependent Activity of the Probe (**ML359**) in the inhibition of PDI-induced insulin aggregation

3.3 Scaffold/Moiety Chemical Liabilities

ML359 is stable in PBS and GSH stability assays. **ML359** is stable in human plasma but not in murine plasma (see **Section 2.2**). **ML359** is not stable in human and mouse liver microsome (**Section 2.2**).

3.4 SAR Tables

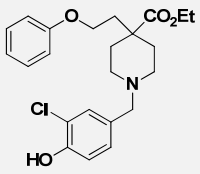
The hit compound ethyl 1-(3-chloro-4-hydroxybenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate (CID23723882) demonstrated a good PDI inhibitory profile in the primary turbidometric assay with no cytotoxicity in mammalian cells. In order to establish minimum pharmacophores and to study SAR, analogues were designed and tested. The summary of the biological assay data of these analogues is described in **Tables 3-4** (PubChem AIDs 652146, 652199, 652119, 652118, 652117). Proton NMR spectra and UPLC chromatograms of these analogues are presented in **Appendix F**. None of the analogues represented in **Tables 3-4** demonstrated any cytotoxicity against HepG2, HEK 293, and HeLa cell lines (PubChem AIDs 652119, 652118, 652117).

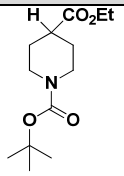
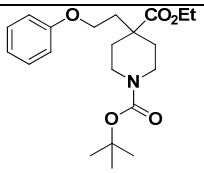
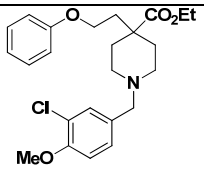
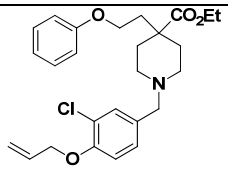
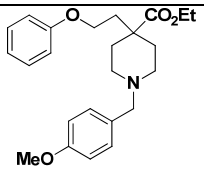
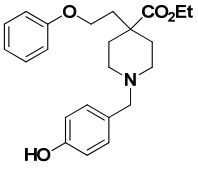
Table 3 depicts the effect on PDI inhibitory activity of various structural modifications of the hit compound. Lack of activity of the piperidine ester (synthetic building block) and phenoxyethyl piperidine ester (synthetic intermediate) proved the incapability of these cores alone to inhibit PDI (Entries 2 and 3, **Table 3**). The influence of the substitution pattern in the 3-chloro-4-hydroxybenzyl core was examined next. Complete loss in potency in the case of 4-alkoxy analogues (Entries 4-6, **Table 3**) suggests that the 4-hydroxy substituent is essential for activity. The presence of the *meta*-chloro substituent is also important, as the corresponding unsubstituted compound has significantly diminished activity (Entry 7, **Table 3**). Efforts to replace the phenolic hydroxyl moiety with various bioisosteres were undertaken as the hydroxyl moiety might have detrimental effect in downstream *in vivo* ADME outcome. However, at the time of this report we have only tested the oxazolidinone analogue (Entry 8, **Table 3**) and it was determined to be inactive. Additionally, replacement of the 3-chloro-4-hydroxybenzyl core with a 3-chloro-4-hydroxybenzoyl moiety (Entry 9, **Table 3**) gave an active compound but it was approximately ten fold less potent. Our early SAR studies suggest that the 2-phenoxyethyl side chain at the upper left part of the molecule is important for potency as compounds with 2-methoxyethyl or benzyl side chains lost all activity (Entries 10-12, **Table 3**). Next, modification of the ester functionality was studied and the results are shown in **Table 4**. Sterically demanding *iso*-propyl and *tert*-butyl ester groups were tolerated (Entries 2 and 3, **Table 4**). Diverse amide derivatives were prepared and no improvements in potency were observed (Entries 4-8, **Table 4**). The hit compound CID23723882 and key analogues were subsequently evaluated for selectivity over other thiol isomerases (**Table 5)**. Gratifyingly, all the analogues were selective and showed no inhibitory activity against thioredoxin, thioredoxin reductase and ERp57. A

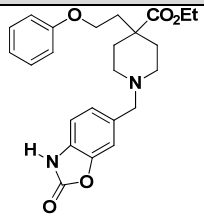
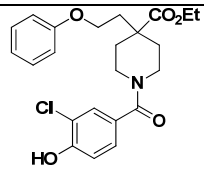
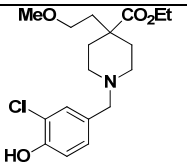
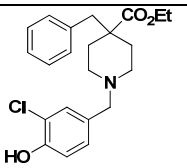
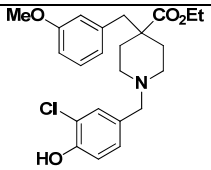
subset of compounds were evaluated against ERp5 and showed no activity. Based on the available data, CID23723882 was the most potent (also selective and non-toxic) PDI inhibitor and was nominated as the probe (**ML359**).

The probe **ML359** and selected analogues were subjected to both mouse and human liver microsome stability assays and was found to be unstable (**Table 6**). The only analogue that demonstrates acceptable human liver microsome stability (*albeit* no PDI inhibitory activity; **Table 3**) was CID42515470 (Entry 8; **Table 6**). We evaluated murine plasma stability (and plasma protein binding) of **ML359** and sets of key analogues and the results are depicted in **Table 7**. It was evident that presence of α-quaternary stereocenter was inadequate to prevent ethyl ester from hydrolysis. Replacement of ester functionality with amide group improved murine plasma stability across the board (Entries 2-6; **Table 7**) but without retaining PDI inhibitory activity. Incremental increase in steric bulk across the ester carbonyl core has a positive outcome and CID70701237 (Entry 8; **Table 7**) is stable in murine plasma. After nominating **ML359** as the probe we found out that replacing ethyl ester with *iso*-propyl or *tert*-butyl ester improved murine plasma stability (with retention of activity and selectivity). Based on this very recent observation, we are currently evaluating a small set of analogues for activity against PDI and other thiol isomerases that will likely have better *in vitro* ADME profile.

Table 3. SAR Analysis: Attempt to identify minimum pharmacophores

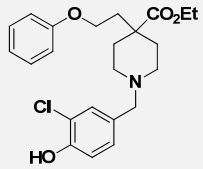
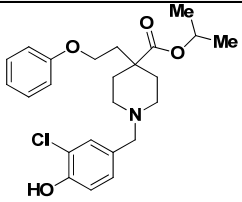
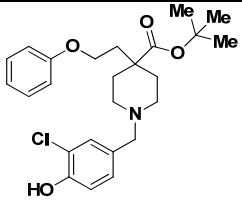
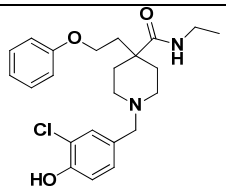
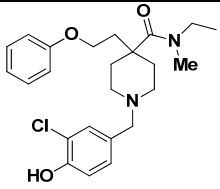
Entry No.#	**CID SID Broad ID**	**Structure**	**PDI_Inhibition _ IC_{50} (uM)†**	**PBS Solubility (uM)**	**Cytotoxicity IC_{50} (uM)*** HEK293/ HepG2/ HeLa
1 (hit)	23723882 160843258 BRD-K20754212-001-10-6 **ML359**		0.25	62.7	>26.0

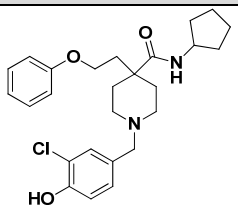
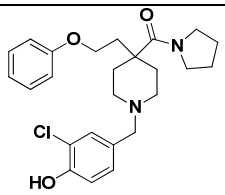
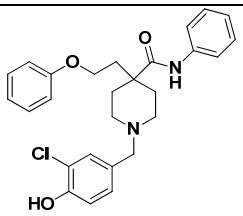
Entry No.[#]	CID SID Broad ID	Structure	PDI_Inhibition _ IC_{50} (uM)[†]	PBS Solubility (uM)	Cytotoxicity IC_{50} (uM)* HEK293/ HepG2/ HeLa
2	2758812 160843261 BRD-K18650194-001-02-9		>30	83.0	>26.0
3	70701267 160843257 BRD-K23121346-001-01-3		>30	0.3	>26.0
4	70701251 160843238 BRD-K13659644-001-01-3		>30	3.3	>26.0
5	70701245 160843256 BRD-K85759585-001-01-5		>30	<0.1	>26.0
6	23723973 160843248 BRD-K94439524-001-07-4		>30	79.4	>26.0
7	23723959 160843243 BRD-K26639447-001-07-6		15.0	85.0	>26.0

Entry No.[#]	CID SID Broad ID	Structure	PDI_Inhibition _ IC_{50} (uM)[†]	PBS Solubility (uM)	Cytotoxicity IC_{50} (uM)* HEK293/ HepG2/ HeLa
8	70701241 160843263 BRD-K09284519-001-01-8		>30	63.1	>26.0
9	70701238 160843250 BRD-K31070838-001-01-7		2.25	20.8	>26.0
10	42515470 160843253 BRD-K53926605-001-02-2		>30	99.7	>26.0
11	25298231 160843245 BRD-K46746533-001-01-3		>30	77.5	>26.0
12	25277312 160843240 BRD-K07804329-001-01-5		>30	67.9	>26.0

[†] Average of two independent experiments (PubChem AIDs 652146, 652199). *For each analogue, cytotoxicity was measured against HEK293, HepG2 and HeLa cell lines and IC_{50} was determined to be >26.0 uM (highest concentration measured) for each cell line (PubChem AIDs 652119, 652118, 652117). [#] Analogues in entries 2-3, 7, 10-12 were commercially procured. All other compounds were synthesized.

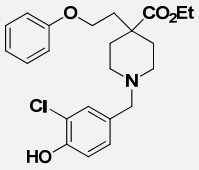
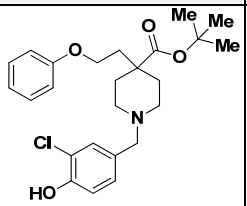
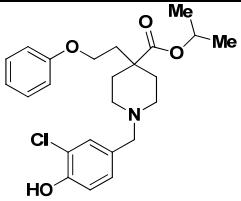
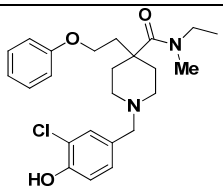
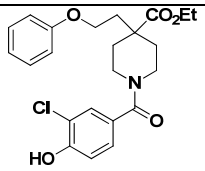
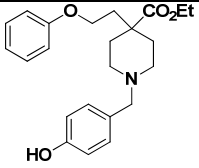
Table 4. SAR Analysis: Modification of ester functionality

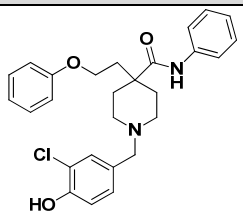
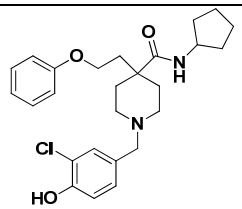
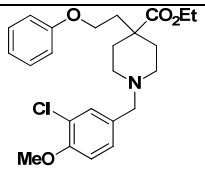
Entry No.	CID SID Broad ID	Structure	PDI_Inhibition _ IC_{50} (uM)†	PBS Solubility (uM)	Cytotoxicity IC_{50} (uM)* HEK 293/ HepG2/ HeLa
1 (hit)	23723882 160843258 BRD-K20754212-001-10-6 **ML359**		0.25	62.7	>26.0
2	70701242 160843255 BRD-K71569005-001-01-6		0.85	16.6	>26.0
3	70701237 160843264 BRD-K31155394-001-01-2		0.65	4.5	>26.0
4	70701263 160843242 BRD-K59037718-001-01-8		>30	95.7	>26.0
5	70701262 160843259 BRD-K10350688-001-01-9		3.0	84.8	>26.0

Entry No.	CID SID Broad ID	Structure	PDI_Inhibition _ IC_{50} (uM)†	PBS Solubility (uM)	Cytotoxicity IC_{50} (uM)* HEK 293/ HepG2/ HeLa
6	70701252 160843244 BRD-K18880881-001-01-2		4.0	78.2	>26.0
7	70701258 160843262 BRD-K26875871-001-01-4		>30	76.6	>26.0
8	70701239 160843247 BRD-K78438862-001-01-9		22.5	12.0	>26.0

† Average of two independent experiments (PubChem AIDs 652146, 652199). *For each analogue, cytotoxicity was measured against HEK293, HepG2 and HeLa cell lines and IC_{50} was determined to be >26.0 uM (highest concentration measured) for each cell line (PubChem AIDs 652119, 652118, 652117).

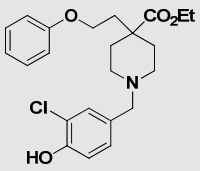
Table 5. Selectivity profile of **ML359** and selective analogues

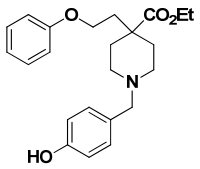
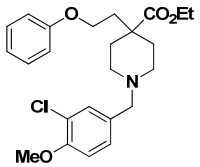
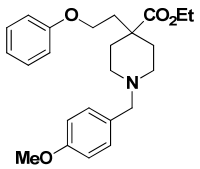
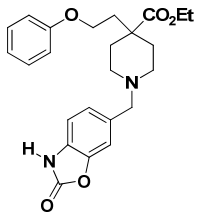
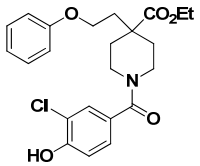
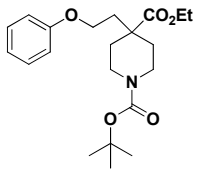
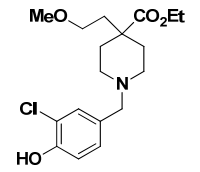
Entry No.	CID SID Broad ID	Structure	PDI_ Inhibition_ IC_{50} (uM)	*Counter Screen: Selectivity against other thiol isomerases* IC_{50} (uM)			
				ERp5*	Thioredoxin	Thioredoxin reductase	ERp57
1 (hit)	23723882 160843258 BRD-K20754212-001-10-6 **ML359**		0.25	>30	>30	>30	>30
2	70701237 160843264 BRD-K31155394-001-01-2		0.65	>30	>30	>30	>30
3	70701242 160843255 BRD-K71569005-001-01-6		0.85	>30	>30	>30	>30
4	70701262 160843259 BRD-K10350688-001-01-9		3.0	N.D	>30	>30	>30
5	70701238 160843250 BRD-K31070838-001-01-7		2.25	N.D	>30	>30	>30
6	23723959 160843243 BRD-K26639447-001-07-6		15.0	N.D	>30	>30	>30

Entry No.	CID SID Broad ID	Structure	PDI_ Inhibition_ IC_{50} (uM)	*Counter Screen: Selectivity against other thiol isomerases* IC_{50} (uM)			
				ERp5*	Thioredoxin	Thioredoxin reductase	ERp57
7	70701239 160843247 BRD-K78438862-001-01-9		22.5	N.D	>30	>30	>30
8	70701252 160843244 BRD-K18880881-001-01-2		4.0	N.D	>30	>30	>30
9	70701251 160843238 BRD-K13659644-001-01-3		>30	N.D	>30	>30	>30

*N.D: not determined. PubChem AIDs 652146, 652199, 624320, 652270. 652269, 652214, 652266

Table 6. Liver Microsome Stability of the Probe (**ML359**) and selected analogues

Entry No.	*Structure*	*PubChem CID*	*Microsomal Stability (% remaining at 1 h)*	
			Human	Murine
1		**23723882 (ML359)**	1.0	1.2

Entry No.	*Structure*	*PubChem CID*	*Microsomal Stability (% remaining at 1 h)*	
			Human	**Murine**
2		23723959 (Analog)	4.3	2.2
3		70701251 (Analog)	0.7	0.0
4		23723973 (Analog)	0.3	0.9
5		70701241 (Analog)	2.7	1.0
6		70701238 (Analog)	0.5	0.0
7		70701267 (Analog/ Starting material)	0.8	0.0
8		42515470 (Analog)	**70.7**	0.0

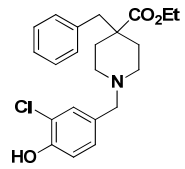
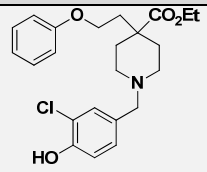
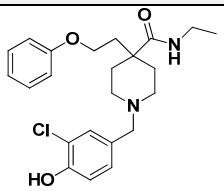
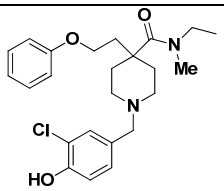
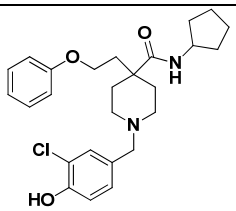
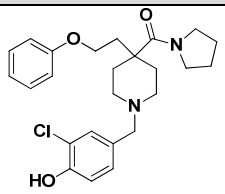
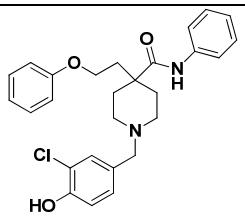
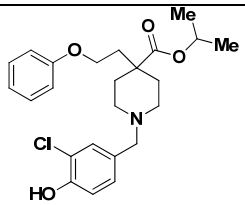
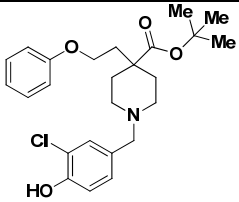
Entry No.	*Structure*	*PubChem CID*	*Microsomal Stability (% remaining at 1 h)*	
			Human	**Murine**
9		25298231 (Analog)	2.0	0.0

Table 7. Plasma Protein Binding and Plasma Stability of **ML359** and selected analogues

Entry No.	CID	Structure	**Plasma stability (% remaining after 5 hours)**		**Plasma protein binding (% bound)**		PDI_Inhibition_ IC_{50} (uM) (See Section 3.4 for details)
			Human	**Murine**	**Human**	**Murine**	
1 (hit)	23723882 **ML359**		100	5.3	98.7	96.5	0.25
2	70701263		100	97.5	79	87.2	>30
3	70701262		100	5.4	98.5	96.8	3.0
4	70701252		100	98.4	92.5	95.3	4.0

Entry No.	CID	Structure	Plasma stability (% remaining after 5 hours)		Plasma protein binding (% bound)		PDI_Inhibition_ IC_{50} (uM) (See Section 3.4 for details)
			Human	Murine	Human	Murine	
5	70701258		95.8	92.2	87.2	91.7	>30
6	70701239		89.5	93.5	98.1	97.8	22.5
7	70701242		97.9	75.3	98.9	98.5	0.85
8	70701237		98.7	95.6	99.7	99.7	0.65

3.5 Cellular Activity

A secondary assay utilizing three human cell lines (HeLa, HEK293, and HepG2) for evaluating compounds for toxicity, and two additional phenotypic secondary assays using human platelets from donors were performed. An overview of these assays is provided in **Section 2.1** and full experimental details can be found in **Appendix B**. The probe **ML359** meets the cellular activity criteria defined for this project.

