MolecularLibraries
Pathways to Discovery

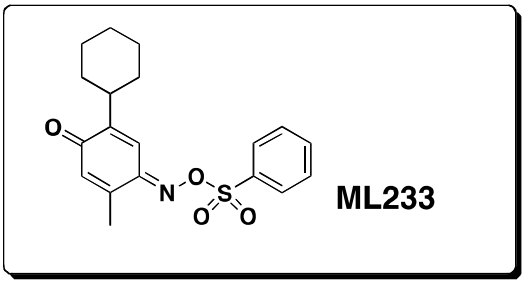
Probe Report

Title: Functional agonists of the Apelin J (APJ) receptor
Authors: Pasha Khan, Patrick R. Maloney, Michael Hedrick, Palak Gosalia, Monika Milewski, Linda Li, Gregory P. Roth, Eduard Sergienko, Eigo Suyama, Eliot Sugarman, Kevin Nguyen, Alka Mehta, Stefan Vasile, Ying Su, Shenghua Shi, Derek Stonich, Hung Nguyen, Fu-Yue Zeng, Arianna Mangravita Novo, Michael Vicchiarelli, Jena Diwan, Thomas D.Y. Chung, Anthony B. Pinkerton and Layton H. Smith
Assigned Assay Grant #: 1R21NS059422-01
Screening Center Name & PI: Sanford Burnham Center for Chemical Genomics (SBCCG) & John C. Reed (PI)
Chemistry Center Name & PI: *same as above*
Assay Submitter & Institution: Layton H. Smith, Sanford-Burnham Medical Research Institute
PubChem Summary Bioassay Identifier (AID): 2580

Abstract. The recently discovered apelin receptor (APJ, AGTRL-1, APLNR) system has emerged as a critical mediator of cardiovascular homeostasis involved in the pathogenesis of hypertension, heart failure, atherosclerosis and other cardiovascular diseases. Herein is presented the discovery and characterization the first non-peptide based potent (3.7 μM) small molecule APJ functional agonist in cell-based assays, that is >21 fold selective over the closely related angiotensin 1 (AT1) receptor, derived from a high throughput screen (HTS) of the ~330,600 compound Molecular Libraries Small Molecule Repository (MLSMR) collection. This agonist showed some binding activity against 4 out of 37 other GPCRs and transporters, including the 5-HT_{1A}, α_{2C} adrenergic, and benzylpiperazine receptors (55%I, 51%I and 65%I at 10 μM, respectively) and the norepinephrine transporter (57%I at 10 μM). The synthetic methodology, development of structure-activity relationships (SAR), and initial *in vitro* pharmacologic characterization are also presented. This probe molecule provides a useful tool compound for investigators interested in understanding apelin receptor pharmacology and function.

Probe Structure & Characteristics:

This Center Probe Report describes the first reported small molecule APJ agonist, **ML233**, which is also selective against the AT1 receptor and cell active.



ML233

CID/ML#	Target Name	EC_{50} (nM) [SID, AID]	Anti-target Name(s)	EC_{50} (μM) [SID, AID]	Fold Selective
CID 46905036 **ML233**	APJ Receptor	3740 nM SID99361200 AID488985	AT1 Receptor	>79 μM SID99361200 AID488986	>21

Recommendations for scientific use of the probe:

The recently discovered apelin system is emerging as a critical mediator of cardiovascular homeostasis (1,2) whose importance in the pathogenesis of hypertension (3-5), heart failure (6-8), atherosclerosis (9, 10) and other cardiovascular diseases is the subject of intense investigation (11). These investigations are limited by the paucity of research tools and reagents needed to fully understand the role of apelin in physiology and pathology. Thus a novel small molecule agonist of the apelin receptor (a.k.a. APJ, AGTRL-1, APLNR) is an essential tool for understanding the pharmacology of APJ and to validate the importance of this system in animal models of cardiovascular disease. Iturrioz *et. al*. (12) have reported E339-3D6 as the first non-peptide apelin receptor agonist. However, examination of the structure of E339-3D6 (see **Fig. 1**, below) weaken this claim as it is clearly a peptidomimetic of ~1400 m.w. Although E339-3D6 binds the apelin receptor competitively (K_i = 0.43 mM) it behaves as a partial agonist towards forskolin-induced cAMP production. Despite this moderate activity, E339-3D6 demonstrated some efficacy *in vivo* and in *ex vivo* tissues. To date, no small molecule has yet been identified as a full agonist. Disclosed in this report is the discovery and characterization of the first potent and selective apelin small molecule receptor agonist. Synthetic methodology, structure-activity relationships (SAR) and activity of our most potent and selective compound in a cell-based assay of apelin receptor activation are included. The probe molecule can be used as a tool compound for investigators interested in understanding apelin receptor pharmacology and function.

1 Introduction

Currently there are no small molecule tools to investigate the biological functions of apelin and its receptor. Apelin is the endogenous peptide ligand for the G-protein coupled receptor (GPCR) APJ (angiotensin II receptor-like 1, AGTRL-1 and APLNR). Until the discovery of apelin, APJ was an orphan GPCR (13, 14). APJ is coupled to Gαi, and has been shown in cell culture to inhibit adenylate cyclase (15). The APJ gene encodes a receptor that most closely resembles the angiotensin receptor AT1. However, the APJ receptor does not bind angiotensin II (13). Underscoring the emerging importance of the apelin/APJ system, recent studies have shown that apelin reduces the extent of atherosclerotic lesions in $ApoE^{-/-}$ mice, and opposes the development of abdominal aortic aneurysms (9). Additionally, work in Dr. Smith's lab revealed that APJ forms a heterodimer with the Ang II receptor AT1, and that this complex facilitates antagonism of Ang II signaling by apelin (LHS personal communication). Despite these exciting results, there remains a multitude of unanswered questions regarding the role of apelin and APJ in normal physiology and the pathogenesis of cardiovascular disease.

An agonist or potentiators of APJ would therefore provide a novel research tool to evaluate the role of apelin in cardiovascular and metabolic disease pathology. The criteria established was to identify (1) a transient and reversible full or partial agonist with an $EC_{50} \leq 5$ μM in a functional assay (cell based) and (2) selective for APJ over AT1 by at least 30-fold.

There has been one recent report of an APJ agonist in the literature, E339-3D6 (**Figure 1**). However the molecular weight exceeds 1000 Da (mw~1400) and the structure is clearly peptide related. Furthermore, E339-3D6 has a highly undesirable fluorescent probe attached to it, in addition to a urea, a methylated imidazole salt and a methylated aminothiazole salt. E336-3D6 is neither

commercially available nor available from the authors. In addition to the report covering E339-3D6, several publications covering high molecular weight peptide derived APJ agonists have been reported in the patent literature (16-18).

E339-3D6

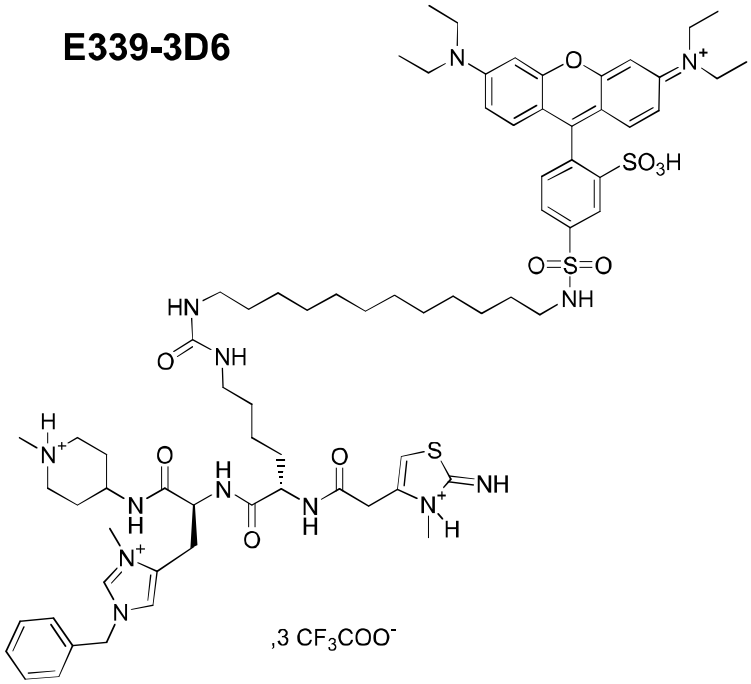


Figure 1: Reported "small molecule" APJ agonist

2 Materials and Methods

The details of the primary HTS and additional assays can be found in the "Assay Description" section in the PubChem BioAssay view under the AIDs as listed in **Table 1** below. Additionally the details for the primary HTS are provided in the Appendix at the end of this probe report.

Primary HTS for APJ receptor agonism: Briefly, the primary assay detect agonists that cause the activation and internalization of the Angiotensin II receptor-like 1 (AGTRL-1; Apelin receptor; APJ) in a CHO-K1 AGTRL-1 Beta-Arrestin Cell Line (DiscoveRx) containing the stably integrated APJ receptor in reference to the canonical peptide APJ receptor agonist, the Apelin-13 peptide. Cells were seeded (1000 cells in 4 μL) in a 1536 well plate, grown overnight, then treated with 20 μM of compounds in 1% final DMSO, incubated at room temperature in the dark, and luminescence read 90 mins after addition of test compounds (See details in the Appendix).

Counterscreen for Angiotensin 1 receptor agonism: Briefly, this assay is performed in a completely analogous manner to the primary APJ agonist assay, except for the use of the angiotensin receptor 1 (AGTR-1; ATR-1) containing CHO-KI-AGTR-1 cell line (DiscoveRx). This assay assesses the selectivity of APJ agonist against this closely related receptor to the APJ receptor. (See details in the Appendix)

2.1 Assays

Table 1 summarizes the details for the assays that drove this probe project.

