Probe Report

Probe project: Selective KOP Receptor antagonists
Title: Agonists and Antagonists for the Kappa Opioid Receptor
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Assigned Assay Grant #: 1X01MH084153-01
Screening Center Name & PI: Burnham Center for Chemical Genomics (BCCG) & John C. Reed
(renamed Conrad Prebys Center for Chemical Genomics at Sanford|Burnham Medical Research Institute in 2010. But still referred to for NIH Roadmap Purposes as BCCG above)
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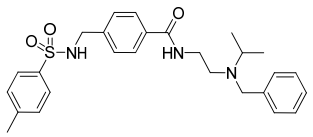
Assay Submitter & Institution: Lawrence Barak, Duke University Medical Center
Collaborating PI: (Laura M. Bohn, The Scripps Research Institute, FL)

PubChem Summary Bioassay Identifier (AID): 1785

Probe Structure & Characteristics:



ML140

CID	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target Name(s)	IC_{50}/EC_{50} (μM) [SID, AID]	Select-ivity	Secondary Assay(s) Name: IC_{50}/EC_{50} (nM) [SID, AID]
3342390 *(sulfonamide)* **ML140**	KOR *κ-opioid receptor*	850 nM IC_{50} SID87218794 AID2285	MOR *μ-opioid receptor*	>32 μM IC_{50} SID87218794 AID2420	> 40X *(Dx/HCS)* >25X *(HCS/HCS)*	KOR Transfluor 1310 nM IC_{50} [SID87218794, [AID2348]
			DOR *δ-opioid receptor*	>32 μM IC_{50} SID87218794 AID2357	> 40X *(Dx/HCS)* >25X *(HCS/HCS)*	MOR Transfluor >32000 nM IC_{50} [SID87218794, [AID2420]
						DOR Transfluor >32000 nM IC_{50} [SID87218794, AID2357]

Recommendations for the scientific use of this probe:

The probe candidate proposed in this report, by selectively inhibiting the human kappa opioid receptor subtype, would provide a scientific tool useful in helping to elucidate individual brain pathways that underlie addictive behavior, thus enabling improved understanding of the molecular basis of dependency and potentially providing a basis for therapeutic development.

1. Scientific Rationale for Project

Specific Aims

The identification of small molecules, each able to block or activate only a distinct receptor underlying an addiction will provide a means to untangle the many pathways resulting in addictive behavior and create detailed pharmacological maps for designing novel targeted treatments. This project proposes screening a G protein-coupled receptor relevant to drug abuse and to the study and treatment of addiction, in a fashion that afford the unique opportunity to discriminate between G protein and arrestin-based signaling modalities. This project will hopefully contribute to understanding and treating addiction by providing chemical probes for dissecting the individual brain pathways that underlie addictive behavior thus enabling improved understanding of the molecular basis of addiction and potentially providing targeted therapeutics for this affliction.

The specific aim of this project is to identify subtype specific small molecule antagonists of the human kappa opioid receptor (KOR). Such antagonists have been shown to prevent reinstatement of drug taking behavior in animal paradigms thought to model relapse. In addition, they have been shown to block aspects of nicotine withdrawal, and have antidepressant effects in animal models. Use of the existing kappa antagonists to explore these effects in vivo has been limited by their very long duration of pharmacological action (3-4 weeks in rhesus monkeys), which appears to be mediated not by pharmacokinetics but by activation of c-Jun N-terminal kinase (JNK) phosphorylation. Known kappa antagonists all appear to share this effect, which may contribute to their long duration of action in vivo. Novel kappa opioid receptor antagonists that do not activate the JNK pathway would be desirable, but their discovery is beyond the scope of the screening project. Therefore, selective kappa antagonists with new chemical structures may represent valuable leads to the discovery of shorter acting compounds. KOR selective antagonists will enable delineation of KOR specific signaling. Subtype selectivity is defined as selective for KOR but not active against μ (MOR) or δ (DOR) subtypes.