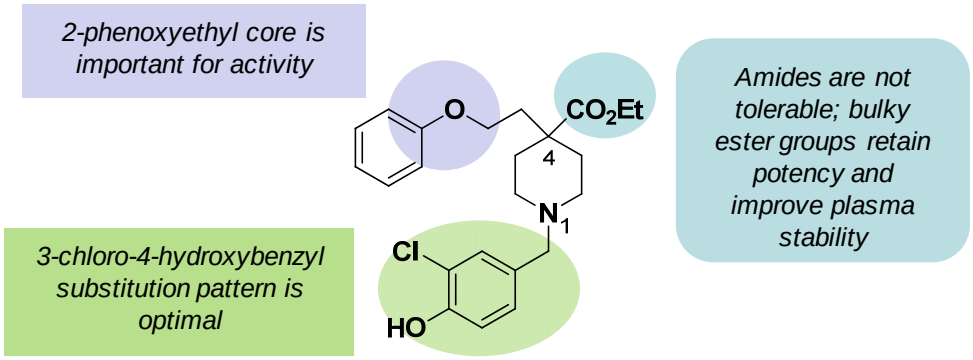
3.6 Profiling Assays

The probe **ML359** will be submitted to Eurofins lead profiling analysis assays against a broad panel of enzymes, receptors and ion channels (67 targets) to evaluate off-target activities.

4 Discussion

An insulin-based turbiometric assay in the presence of PDI was used to screen 348,505 compounds in the MLSMR collection to identify inhibitors to PDI. Several compounds were chosen for additional analysis, however after conformation of activity with dry powders and several secondary assays, CID23723882 was advanced for chemical optimization and SAR analysis. Three primary points of diversification (**Figure 5**; highlighted in green, purple and blue) were identified to initiate the early medicinal chemistry optimization. The analogs presented in **Section 3.4** for each sub-core is shown in **Figure 5**.

Figure 5. Summary of the SAR profile of hit CID23723882 (Key SAR findings for each site of diversification are provided in italics)



CID23723882: ethyl 1-(3-chloro-4-hydroxybenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate

The presence of the 3-chloro-4-hydroxybenzyl substitution pattern is essential for potency and the 2-phenoxyethyl side chain is also necessary for activity against PDI. Sterically demanding ester functionalities are tolerable and give an improved plasma stability profile. Replacement of ester substituents with various amides proved to be detrimental towards PDI inhibitory activity. In the end, **ML359** was nominated as the probe as it was the most potent PDI inhibitor and maintained excellent selectivity over other thiol isomerases. **ML359** has been listed as active in five other assays in PubChem and inactive in 410 bioassays. Hence, **ML359** is not promiscuous. Further, **ML359** is not toxic to HEK 293, HepG2, or HeLa human cell lines. The probe is also stable in PBS and GSH stability assays. Based on the available activity, selectivity, toxicity data and stability profile, **ML359** will serve as a suitable tool compound to study the role of PDI in arterial thrombosis and other important biological processes.

A comparison of bioactivities, selectivity data and the toxicity profile of the probe (**ML359**) with the predefined probe criteria specified in the chemical probe development plan is presented in **Table 8.** Based on the available data summarized in **Table 8**, the probe, **ML359**, fulfills all the goals set out at the beginning of the probe development program.

Table 8. Comparison of the Probe **ML359** to Project Criteria

No.	Property	CPDP Requirement	Probe
1	Target Activity: Inhibition of PDI in insulin-based turbidometric assay	IC_{50}<1 uM	0.25 uM
2	Selectivity: Inhibition over ERP5, Thioredoxin Reductase, Thioredoxin anti-targets	>10X selective	>100X
3	Cellular toxicity (HeLa, HEK293, HepG2)	IC_{50}≥1uM or 25X effective IC_{50}	>30 uM
4	Biological mode of action	Inhibitor to platelets aggregation, reversible	See section 4.2
4	Functional groups	Avoid functional groups which are chemically reactive, pH sensitive or hydrolytically unstable	No reactive functionality
5	Solubility	Soluble in aqueous buffer	62.7 uM in PBS (pH 7.4, 23°C)

4.1 Comparison to existing art and how the new probe is an improvement

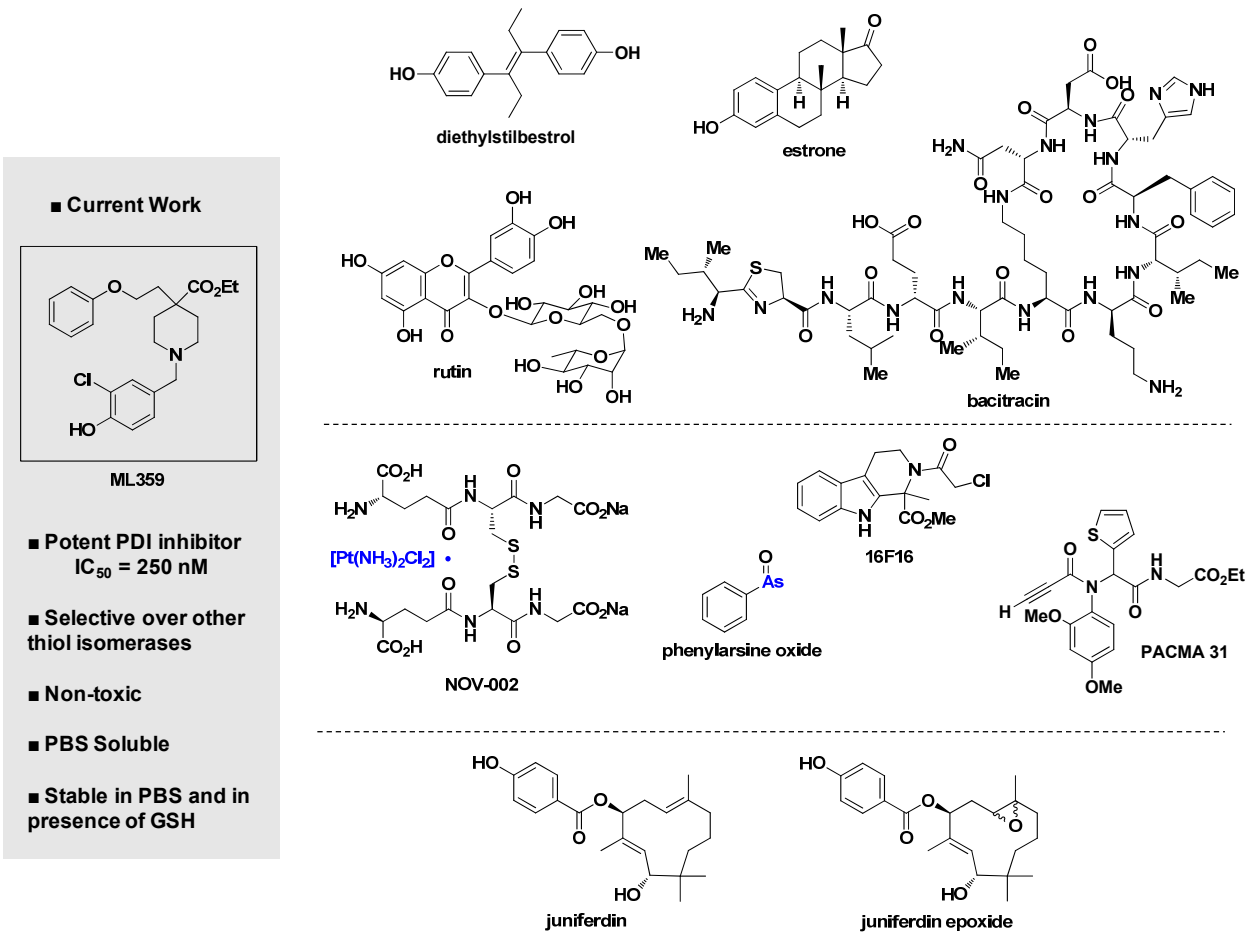
Investigation into relevant prior art entailed searching the following databases: SciFinder, PubChem, PubMed, Patent Lens databases. The search terms applied and hit statistics for the prior art search are provided in **Table 9**. Abstracts were obtained for all references returned and were analyzed for relevance to the current project. The searches were performed on April 4, and are current as of April 4, 2013.

Table 9: Prior Art Database Search

Search String (topic)	Database	Number of Hits Found
"protein disulfide isomerase inhibitors"	SciFinder	254
"PDI inhibitors"	SciFinder	338
"inhibitors of protein disulfide isomerase"	SciFinder	254
"protein disulfide isomerase inhibitors"	PubMed	282
"PDI inhibitors"	PubMed	280
"inhibitors of protein disulfide isomerase"	PubMed	282
"protein disulfide isomerase inhibitors"	PubChem	114
"PDI inhibitors"	PubChem	20
"inhibitors of protein disulfide isomerase"	PubChem	114
"protein disulfide isomerase inhibitor" (full text search)	Patent Lens	80 (>50% score)
"PDI inhibitor" (full text search)	Patent Lens	30 (>50% score)
"inhibitor of protein disulfide isomerase" (full text search)	Patent Lens	50 (>50% score)

Despite its potential importance in disease processes, selective and potent non-toxic small molecule inhibitors of PDI are lacking. Sulfhydryl blockers, antibodies, and the macrocyclic dodecapeptide antibiotic, bacitracin are the three types of inhibitors constitute the vast majority of antagonists reported in literature for studying PDI in cellular systems. Sulfhydryl blockers and antibodies to PDI have obvious liabilities and limitations (see **section 1**). Bacitracin **(Figure 6)** is a cyclic dodecapeptide widely used to inhibit PDI (2). However, it is not potent (IC_{50} of approximately 150-200 uM) and demonstrate poor selectivity, nephrotoxicity (see **section 1)** and low membrane permeability (32).

Figure 6: Structures of Small Molecule PDI Inhibitors



A small number of small molecule modulators of PDIs have been reported recently and deserve comment. First is the polyphenol natural product rutin (**Figure 6**), which was identified by the Flaumenhaft lab in a pilot screen of the Known Bioactives Collection at the Institute for Chemistry and Cell Biology, Harvard Medical School. Rutin is a flavonol glycoside found in large quantities in a variety of fruits and vegetables. It possesses decent potency against PDI (IC_{50} ~ 6 uM), and it inhibited the agonist-induced aggregation of platelets at 30 uM, however its utility is limited by its low solubility and its interaction with other reported cellular targets. Additionally, it undergoes rapid and extensive glucuronidation and sulfation, which make it undesirable for antithrombotic or other *in vivo* studies. Estrogenic compounds have been reported to be inhibitors of PDI, including diethylstilbestrol and estrone (35). Such compounds are by definition active against the estrogen receptor and other biological targets, and so are of little interest presently. NOV-2002 is a novel glutathione-disulfide mimic complexed to platinum that was

studied for use as a chemotherapeutic for lung cancer (36). It was reported to inhibit PDI, but it was also reported to be a substrate for glutathione reductase. Its likely toxicity, irreversibility, and lack of selectivity make it undesirable for antithrombotic studies.

A beta-carboline/chloroacetamide derivative ("16F16") has recently been reported by the Stockwell group (**Figure 6**) to suppress apoptosis of neurons in a Huntington's disease model (37). The target of the molecule was identified as PDI, and the target ID was facilitated by labeled analogs that reacted via the chloroamide to form covalent adducts with PDI. The Petasis and Neamati groups recently disclosed the irreversible small-molecule PDI inhibitor PACMA31 (**Figure 6**) that forms a covalent bond with the active site cysteines of PDI (28).

Very recently, Osada and coworkers at RIKEN screened a 10,000-member natural product library against PDI, and they found a number of phenolic inhibitors including juniferdin and juniferdin epoxide (**Figure 6**). We procured juniferdin and re-synthesized juniferdin epoxide and determined their IC_{50s} against PDI as 3.5 uM and 1.5 uM respectively (33). Juniferidin has significant cytotoxicity against numerous cell lines and is not commercially available in significant quantities (37). The juniferdin epoxide (**1:1** diastereomeric mixure**)** is significantly less toxic but also less selective over other thiol isomerases (ERp5 IC_{50} = 5.0 uM; thioredoxin IC_{50} = 3.0 uM*)*. Also it is unclear at this moment if its inhibition is reversible or not.

ML359 is a potent (IC_{50} = 250 nM) reversible inhibitor of PDI and demonstrates excellent selectivity over other thiol isomerases (ERp5, thioredoxin, thioredoxin reductase, ERp57). **ML359** is non-toxic and stable in PBS and GSH stability assays. **ML359** will serve as a valuable tool molecule for the scientific communities to study PDI-mediated biological processes including arterial thrombosis.

4.2 Mechanism of Action Studies

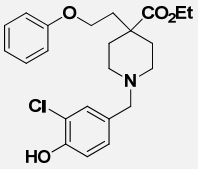
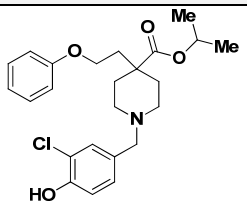
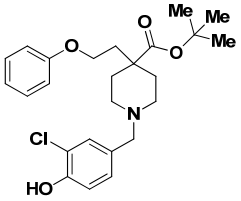
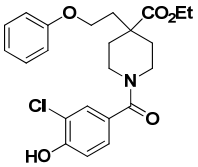
PDI is secreted by stimulated platelets where it binds the extracellular membrane, and plays a role in platelet activation, which includes the aggregation of platelets (see **Section 1**). **ML359** and some analogs were evaluated for their ability to inhibit platelet aggregation as described in **Section 2.1.9.** The probe, **ML359** only shows nominal inhibition of platelet aggregation, as compared to analogs CID 70701242, CID 70701237, and CID 70701238 (**Table 10**) and prior

art compound juniferdin (**Figure 6, PubChem AID 652249**). The threshold for activity was set for >50%.

The analogs CID 70701242, CID 70701237, and CID 70701238 that showed full platelet inhibition were further tested for reversibility by washing the platelets after incubation with the compounds. The assay is described in (**Section 2.1.10**). These studies showed that 2 of the analogs, CID 70701242 and CID 70701238 were partially reversible, showing inhibition ~45% after platelet washing, where the threshold for activity is >50%. Although the third analog CID 70701237 showed only 15% activity **(PubChem AID 652274)**, the results are questionable since that compound failed to show activity in initial platelet aggregation, prior to washing the samples (data not shown). Therefore, the activity of the compound is marked inconclusive in **Table 10**. The prior art compound juniferdin was also tested and demonstrated irreversibility in platelet aggregation in this assay with an activity of 100% after the washing the platelets **(PubChem AID 652274)**.

At this time, it is not clear why the results are inconsistent. It is important to note that the platelets aggregation assays are variable in nature as each test is performed with platelets isolated from different donors, which are washed and activated with exogenously added thrombin receptor activating peptide SFLRRN. The response seen in each batch of platelets can vary. Additional studies with multiple replicates will be required to better understand the mechanism of action of these compounds on platelets.

Table 10. Platelet Aggregation Activity and Platelet Aggregation Reversibility: Profile of **ML359**, CID70701242 and CID70701237

Entry No.	CID SID Broad ID	Structure	PDI_Inhibition _ IC_{50} (uM)	Platelet Aggregation (% inhibition at 30 uM)	Platelet Aggregation Reversibility (% inhibition at 30 uM)[#]
1 (hit)	23723882 160843258 BRD-K20754212-001-10-6 **ML359**		0.25	25	N.D
2	70701242 160843255 BRD-K71569005-001-01-6		0.85	97	Inconclusive
3	70701237 160843264 BRD-K31155394-001-01-2		0.65	100	44
10	70701238 160843250 BRD-K31070838-001-01-7		2.25	100	41

[#]N.D: Not Determined

4.3 Planned Future Studies

4.3.1. Target ID and *in vivo* mouse model studies. The Flaumenhaft lab is presently working with Dr. Mingdong Huang, Division Haemostasis and Thrombosis, Beth Israel Deaconess Medical Center, to determine the crystal structure of the probe **ML359** in complex with PDI. Dr. Huang recently established conditions for growing crystals of a PDI construct (amino acids 1-479) that contains all four PDI domains (i.e., a, a', b', b). Dr. Huang has an interest in small molecule inhibition of thiol isomerases and will develop and analyze crystals of inhibitor:PDI complexes using the probe **ML359** to compare to other PDI inhibitors, such as rutin. The structural information obtained from these studies could be used by synthetic chemists to optimize lead compounds discovered from the screen.

4.3.2. *in vivo* mouse model studies. The ability of the probe **ML359** to inhibit thrombus formation in mice following laser-induced injury of arterioles will be tested. The Flaumenhaft laboratory has considerable experience in testing compounds identified by HTS *in vivo* (38). Tolerability of compounds in mice will be first established, and then followed by determination of the ability of the compounds to inhibit laser-induced thrombus formation in mouse cremaster arterioles. Inhibition of PDI has previously been demonstrated to block both platelet accumulation and fibrin generation during thrombus formation (16). A multifluor fluorescence microscope coupled to a Sutter Lambda DG-4 high-speed wavelength changer with a changing time of 1.2 msec will be used for these studies, allowing for near simultaneous capture of both platelet and fibrin signal, thus enabling comparison of antiplatelet activity and antifibrin activities of **ML359**. Since the assay is quantitative, IC_{50} values will be determined for inhibitory compounds like **ML359**, which will allow for a direct comparison with other PDI inhibitors. Use of a reversible inhibitor of PDI like **ML359** will determine whether or not reversal of PDI inhibition following laser injury enables recovery of thrombus formation.