Table 1. Summary of Assays and AIDs

PubChemBioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
Summary assay for small molecule agonists of the APJ receptor. [Summary]	2580	Agonist	Summary	N/A	N/A
uHTS identification of small molecule agonists of the APJ receptor via a luminescent beta-arrestin assay [Primary Screening]	2520	Agonist	Primary	Cell-based	Luminescence & 1536-well
Single concentration confirmation of uHTS hits from a small molecule agonists of the APJ receptor via a luminescent beta-arrestin assay [Primary Screening]	2764	Agonist	Confirmatory (Cherry Pick)	Cell-based	Luminescence & 1536-well
Dose Response confirmation of uHTS hits from a small molecule agonists of the APJ receptor via a luminescent beta-arrestin assay [Confirmatory]	488748	Agonist	Confirmatory (DMSO Dose Response)	Cell-based	Luminescence & 1536-well
SAR analysis of a small molecule agonists of the APJ receptor via a luminescent beta-arrestin assay [Confirmatory]	488811 488985 492985 493183 504524	Agonist	Confirmatory (Dry Powder)	Cell-based	Luminescence & 1536-well
SAR analysis of small molecule agonists of the Angiotensin II Receptor Type 1 to assess selectivity of uHTS small molecule agonists hits of the APJ receptor [Confirmatory]	488865 488881 488986 492982 493202 504532	Agonist	Secondary (Dry Powder)	Cell-based	Luminescence & 1536-well

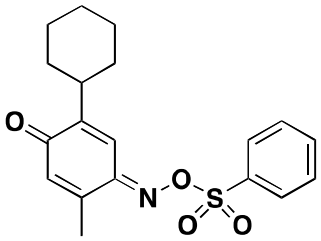
2.2 Probe Chemical Characterization

a) **Chemical name of probe compound**

The IUPAC name of the probe is (E)-2-cyclohexyl-5-methyl-4-(phenylsulfonyloxyimino)cyclohexa-2,5-dienone. The specific batch prepared, tested and submitted to the MLSMR is archived as SID99361200 corresponding to CID46905036.

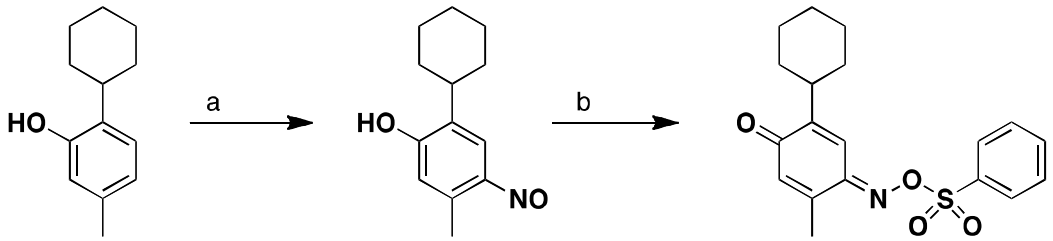
b) **Probe chemical structure including stereochemistry if known**

The probe **ML233** was obtained as a single observed *anti* isomer. The assignment is consistent with precedent literature (19).



ML233

c) **Synthesis and Structural Verification Information of probe SID99361200 corresponding to CID46905036**



Scheme 1: Synthesis of **ML233**, conditions: a. Sodium nitrite (1.5 eq.), HCl (conc.), EtOH; b. benzenesulfonyl chloride (1 eq.), DMAP (cat.), pyridine.

For detailed procedures see **Section 2.3**. Images of spectral data (^{1}HNMR, ^{13}CNMR, and LC/MS) used to support the structural assignment of **ML233** can be found in **Section 6** (Supplementary Information).

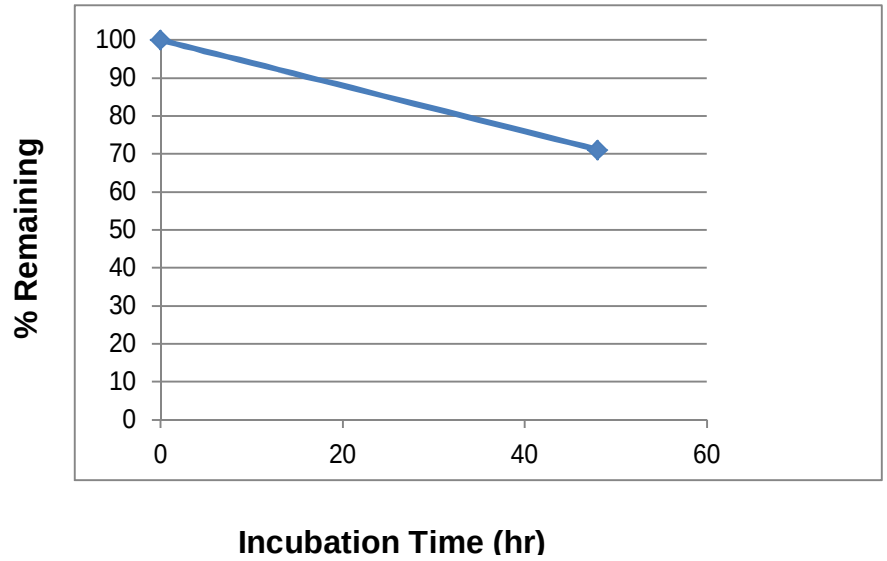
d) **If available from a vendor, please provide details.**

This probe is not commercially available. A 25 mg sample of **ML233** synthesized at SBCCG has been deposited in the MLSMR (see Probe Submission **Table 4** below).

e) **Solubility and Stability of probe in PBS at room temperature:**

The stability and solubility of **ML233** was investigated in PBS buffer at room temperature (**Figure 2**). Initial experiments examining the stability of **ML233** revealed no quantifiable level of compound after 1 hr in PBS buffer due to low solubility. Subsequent examination with 50% showed the compound was mostly stable out to 48 hr (71% remaining).

Figure 2: Stability of **ML233** in PBS



f) **A tabulation of calculated and known probe properties**

Table 2. ML233 (CID46905036)	
Molecular Weight	359.4393 [g/mol]
Molecular Formula	C19H21NO4S
AlogP	4.697
H-Bond Donor	0
H-Bond Acceptor	5
Rotatable Bond Count	4
Exact Mass	359.43933
MonoIsotopic Mass	359.43933
Topological Polar Surface Area	81.18
Heavy Atom Count	25
Formal Charge	0
Complexity	646
Isotope Atom Count	0
Defined Atom StereoCenter Count	0
Undefined Atom StereoCenter Count	0
Defined Bond StereoCenter Count	1
Undefined Bond StereoCenter Count	0
Covalently-Bonded Unit Count	1

g) **Provide MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection:**

Samples of the probe (25 mg) and each of five analogs (20 mg) synthesized at SBCCG were submitted to MLSMR **(Table 3)**, and 5 mg of the probe was provided to Dr. Smith.

Table 3. Probe and Analog Submissions to MLSMR (BioFocus DPI) for APJ Agonists

Probe ML233 - CID46905036

Probe /Analog	MLS_ID (SBCCG)	MLS_ID (MLSMR)	CID	SID	Source (vendor or SBCCG syn)	Amt (mg)	Date Submitted
Probe **ML233**	0437644	MLS003429729	46905036	99361200	SBCCG syn	25	3/17/11
Analog 1	0445745	MLS003429730	49787121	103061724	SBCCG syn	20	3/17/11
Analog 2	0437377	MLS003429731	5601088	99361194	SBCCG syn	20	3/17/11
Analog 3	0437376	MLS003429732	6508095	99361193	SBCCG syn	20	3/17/11
Analog 4	0437527	MLS003429733	5731374	99361197	SBCCG syn	20	3/17/11
Analog 5	0445746	MLS003429734	49787114	103061725	SBCCG syn	20	3/17/11

2.3 Probe Preparation

Step 1: 2-cyclohexyl-5-methylphenol (0.5g, 2.63mmol) was dissolved in 10 ml ethanol followed by addition of 10 ml concentrated HCl. The resultant mixture was cooled to 0 °C and sodium nitrite

(272mg, 3.94mmol) was added to it in two portions of about 140mg each. After addition of sodium nitrite, the reaction mixture turned green. The reaction mixture was stirred overnight at room temperature followed by addition of ice-cold water, which resulted in precipitation of a yellow solid. The aqueous solution was filtered under vacuum to yield 0.55g (96% yield) of 2-cyclohexyl-5-methyl-4-nitrosophenol (**2**) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 (s, 1H), 7.47 (s, 1H), 6.30 (d, *J* = 1.2 Hz, 1H), 2.74 (t, *J* = 12.0 Hz, 1H), 2.15 (d, *J* = 1.1 Hz, 3H), 1.88 – 1.65 (m, 5H), 1.49 – 1.31 (m, 2H), 1.29 – 1.07 (m, 3H).

Step 2: 2-cyclohexyl-5-methyl-4-nitrosophenol (50mg, 0.23mmol) was dissolved in 2ml pyridine followed by addition of catalytic amount of DMAP (N, N'-Dimethylaminopyridine). Benzenesulfonyl chloride (40mg, 0.23mmol) was added to the reaction mixture and the reaction mixture was stirred for 4-6h. The reaction mixture was partitioned with approximately 20 ml of 1:1 ethyl acetate and water and the organic layer was washed with 10% HCl (2×20ml) and brine (1×20ml). The organic layer was concentrated *in vacuo* and the crude product was purified by column chromatography to yield 60mg (73%) of 4-methyl-5-(((phenylsulfonyl)oxy)imino)-[1,1'-bi(cyclohexane)]-3,6-dien-2-one as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 – 7.99 (m, 2H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.62 (t, *J* = 7.8 Hz, 2H), 7.28 (d, *J* = 0.8 Hz, 1H), 6.33 (d, *J* = 1.3 Hz, 1H), 2.75 (dt, *J* = 21.2, 7.4 Hz, 1H), 2.10 (d, *J* = 1.3 Hz, 3H), 1.92 – 1.69 (m, 5H), 1.47 – 1.31 (m, 2H), 1.32 – 1.12 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.66, 154.32, 151.39, 144.11, 134.74, 134.60, 131.50, 129.23, 129.11, 118.61, 36.57, 32.29, 26.37, 26.01, 16.77.