Background and Significance

For normal activities that produce rewards, there is a rapid habituation of the circuits involved and the behaviors will wane. However, for addictive drugs habituation does not occur and dopamine release persists despite repetitive trials. Upon withdrawal of the drug, a decrease of dopamine levels in the nucleus accumbens results, and this has been observed for opioids, cannabinoids, alcohol, amphetamines, and nicotine (1). This loss of dopamine accounts for the withdrawal syndromes observed with these drugs. The prototype opioid drug is morphine. It produces many effects typical of most opioids including analgesia, euphoria, nausea, and respiratory depression. Repeated use of opioids produces physical dependence and tolerance. These manifestations of opioid use are due to the three recognized types of opioid receptors that are members of the GPCR family, the mu (μ), delta (δ), and kappa (κ) subtype receptors. While stimulation of the mu and delta receptors increases dopamine release in the nucleus accumbens, κ opioid receptor (KOR) activation by its endogenous ligand dynorphin-A reduces extracellular dopamine. It has been suggested that stimulation of KOR by endogenous opioids like dynorphins will produce an aversive

state and thereby counter the effects of rewarding and addictive compounds like alcohol, cocaine and nicotine. Moreover, exogenous KOR agonists have also been observed to attenuate drug-taking behavior (2-6). However, it may be difficult to strike a balance between opposing the sense of reward gained by drugs of abuse and producing an aversive state; therefore, activation of the KOR may not be therapeutically preferable. Although these statements appear contrary, KOP agonists can both alleviate drug self administration in animal models (most likely via dopamine regulation) and also trigger relapse. This conflicting dual action of KOP agonists alludes to the complex physiological role of K receptors and underscores the need for their further investigation.

Intracranial self-stimulation has become a useful means of assessing reward thresholds in rodents and nonhuman primates. In essence, an animal will press a lever to electrically stimulate the brain via implanted probes. This "self stimulation" will be performed to a certain extent in training and that extent is an indication of the animal's "reward threshold." Administration of drugs of abuse have been shown to decrease this reward threshold such that the animal will seek less stimulation to achieve the desired effect. This model paradigm has been likened to positive hedonic states produced by drugs of abuse in human addicts. In rodents, the direct activation of KOP using selective agonists increases reward thresholds (mimicking the withdrawal state) and creating a "depressive-like" state (where more self stimulation is required to achieve the desired effect). Treatment with antagonists has been shown to restore reward thresholds in this model (7). The restoration of reward thresholds may be a very important step in drug abuse treatment as drug cessation is strongly negatively reinforced by aversive feelings, which may be due to a increased reward threshold. Therefore, the development of KOR antagonists may be particularly beneficial in "resetting" this threshold. Furthermore, since an increased reward threshold may manifest as a "depressive state," then KOR antagonists may also be beneficial for the treatment of depressive disorders. Currently, there are currently no approved agents or compounds for treating the altered reward pathways associated with drug addiction (2).

In this probe report, we describe the discovery and optimization of a novel antagonist for the kappa-(κ)opioid receptor (KOR) that is >40-fold selective over the mu-(μ) (MOR) and the delta-(δ) (DOR) opioid receptors. We sent the probe for broad GPCR paneling and these data are discussed below (see **Table 6**). Importantly, this probe and its analogs represent a novel chemical class compared to current literature antagonists and displays potentially novel pharmacology. Accordingly, this probe and its analogs may serve as interesting tools to advance addiction research. Additionally, this new chemotype is less complicated compared to known KOP antagonist compounds. The structure contains no stereochemical centers and the short, versatile synthetic route enables both the synthesis of potential analogs and the production of the compound on larger scale.

Follow up studies subsequent to the probe report to investigate the properties of the new probes will be required and will employ confirmatory assays to validate that the compounds are interacting directly with the KOR. To demonstrate selective subtype specific binding to the KOR in competitive assays we will utilize a set of radiolabeled compounds that are commercially available for this purpose including, [3H]-Diprenorphine (KOR and MOR), DAMGO (MOR) and Naltrindole [5`,7`-3H] (DOR). Of particular interest is whether the probe is long or short acting at the KOR, and studies of this question in cells and animal models may clarify as to whether the underlying mechanism for antagonist anti-addictive behavior requires activation of JNK.

2. Project Description

a. Describe the original goal for probe characteristics as identified in the CPDP.

The original goal from the CPDP was to find antagonists structurally distinct from current literature probes, with potencies of less than 1 μM for the kappa-opioid receptor (KOR), with greater than 100-fold selectivity against the mu-opioid receptor (MOR) and 10-fold

selectivity against the delta-opioid receptor (DOR), or to the extent achievable by test concentration limitations**.**

b. For each assay implemented and screening run please provide

i. **PubChem Bioassay Name(s), AID(s), Assay-Type (Primary, DR, Counterscreen, Secondary**)—