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Appendix A: Assay Summary Table

Table A1. Summary of Completed Assays and AIDs

PubChem AID No.	Type	Target	Concentration Range (UM)	Samples Tested
588424	Summary	NA	NA	NA
588391	Primary	PDI	10uM	348,505
602350	Cherry Pick	PDI	20uM-0.009uM	441
624274	Cherry Pick	PDI	20uM-0.009uM	441
624274	Cherry Pick	HeLa Cells	38uM-0.005uM	442
624310	Dry Powder	PDI	20uM-0.009uM	14
652146	Dry Powder	PDI	33.3uM-0.015uM	29
652199	Dry Powder	PDI	33.3uM-0.015uM	12
624311	Dry Powder	PDI	20uM-0.009uM	14
624320	Dry Powder	ERp5	20uM-0.009uM	14
652270	Dry Powder	ERp5	33.3uM-0.015uM	4
652269	Dry Powder	Thioredoxin Reductase	33.3uM-0.015uM	13
652214	Dry Powder	Thioredoxin	33.3uM-0.015uM	12
652249	Dry Powder	Platelet Aggregation	30uM	12
652274	Dry Powder	Platelet Aggregation Reversibility	30uM	4
652119	Dry Powder	HEK293 Cells	26uM-0.004uM	29
652118	Dry Powder	HepG2 Cells	26uM-0.004uM	29
652117	Dry Powder	HeLa Cells	26uM-0.004uM	29
652266	Dry Powder	ERp57	33.3uM-1.2uM	12

NA= not applicable

Appendix B: Detailed Assay Protocols

Turbidometric primary and reconfirmation HTS to detect the inhibition of insulin aggregation in the presence of PDI (AID 588391, 602350, 624274, 624310, 652146, 652199)

Materials and Reagents

- Human PDI: expressed and purified from *E. coli* by the Flaumenthaft Laboratory and aliquoted at a concentration of 1.85 mg/mL
- Bovine insulin (Sigma): reconstituted to 10 mg/mL with 1N HCl
- DTT (Sigma): prepared as a 1M stock solution in water
- Rutin Hydrate (Sigma): used as a positive control, reconstituted to 20 mM in DMSO
- Assay Buffer: 100 mM Potassium Phosphate, pH 7.0; 2 mM EDTA
- Aurora, 1536-well, clear-bottomed plates, with 7.5nl or 15nl of compounds previously added using an Echo Liquid Handler (Labcyte). The final concentration of compounds in these assay ready plates (ARPs) was 10uM for the primary assay or 20 uM with 8, 3-fold serial dilutions for retests.
- Nunc 384-well, clear-bottomed plates

Procedures:

Primary Assay (Broad):

1. Prepare reagents:
    a. PDI/Insulin Mix: Reconstituted insulin is first diluted 1:14 with assay buffer to a concentration of 125 uM. The insulin solution is then used to dilute PDI 1:231, for a final concentration of 8 ug/mL
    b. DTT: The 1M stock solution is diluted to 5.6mM in assay buffer
    c. Rutin Hydrate: The 20 mM stock solution is diluted to 1.6 mM with assay buffer
2. Dispense 7.0 uL of the PDI/Insulin mix to all wells of the 1536-well ARPs, and 0.5 uL of 1.6mM Rutin Hydrate to 128 designated positive control wells simultaneously, using a BioRAPTR (Beckman) liquid handler. The final concentration of Rutin Hydrate is 100 uM
3. Dispense 0.5 uL 5.6 mM DTT to all wells, using a Thermo Multidrop Combi nL, to a final concentration of 350 uM
4. The plates are incubated for 90 min. at room temperature
5. The plates are then read for absorbance at 650 nm, using a PerkinElmer Envision

Reconfirmation Assay at dose, from DMSO stocks (Broad):

1. Prepare reagents:
    a. PDI/Insulin Mix: Reconstituted insulin is first diluted 1:14 with assay buffer to a concentration of 125 uM. The insulin solution is then used to dilute PDI 1:231, for a final concentration of 8 ug/mL
    b. DTT: The 1 M stock solution is diluted to 5.6 mM in assay buffer
    c. Rutin Hydrate: The 20 mM stock solution is diluted to 1.6 mM with assay buffer

2. Dispense 7.0 uL of the PDI/Insulin mix to all wells of 1536-well ARPs, and 0.5 uL of 1.6 mM Rutin Hydrate to designated positive control wells simultaneously, using a BioRAPTR (Beckman) liquid handler. The final concentration of Rutin Hydrate is 100 uM
3. Dispense 0.5 uL of 5.6 mM DTT to all wells, using a Thermo Multidrop Combi nL, to a final concentration of 350 uM
4. The plates are incubated for 90 min. at room temperature
5. The plates are then read for absorbance at 650 nm, using a PerkinElmer Envision

Reconfirmation Assay at dose, from dry powders (Broad):

1. Prepare reagents:
    a. PDI/Insulin Mix: Reconstituted insulin is first diluted 1:14 with assay buffer to a concentration of 125 uM. The insulin solution is then used to dilute PDI 1:231, for a final concentration of 8 ug/mL
    b. DTT: The 1 M stock solution is diluted to 10.5 mM in assay buffer
    c. Bacitracin (From Enzo Life Sciences' ProteoStat™ PDI assay kit (catalog # ENZ-51024-KP002)): Positive control, used in place of Rutin Hydrate
2. Dispense 30 uL of the PDI/Insulin mix to 384-well plates by hand pipet
3. Dispense 1 uL DTT to wells, by hand pipet. The final concentration of DTT is 350 uM
4. Compounds are arrayed at dose, and are added by pin transfer of 100 nL into an assay volume of 31uL. The final concentration of compounds ranges from 20 uM to 1 pM. Bacitracin is also added to designated positive control wells, at a final concentration of 1 mM
5. The plates are incubated for 90 min. at room temperature
6. The plates are then read for absorbance at 650 nm, using a PerkinElmer Envision

Cytotoxicity in HEK293 cells (AID 652119)

Assay Protocol:

1. Day 0, HEK293 cells grown in Triple flask (NUNC) to ~95% confluence (TrypLE Phenol Red free) and resuspended for dispensing at 125,000 cells/mL of DMEM, 10% FBS/Pen/Strep/L-Glutamine (Compact SelecT).
2. Day 1: Plate cells @5000 per well in 40 uL media (DMEM/10% FBS/Pen/Strep/L-Glutamine) using Corning 8867BC 384 well plates; incubate in standard TC conditions (5% CO_2; 95% humidity, 37°C) for 24 hours (Compact SelecT).
3. Day 2: Add 100 nL compound per well at dose into 40 uL assay volume using a pin tool (CyBi Well). Pin 100 nL cytotoxic compounds, Staurosporine to positive control wells to a final concentration of 10 uM (100 nL 4 mM DMSO stock).. Incubate for 48 hours at 37°C in Liconic incubator, 95% humidity 5% CO_2.

4. Day 4: Remove plate from incubator to cool for 15 minutes to room temperature; add 20 uL 50% Promega CellTiterGlo (diluted 1:1 with PBS, pH 7.4) with Thermo Combi.
5. Incubate at RT for 5 minutes.
6. Read on Perkin-Elmer EnVision with US LUM settings for 0.1 sec per well.

Cytotoxicity in HepG2 cells (AID 652118)

Assay Protocol:

1. Day 0, HepG2 cells grown in Triple flask (NUNC) to ~95% confluence (TrypLE Phenol Red free) and resuspended for dispensing at 125,000 cells/mL of DMEM, 10% FBS/Pen/Strep/L-Glutamine (Compact SelecT).
2. Day 1: Plate cells @5000 per well in 40 uL media (DMEM/10% FBS/Pen/Strep/L-Glutamine) using Corning 8867BC 384 well plates; incubate in standard TC conditions (5% CO_2; 95% humidity, 37°C) for 24 hours (Compact SelecT).
3. Day 2: Add 100 nL compound per well at dose into 40 uL assay volume using a pin tool (CyBi Well). Pin 100 nL cytotoxic compounds, Staurosporine to positive control wells to a final concentration of 10 uM (100 nL 4 mM DMSO stock).. Incubate for 48 hours at 37°C in Liconic incubator, 95% humidity 5% CO_2.
4. Day 4: Remove plate from incubator to cool for 15 minutes to room temperature; add 20 µL 50% Promega CellTiter-Glo (diluted 1:1 with PBS, pH 7.4) with Thermo Combi.
5. Incubate at RT for 5 minutes.
6. Read on Perkin-Elmer EnVision with US LUM settings for 0.1 sec per well.

Cytotoxicity in HeLa cells (AID 652117)

Assay Protocol:

1. Day 0, HeLa cells grown in Triple flask (NUNC) to ~95% confluence (TrypLE Phenol Red free) and resuspended for dispensing at 125,000 cells/mL of DMEM, 10% FBS/Pen/Strep/L-Glutamine (Compact SelecT).
2. Day 1: Plate cells @5000 per well in 40 uL media (DMEM/10% FBS/Pen/Strep/L-Glutamine) using Corning 8867BC 384 well plates; incubate in standard TC conditions (5% CO_2; 95% humidity, 37°C) for 24 hours (Compact SelecT).
3. Day 2: Add 100 nL compound per well at dose into 40 uL assay volume using a pin tool (CyBi Well). Pin 100 nL cytotoxic compounds, Staurosporine to positive control wells to a final concentration of 10 uM (100 nL of 4 mM DMSO stock). Incubate for 48 hours at 37°C in Liconic incubator, 95% humidity 5% CO_2.
4. Day 4: Remove plate from incubator to cool for 15 minutes to room temperature; add 20 uL 50% Promega CellTiterGlo (diluted 1:1 with PBS, pH 7.4) with Thermo Combi.
5. Incubate at RT for 5 minutes.

Selectively screen to determine compound activity against ERp5 (AID 624320)

Materials and Reagents

- Human ERp5: received from the Flaumenthaft Laboratory and aliquoted at a concentration of 5.3 mg/mL
- Bovine insulin (Sigma): reconstituted to 10 mg/mL with 1N HCl
- DTT (Sigma): prepared as a 1M stock solution in water
- Assay Buffer: 100 mM Potassium Phosphate, pH 7.0; 2 mM EDTA
- Bacitracin (From Enzo Life Sciences' ProteoStat™ PDI assay kit (catalog # ENZ-51024-KP002)): Positive control

Reconfirmation Assay at dose, from DMSO stocks (Broad):

1. Prepare reagents:
    a. ERp5/Insulin Mix: Reconstituted insulin is first diluted with assay buffer to a concentration of 142 uM. The insulin solution is then used to dilute ERp5 1:200, for a final concentration of 26.5 ug/mL
    b. DTT: The 1 M stock solution is diluted to 5.6 mM in assay buffer
    c. Bacitracin
2. Dispense 30 uL of the ERp5/Insulin mix to all wells of 384-well plates
3. Add compounds via pin transfer, at 100 nL. Compounds are arrayed at dose so that final concentrations range from 20 uM to 0.017 pM. Add Bacitracin positive control to designated wells, at a final concentration of 1mM
4. Dispense 2 uL 5.6 mM DTT to all wells, using a Thermo Multidrop Combi nL, to a final concentration of 350 uM
5. The plates are incubated for 120 min. at room temperature
6. The plates are then read for absorbance at 650nm, using a PerkinElmer Envision

Selectively screen to determine compound activity against Thioredoxin Reductase (AID 652269)

Materials and Reagents

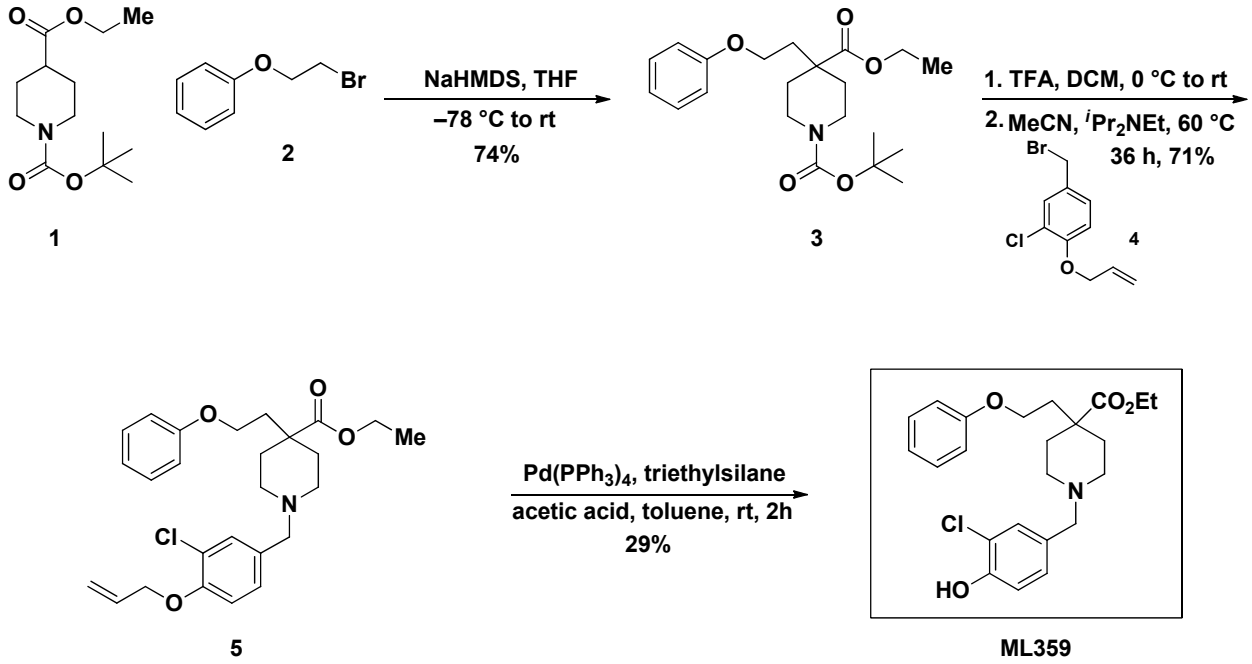
- Rat recombinant thioredoxin reductase I (IMCO Corp, Stockholm, Sweden)
- Human recombinant His-tagged thioredoxin-1 (R & D Systems, Minneapolis, MN)
- NADPH (Sigma, St. Louis, MO)
- Bovine insulin (Sigma, St. Louis, MO)
- DTT (Sigma, St. Louis, MO)
- Assay buffer: 100 mM sodium phosphate, 2 mM EDTA (pH 7.4)
- Compounds diluted to 1 mM in 50%DMSO/50% H_2O

Procedures:

1. Thioredoxin reductase (140 nM) is pre-incubated with compound (at concentrations of 0.005, 0.015, 0.046, 0.137, 0.411, 1.23, 3.7, 11.1 and 33.33 uM) or control buffer for 30 min at RT in 96-well plates.
2. NADPH is then added at a concentration of 0.12 mM, followed by thioredoxin 0.83 uM and insulin 100 uM in assay buffer. The final reaction volume is 100 uL.
3. The precipitation of insulin B chains is monitored as at OD 650nm. As background, insulin plus thioredoxin-1/ NADPH alone (no thioredoxin reductase) is used. As positive control, insulin reduction by thioredoxin/ DTT is used.

Appendix C: Experimental Procedures for the Synthesis of the Probe

General Details. All reagents and solvents were purchased from commercial vendors and used as received. NMR spectra were recorded on a Bruker 300 MHz or Varian UNITY INOVA 500 MHz spectrometer as indicated. Proton, fluorine, and carbon chemical shifts are reported in parts per million (ppm; δ) relative to tetramethylsilane or $CDCl_3$ solvent (^{1}H δ 0, ^{19}F δ 0, ^{13}C δ 77.16, respectively). NMR data are reported as follows: chemical shifts, multiplicity (obs = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25°C. Flash chromatography was performed using 40-60 um Silica Gel (60 Å mesh) on a Teledyne Isco Combiflash Rf system. Tandem liquid chromatography/mass spectrometry (LCMS) was performed on a Waters 2795 separations module and Waters 3100 mass detector. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and aqueous potassium permanganate ($KMnO_4$) stain followed by heating. Liquid chromatography/mass spectrometry (LCMS) was performed on an Agilent 1290 Infinity separations module and 6230 time-of-flight (TOF) mass detector operating in ESI+ mode. Compound purity and identity were determined by UPLC-MS (Waters, Milford, MA). Purity was measured by UV absorbance at 210 nm. Identity was determined on a SQ mass spectrometer by positive electrospray ionization. Mobile Phase A consisted of either 0.1% ammonium hydroxide or 0.1% trifluoroacetic acid in water, while mobile Phase B consisted of the same additives in acetonitrile. The gradient ran from 5% to 95% mobile Phase B over 0.8 minutes at 0.45 mL/min. An Acquity BEH C18, 1.7 um, 1.0 x 50 mm column was used with column temperature maintained at 65°C. Compounds were dissolved in DMSO at a nominal concentration of 1 mg/mL, and 0.25 uL of this solution was injected.



1 2 3 4 5 ML359

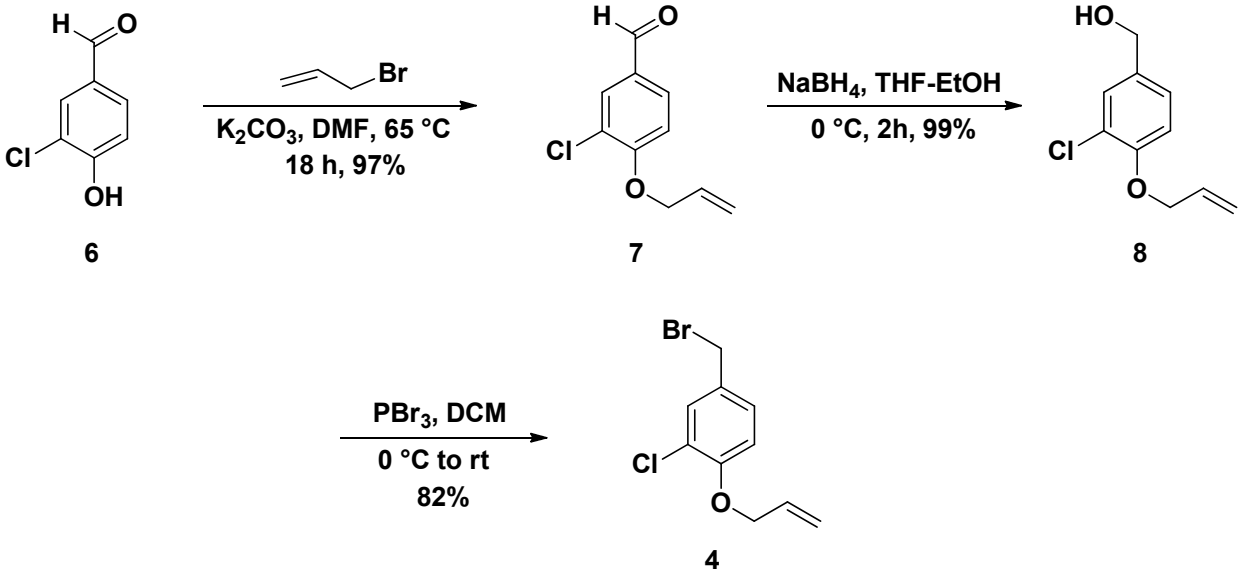
1-tert-butyl 4-ethyl 4-(2-phenoxyethyl)piperidine-1,4-dicarboxylate (3): To a solution of 1-tert-butyl 4-ethyl piperidine-1,4-dicarboxylate (10.2 g, 39.6 mmol) in anhydrous THF (60 mL) at –78°C under nitrogen atmosphere was added NaHMDS (1 M solution, 51.5 mL, 51.5 mmol) dropwise. The mixture was stirred at –78°C for 10 min, warmed to rt for 10 min, and stirred at –78°C for 20 minutes. 2-bromoethoxybenzene (9.56 g, 47.6 mmol) was added in one portion. The yellow solution was stirred at –78°C for 30 min then warmed to rt. The mixture was stirred at rt for 16 h. The reaction was quenched by slow addition of satd aq NH_4Cl solution (50 mL) and the mixture was then diluted with water (100 mL), extracted with ethyl acetate (3 x 80 mL). The combined organic layer was washed with brine (80 mL, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography over 80 g of silica and eluted with ethyl acetate/hexane (0-20%) to provide the title compound (11.4 g, 74% yield) as yellow oil. **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.27 (m, 2H), 6.93 (m, 1H), 6.84 (m, 2H), 4.15 (m, 3H), 3.98 (t, *J*= 6.6 Hz, 2H), 3.86 (m, 2H), 2.94 (m, 2H), 2.15 (m, 2H), 2.05 (m, 3H), 1.44 (s, 9H), 1.21 (t, *J*=7.1 Hz, 3H). **MS** (ESI^+): 278.2 (M+H–100).