3 Results

3.1 Summary of Screening Results

The flowchart on the right summarizes the screening results (**Figure 3**). Following primary HTS of approximately 330,000 Molecular Libraries Small Molecules Repository (MLSMR) compounds at 20 μM against the APJ receptor (AID2520), 347 initial actives (~0.1% hit rate) were obtained at a 30% activity cut-off which corresponded to a Z-score of 6.0. For a cell-based assay, the screen was robust with an average Z' of 0.56.

Fresh stock solution "cherry picks" of the 347 initial hits were requested from the MLSMR and 311 were received (89.6%) and tested (AID2764) and activities (EC_{50}) were confirmed in full 10-point dose-response titrations (AID488748). In parallel, the selectivity against the angiotensin receptor 1 (ATR1) was also obtained (AID488865).

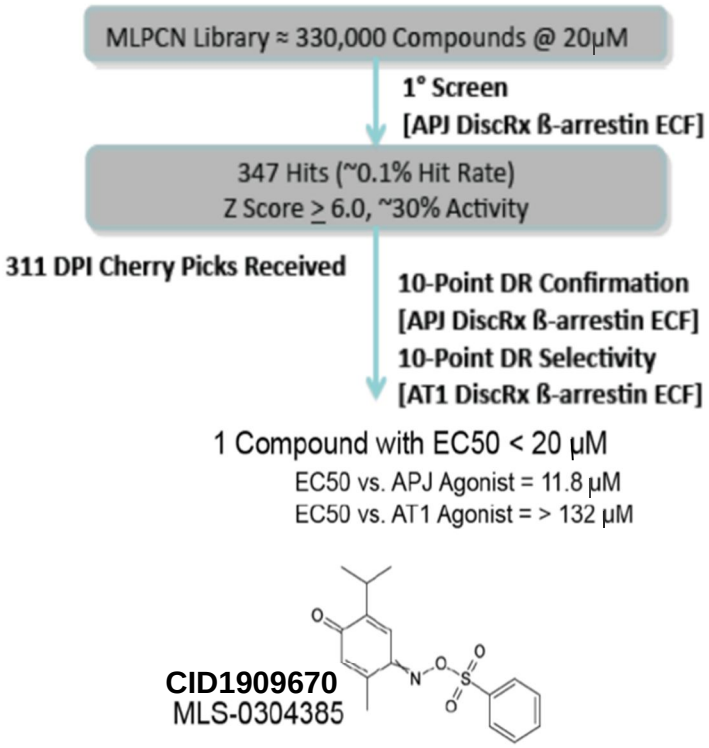


Figure 3. Flowchart Summary of Screening Results

Only one tractable and reasonable compound/scaffold (quinone-oxime sulfonate) was obtained, that appeared selective against the AT1 receptor. However the potency (11.8 μM EC_{50} from liquid reorder) did not meet the desired 5 μM potency, and the level of agonism of the most potent compounds were in the 30 – 60% efficacy range. Interestingly, this compound was listed in PubChem with undefined stereochemistry. Upon reorder of the dry powder, the compound was found to be a single isomer CID5601088 (MLS-0437377), although reordered CID1909670 (MLS-0304385) had the same spectral properties and biological activity.

Over 40 additional analogs were available for purchase, which helped to guide the SAR and synthesis plan as detailed in the SAR sections below.

3.2 Dose Response Curves for Probe

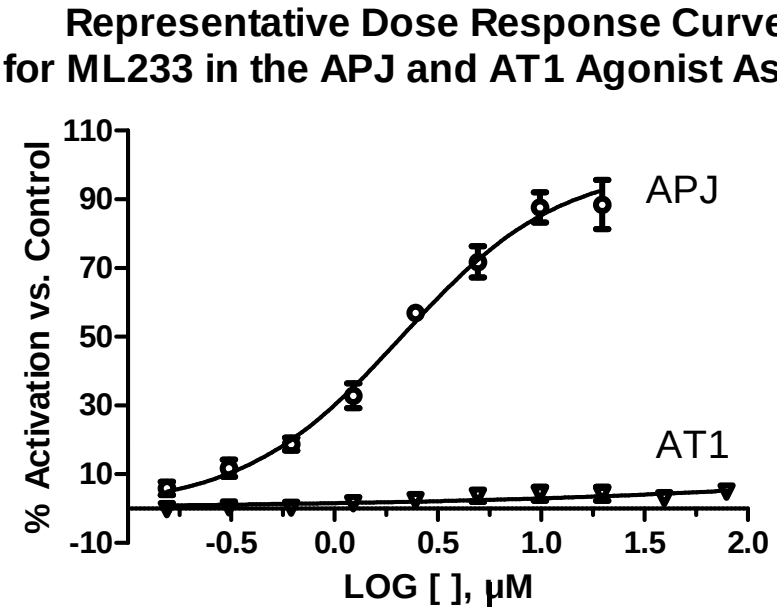


Figure 4: Dose response curves for **ML233**

3.3 Scaffold/Moiety Chemical Liabilities

Unlike the related quinone imine sulfonate scaffold, which is generally reactive and appears as a frequent hitter in bioassays, the quinone oxime sulfonate is stable and does not appear as a promiscuous compound from a Scifinder® search performed March 22, 2011.

To address concerns of potential hydrolytic stability or reactivity of **ML233**, an aliquot of the compound was prepared as a solution in 50% aqueous acetonitrile and was analyzed by LC/MS. A comparison at time zero, six, fifteen and thirty hours indicates the oxime and sulfonamide moiety is stable in aqueous solution (see Supplementary Information). In addition, an aliquot of probe **ML233** was treated with a solution of glutathione to evaluate potential reactivity as an electrophile. The

results indicate **ML233** to be unreactive to glutathione over a seven-hour period (see Supplementary Information).

3.4 SAR Tables

The general SAR strategy we pursued around the quinone oxime sulfonate scaffold is depicted in **Figure 5**. The structure represented by MLS-0304385 (as noted above, upon reorder of the dry powder, the compound was found to be a single isomer CID5601088 (MLS-0437377)) emerged as the only scaffold from the HTS to demonstrate selectivity over the AT-1 receptor, which was a key element of the desired probe criteria. Once hydrolytic stability and lack of reactivity towards GSH were confirmed (see section 3.3), analog synthesis was initiated to develop SAR with a primary focus on aliphatic substitutions on the core quinone moiety (in green) and a range of substituted aryl sulfonates (in blue). The results are summarized in the table below (**Table 4**).

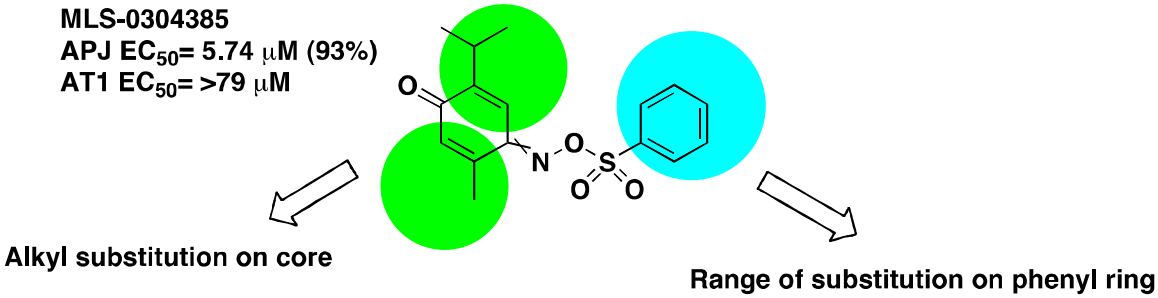


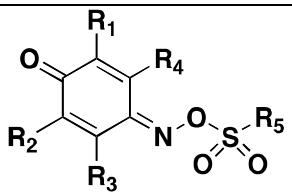
Figure 5: General SAR strategy around quinone-oxime sulfonate scaffold

For the core quinone portion it was found that the optimal substitution consisted of a single larger aliphatic (for example isopropyl, t-butyl or cyclohexyl) flanking the oxygen (R_1) combined with a smaller aliphatic group (such as methyl) flanking the imine (R_3). For example, one of the most potent full agonists was achieved with R_1= cyclohexyl and R_3= methyl (Entry 6, **ML233**, CID46905036). Other combinations with R_3=methyl that were potent were R_1=isopropyl (Entries 5, 8, 10, 11, 13, 15 28-30) and R_1=t-butyl (Entry 7). Interestingly, the reverse substitution with R_3= isopropyl and R_1= methyl gave less potent (Entry 18) or partial agonists (Entries 16-17).

In general, singly substituted analogs were inactive (Entries 21-27 and 40-42). Other combinations gave compounds that were either less active or showed only partial agonism. For example, with R_1 and R_2= methyl, only partial agonists were obtained (Entries 1-4), as with combinations of R_1 and R_3= methyl (Entries 9 and 12).

A range of substitution was tolerated on the pendant phenyl ring, including chloro, bromo, cyano, methyl and methoxy groups (Entries 32-39). Interestingly, a 3-pyridyl was found to be a suitable replacement for phenyl (Entry 31, compared to entry 11), providing opportunities to further improve the solubility of this series.