Table 1. Summary of Assays and AIDs

PubChemBioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
Summary of small molecule antagonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Summary]	1785	Antagonist	Summary	N/A	N/A
uHTS identification of small molecule antagonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Confirmatory]	1778	Antagonist	Primary	Cell-based	Luminescence-DiscoveRx β-arrestin & 1536
SAR analysis of small molecule antagonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Confirmatory]	2285	Antagonist	SAR	Cell-based	Luminescence-DiscoveRx β-arrestin & 1536
HTS Dose response counterscreen for assays utilizing the enzyme, b-galactosidase [Confirmatory]	1966	Antagonist	Counter-screen	Enzymatic	Luminescence &1536
HTS Image-Based Screen for Selective Antagonists of the KOR Receptor [Confirmatory]	2136	Antagonist	Alternate	Cell-based	HCS – Transfluor & 384
HTS Image-Based Screen for Antagonists of the DOR Receptor [Confirmatory]	2356	Antagonist	Selectivity	Cell-based	HCS – Transfluor & 384
SAR analysis of Antagonists of the Kappa Opioid Receptor (KOR) using an Image-Based Assay [Confirmatory]	2348 2491	Antagonist	Alternate	Cell-based	HCS – Transfluor & 384
SAR Analysis of Antagonists of the MOR Receptor using an Image-Based Assay [Confirmatory]	2420	Antagonist	Selectivity	Cell-based	HCS – Transfluor & 384
SAR Analysis of Antagonists of the DOR Receptor using an Image-Based Assay [Confirmatory]	2357	Antagonist	Selectivity	Cell-based	HCS – Transfluor & 384

ii. Assay Rationale & Description (when describing primary screen it would be useful to see standard metrics like, Z', S:B for the optimized assay). Table of reagents and source.

Unlike imaging or other second messenger assays, the DiscoveRx β-arrestin assay allows for a direct measure of GPCR activation by detection of β-arrestin binding to the KOR1 receptor. In this system, β-arrestin is fused to an N-terminal deletion mutant of β-gal (termed the enzyme acceptor of EA) and the GPCR of interest is fused to a smaller (42 amino acids), weakly complementing fragment termed ProLink™. In cells that stably express these fusion proteins, ligand stimulation results in the interaction of β-arrestin and the Prolink-tagged GPCR, forcing the complementation of the two β-gal fragments and resulting in the formation of a functional enzyme that converts substrate to detectable signal.

Assay materials:

1) OPRK1 b-Arrestin (DiscoveRx)
2) Assay Medium: Opti-MEM Medium supplemented with 1% hiFBS, 1X Pen/Strep/Glu, 125 ug/mL Hygromycin (1/2 recommended), 250 ug/mL Geneticin (1/2 recommended)

3) Growth Medium: MEM supplemented with 10% hiFBS, 1X Pen/Strep/Glu, 125 ug/mL Hygromycin (1/2 recommended), 250 ug/mL Geneticin (1/2 recommended)

Table 2. Reagents used for the uHTS experiments	
Reagent	**Vendor**
OPRK1 beta-Arrestin Cell Line	DiscoveRx
Assay Medium: Opti-MEM Medium supplemented with 1% hiFBS, 1X Pen/Strep/Glu, 125 ug/mL Hygromycin, 250 ug/mL Geneticin	Invitrogen
Growth Medium: MEM supplemented with 10% hiFBS, 1X Pen/Strep/Glu, 125 ug/mL Hygromycin, 250 ug/mL Geneticin	Invitrogen

The following uHTS protocol was implemented at single point concentration confirmation:

uHTS protocol:

Day 1
1) Harvest cells using Enzyme-Free Dissociation Buffer (Invitrogen Cat#13151-14). Add 500 cells/well in 5 uL of media to each well of a white, 1536 well plate.
2) Spin cells at 500 rpm for 1 min, then wrap plates in Saran Wrap.
3) Incubate overnight at 37C with 5% CO2.

Day 2
1) Using a Highres Biosolutions pintool pin 30 nL to wells. Columns 1-4 should be DMSO only (Control wells), Columns 5-48 contain test compounds (10μM final in well concentration).
2) Immediately following pintool addition, add 1.0 uL of assay media to columns 1-2 and 1.0 uL of assay media containing 240 nM dynorphin A for a final assay concentration of 40 nM. Centrifuge plates at 500 rpm for 1 min immediately following additions.
3) Incubate for 1hr and 30 minutes.
4) During test incubation, prepare Detection Reagent Solution from DiscoveRx (1 part Galacton Star: 5 parts Emerald II and 19 parts Cell Assay Buffer)
5) Add 2.5ul of detection reagent solution to each well.
6) Incubate at room temperature for 60 min in the dark
7) Read plates in a Perkin Elmer Envision using a luminescence protocol

Dose Response protocol:
Day 1
1) Harvest cells using Enzyme-Free Dissociation Buffer (Invitrogen Cat#13151-14). Add 500 cells/well in 5 uL of media to each well of a white, 1536 well plate.
2) Spin cells at 500 rpm for 1 min, then wrap plates in Saran Wrap.
3) Incubate overnight at 37C with 5% CO2.