Ethyl 1-(4-(allyloxy)-3-chlorobenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate (5): To a solution of 1-tert-butyl 4-ethyl 4-(2-phenoxyethyl)piperidine-1,4-dicarboxylate (1.6 g, 4.33 mmol) in anhydrous methylene chloride (10 mL) at 0°C under nitrogen atmosphere was added trifluoroacetic acid (1.5 mL, 26.5 mmol) dropwise. The mixture was stirred at 0°C for 1 h then at rt for 1 h. The solvent was removed under reduced pressure and then evaporated with toluene

(3 x 10 mL). The crude product was put under high vacuum pump for 1 h. The crude oil was used in the next step without further purification.

To a solution of piperidine carboxylate (1.2 g, 4.33 mmol) in anhydrous acetonitrile (20 mL) was added 1-(allyloxy)-4-(bromomethyl)-2-chlorobenzene (1.7 g, 6.50 mmol) and *N*,*N*-diisopropylethylamine (2.3 mL, 13.00 mmol). The mixture was stirred at 60°C for 36 h. The mixture was cooled down to rt and was diluted with water (50 mL), extracted with ethyl acetate (3 x 40 mL). The combined organic layer was washed with brine (30 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography over 40 g of silica and eluted with ethyl acetate/hexane (10-100%) to provide the title compound (1.4 g, 71% yield) as yellow oil. **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.33 (d, *J*=1.9 Hz, 1H), 7.29 (m, 2H), 7.11 (d, *J*=8.3 Hz, 1H), 6.92 (t, *J*=7.3 Hz, 1H), 6.84 (m, 3H), 6.18-5.94 (m, 1H), 5.46 (d, *J*=17.3 Hz, 1H), 5.31 (d, *J*=10.5 Hz, 1H), 4.60 (d, *J*=5.0 Hz, 2H), 4.17 (q, *J*=7.1Hz, 2H), 3.95 (t, *J*=6.8 Hz, 2H), 3.36 (s, 2H), 2.67 (s, 2H), 2.21 (d, *J*=13.5 Hz, 2H), 2.04 (t, *J*=6.8 Hz, 4H), 1.58 (m, 2H), 1.23 (t, *J*=7.1 Hz, 3H). **MS** (ESI^+): 458.3 (M+H).

Ethyl 1-(3-chloro-4-hydroxybenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate (ML359): To a solution of ethyl 1-(4-(allyloxy)-3-chlorobenzyl)-4-(2-phenoxyethyl)piperidine-4-carboxylate (0.20 g, 0.45 mmol) in anhydrous toluene (10 mL) at rt was degassed with nitrogen for 30 min and was added $Pd(PPh_3)_4$ (23 mg, 0.023 mmol), triethylsilane (0.36 mL, 2.252 mmol) and acetic acid (0.129 mL, 2.252 mmol) was added. The mixture was stirred at rt for 2 h. The mixture was diluted with ethyl acetate (30 mL), washed with satd aq $NaHCO_3$ solution (30 mL), extracted with ethyl acetate (2 x 30 mL). The combined organic layer was washed with brine (30 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography over 12 g of silica and eluted with methanol/dichloromethane (0-20%) to provide the title compound (52 mg, 29% yield) as off-white solid. **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.27 (m, 3H), 7.10 (d, *J*=8.2 Hz, 1H), 6.91 (m, 4H), 4.05 (t, *J*=7.3 Hz, 2H), 3.45 (q, *J*=7.1 Hz, 2H), 3.41 (s, 2H), 3.28 (s, 2H), 2.41 (m, 4H), 1.89 (t, *J*= 7.3 Hz, 2H), 1.57 (m, 2H), 1.18 (t, *J*=7.0 Hz, 3H). **HRMS** (ESI^+): calculated for C23H28ClNO4 [M+H] 418.1785, found 418.1781.



4-(allyloxy)-3-chlorobenzaldehyde (7): To a solution of 3-chloro-4-hydroxybenzaldehyde (5.0 g, 31.9 mmol) in anhydrous DMF (20 mL) at rt under nitrogen atmosphere was added K_2CO_3 (13.2 g, 96.0 mmol) in one portion followed by addition of allyl bromide (4.2 mL, 47.9 mmol). The mixture was stirred at 65°C for 18 hr. The mixture was cooled down to rt followed by addition of water (100 mL). The mixture was extracted with ethyl acetate (3 x 80 mL). The combined organic layer was washed with brine (80 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography over 80 g of silica and eluted with ethyl acetate/hexane (0-30%) to provide the 4-(allyloxy)-3-chlorobenzaldehyde (6.1 g, 97% yield) as colorless oil. **^{1}H NMR (300 MHz, $CDCl_3$):** δ 9.84 (s, 1H), 7.91 (s, 1H), 7.76 (d, *J*= 8.4 Hz, 1H), 7.03 (d, *J*= 8.3 Hz, 1H), 6.07 (m, 1H), 5.53 (dd, *J*= 17.1, 1.3 Hz, 1H), 5.35 (dd, *J*=17.0, 1.4 Hz, 1H), 4.71 (m, 2H). **MS** (ESI^+): 197.2 (M+H).

(4-(allyloxy)-3-chlorophenyl)methanol (8): To a solution of 4-(allyloxy)-3-chlorobenzaldehyde (4.7 g, 23.9 mmol) in THF and EtOH (10 mL/10 mL) at 0°C was added sodium borohydride (0.9 g, 23.9 mmol). The mixture was stirred at 0 °C for 2 h. The reaction was quenched by slow addition of satd aq $NaHCO_3$ solution (80 mL) at 0 °C. The mixture was extracted with ethyl acetate (3 x 60 mL). The combined organic layer was washed with water (50 mL), brine (80 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude oil was put under high vacuum for 1 h. The crude product was used in the next step without further purification. **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.35 (s, 1H), 7.16 (d, *J*= 8.4 Hz, 1H), 6.88 (d, *J*= 8.4

Hz, 1H), 6.06 (m, 1H), 5.46 (dd, *J*= 17.1, 1.5 Hz, 1H), 5.31 (dd, *J*=17.0, 1.3 Hz, 1H), 4.61 (m, 2H), 4.56 (s, 2H), 2.25 (brs, 1H).

1-(allyloxy)-4-(bromomethyl)-2-chlorobenzene (4): To a solution of (4-(allyloxy)-3-chlorophenyl) methanol (4.8 g, 24.2 mmol) in anhydrous methylene chloride (40 mL) at 0°C under nitrogen atmosphere was added PBr_3 (2.5 mL, 26.6 mmol) dropwise. The mixture was stirred at 0 °C for 30 min then at rt for 1 h. The reaction mixture was slowly poured into ice and was stirred for 30 min. The mixture was extracted with methylene chloride (3 x 50 mL). The combined organic layer was washed with brine (50 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography over 40 g of silica and eluted with ethyl acetate/hexane (0-20%) to provide the title compound (5.2 g, 82% yield) as yellow oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.42 (d, J=2.20 Hz, 1H), 7.22 (dd, *J*=2.2, 8.5Hz, 1H), 6.87 (d, *J*=8.5 Hz, 1H), 6.05 (m, 1H), 6.46 (dd, *J*=1.5, 17.3 Hz, 1H), 5.32 (*J*=1.4, 10.5 Hz, 1H), 4.62 (m, 2H), 4.41 (s, 2H). **MS** (ESI^+): 262.5 (M+H).

Appendix D: Experimental Procedure for Analytical Assays

Solubility. Solubility was determined in phosphate buffered saline (PBS) pH 7.4 with 1% DMSO. Each compound was prepared in triplicate at 100 uM in both 100% DMSO and PBS with 1% DMSO. Compounds were allowed to equilibrate at room temperature with a 350 rpm orbital shake for 18 hours. After equilibration, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. The DMSO samples were used to create a two-point calibration curve to which the response in PBS was fit.

PBS Stability. Stability was determined in the presence of PBS pH 7.4 with 1% DMSO. Each compound was prepared in triplicate at 1 uM on six separate plates and allowed to equilibrate at room temperature with a 350-rpm orbital shake for 48 hours. One plate was removed at each time point (0, 2, 4, 8, 24, and 48 hours). An aliquot was removed from each well and analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. Additionally, to the remaining material at each time point, methanol was added to force dissolution of compound (to test for recovery of compound). An aliquot of this was also analyzed by UPLC-MS.

GSH Stability. Stability was determined in the presence of PBS pH 7.4 uM and 50 uM glutathione with 1% DMSO. Each compound was prepared in duplicate at 1 uM on six separate plates and allowed to equilibrate at room temperature with a 350-rpm orbital shake for 48 hours. One plate was removed at each time point (0, 2, 4, 8, 24, and 48 hours). An aliquot was removed from each well and analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. Additionally, to the remaining material at each time point, methanol was added to force dissolution of compound (to test for recovery of compound). An aliquot of this was also analyzed by UPLC-MS.

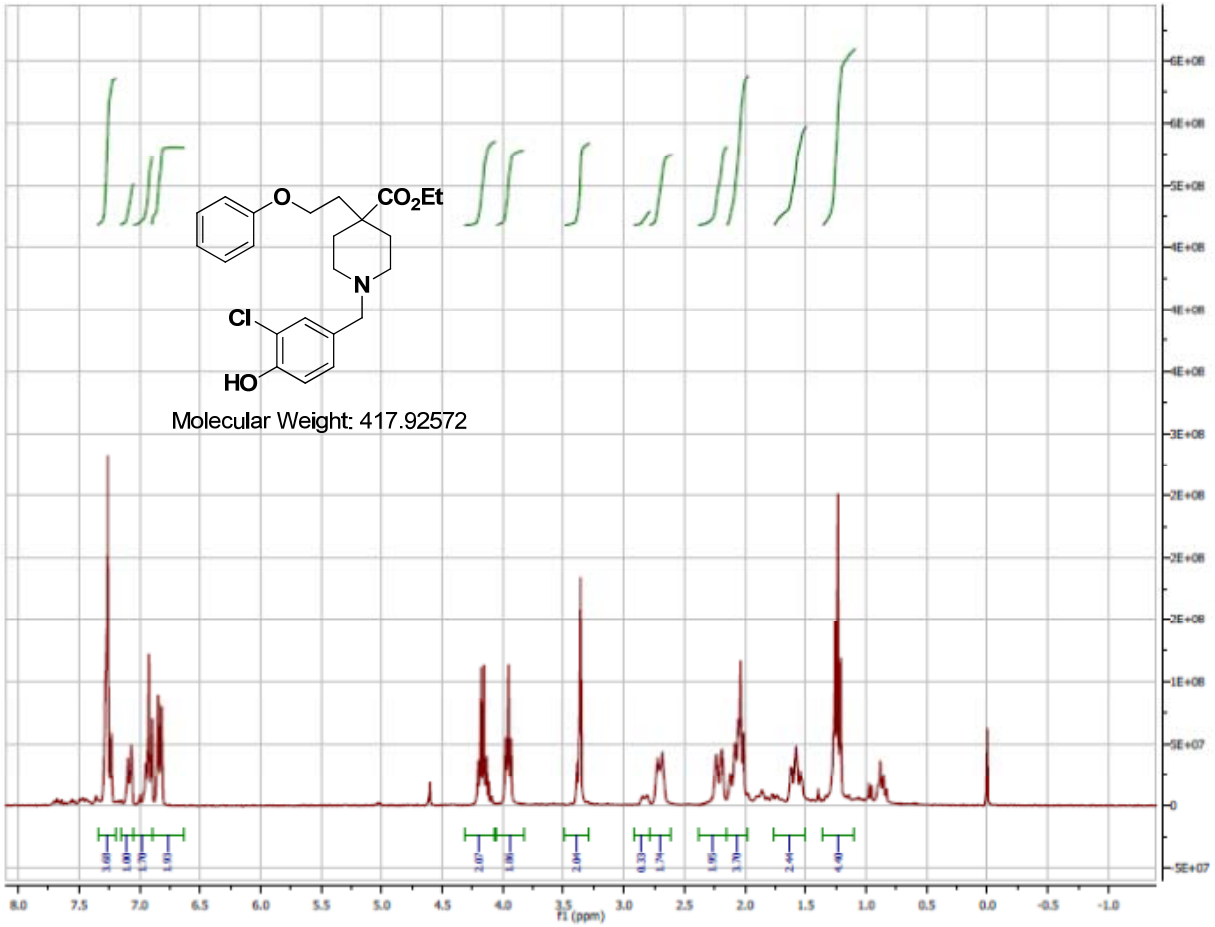
Plasma Protein Binding. Plasma protein binding was determined by equilibrium dialysis using the Rapid Equilibrium Dialysis (RED) device (Pierce Biotechnology, Rockford, IL) for both human and mouse plasma. Each compound was prepared in duplicate at 5 uM in plasma (0.95% acetonitrile, 0.05% DMSO) and added to one side of the membrane (200 uL) with PBS pH 7.4 added to the other side (350 uL). Compounds were incubated at 37ºC for 5 hours with a 250-rpm orbital shake. After incubation, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

Plasma Stability. Plasma stability was determined at 37ºC at 5 hours in both human and mouse plasma. Each compound was prepared in duplicate at 5 uM in plasma diluted 50/50 (v/v) with PBS pH 7.4 (0.95% acetonitrile, 0.05% DMSO). Compounds were incubated at 37ºC for 5 hours with a 250-rpm orbital shake with time points taken at 0 hours and 5 hours. Samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

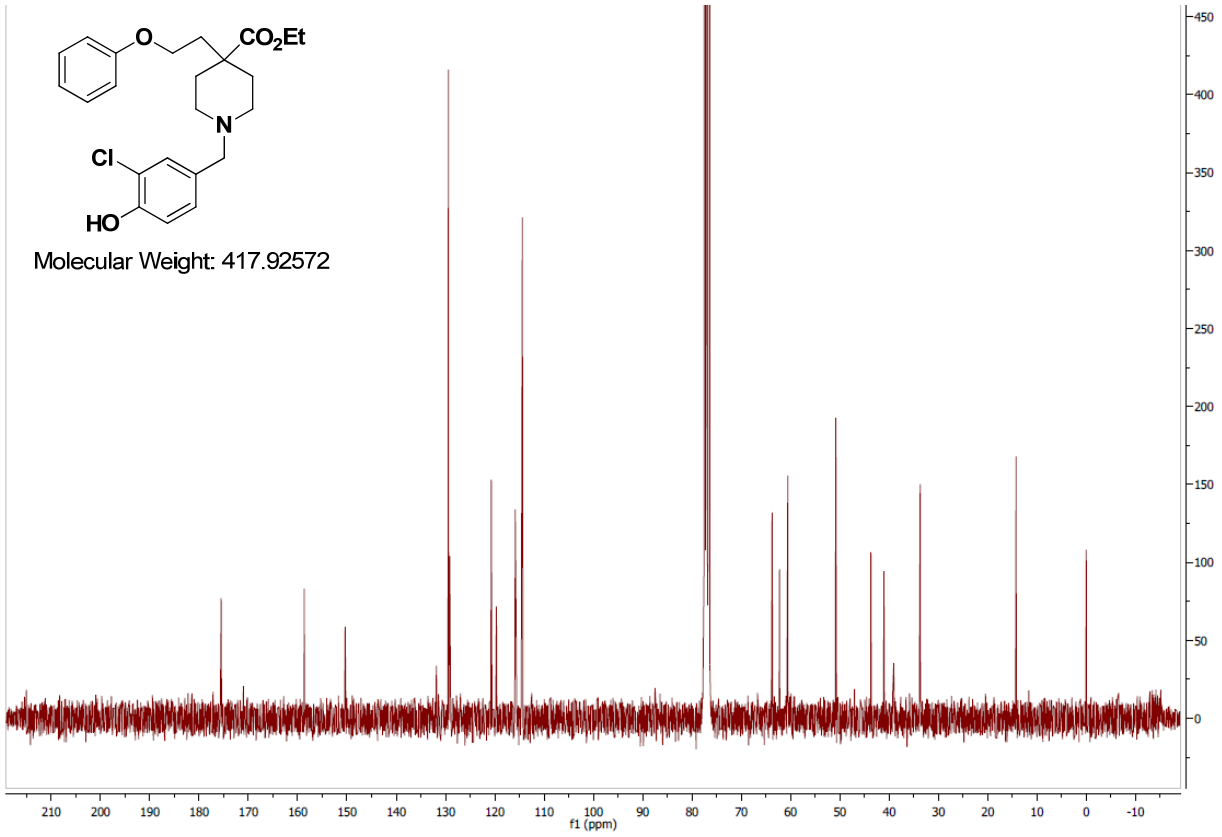
Microsomal Stability. Microsomal stability was determined by measuring degradation of the compound over 1-hour in human and/or mouse liver microsomes (Xenotech LLC, Lenexa, KS). Each compound was prepared in duplicate and added to a microsomal solution in PBS pH 7.4 so that the final concentration was 1 uM compound, 0.3 mg/mL microsomes, 0.5 mM NADPH and 1% DMSO. Compounds were incubated at 37ºC for 1 hour with 250 rpm orbital shake with time points taken at 0 hours and 1 hour. Samples were analyzed by UHPLC-MS (Agilent, Santa Clara, CA) with compounds detected on a time-of-flight mass spectrometer and analyzed using Find By Formula.