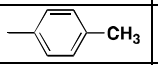
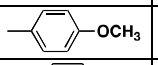
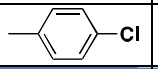
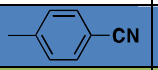
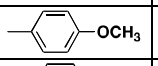
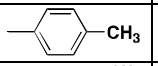
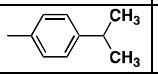
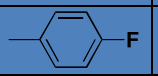
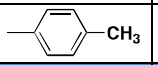
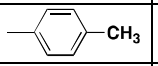
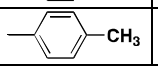
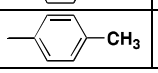
Table 4: SAR Analysis of APJ Agonists: Quinone-oxime sulfonates

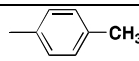
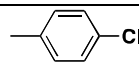
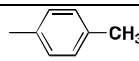
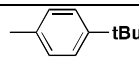
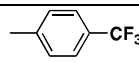
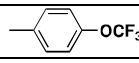
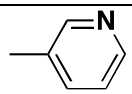
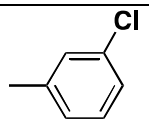
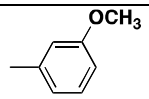
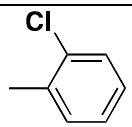
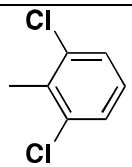
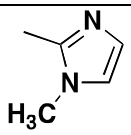
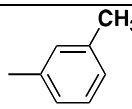
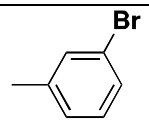
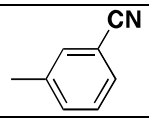
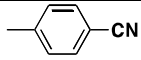


Potency (μM)

Ave. ± *S.E.M.* (stdv/sqrt (n))
(n = 4)

if number of replicates is different than the default it is noted in parentheses

Entry	CID	SID	MLS	S/ P*	R_1	R_2	R_3	R_4	R_5	APJ	Ave % Ap-13	AT1	SI APJ/AT 1
1	2906074	99361256	0437248	P	$-CH_3$	$-CH_3$	-H	-H	-Ph	2.50 ± 0.22 (3)	38	>79	>32
2	2906244	99361258	0437250	P	$-CH_3$	$-CH_3$	-H	-H		2.52 ± 0.20 (3)	40	>79	>31
3	2221149	99361257	0437249	P	$-CH_3$	$-CH_3$	-H	-H		2.56 ± 0.29 (3)	35	>79	>32
4	2217688	99361252	0437246	P	$-CH_3$	$-CH_3$	-H	-H		2.90 ± 0.47 (3)	39	>79	>27
5	49787121	103061724	0445745	S	iPr	-H	$-CH_3$	-H		3.52 ± 0.72	100	>79	>22
6	46905036	99361200 (**ML233**)	0437644	S	Chex	-H	$-CH_3$	-H	-Ph	3.74 ± 0.80	100	>79	>21
7	46905035	99361199	0437590	S	tBu	-H	$-CH_3$	-H	-Ph	4.20 ± 1.12	100	>79	>19
8	5932070	99361260	0437252	P	iPr	-H	$-CH_3$	-H		4.93 ± 0.50 (3)	42	>79	>16
9	5649929	99361253	0437247	P	$-CH_3$	-H	$-CH_3$	-H		5.40 ± 0.56 (3)	27	>79	>15
10	49787116	103061726	0445747	S	iPr	-H	$-CH_3$	-H		5.56 ± 0.94	100	>79	>14
11	5601088	99361194 (screen hit)	0437377	S	iPr	-H	$-CH_3$	-H	-Ph	5.98 ± 0.53	100	>79	>13
12	2303313	99361274 (screen hit)	0105911	P	$-CH_3$	-H	$-CH_3$	-H	-Ph	6.07 ± 0.48	42	>79	>13
13[a]	1909670	99361279	0304385	P	iPr	-H	$-CH_3$	-H	-Ph	5.74 ± 0.92	93	>79	>14
14	6508095	99361193	0437376	S	$-CH_3$	-H	$-CH_3$	-H	-Ph	6.82 ± 1.07	77	>79	>12
15	49787114	103061725	0445746	S	iPr	-H	$-CH_3$	-H		7.15 ± 1.54	100	>79	>11
16	2221138	99361255	0221001	P	$-CH_3$	-H	iPr	-H	-Ph	7.43 ± 1.09 (3)	38	>79	>11
17	5941439	99361259	0437251	P	$-CH_3$	-H	iPr	-H		9.90 ± 2.39 (3)	45	>79	>8
18	5731374	99361197	0437527	S	$-CH_3$	-H	iPr	-H	-Ph	11.90 ± 2.14 (3)	100	>79	>7
19	5258778	99361201	0437652	S	iPr	iPr	H	-H	-Ph	13.10 ± 3.00	100	>79	>6
20	2221133	99361254	0081733	P	H	H	$-CH_3$	$-CH_3$	-Ph	>79	ND	>79	ND
21	2246289	99361251	0084855	P	$-CH_3$	H	H	H	-Ph	>79	ND	>79	ND
22	1587414	99361262	0226770	P	$-CH_3$	H	H	$-CH_3$		>79	ND	>79	ND
23	2245190	99361248	0437242	P	H	H	H	H		>79	ND	>79	ND
24	5339511	99361249	0437243	P	H	H	$-CH_3$	H		>79	ND	>79	ND

25	5339523	99361250	0437244	P	-CH_3	H	H	H		>79	ND	>79	ND
26	5547945	99361261	0437253	P	-CH_3	H	H	H		>79	ND	>79	ND
27	5721885	99361275	0437287	P	H	H	-CH_3	H		>79	ND	>79	ND
28	49842909	104169554	0446034	S	iPr	H	-CH_3	H		14.70 ± 2.03 (3)	100	>79	>5
29	49842901	104169555	0446035	S	iPr	H	-CH_3	H		3.69 ± 0.96 (3)	100	>79	>21
30	49842904	104169556	0446036	S	iPr	H	-CH_3	H		3.82 ± 0.53	100	>79	>21
31	49842907	104169558	0446038	S	iPr	H	-CH_3	H		6.45 ± 2.64 (3)	100	>79	>12
32	49852493	104222787	0454424	S	Chex	H	-CH_3	H		2.61 ± 0.58 (4)	100	>79	>30
33	49852488	104222788	0454425	S	Chex	H	-CH_3	H		5.53 ± 1.85 (6)	100	>79	>14
34	50898268	110167715	0454448	S	Chex	H	-CH_3	H		6.50 ± 2.45 (6)	100	>79	>12
35	50898258	110167716	0454449	S	Chex	H	-CH_3	H		3.59 ± 1.22 (3)	100	>79	>22
36	50898272	110167717	0454450	S	Chex	H	-CH_3	H		6.36 ± 3.26 (6)	100	>79	>12
37	50898262	110167722	0454506	S	Chex	H	-CH_3	H		5.49 ± 2.11 (6)	100	>79	>14
38	50898264	110167723	0454507	S	Chex	H	-CH_3	H		6.03 + 2.31 (6)	100	>79	>13
39	50898270	110167728	0454512	S	Chex	H	-CH_3	H		5.13 ± 2.47 (6)	100	>79	>15
40	50898267	110167729	0454513	S	2-Ph	H	H	H	-Ph	>79	ND	>79	ND
41	50898254	110167730	0454514	S	2-Ph	H	H	H		>79	ND	>79	ND
42	50898260	110167732	0454515	S	2-iPr	H	H	H	-Ph	4.96 ± 1.25 (4)	100	>79	>16

* S = Synthesized P = purchased ND = not determined Ave % Ap-13= percent response compared to Apelin-13

[a]compound listed as mixture of isomers **probe** **submitted analog**

3.5 Cellular Activity

All of the primary and selectivity assays are cell based. Specific measures of permeability and toxicity are discussed in section 3.6 below.

3.6 Profiling Assays

The nominated probe was evaluated in a detailed *in vitro* pharmacology screen as shown in **Table 5**.

Table 5: Summary of *in vitro* ADME Properties of APJ Antagonist probe **ML233**

Probe **Probe ML#** SBCCG MLS-#	**Aqueous Solubility** (µg/mL) (pH5.0/6.2/7.4) *[µM]*[a]	**Aqueous Solubility** in 1x PBS (µg/mL) **pH 7.4** *[µM]*	**PAMPA** **Pe** ($x10^{-6}$ cm/s) **Donor pH:** 5.0/6.2/7.4 **Acceptor** pH: 7.4	**Plasma Protein Binding** **(% Bound)** **Human** 1µM	 **Mouse** 1µM	**Plasma Stability** (%Remaining @3hrs) Human/Mouse Plasma:1x PBS, pH 7.4, 1:1 1x PBS, pH 7.4	**Hepatic Microsome Stability** Human/ Mouse	**Hepatic Toxicity** LC_{50} (µM)
CID46905036 **ML233** MLS-00437644	<0.1/<0.1/<0.1 *[<0.3/<0.3/<0.3]]*	<0.1 *[<0.3]*	(1299/1159/882)* *with 20% acetonitrile	97.84	99.68	18.68/100 5.21/6.12	<1/<1	25.8

[a] Solubility also expressed in molar units (µM) as indicated in *italicized [bracketed values],* in addition to more traditional µg/mL units.

ML233 is poorly soluble in aqueous media at pH 5.0/6.2/7.4.

The PAMPA (**P**arallel **A**rtificial **M**embrane **P**ermeability **A**ssay) assay is used as an *in vitro* model of passive, transcellular permeability. An artificial membrane immobilized on a filter is placed between a donor and acceptor compartment. At the start of the test, drug is introduced in the donor compartment. Following the permeation period, the concentration of drug in the donor and acceptor compartments is measured using UV spectroscopy. Consistent with the predicted LogP (see **Table 3**), **ML233** is a highly permeable compound in this assay. This data suggests that, like many therapeutic molecules, the poor aqueous solubility of **ML233** will have little impact on its activity in cells and tissues.

Plasma Protein Binding is a measure of a drug's efficiency to bind to the proteins within blood plasma. The less bound a drug is, the more efficiently it can traverse cell membranes or diffuse. Highly plasma protein bound drugs are confined to the vascular space, thereby having a relatively low volume of distribution. In contrast, drugs that remain largely unbound in plasma are generally available for distribution to other organs and tissues. **ML233** is highly bound to plasma proteins in mouse plasma (99% bound). Similarly, although to a lesser extent, **ML233** is highly protein bound in human plasma (98% bound). This interaction may confer stability to the molecule in plasma, protecting it from metabolizing enzymes.

Plasma Stability is a measure of the stability of small molecules and peptides in plasma and is an important parameter, which can strongly influence the *in vivo* efficacy of a test compound. Drug candidates are exposed in plasma to enzymatic processes (proteinases, esterases), and they can undergo intramolecular rearrangement or bind irreversibly (covalently) to proteins. Although **ML233** is extensively bound to plasma proteins, the compound exhibits very poor stability in human plasma (18.68% remaining) after 3 hr. In contrast, **ML233** is very stable in mouse plasma (100% remaining). This may be explained by the relative activities of metabolizing enzymes in the differing plasmas.