Day 2
1) Using a Labcyte Echo, DMSO and test compounds are transferred to wells. DMSO only is transferred to columns 1-3 and 46-48(Control wells), while varying volumes of test compounds are transferred to columns 4-45 to achieve the desired test concentrations. Test compound wells in the assay plate are back-filled with DMSO to equalize final assay concentrations.
2) Immediately following Echo transfer, 1.0 uL of assay media is added to columns 1-3 and 1.0 uL of assay media containing 240 nM dynorphin A is added to columns 4-48 for a final assay concentration of 40 nM. Centrifuge plates at 500 rpm for 1 min immediately following additions.
3) Incubate for 1hr and 30 minutes.
4) During test incubation, prepare Detection Reagent Solution from DiscoveRx (1 part Galacton Star: 5 parts Emerald II and 19 parts Cell Assay Buffer)
5) Add 2.5ul of detection reagent solution to each well.
6) Incubate at room temperature for 60 min in the dark
7) Read plates in a Perkin Elmer Envision using a luminescence protocol

The average Z' for the screen was 0.51, the signal to background (S/B) was 4.41, signal to noise (S/N) was 28.9 and signal to window was 4.26.

Rationale for confirmatory, counter and selectivity assays:

The initial frontline counterscreen that was performed shortly following dose response confirmations on both the agonist and antagonist KOR1 primary screens was the β-galactosidase dose response assay. Each confirmed hit (EC50 < 10 μM) was run in a β-gal dose response assay. Because the primary screen is based upon the formation of a functional β-gal enzyme upon β-arrestin migration to the GPCR, we wanted to rule out compound interaction, either stimulatory or inhibitory, with the β-gal enzyme in the absence of GPCR interaction.

The High-Content Imaging-based confirmatory (KOR) and selectivity assays (MOR, DOR) which are based upon the translocation of β-arrestin linked to GFP to other receptor subtypes were developed and performed to confirm antagonist activity in the KOR antagonist primary assay, as well as to ascertain the selectivity of compounds for the KOR receptor vs. the MOR and DOR receptor sub-types.

Improved potency for KOR and increased selectivity against MOR and DOR were primary drivers for compound selection and optimization.

Confirmation assays

The initial confirmatory assays were performed in full dose-response for compounds from solvated DPI stock solutions to confirm activity seen first in test agents from screening library in the initial primary screen. Active compounds were then tested in an alternative format for inhibition of GPCR activation, via the imaging-based KOR High-Content Transfluor Antagonist Assay. In the Transfluor assay, GPCR activation is measured indirectly by via the detection of β-Arrestin-GFP redistribution from the cytosolic compartment to the plasma membrane to coated pits and finally endosomal vesicles. The image-based KOR assays allowed for independent confirmation of KOR activation utilizing an alternative technology.

The following are confirmation assays for this project:

Assay 1: HTS identification of small molecule antagonists of the kappa opioid receptor via a luminescent beta-arrestin assay *(AID 1778)*

Assay 2: SAR analysis of small molecule antagonists of the kappa opioid receptor via a luminescent beta-arrestin assay *(AID* 2285*)*

Assay 3: HTS Image-Based Screen for Selective Antagonists of the KOR Receptor *(AID* 2136*)*

Assay 4: SAR analysis of Antagonists of the Kappa Opioid Receptor (KOR) using an Image-Based Assay (AID 2359)

Counterscreen assays

The β-Galactosidase Counterscreen Assay was utilized to ascertain possible enzyme inhibition, which might present the opportunity for false positives from the initial primary assay. The inhibition of activity of the b-galactosidase fragment complementation in the primary KOR1 β-Arrestin Assay in the presence of test agent could lead to decreased signal formation and therefore a false positive result. This counterscreen assay would allow for the detection of these artifactual compounds.