Appendix E: Chemical Characterization Data for Probe

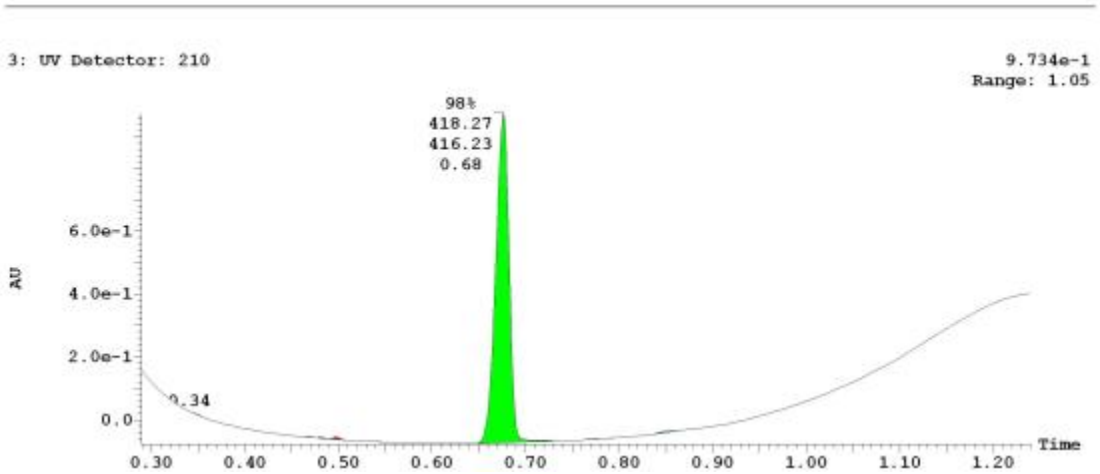
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of Probe ML359



^{13}C NMR Spectrum (75 MHz, $CDCl_3$) of Probe ML359

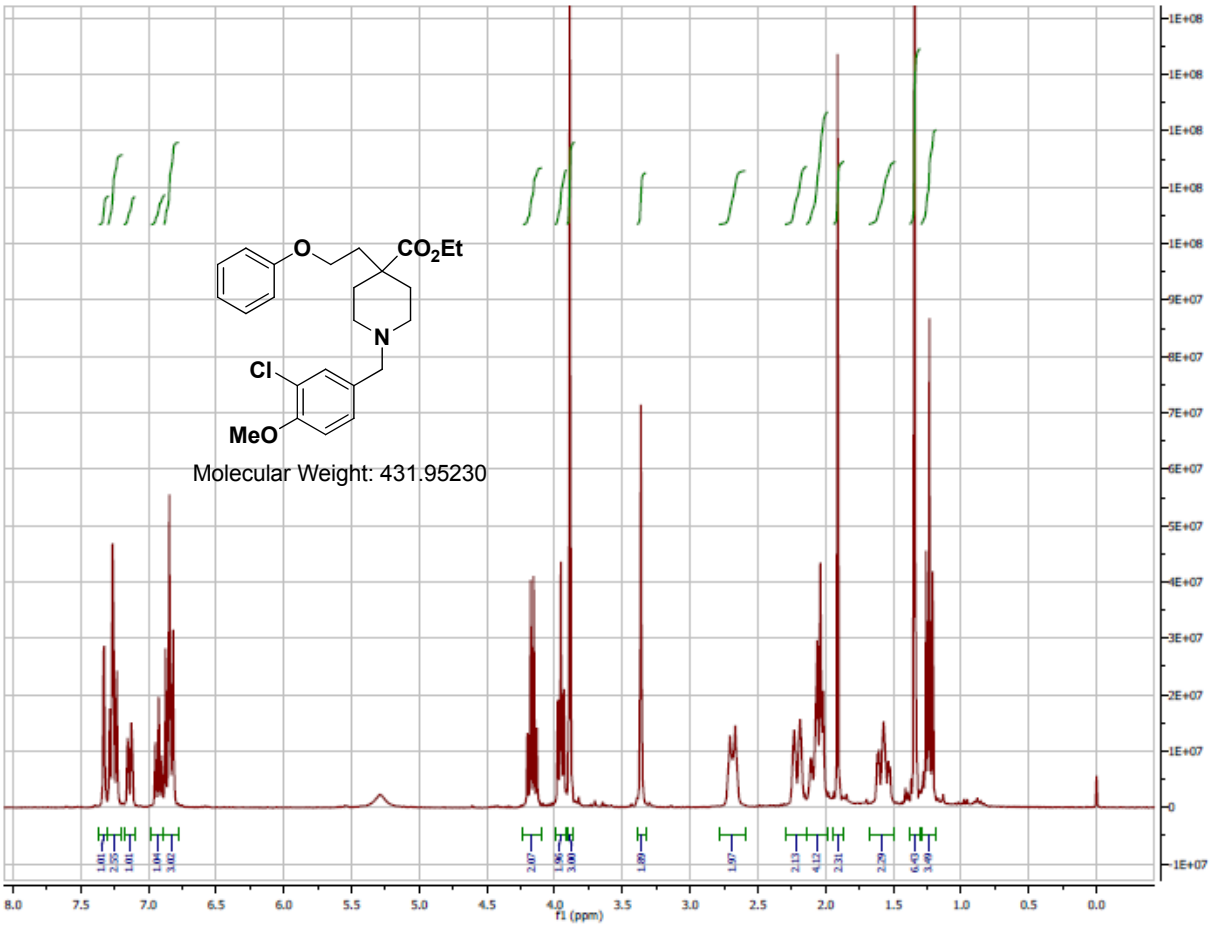


UPLC-MS Chromatogram of Probe ML359 (FW = 417.93)

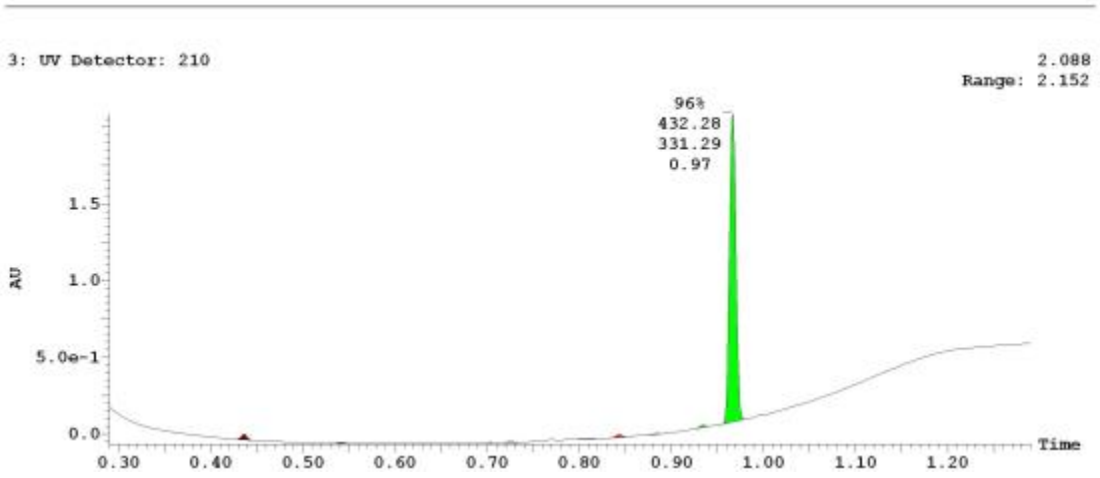


Appendix F: Chemical Characterization Data for All Analogs

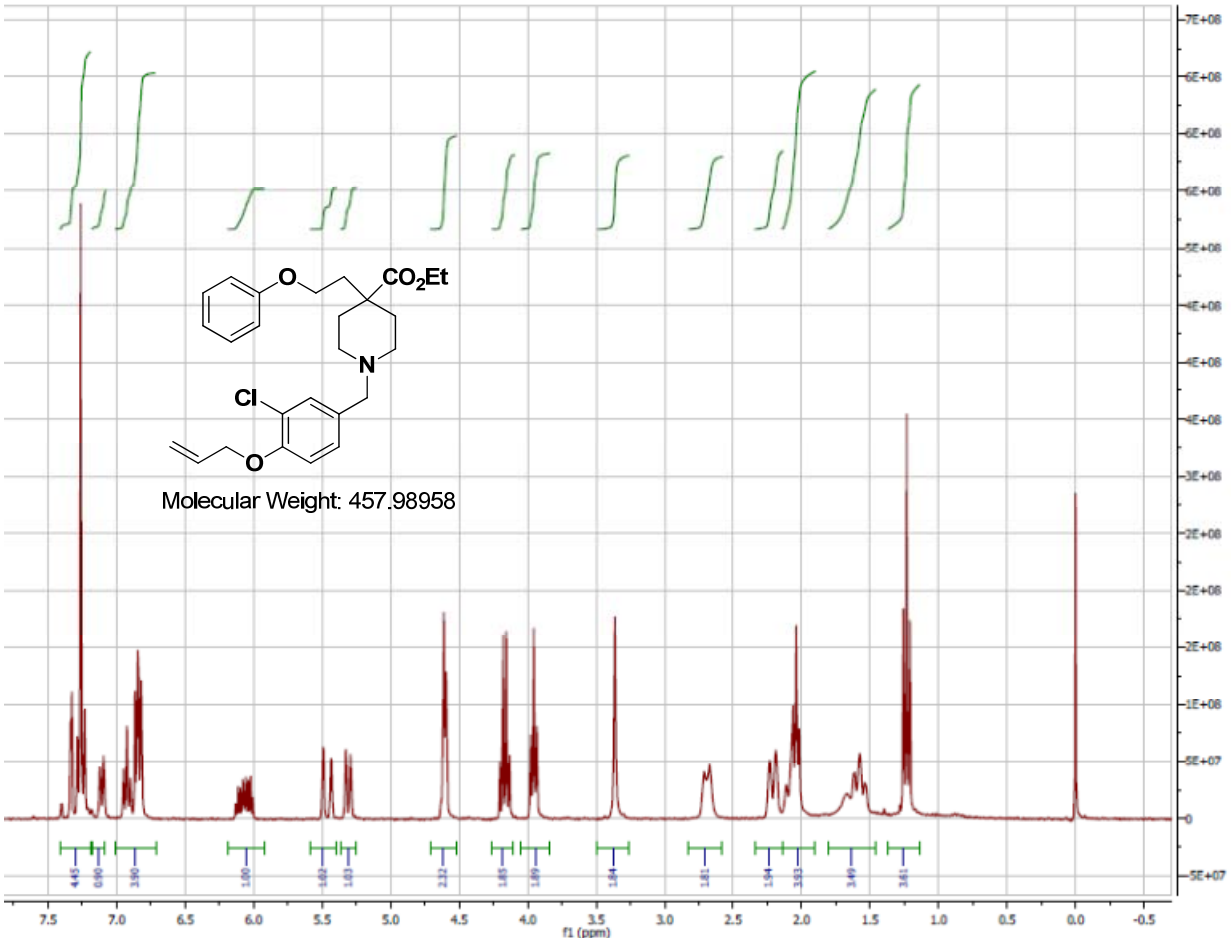
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701251



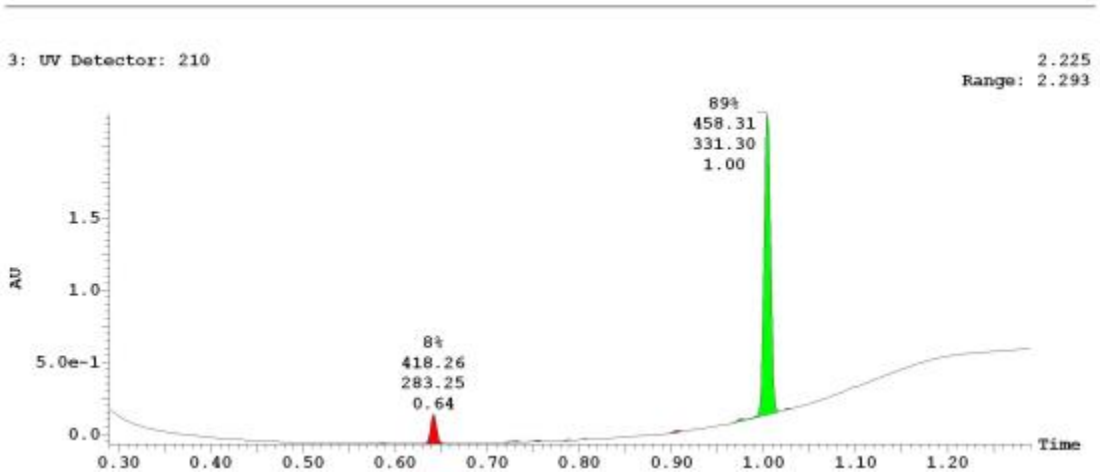
UPLC-MS Chromatogram of CID70701251 FW = 431.95



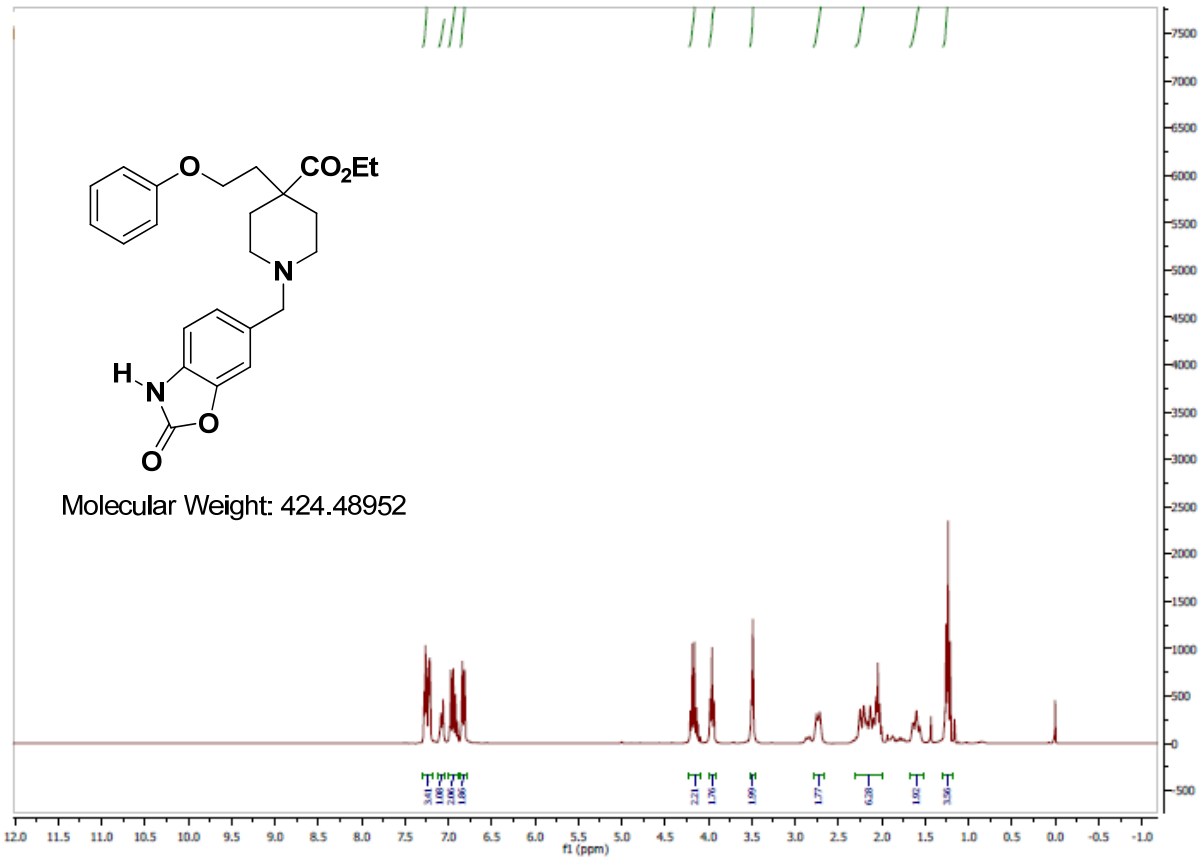
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701245



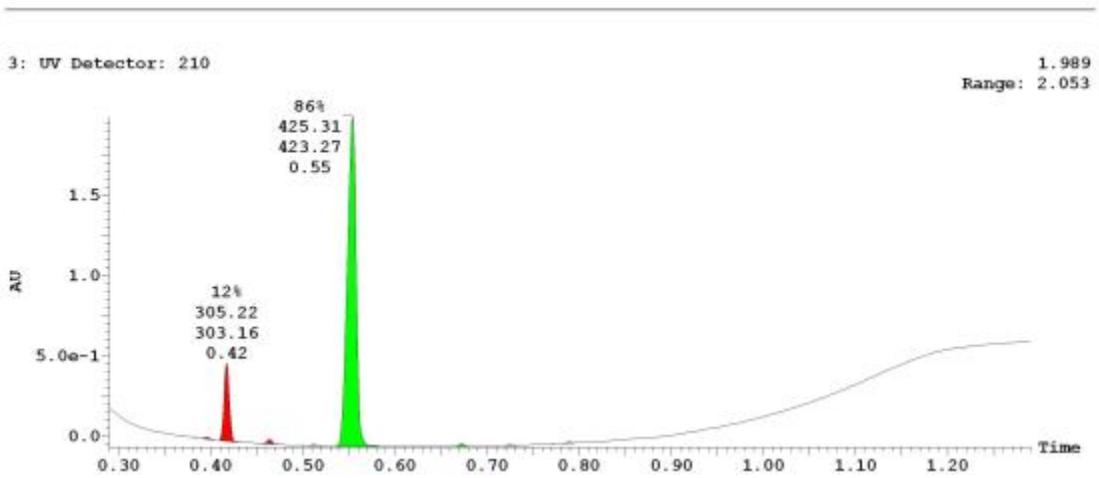
UPLC-MS Chromatogram of CID70701245 FW = 457.99