Alternatively, these disparate results may be explained by the subtle differences in plasma protein binding in mouse versus human plasma. Although the difference in percent compound bound in mouse versus human plasma appears minor (99% vs. 98%), the plasma protein-binding assay is not performed in a way that is suitable for assessing the kinetics of **ML233** binding to plasma proteins. Therefore, we cannot exclude the possibility that as **ML233** dissociates from human plasma proteins, it is rapidly metabolized.

The microsomal stability assay is commonly used to rank compounds according to their metabolic stability. This assay addresses the pharmacologic question of how long the parent compound will remain circulating in plasma within the body. **ML233** shows poor stability (<1.0% remaining after 60 minutes) in both human and mouse liver homogenates. Ultimately this limits the utility of this probe to *in vitro* studies or apelin receptor or *in vivo* studies using acute intravenous doses to avoid hepatic metabolism.

ML233 shows some toxicity (LC_{50} = 25.8 μM) toward human hepatocytes.

Profiling against other GPCRs. The probe, **ML233** (CID46905036), was submitted to the Psychoactive Drug Screening Program (PDSP) at the University of North Carolina (PDSP, Bryan Roth, PI) and the data against a GPCR binding assay panel is shown in **Figure 6**. **ML233** shows potentially significant binding against several other receptors, including the 5-HT_{1A}, α_{2C} adrenergic, and benzylpiperazine receptors (55%I, 51%I and 65%I at 10 μM, respectively) and norepinephrine transporter (57%I at 10 μM). It is not known whether these activities in binding assays are translated into functional modification of the activities of these receptors. In addition, these potential cross-reactivities should not confound any *in vitro* data obtained with **ML233**.

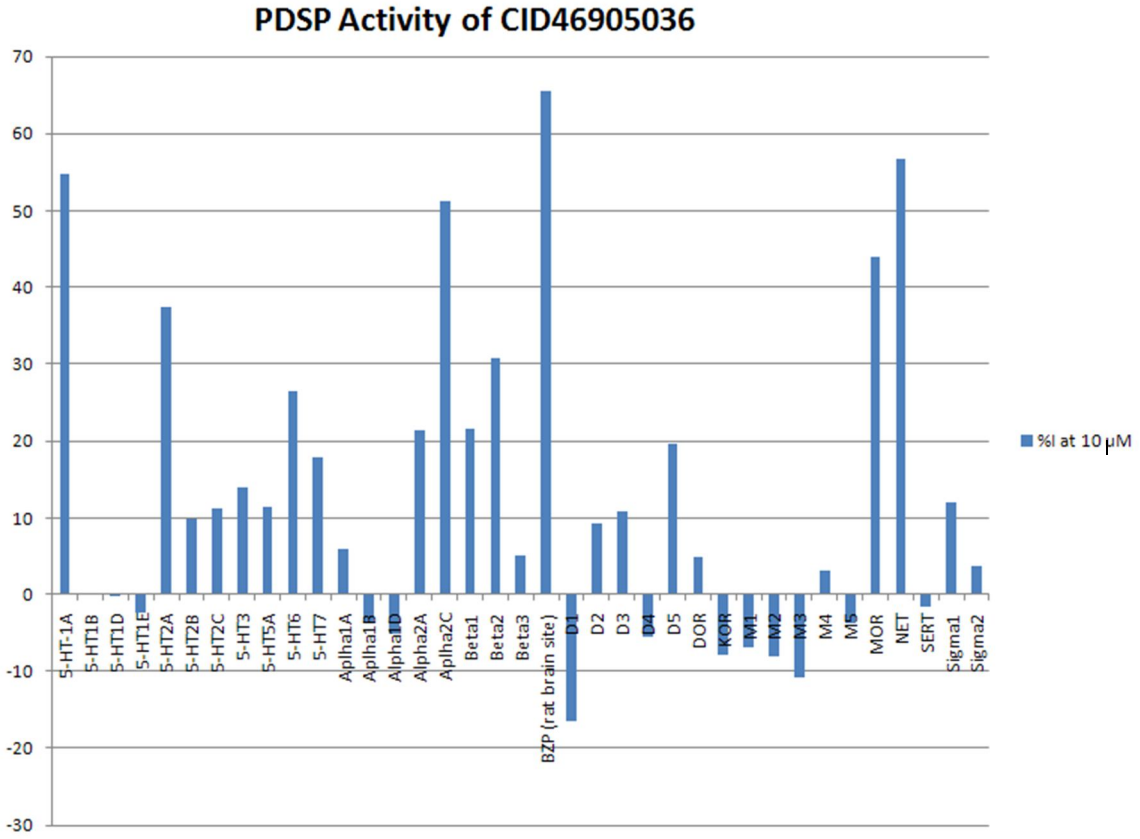


Figure 6: GPCR profiling panel for **ML233**

As a follow up experiment, **ML233** was submitted for further testing by DiscoveRx (as a CRO) in a panel of functional assays of selected GPCRs (see **Table 6** below). Those GPCRs tested were chosen based on their logical association with apelin/APJ due to their effects on cardiovascular function. **Table 6** shows that **ML233** has little to no agonist activity at 14 related receptors when assayed at 10µM. Those receptors tested include the adrenergic receptor family, the endothelin and bradykinin receptor families, as well as the vasopressin receptors. In this panel of GPCRs, the AT1 receptor was also profiled (AGTR1, line 7). The data returned from this externally executed panel is shown below in **Table 6**.

Table 6. ML233 Functional profiling data against the DiscoveRx GPCR Panel tested at a single 10 µM concentration.

GPCR ID	Control 1	Mean RLU	SD	%CV	Control 2	Mean RLU	SD	Compound ID	Assay Mode	Conc (µM)	Mean RLU	SD	% Activity
ADRA1B	Baseline	156020	6833	4%	Max	764870	32273	ML233	Agonist	10	188740	13944	5%
ADRA2A	Baseline	219850	11737	5%	Max	1301750	40101	ML234	Agonist	10	612660	7608	36%
ADRA2B	Baseline	108230	3615	3%	Max	588120	26356	ML235	Agonist	10	216040	622	22%
ADRA2C	Baseline	7475	1030	14%	Max	51800	3784	ML236	Agonist	10	10720	170	7%
ADRB1	Baseline	45510	5029	11%	Max	193540	4607	ML237	Agonist	10	83740	17508	26%
ADRB2	Baseline	18255	1580	9%	Max	454380	5568	ML238	Agonist	10	24440	1697	1%
AGTR1	Baseline	276870	8275	3%	Max	1722980	90271	ML239	Agonist	10	204160	9899	-5%
AVPR1A	Baseline	10905	457	4%	Max	40520	3835	ML241	Agonist	10	10500	651	-1%
AVPR1B	Baseline	13445	1027	8%	Max	127650	5927	ML242	Agonist	10	12820	481	-1%
AVPR2	Baseline	132140	5819	4%	Max	996790	42586	ML243	Agonist	10	156320	2376	3%
BDKRB1	Baseline	24125	1423	6%	Max	153520	6898	ML244	Agonist	10	48140	2121	19%
BDKRB2	Baseline	223210	10360	5%	Max	1296580	57120	ML245	Agonist	10	241920	47122	2%
EDNRA	Baseline	15005	2130	14%	Max	147020	14019	ML246	Agonist	10	35980	820	16%
EDNRB	Baseline	6015	968	16%	Max	61690	8013	ML247	Agonist	10	24360	566	33%

4 Discussion

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

There has been only one report of a “small molecule” APJ agonists in the open or patent literature (12), but, as discussed in the introduction, this is a peptide derived compound with mw>1000. Thus, **ML233** represents the first true small molecule (mw= 359) APJ agonist that will be available to the scientific community.

4.2 Mechanism of Action Studies

Dr. Smith’s laboratory has performed a series of exploratory studies to investigate the mechanism of action of this probe. The mechanism of action of **ML233** is that of a classic full agonist of APJ in the cell-based assays employed in this project. Because APJ is capable of both G-protein-dependent and β-arrestin-dependent signaling, and the primary assay utilized in the project measured the activity of **ML233** as a function of β-arrestin recruitment to APJ, we assessed the activity of **ML233** in an assay of G-protein signaling by APJ. This MOA assay uses the High Sensitivity Lance TR-FRET cAMP assay (Perkin Elmer). Consistent with its role as an APJ agonist, **ML233** reduced forskolin stimulated increases in intracellular cAMP (**Figure 7A**). Interestingly, the observed potency of **ML233** to reduce cAMP was significantly higher than the maximal concentration studied (100µM). In addition, **ML233** induced APJ internalization (**Figure 7B**) taken together these probe

characterization studies suggest that, like most GPCRs, the apelin receptor is capable of pleiotropic signaling, and that **ML233** is a useful tool probe β-arrestin-mediated signaling and receptor internalization of APJ. Those investigators specifically interested in these aspects of APJ pharmacology and function will be well served by using **ML233**. However, the utility of **ML233** in probing G-protein signaling of APJ is yet to be fully explored. Investigators using **ML233** for this purpose should further explore the effects of **ML233** in their assay of choice.