Assay 1: HTS Dose response counterscreen for assays utilizing the enzyme, β-galactosidase *(AID* 1966*)*

Secondary Assays:

The imaging-based MOR and DOR High-Content Transfluor Antagonist Assays provide for the determination of KOR receptor selectivity. The probe criteria specifies the necessity of at

least 100-fold selectivity against MOR and DOR, or within the reasonable limitations imposed for testing compounds at high concentrations, i.e. 100 μM selectivity for a 1 μM compound.

Assay 1: HTS Image-Based Screen for Antagonists of the MOR Receptor *(AID* 2344*)*

Assay 2: HTS Image-Based Screen for Antagonists of the DOR Receptor *(AID 2356)*

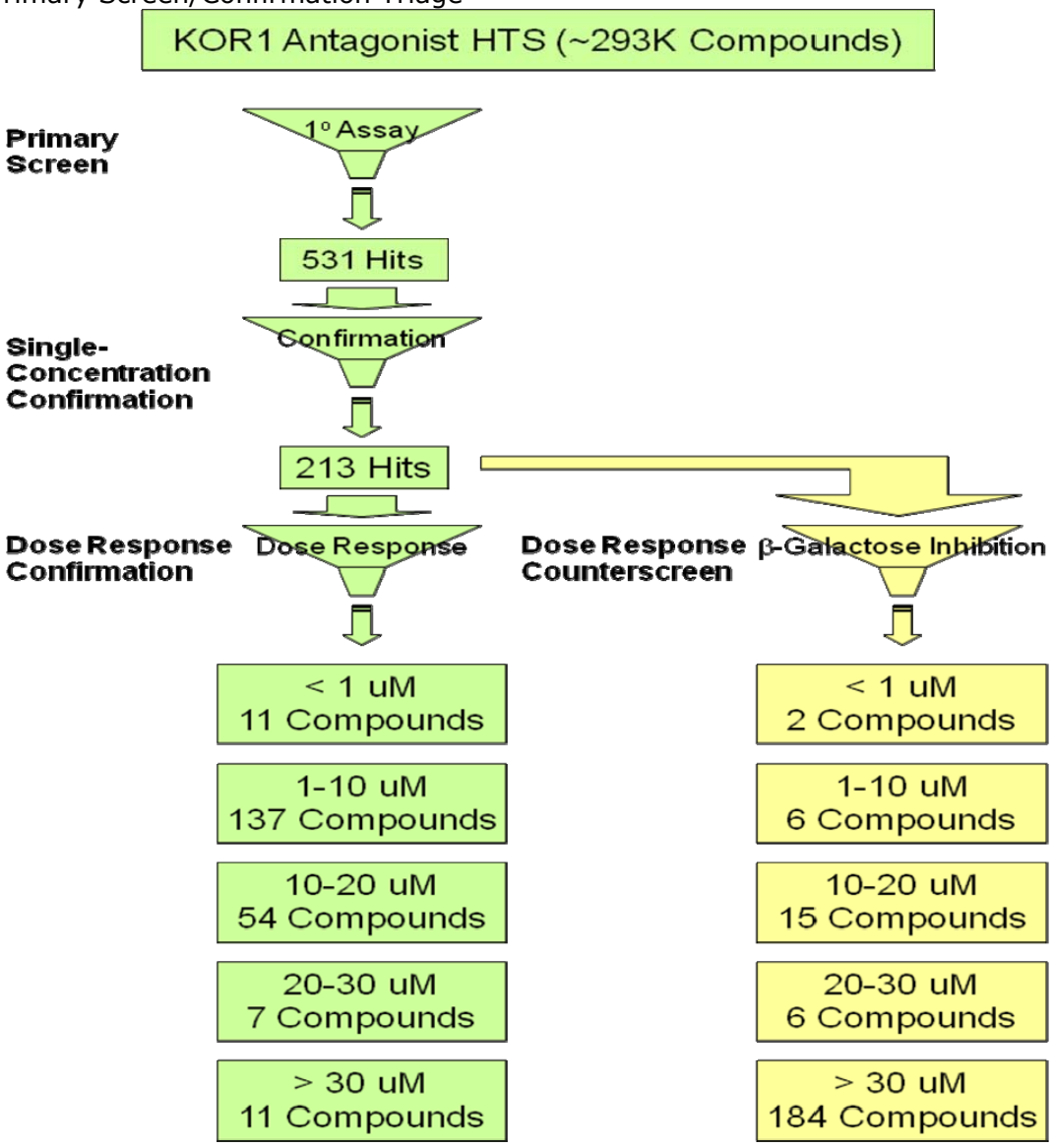
Assay 3: SAR Analysis of Antagonists of the MOR Receptor using an Image-Based Assay *(AID 2420)*