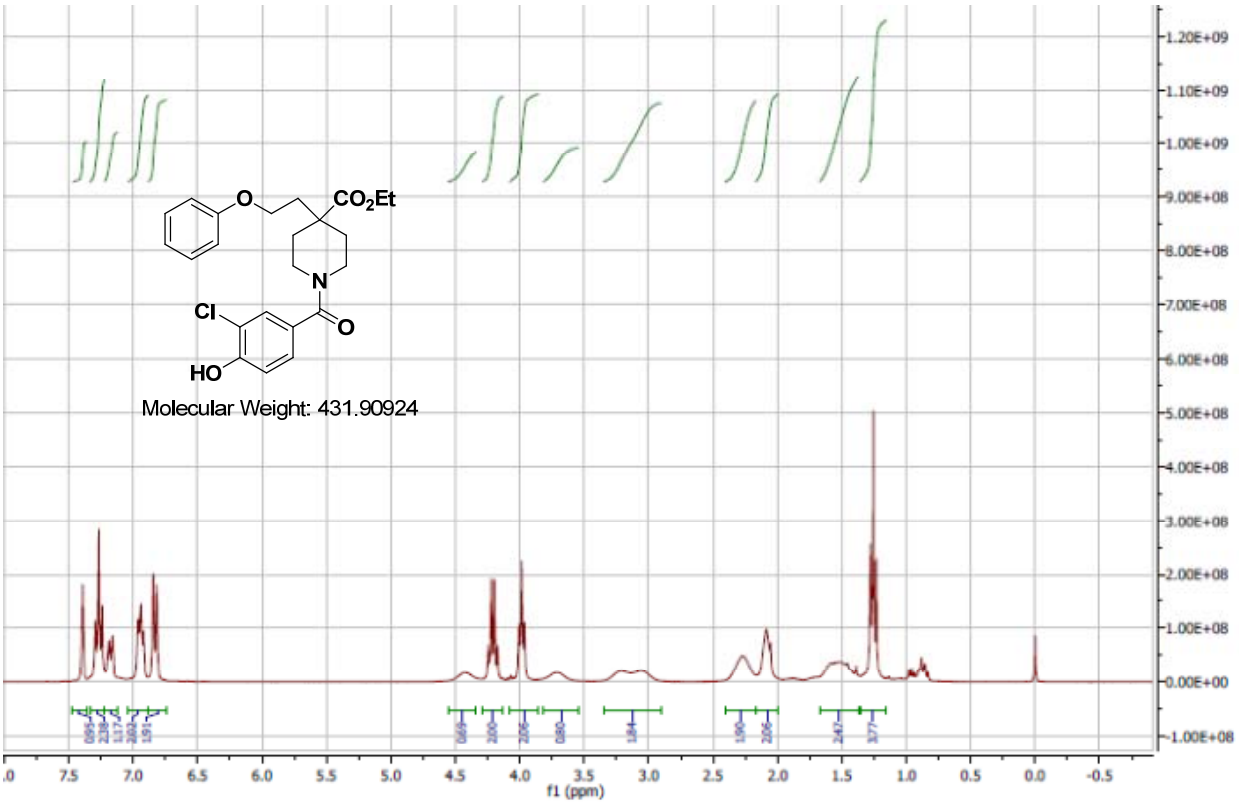
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701241



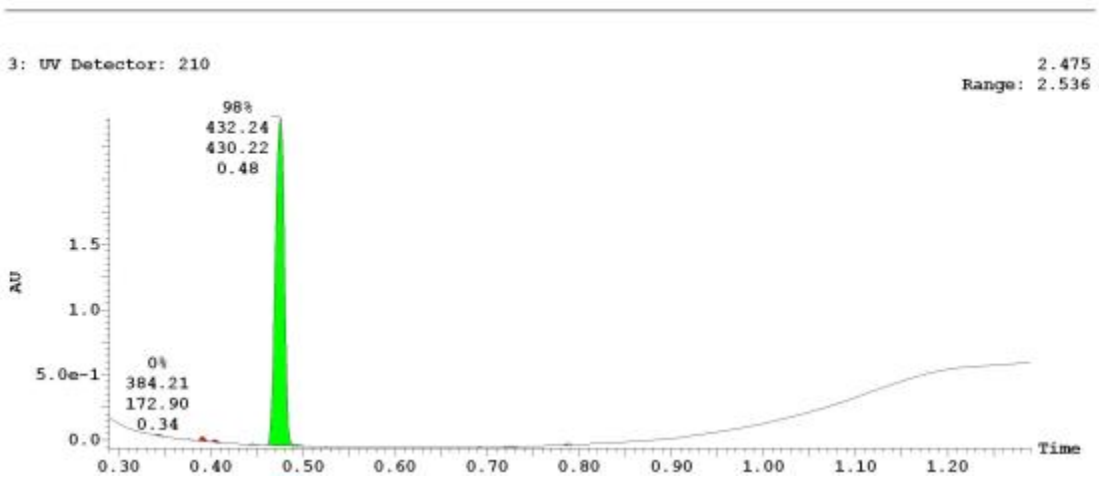
UPLC-MS Chromatogram of CID70701241 FW = 424.49



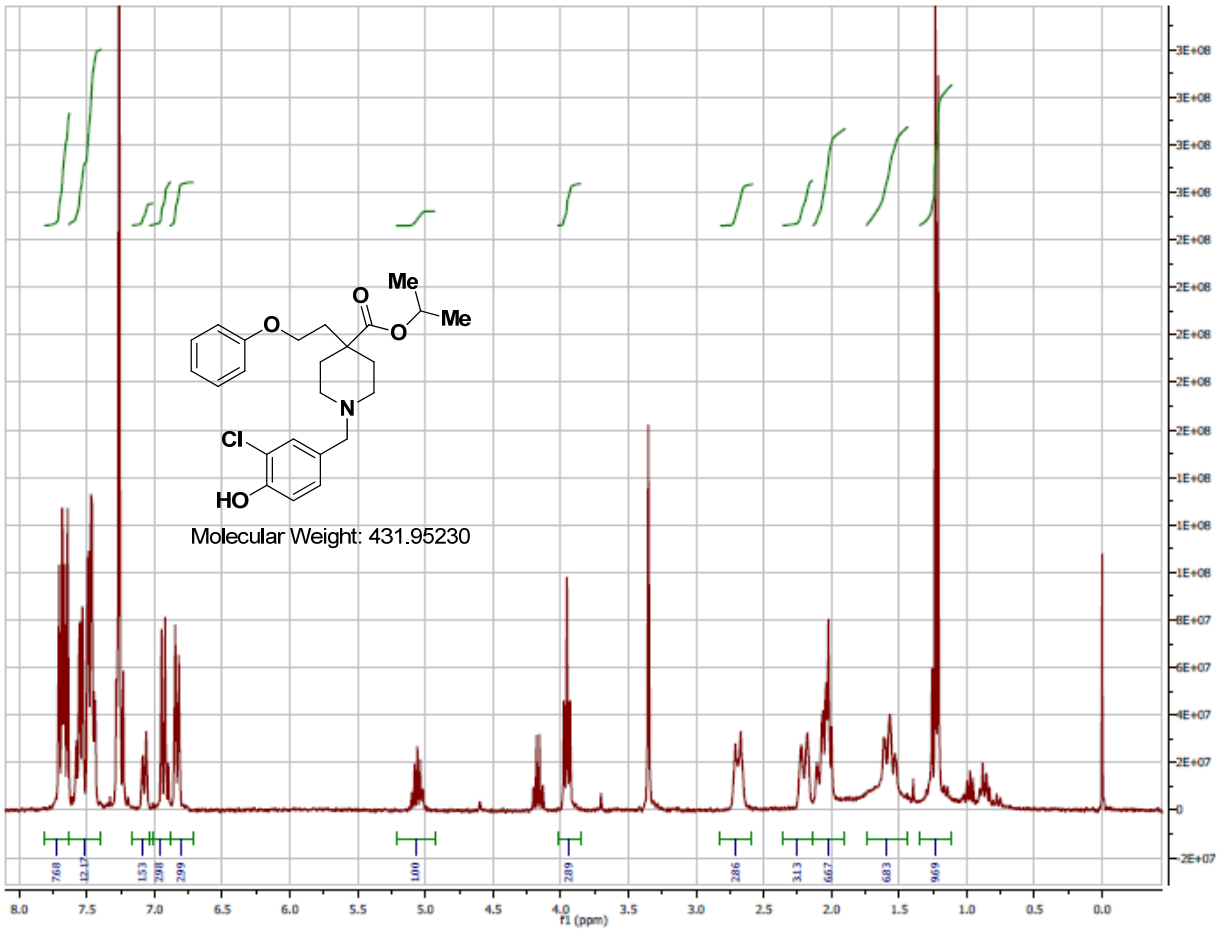
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701238



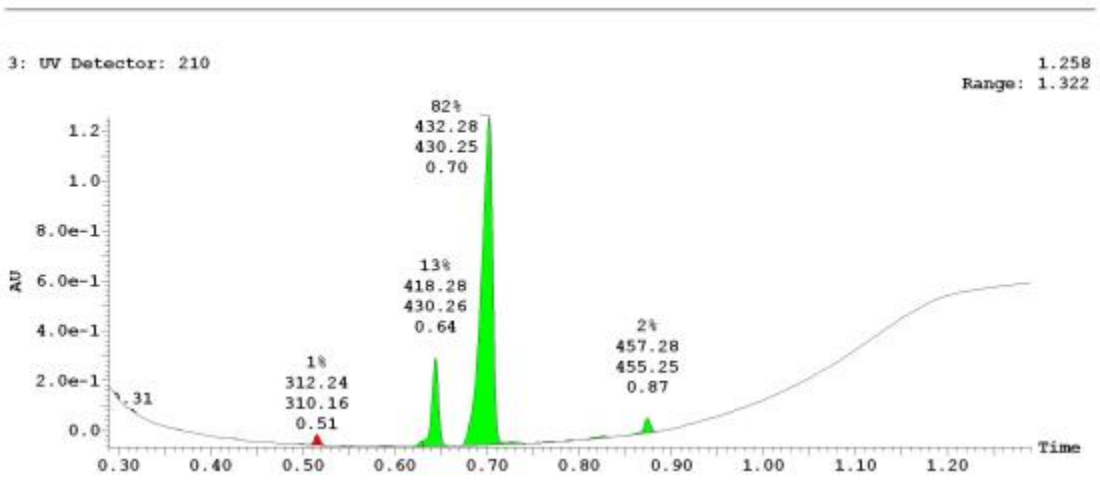
UPLC-MS Chromatogram of CID70701238 FW = 431.91



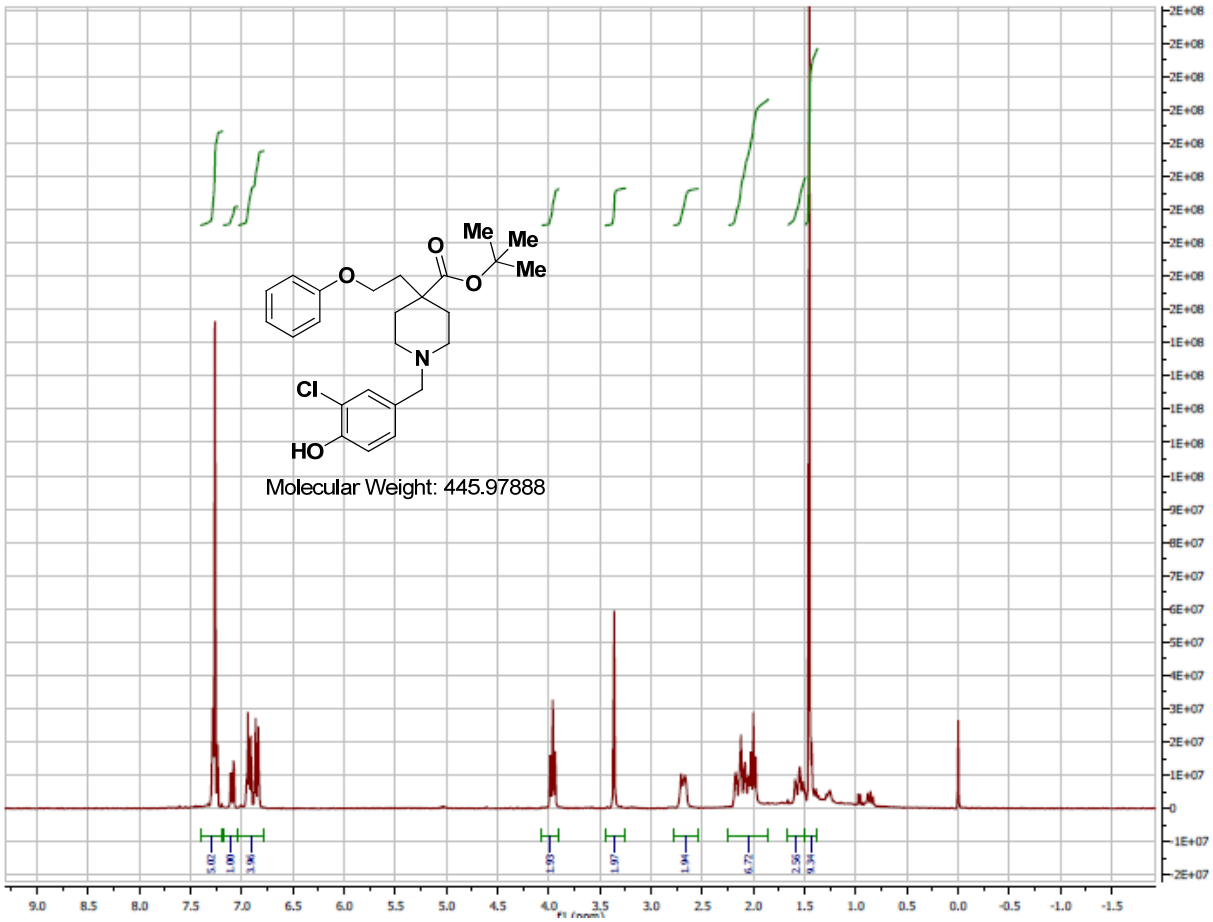
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701242



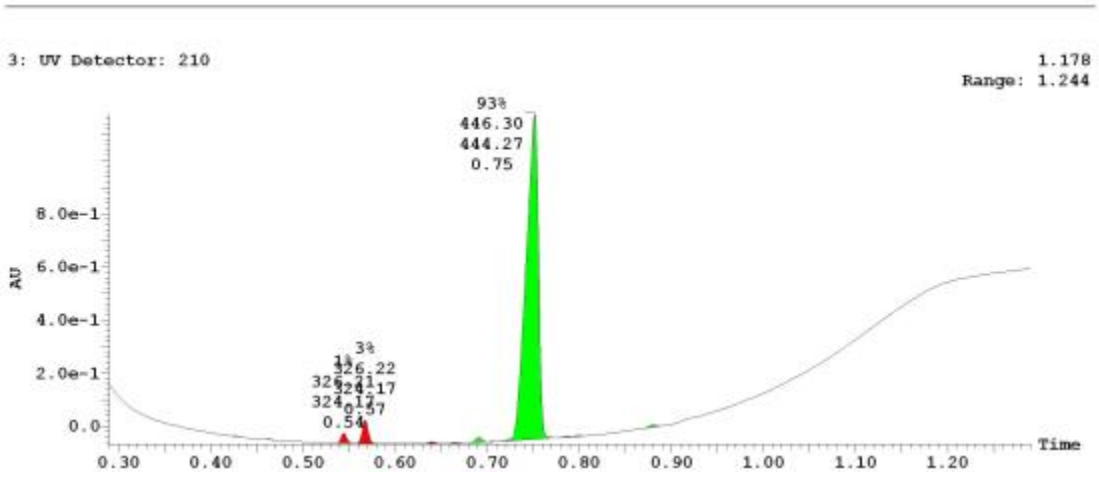
UPLC-MS Chromatogram of CID70701242 FW = 431.95



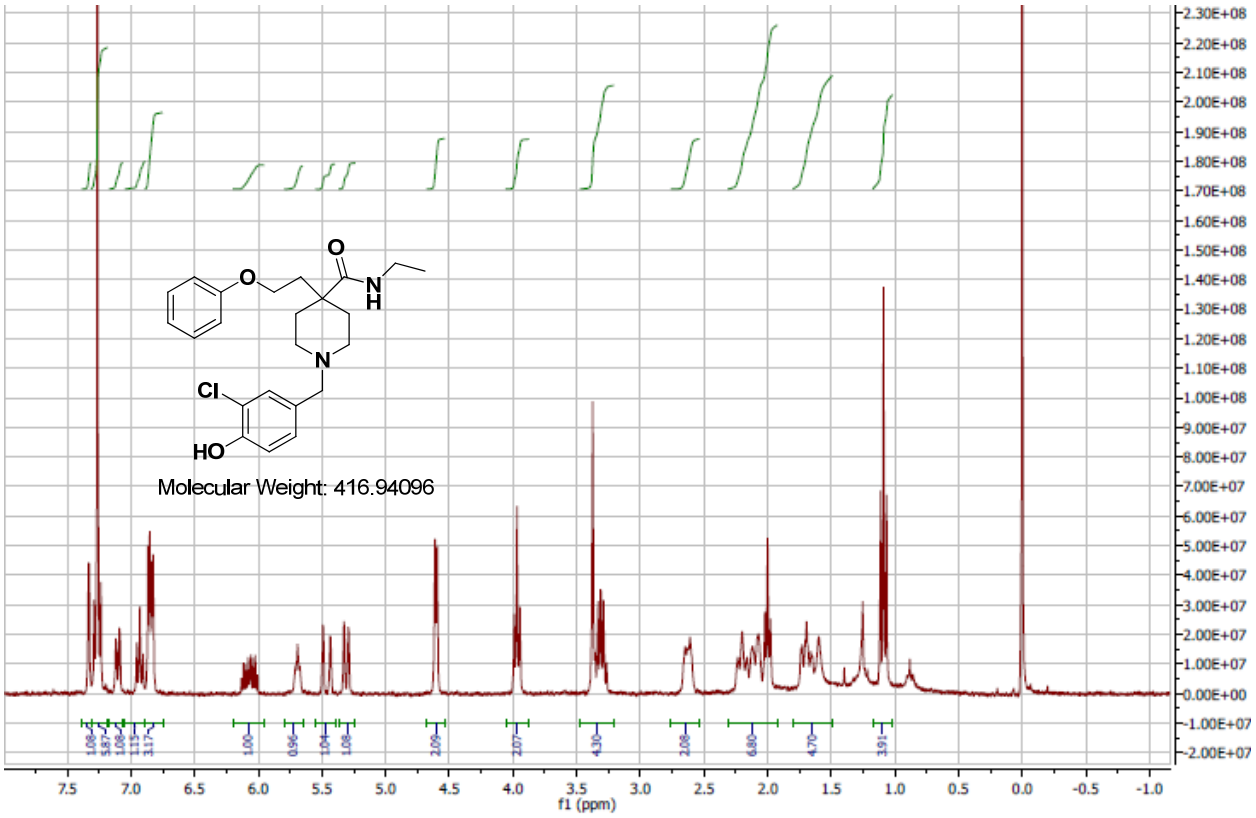
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701237