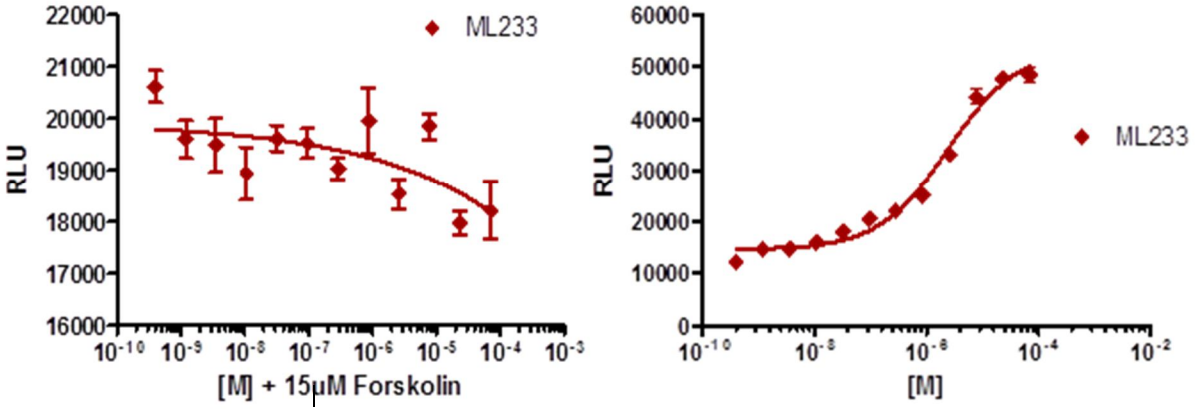


Figure 7: ML233 reduces forskolin stimulated intracellular cAMP and stimulates APJ internalization. (A) CHO-K1 cells heterologously expressing the human apelin receptor APJ were exposed to forskolin (15 μM) and a range of concentration of ML233. ML233 weakly reduced (>10% of max) intracellular cAMP at 100μM .(B) ML233 induced APJ internalization with a potency that is consistent with those observed in the β-arrestin recruitment assay (EC50=2.4μM). These figures are representative of experiments that were repeated twice with a n=3. Nonlinear regression analysis was performed using GraphPad Prism5 and a 4 point logistic curve fit.

4.3 Planned Future Studies

As described above, studies to support the proposed mechanism of action are underway in Dr. Smith's laboratory using the probe molecule described in this report. Future studies also planned will involve testing **ML233** in genetically tractable model organisms including zebra fish and mice. Additional medicinal chemistry efforts to improve the drug-like properties of **ML233** are under way. Specific efforts will be directed at improving the metabolic stability and especially solubility. Improvements in these properties will improve the *in vivo* utility of **ML233** *in vivo*.

Future *in vitro* studies to be performed by Dr. Smith will determine if **ML233** is a direct competitor of apelin binding using standard competition binding studies and radiolabeled apelin-13. Consistent with our Chemical Probe Development Plan, we will also evaluate the affinity of **ML233** to the angiotensin II type 1 receptor, the receptor most closely related to APJ. Additional studies will utilize existing cell based assays to determine if **ML233** is an allosteric modulator of APJ

5 References

1. Quazi, R., Palaniswamy, C., and Frishman, W.H. 2009. The emerging role of apelin in cardiovascular disease and health. *Cardiol Rev* 17:283-286. PMID: 19829178.
2. Sorli, S.C., van den Berghe, L., Masri, B., Knibiehler, B., and Audigier, Y. 2006. Therapeutic potential of interfering with apelin signalling. *Drug Discov Today* 11:1100-1106. PMID: 17129829.
3. Chandra, S.M., Razavi, H., Kim, J., Agrawal, R., Kundu, R.K., de Jesus Perez, V., Zamanian, R.T., Quertermous, T., and Chun, H.J. 2010. Disruption of the Apelin-APJ System Worsens Hypoxia-Induced Pulmonary Hypertension. *Arterioscler Thromb Vasc Biol* 31:814-820. PMID: 21233449.
4. Niu, W., Wu, S., Zhang, Y., Li, W., Ji, K., Gao, P., and Zhu, D. 2010. Validation of genetic association in apelin-AGTRL1 system with hypertension in a larger Han Chinese population. *J Hypertens* 28:1854-1861. PMID: 20485192.
5. Przewlocka-Kosmala, M., Kotwica, T., Mysiak, A., and Kosmala, W. 2011. Reduced circulating apelin in essential hypertension and its association with cardiac dysfunction. *J Hypertens*. PMID: 21346619.
6. Ashley, E.A., Powers, J., Chen, M., Kundu, R., Finsterbach, T., Caffarelli, A., Deng, A., Eichhorn, J., Mahajan, R., Agrawal, R., *et al.* 2005. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading *in vivo*. *Cardiovasc Res* 65:73-82. PMID: 15621035.
7. Chen, M.M., Ashley, E.A., Deng, D.X., Tsalenko, A., Deng, A., Tabibiazar, R., Ben-Dor, A., Fenster, B., Yang, E., King, J.Y., *et al.* 2003. Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. *Circulation* 108:1432-1439. PMID: 12963638.
8. Jia, Y.X., Pan, C.S., Zhang, J., Geng, B., Zhao, J., Gerns, H., Yang, J., Chang, J.K., Tang, C.S., and Qi, Y.F. 2006. Apelin protects myocardial injury induced by isoproterenol in rats. *Regul Pept* 133:147-154. PMID: 16278022.
9. Chun, H.J., Ali, Z.A., Kojima, Y., Kundu, R.K., Sheikh, A.Y., Agrawal, R., Zheng, L., Leeper, N.J., Pearl, N.E., Patterson, A.J., *et al.* 2008. Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J Clin Invest* 118:3343-3354. PMID: 18769630.
10. Hashimoto, T., Kihara, M., Imai, N., Yoshida, S., Shimoyamada, H., Yasuzaki, H., Ishida, J., Toya, Y., Kiuchi, Y., Hirawa, N., *et al.* 2007. Requirement of apelin-apelin receptor system for oxidative stress-linked atherosclerosis. *Am J Pathol* 171:1705-1712. PMID: 17884970.
11. Barnes, G., Japp, A.G., and Newby, D.E. 2010. Translational promise of the apelin--APJ system. *Heart* 96:1011-1016. PMID: 20584856.
12. Iturrioz, X., Alvear-Perez, R., De Mota, N., Franchet, C., Guillier, F., Leroux, V., Dabire, H., Le Jouan, M., Chabane, H., Gerbier, R., *et al.* 2010. Identification and pharmacological properties of E339-3D6, the first nonpeptidic apelin receptor agonist. *FASEB J* 24:1506-1517. PMID 20040517.
13. O'Dowd, B.F., Heiber, M., Chan, A., Heng, H.H., Tsui, L.C., Kennedy, J.L., Shi, X., Petronis, A., George, S.R., and Nguyen, T. 1993. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 136:355-360. PMID: 8294032.
14. Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M.X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C., *et al.* 1998. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 251:471-476. PMID: 9792798.
15. Hosoya, M., Kawamata, Y., Fukusumi, S., Fujii, R., Habata, Y., Hinuma, S., Kitada, C., Honda, S., Kurokawa, T., Onda, H., *et al.* 2000. Molecular and functional characteristics of

APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem* 275:21061-21067. PMID: 10777510.

16. Janz, J.; Kuliopulos, A.; McMurry, T. J., WO2010/053545, "APJ Receptor Compounds"
17. Llorens-Cortes, C.; Guillier, F.; Franchet, C., EP1903052 (2006) "APJ receptor ligands and uses thereof"
18. Hamada, J.; Kimura, J.; Ishida, J.; Kohda, T.; Morishita, S.; Ichihara, S.; Fukamizu, A., Evaluation of novel cyclic analogues of apelin, International Journal of Molecular Medicine, 2008, 22, 547-552. PMID: 18813863.
19. Ishikawa, T., Saito, T., Ishii, H. **1995**. Synthesis of Macarpine and its Cytotoxicity: Toward a Synthetic Route for 12-Alkoxybenzo[*c*]phenanthridine Alkaloids through Aromatic Nitrosation under Basic Condition. *Tetrahedron* 51: 8447-8458. PMID: 18813863.

6 Supplementary Information

6.1 Assay Details: *APJ Beta-Arrestin 1536-Well Agonist Assay Protocol*

A. Brief Description of the Assay: The purpose of this assay is to detect agonists that cause the activation and internalization of the Angiotensin II receptor-like 1 (Apelin receptor) in the CHO-K1 AGTRL-1 Beta-Arrestin Cell Line in 1536-well plate format.

B. Materials:

- Angiotensin II receptor-like 1 (AGTRL-1) Cell Line (DiscoveRx, Cat# 93-0250C2)
- F12 nutrient mix HAMs (Invitrogen, Cat# 11765)
- Fetal Bovine Serum, heat-inactivated (Hyclone, Cat# SH30396)
- 100X Penicillin/Streptomycin Solution (Invitrogen, Cat#15140-122)
- Hygromycin B (Roche, Cat# 10843555001)
- Geneticin (MPBiomedicals, Cat # 1672548)
- Trypsin-EDTA 0.25% (Invitrogen, Cat# 25200-056)
- Cell Dissociation Buffer (Invitrogen, Cat # 13151)
- DPBS (Hyclone, Cat# 30028.02)
- T225 TC Flask (Nunc, Cat# 159934)
- Cell strainer, 40 um (BD, Cat# 352340)
- 1536-well, white, solid-bottom, Kalypsys compatible, TC plate (Corning)
- Apelin-13 (Sigma-Aldrich, Cat # A6469)
- PathHunter Detection Reagents (DiscoveRx, Cat# 93-0001)
- Galacton Star
- Emerald 11
- Cell Assay Buffer

C. Procedures:

Day1 –Cell Seeding

1. Plate 1000 cells/well in 4 μL of assay media into columns 1-48 of a 1536-well assay plate, using straight tip dispense on a Kalypsys dispenser.
2. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge. Use Kalypsys metal lids.
3. Incubate overnight at 37 degrees, 100% relative humidity, 5% CO_2 for 16-18 hours.