Assay 4: SAR Analysis of Antagonists of the DOR Receptor using an Image-Based Assay (AID 2357)

3. Center Summary of Results

The following flowchart summarizes the compound triage and decision tree for advancement of compounds:

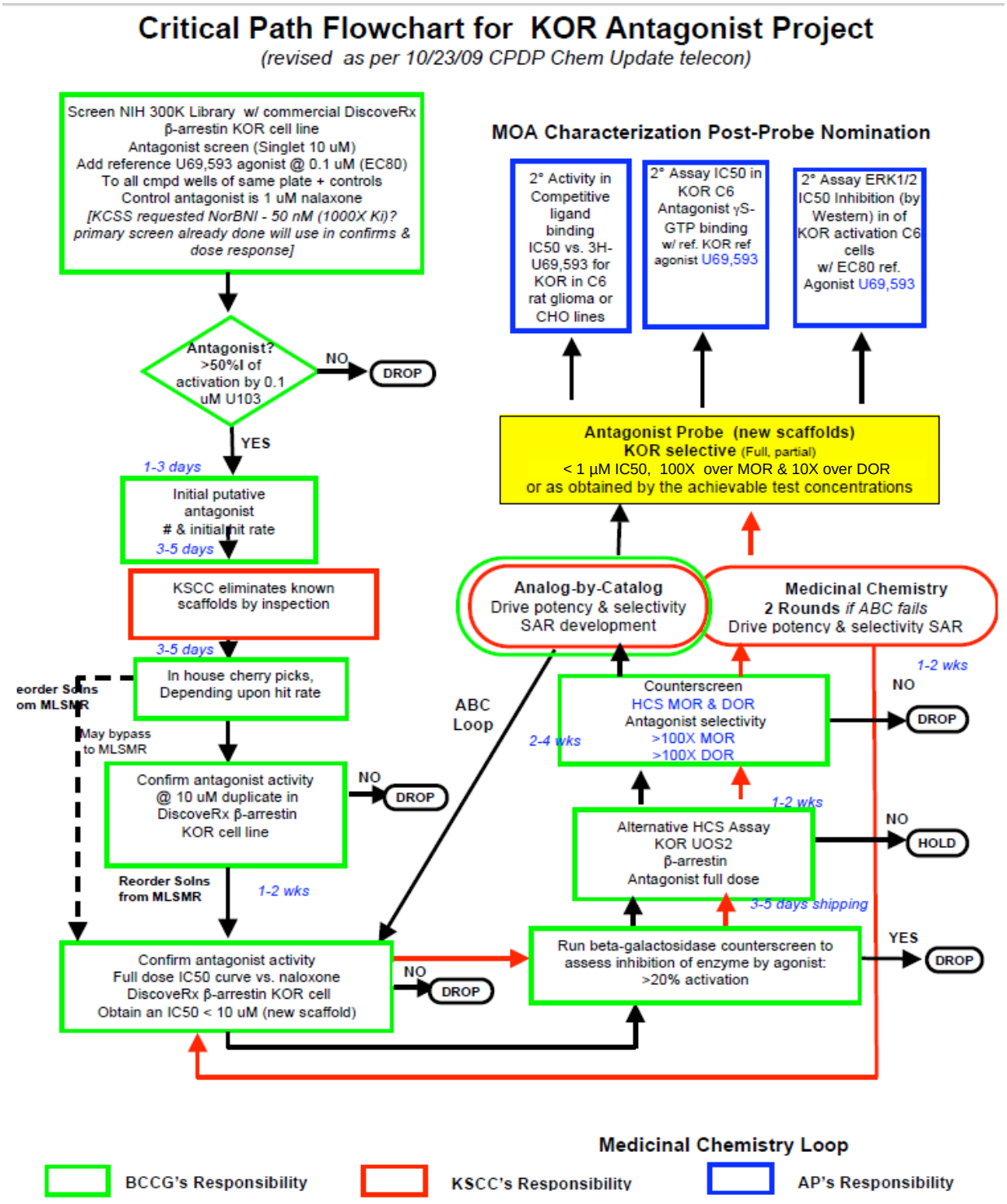
Primary Screen/Confirmation Triage



A library of approximately 290,000 compounds was tested in the KOR1 DiscoveRx b-Arrestin primary screen. Upon data analysis, 606 hits with activity >50% at a single concentration

point of 10 μM were identified. Liquid samples were then ordered through DPI and 531 compounds were received.