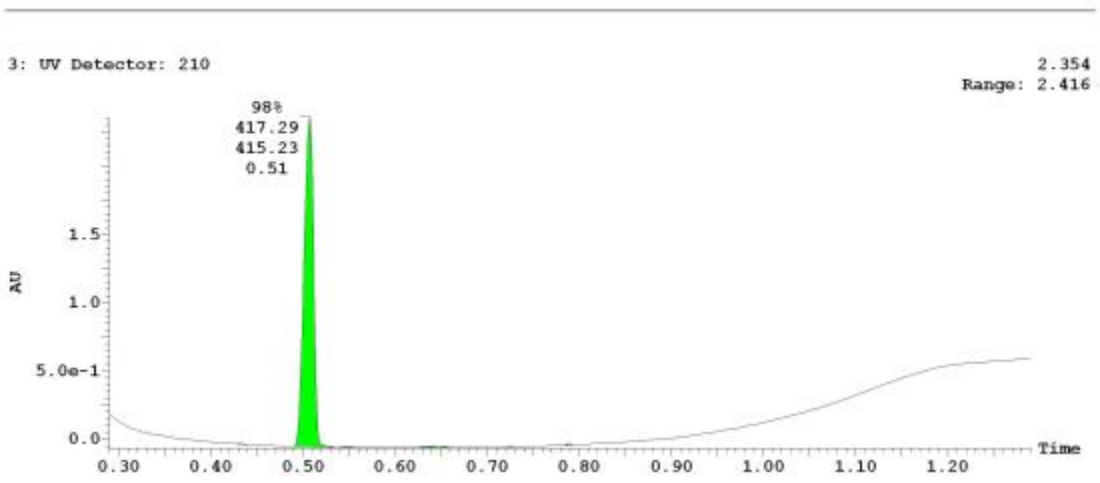
UPLC-MS Chromatogram of CID70701237 FW = 445.98



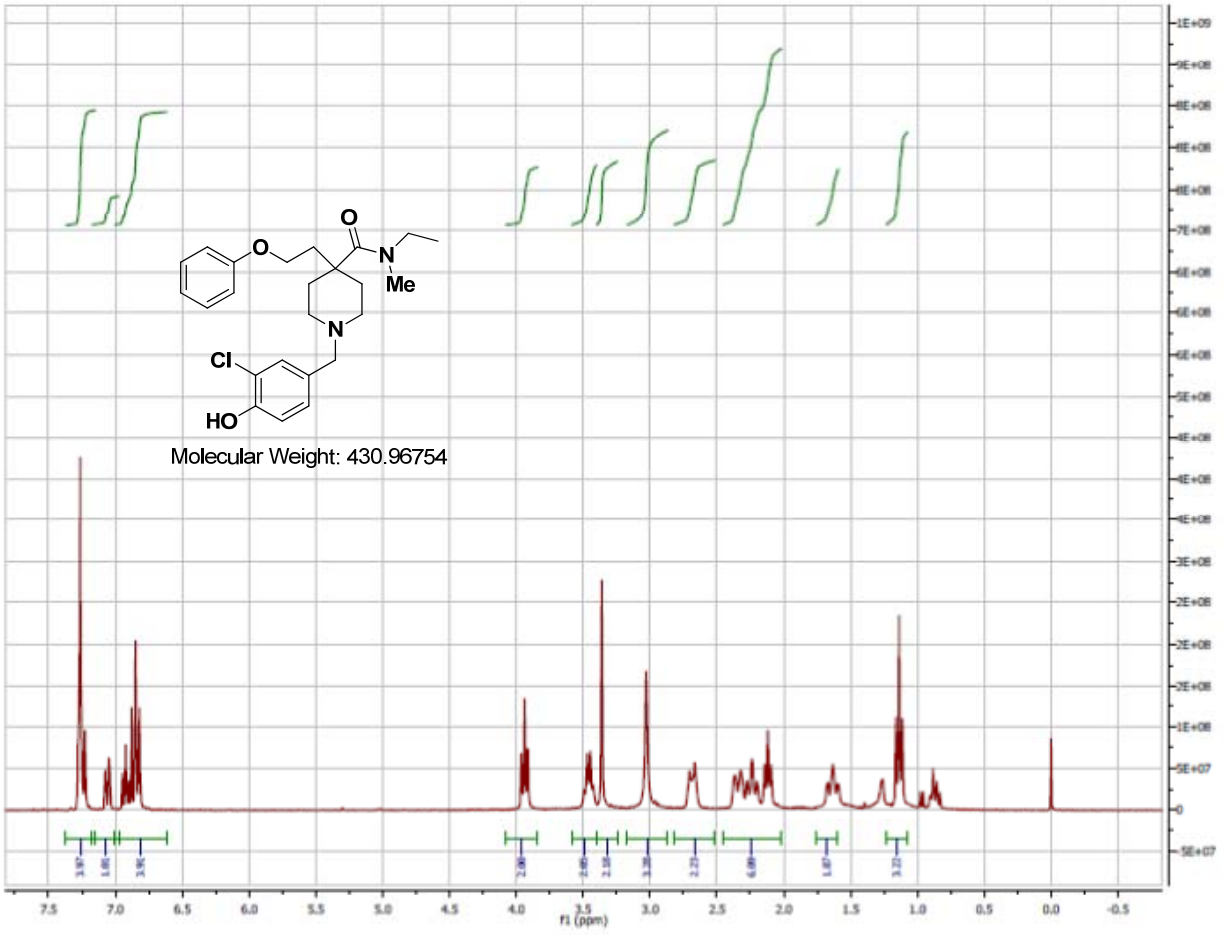
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701263



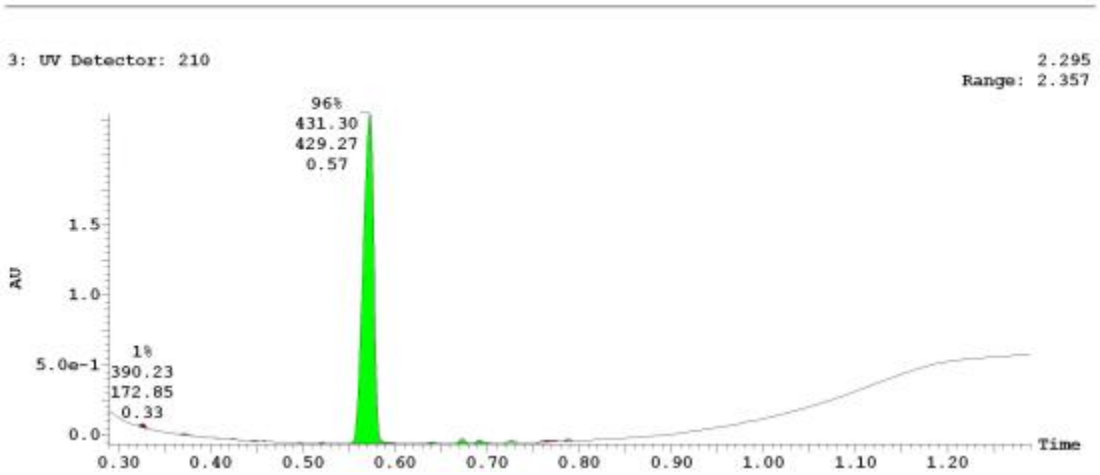
UPLC-MS Chromatogram of CID70701263 FW = 416.94



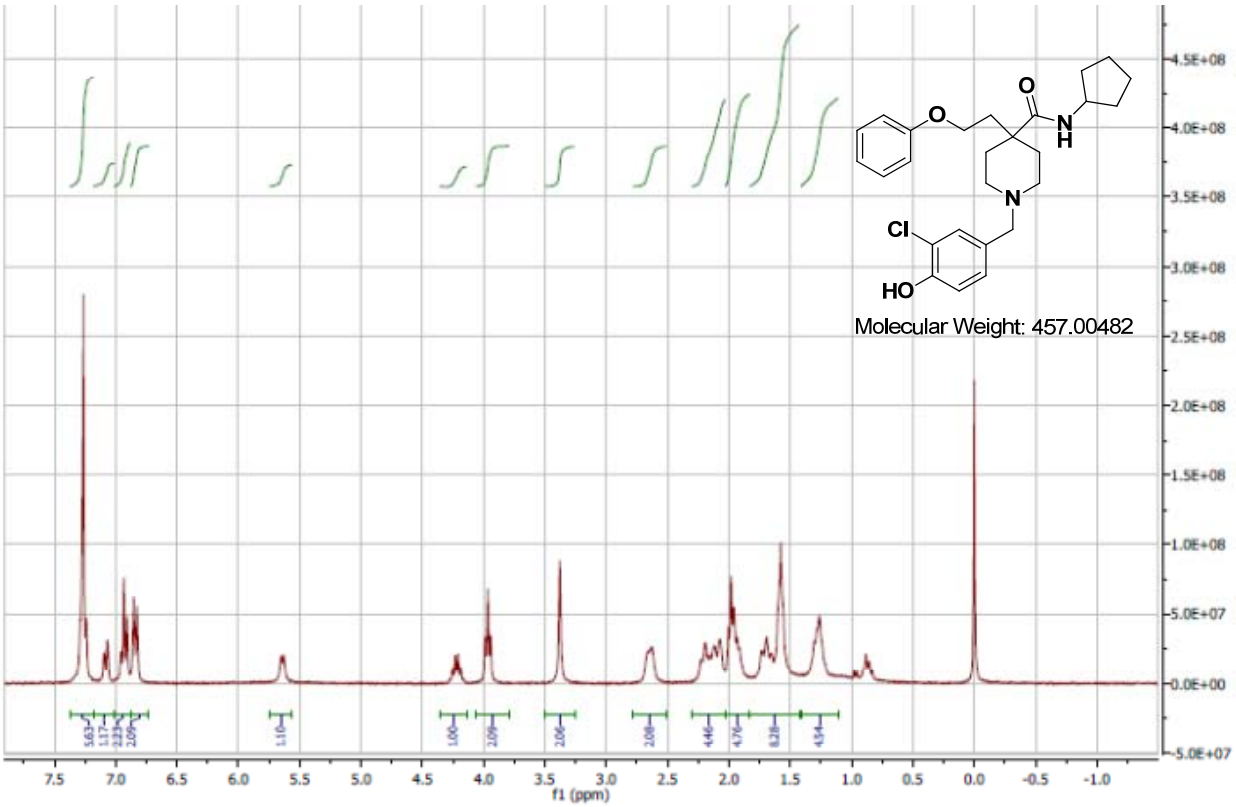
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701262



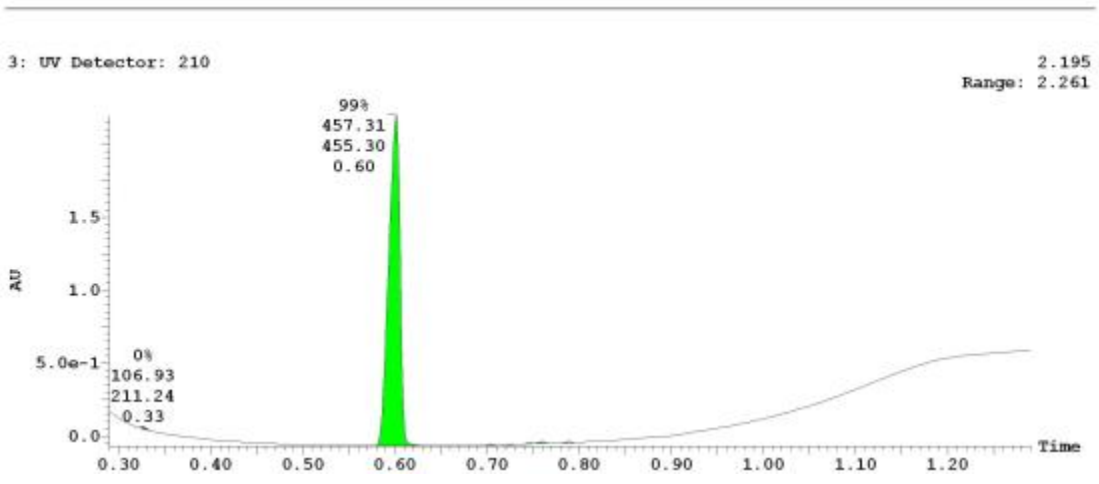
UPLC-MS Chromatogram of CID70701262 FW = 430.97



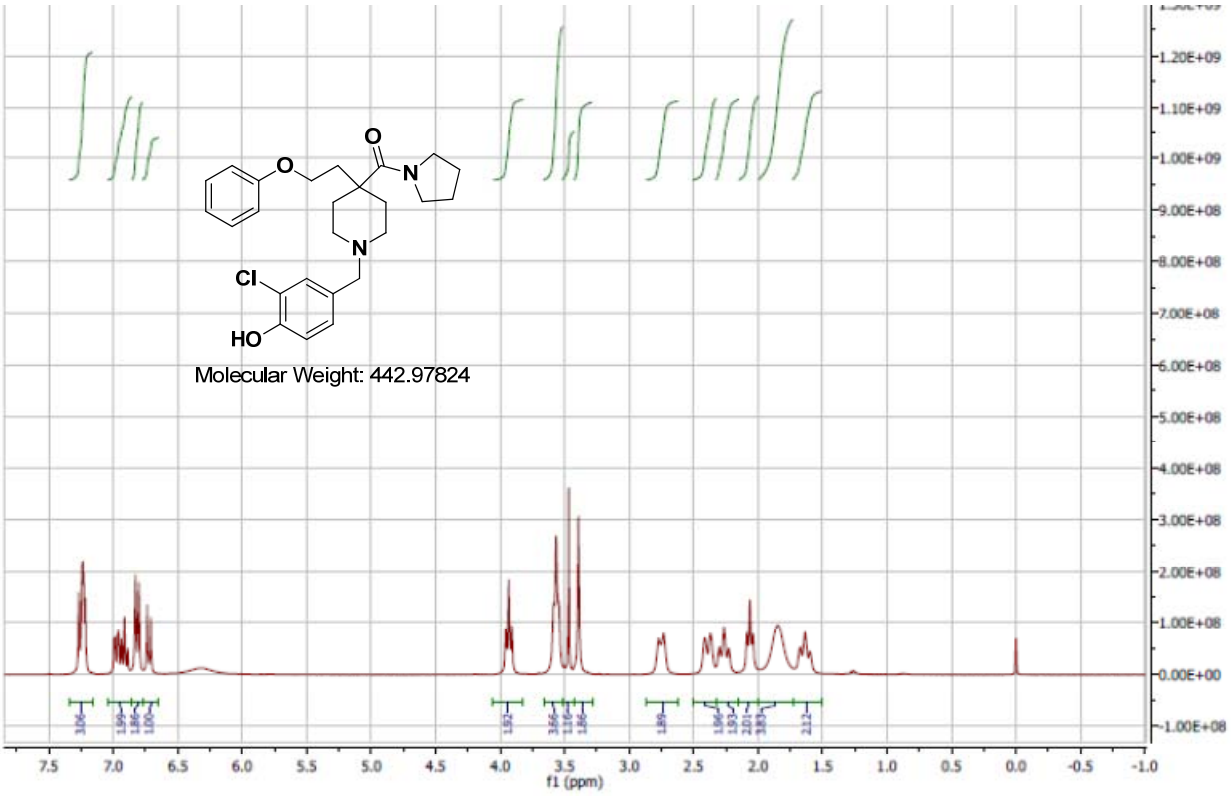
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701252



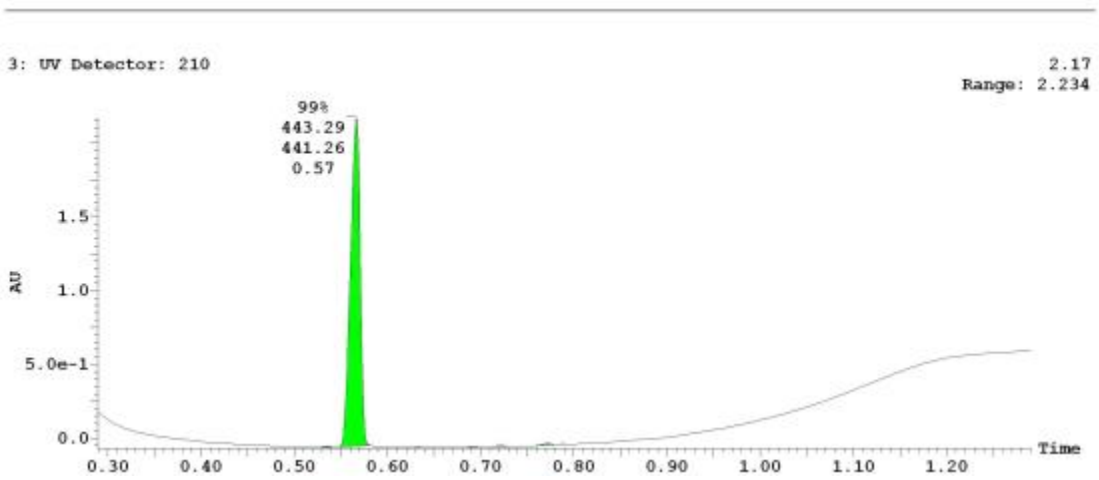
UPLC-MS Chromatogram of CID70701252 FW = 457.00