Day2 –Compound Addition

4. Using the Kalypsys Dispenser, add 2μl/well of Assay media to Col. 3-48 for the negative control and test compound wells.
5. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge.
4. Using LabCyte Echo, transfer appropriate volume of test compounds in DMSO into assay plate Col. 5 – 48, then backfill with DMSO to equalize DMSO concentration if necessary (Final concentration primary assay= 1.0% DMSO, Final concentration dose response= 1.32%).
6. Transfer equal volume of DMSO to positive and negative control wells in Columns 1 – 4.
7. Immediately following compound/DMSO transfer via the Echo, transfer 2μl/well of 6 μM Apelin-13 in assay media to the positive control wells.
8. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge.
9. Incubate plates at room temperature in the dark for 90 minutes.
10. Following 90 minute incubation, deliver 3.0 µL of Detection Reagent solution to each assay plate (Columns 1 – 48) using a Kalypsys dispenser.
11. Centrifuge plates at 1000 rpm for 1 minute on a Vspin centrifuge.
12. Incubate plates for 60 minutes at room temperature in the dark.
13. Read plates using a Perkin Elmer Envision using a luminescence

D. Recipe:

- Growth Media: F12 nutrient mix HAMs supplemented with 10% hi-FBS, 1X Penicillin/Streptomycin, 300μg/ml Hygromycin B, 800μg/ml Geneticin
- Assay Media: Same as Growth Media
- Trypsin: Dilute 0.25% Trypsin/EDTA to 0.05% Trypsin/EDTA using DPBS
- Positive Control: Assay Media with Apelin-13 (10 nM FAC)
- Negative Control: Assay Media with no Apelin-13
- Detection Reagent: Use the following ratio to prepare the detection reagent: Galacton Star: Emerald: Cell Assay Buffer (1:5:19)

6.2 Assay Details: *AT1 Beta-Arrestin 1536-Well Agonist Assay Protocol*

A. Brief Description of the Assay: The purpose of this assay is to detect agonists that cause the activation and internalization of the Angiotensin II receptor type 1 (AT1 receptor) in the CHO-K1 AGTR-1 Beta-Arrestin Cell Line in 1536-well plate format.

B. Materials:

- Angiotensin II receptor type 1 (AGTRL-1) Cell Line (DiscoveRx, Cat# 93-0312C2)
- F12 nutrient mix HAMs (Invitrogen, Cat# 11765)
- Fetal Bovine Serum, heat-inactivated (Hyclone, Cat# SH30396)
- 100X Penicillin/Streptomycin Solution (Invitrogen, Cat#15140-122)
- Hygromycin B (Roche, Cat# 10843555001)
- Geneticin (MPBiomedicals, Cat # 1672548)
- Trypsin-EDTA 0.25% (Invitrogen, Cat# 25200-056)
- Cell Dissociation Buffer (Invitrogen, Cat # 13151)
- DPBS (Hyclone, Cat# 30028.02)
- T225 TC Flask (Nunc, Cat# 159934)
- Cell strainer, 40 μm (BD, Cat# 352340)
- 1536-well, white, solid-bottom, Kalypsys compatible, TC plate (Corning)
- Apelin-13 (Sigma-Aldrich, Cat # A6469)
- PathHunter Detection Reagents (DiscoveRx, Cat# 93-0001)
- Galacton Star

- Emerald 11
- Cell Assay Buffer

C. Procedures:

Day1 –Cell Seeding

1. Plate 500 cells/well in 5 μL of assay media into columns 1-48 of a 1536-well assay plate, using straight tip dispense on a Kalypsys dispenser.
2. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge. Use Kalypsys metal lids.
3. Incubate overnight at 37 degrees, 100% relative humidity, 5% CO_2 for 16-18 hours.

Day2 –Compound Addition

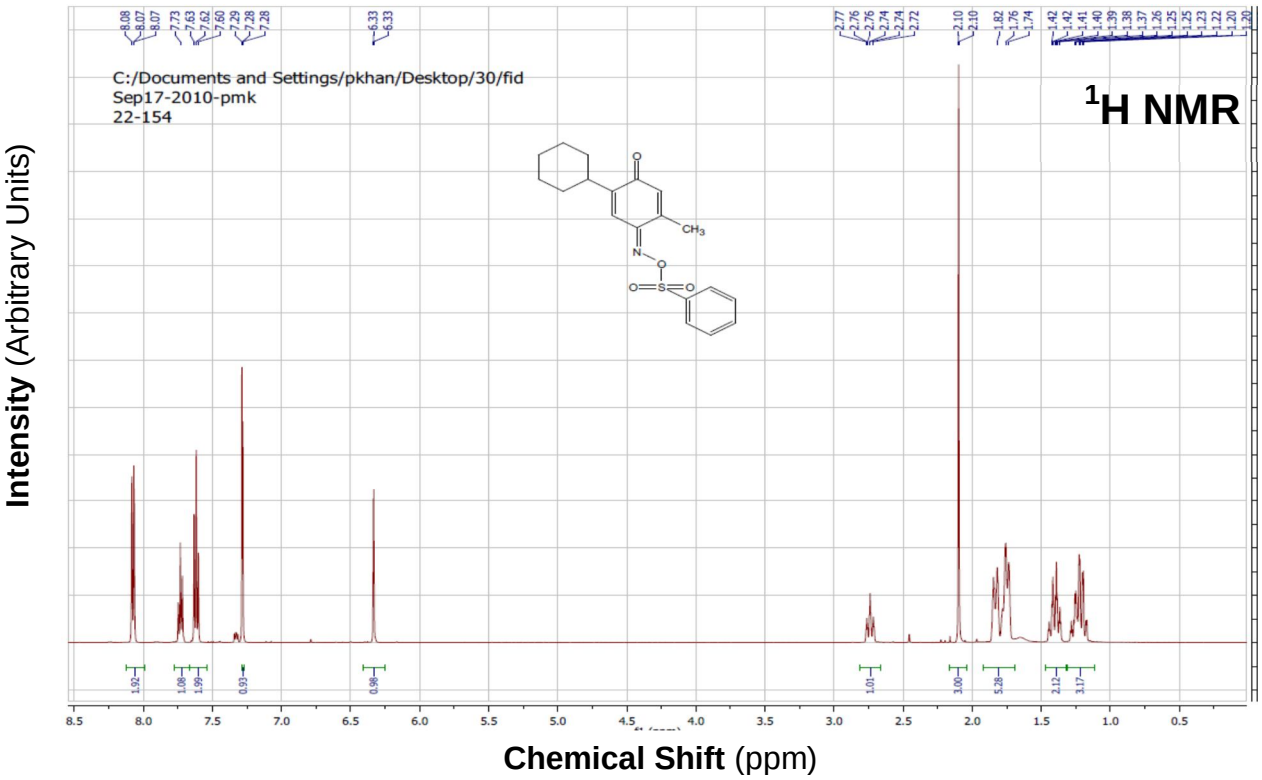
5. Using the Kalypsys Dispenser, add 1μl/well of Assay media to Col. 3-48 for the negative control and test compound wells.
6. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge.
7. Using LabCyte Echo, transfer appropriate volume of test compounds in DMSO into assay plate Col. 5 – 48, then backfill with DMSO to equalize DMSO concentration (Final concentration = 1.32% DMSO).
8. Transfer equal volume of DMSO to positive and negative control wells in Columns 1 – 4.
9. Immediately following compound/DMSO transfer via the Echo, transfer 1μl/well of 6 μM Angiotensin II in assay media to the positive control wells.
10. Centrifuge plates at 500 rpm for 1 minute on a Vspin centrifuge.
11. Incubate plates at room temperature in the dark for 90 minutes.
12. Following 90 minute incubation, deliver 3.0 μL of Detection Reagent solution to each assay plate (Columns 1 – 48) using a Kalypsys dispenser.
13. Centrifuge plates at 1000 rpm for 1 minute on a Vspin centrifuge.
14. Incubate plates for 60 minutes at room temperature in the dark.
15. Read plates using a Perkin Elmer Envision using a luminescence

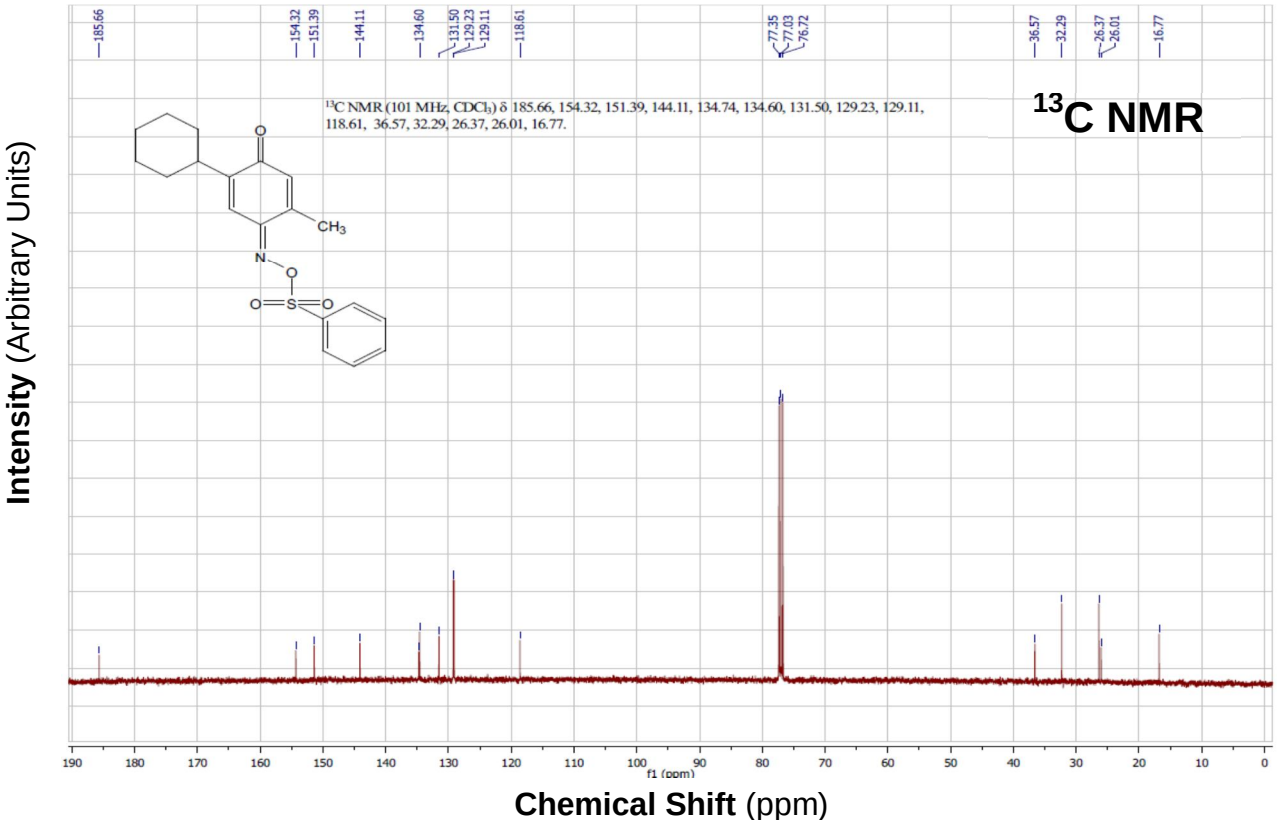
D. Recipe:

- Growth Media: F12 nutrient mix HAMs supplemented with 10% hi-FBS, 1X Penicillin/Streptomycin, 300μg/ml Hygromycin B, 800μg/ml Geneticin
- Assay Media: Same as Growth Media except 6% hi-FBS
- Trypsin: Dilute 0.25% Trypsin/EDTA to 0.05% Trypsin/EDTA using DPBS
- Positive Control: Assay Media with Angiotensin II (FAC = 1 μM)
- Negative Control: Assay Media with no Angiotensin II
- Detection Reagent: Use the following ratio to prepare the detection reagent: Galacton Star: Emerald: Cell Assay Buffer (1:5:19)

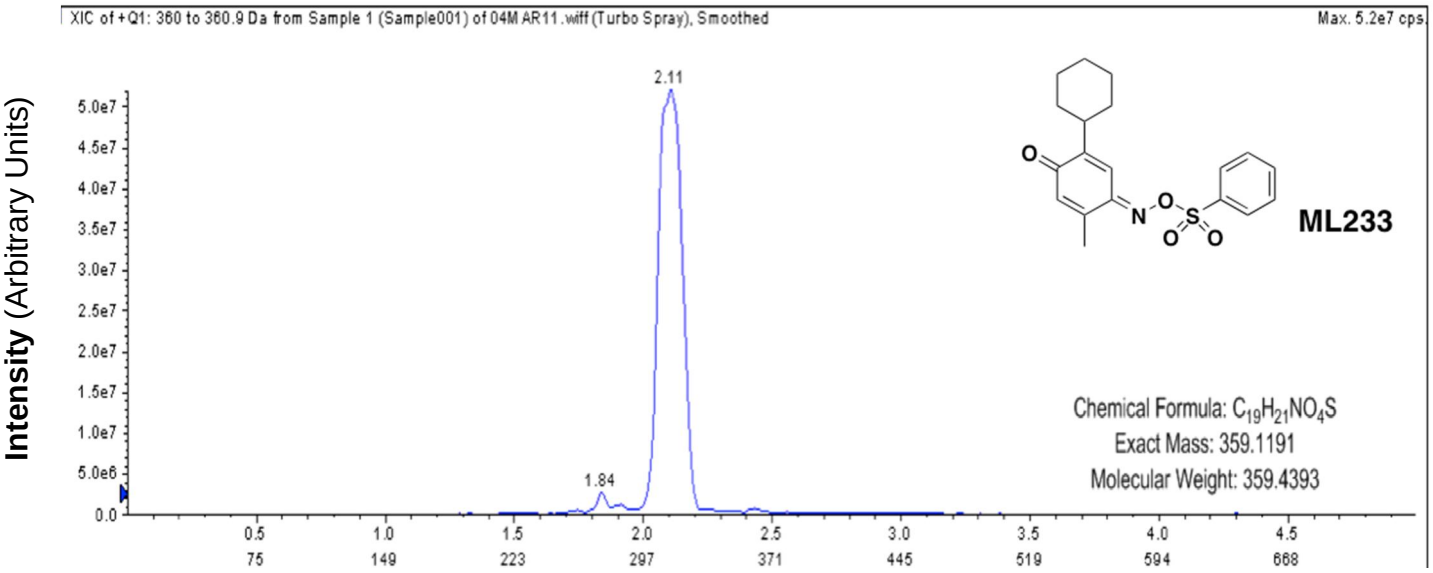
6.3 Supplementary Information for Probe Characterization.

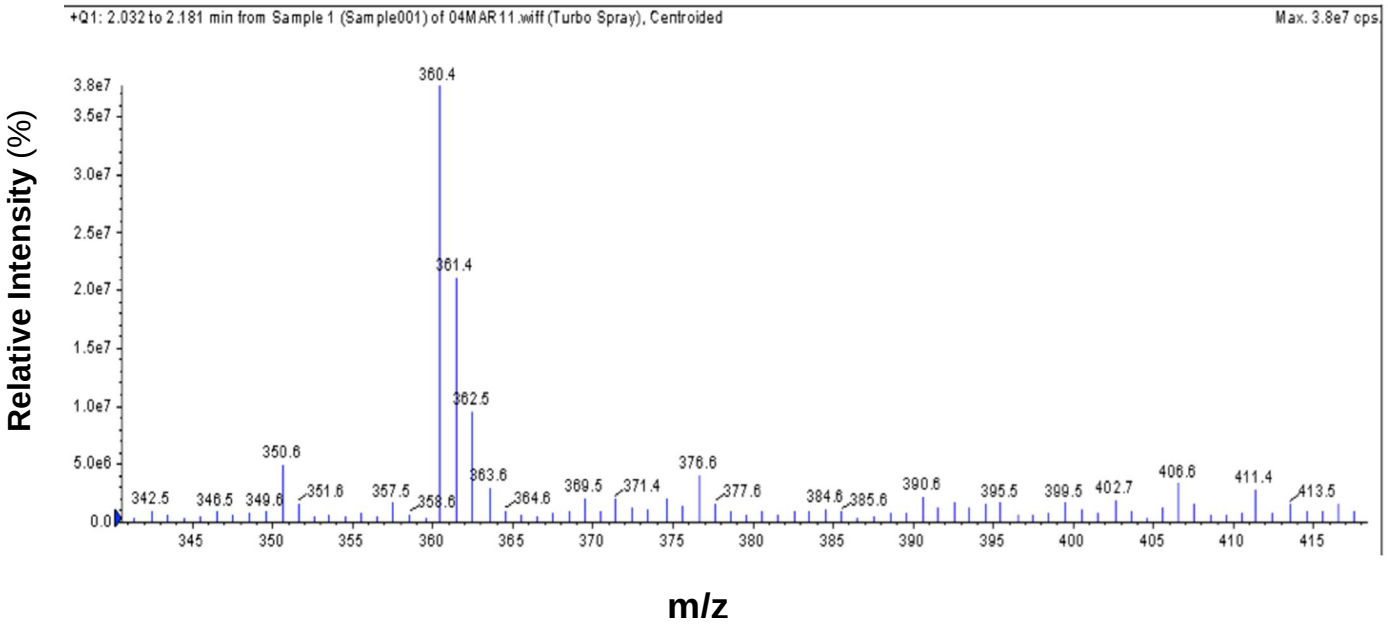
^{1}H & ^{13}C NMR Spectra and LC/MS Data for Structural Determination of **ML233**



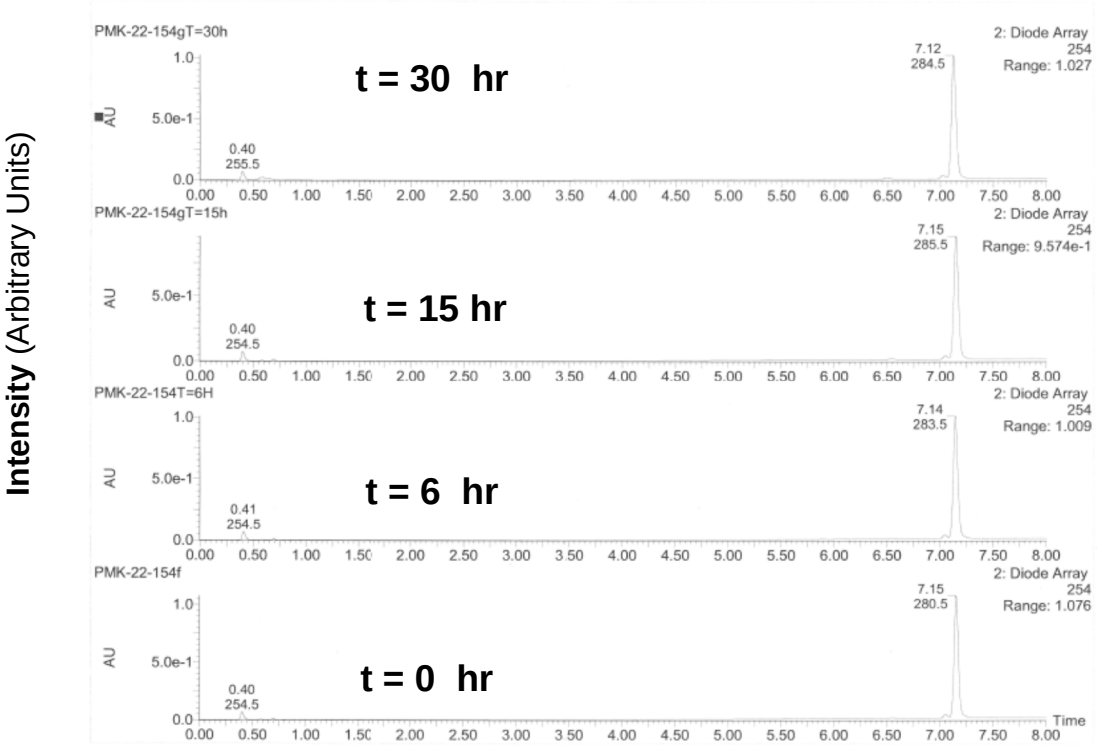


LC/MS Data for Structural Determination of ML233





Stability study of ML233 in 50% aqueous acetonitrile



LC/MS of ML233 with glutathione after 0, 30 min, 2 and 7 hours