The compound solutions resupplied by the MLSMR were first confirmed in 10 μM single-point duplicate in the KOR1 DiscoveRx b-Arrestin primary assay. Of these, 213 compounds were confirmed to have at least 50% activity at a 10 μM assay concentration (see Critical Path flowchart below).



The confirmed compounds were further tested in dose response in the KOR1 DiscoveRx b-Arrestin primary assay to obtain EC50 values and were also tested in a b-Galactosidase

Counterscreen assay to assess the possibility that these compounds might inhibit the enzyme. The KOR1 antagonist dose response experiments revealed 148 compounds with EC50 potencies at or below 10 μM. Twenty-nine of the compounds were eliminated from future consideration because they were found to inhibit b-galactosidase activity in the enzymatic counterscreen.

The active, confirmed compounds were then tested in the KOR1 High-Content Transfluor Antagonist assay for further confirmation, then in the MOR and DOR High-Content Transfluor Antagonist assays to determine subtype selectivity.

Chemistry and cheminformatics resources were then employed in the selection of both novel and chemically tractable molecules to pursue for a KOR1 selective probe. Structures of interest and analogs thereof were either purchased as commercial dry powders. In total, 32 structures were received from commercial vendors. These constituted the SAR driving chemistries from which the KOR1 antagonist probe candidate and analogs emerged.

SAR testing of re-constituted powders encompassed dose response testing of compounds in four assays: KOR1 DiscoveRx β-Arrestin Antagonist assay, the KOR High-Content Transfluor Antagonist assay, and the MOR and DOR High-Content Transfluor Antagonist assays.

Probe Optimization

i. Describe SAR & chemistry strategy (including structure and data) that led to the probe.

The screening of the MLPCN compound collection identified three promising chemotypes (Figure 1). The Series 1 chemotype although sufficiently potent, did not confirm as a selective KOP (over DOP and MOP) antagonist series. The Series 2 scaffold data contained an existing compound meeting probe criteria for potency and selectivity and was selected for resynthesis of the compound as well as a set of 32 analogs to probe the SAR of this series. The Series 3 chemotype contained the compound shown in Figure 1 (IC_{50} = 1.67 μM) and an additional analog (IC_{50} = 4.32 μM). With this limited yet promising data as a starting point, we investigated a synthetic strategy towards this class of compounds. Concurrently, we purchased ten analogs to establish additional SAR around this chemotype.

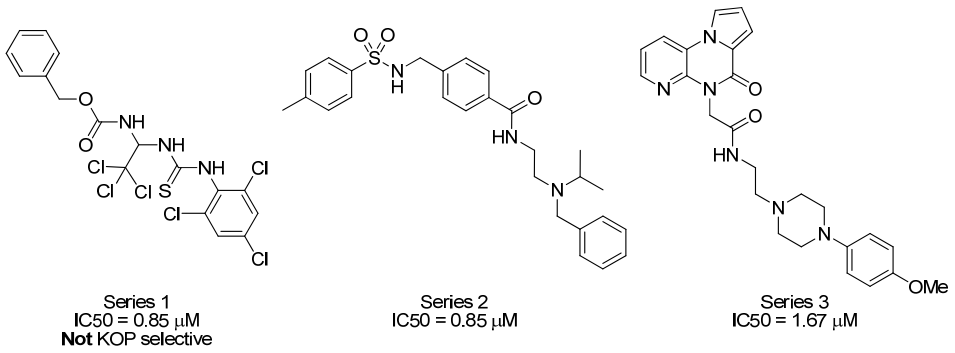
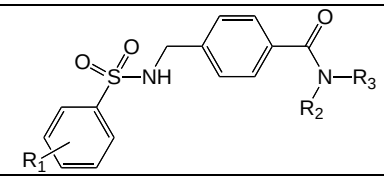
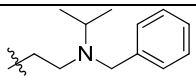
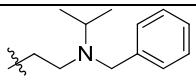
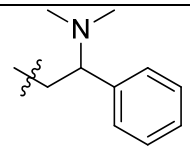
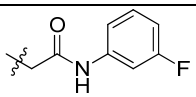
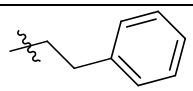
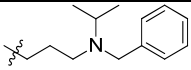
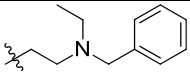
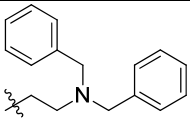
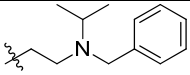
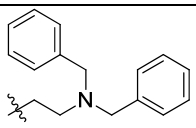
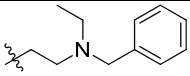