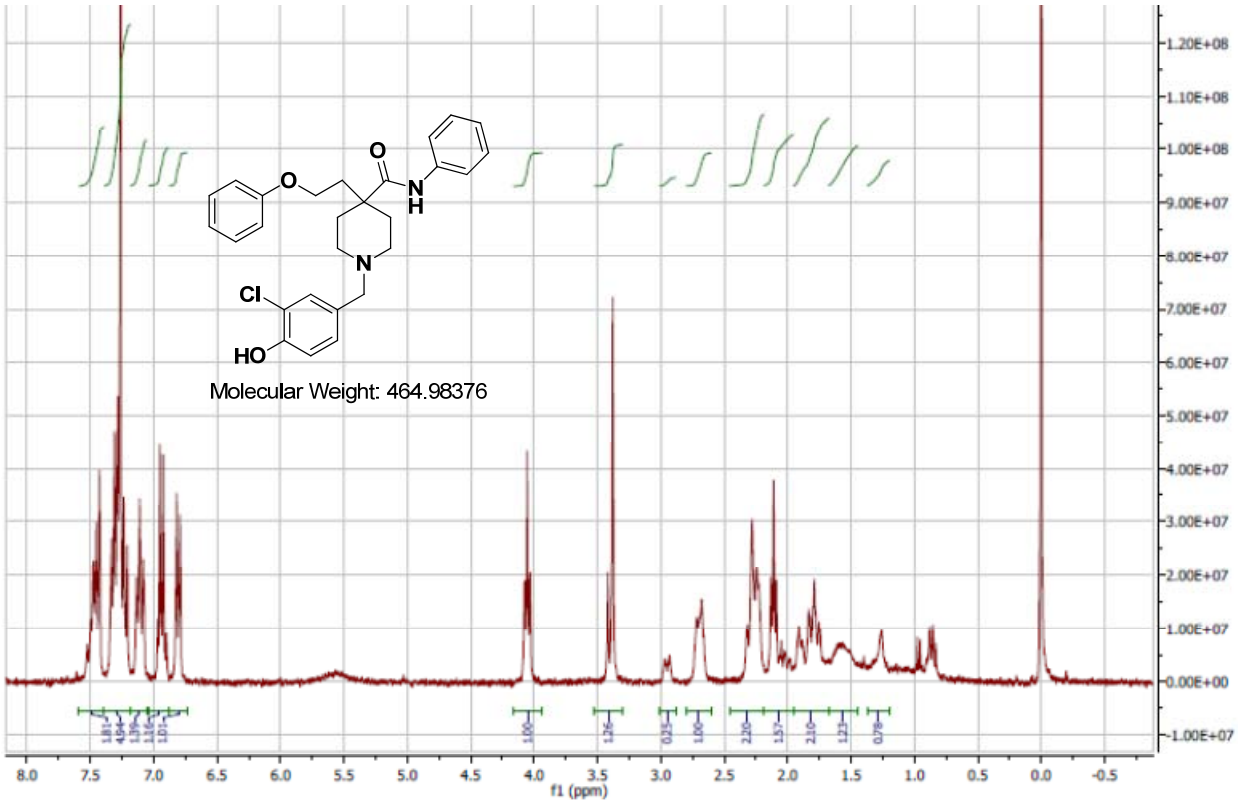
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701258



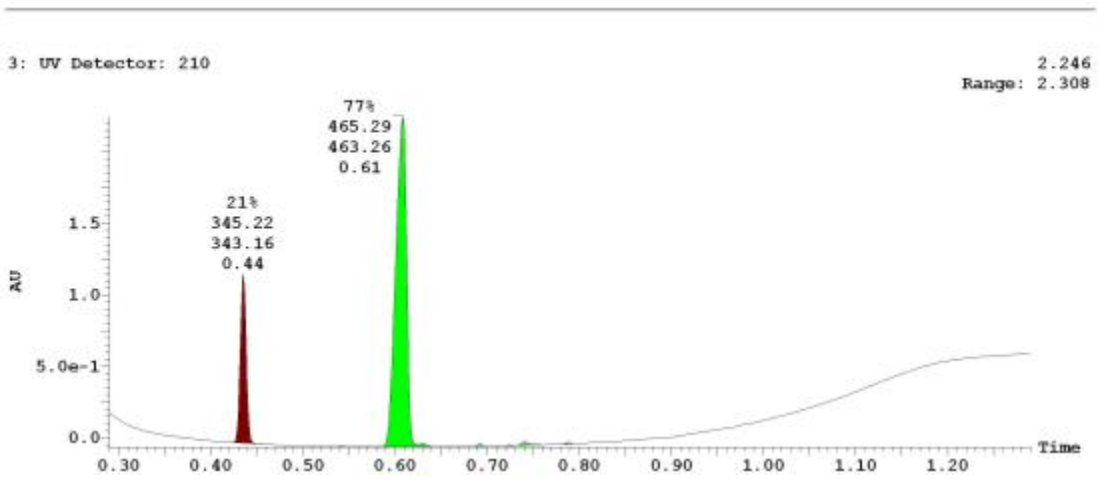
UPLC-MS Chromatogram of CID70701258 FW = 442.98



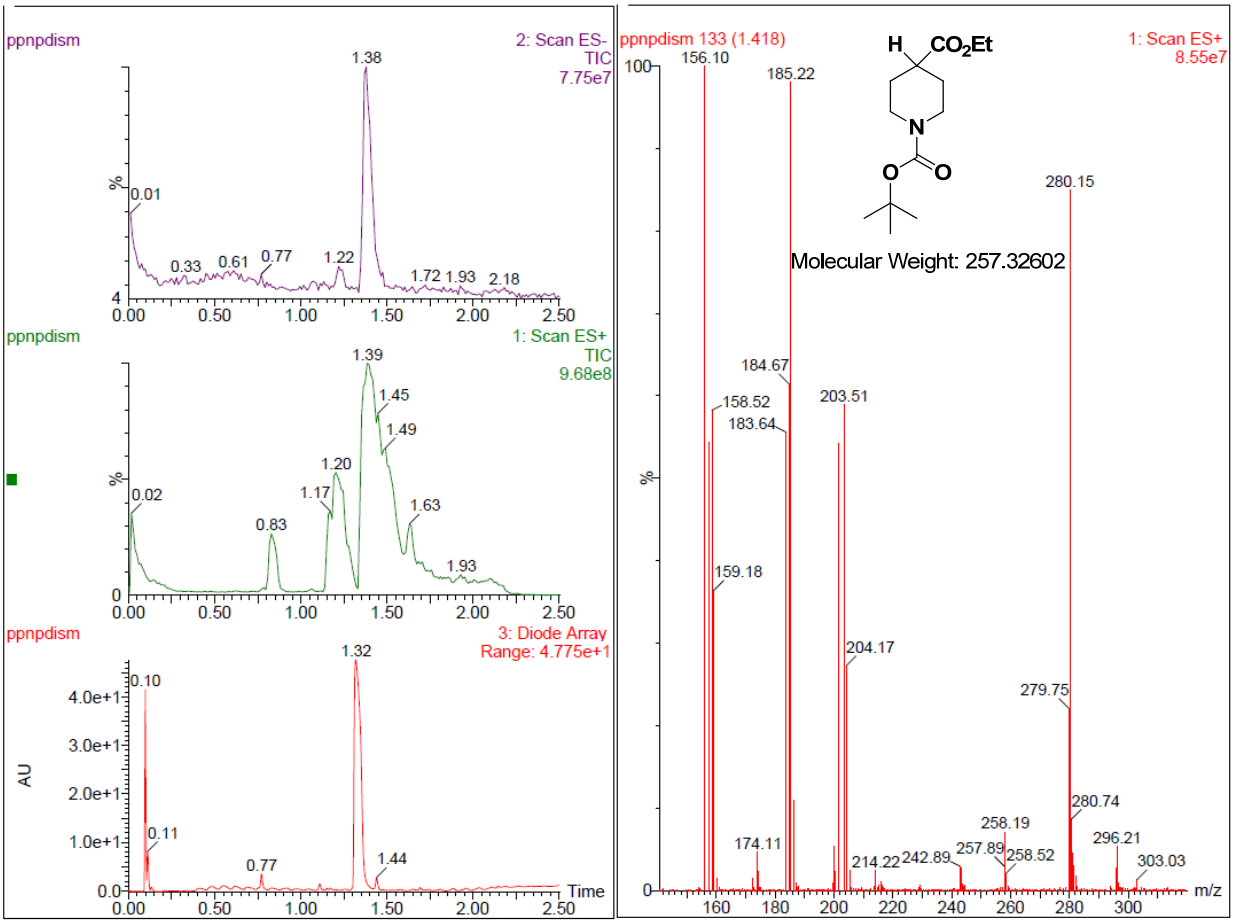
^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of CID70701239



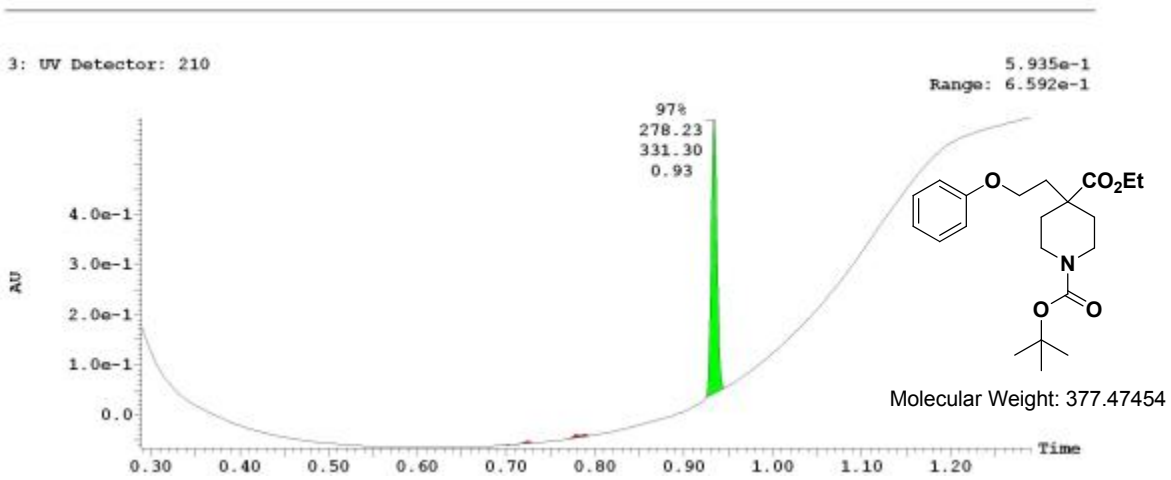
UPLC-MS Chromatogram of CID70701239 FW = 464.98



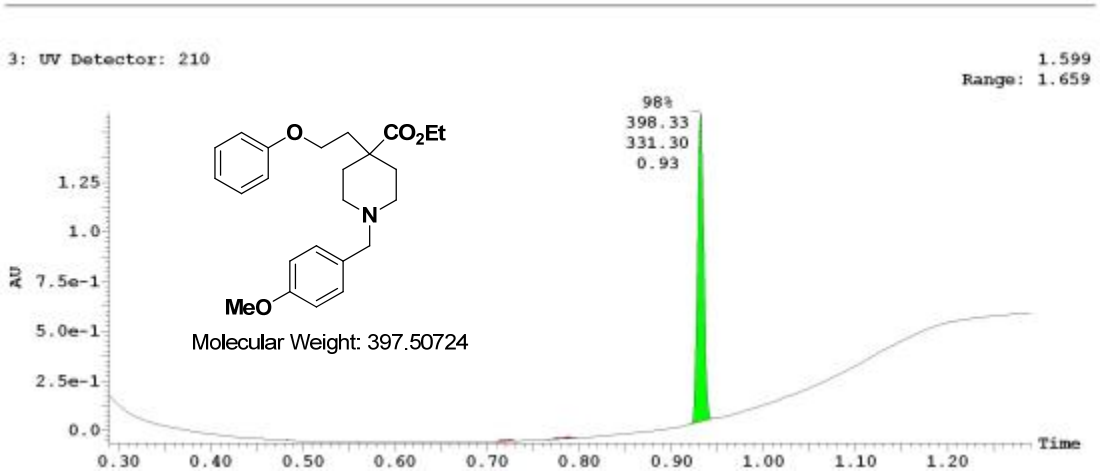
UPLC-MS Chromatogram of CID2758812 FW = 257.33 *(Boc-protecting group fragmentation)*



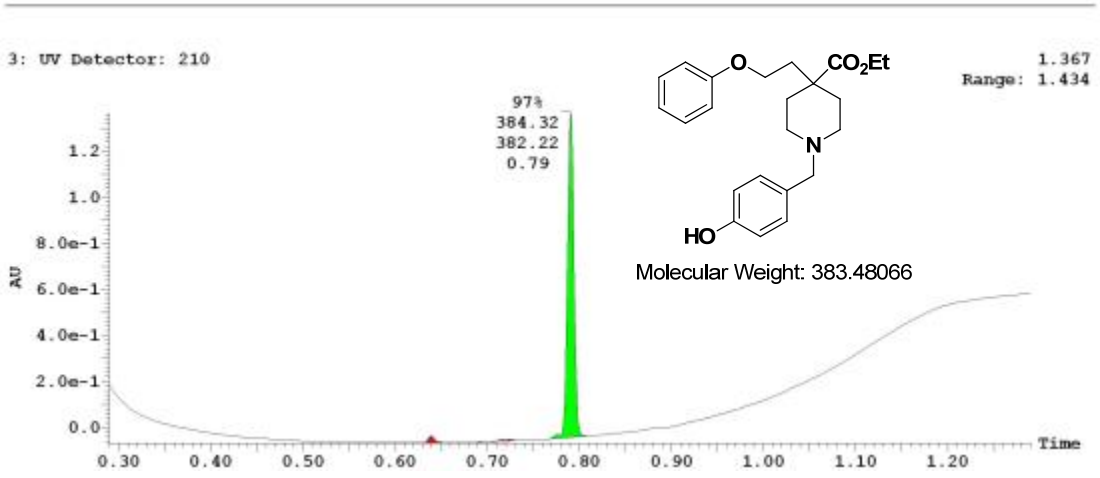
UPLC-MS Chromatogram of CID70701267 FW = 377.47 *(Boc-protecting group fragmentation)*



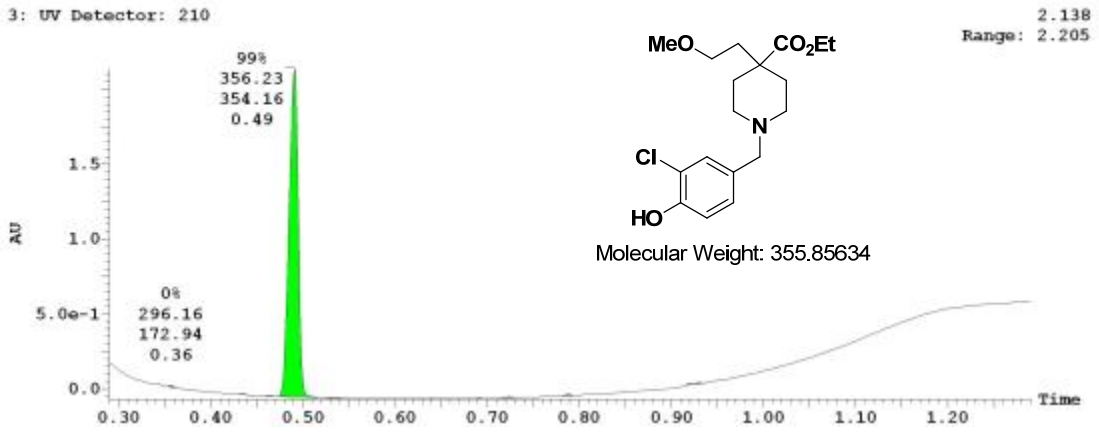
UPLC-MS Chromatogram of CID23723973 FW = 397.51



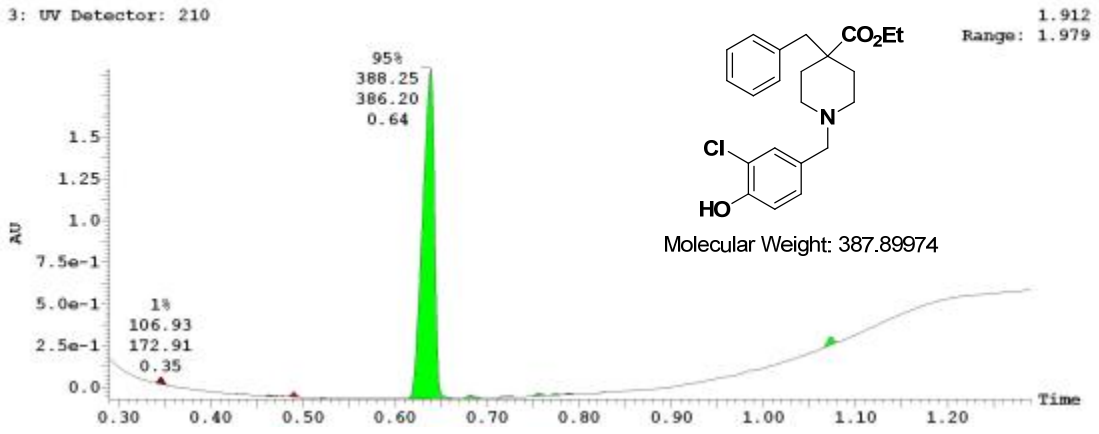
UPLC-MS Chromatogram of CID23723959 FW = 383.48



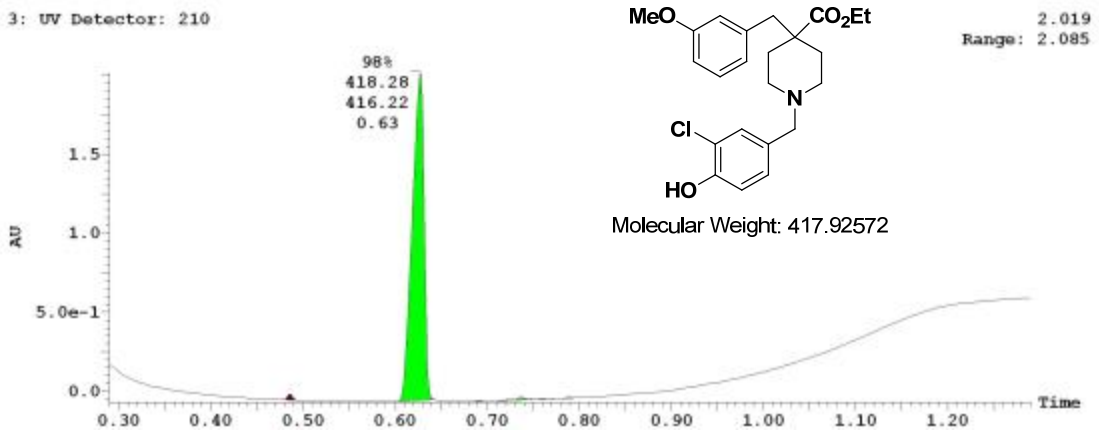
UPLC-MS Chromatogram of CID42515470 FW = 355.86



UPLC-MS Chromatogram of CID25298231 FW = 387.90



UPLC-MS Chromatogram of CID25277312 FW = 417.93



Appendix G: Compounds Provided to Evotec

Table A3. Probe and Analog Information

BRD	SID	CID	P/A	MLSID	ML
BRD-K20754212-001-10-6	160843258	23723882	P	MLS004820109	**ML359**
BRD-K13659644-001-01-3	160843238	70701251	A	MLS004820110	NA
BRD-K10350688-001-01-9	160843259	70701262	A	MLS004820108	NA
BRD-K59037718-001-01-8	160843242	70701263	A	MLS004820103	NA
BRD-K18880881-001-01-2	160843244	70701252	A	MLS004820107	NA
BRD-K31070838-001-01-7	160843250	70701238	A	MLS004820102	NA

A = analog; NA= not applicable; P = probe