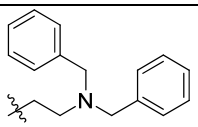
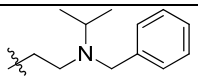
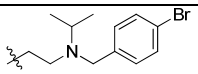
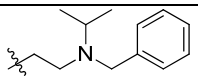
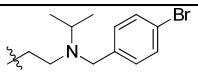
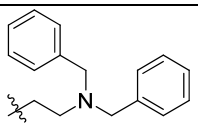
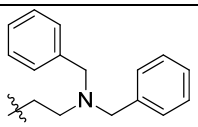
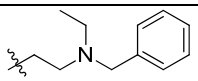
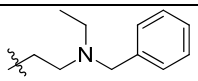
Figure 1. Summary of the hit structures for the KOP antagonist project.

SAR Analysis. Screening of the MLPCN compound collection uncovered a sulfonamide containing (Series 2) compound possessing sufficient potency to meet probe criteria. However, only one additional compound in this series with measurable activity (less than 10 μM) was known. In an effort to uncover additional supporting analogs and to broaden the

known SAR of this chemotype we synthesized the 51 analog compounds mentioned in **Tables #3A-3B**. In addition, we synthesized and submitted for screening the compound depicted in **Figure 1** to confirm the activity of this synthetic material. The lead compound (CID03342390, entry **1** in **Table 3A**) for this series remained the most potent compound and is therefore nominated as the probe candidate. The R_1 = ethyl analog (CID44601476, entry **2** in **Table 3A**) was found to be slightly less potent than the lead compound where R_1 = methyl. The other synthetic analogs had more substantial changes relative to the lead and unfortunately, turned out to be far less potent.

Table 3A: SAR Analysis for Selective κ–opioid receptor antagonists of the sulfonamide sub-scaffold 1

SAR Analysis for KOR Selective Scaffolds (Medicinal Chemistry & Cheminformatics Analysis)									**Potency (μM)** Ave. ± *S.E.M.* (stdv/sqrt (n)) (n = replicates)			
#	**CID**	**SID**	**BCCG MLS-**	*****	**Purity** *(%)*	**R1**	**R2**	**R3**	**KOR** *DRx (n=3)*	**KOR** *HCS (n=2)*	**MOR** *HCS (n=2)*	**DOR** *HCS (n=2)*
1	03342390	87218794**	0239991	P	ND	4-Me	H		0.85 *±0.08*	1.31	>32	>32
2	44601476	87334050	0435600	S	98.0	4-Et	H		1.12 *±0.17 (n=4)*	2.55	>32	>32
3	16294686	87218749	0355913	P	ND	4-$COCH_3$	H		6.72 *±0.75*	4.73	1.92	>32
4	16295215	87218750	0332830	P	ND	4-$COCH_3$	Me		>10	>32	>32	>32
5	44601477	87334051	0435601	S	99.0	4-Et	H		>20 *(n=4)*	>32	>32	>32
6	20968607	87334052	0435602	P	97.2	4-Me	H		>20 *(n=4)*	>32	>32	>32
7	44608034	87457826	0435639	S	>99.0	4-Me	H		5.76 (n=4)	2.93	10.33	>32
8	44608028	87457827	0435640	S	98.9	4-Me	H		18.7 (n=4)	0.60	>32	>32
9	44608030	87457829	0435642	S	97.8	3-Me	H		2.68 (n=4)	0.87	5.69	>32
10	44608038	87457834	0435647	S	99.1	3-Me	H		>20 *(n=4)*	>32	>32	>32
11	44608039	87457835	0435648	S	92.2	4-Et	H		17.9 (n=4)	1.99	25.7	>32

12	44608033	87457838	0435651	S	>99.0	4-Et	H		>20 *(n=4)*	>32	>32	>32
13	44620904	87544129	0435687	S	96.3	4-OMe	H		2.92 (n=4)	0.81	9.60	>32
14	44620903	87544131	0435689	S	96.1	4-OMe	H		15.7 (n=4)	1.38	>32	>32
15	44620910	87544132	0435690	S	98.6	4-Br	H		1.28 (n=4)	0.42	3.22	>32
16	44620900	87544134	0435692	S	98.0	4-Br	H		>20 (n=4)	2.03	>32	>32
17	44620905	87544137	0435695	S	99.2	4-OMe	H		>20 (n=4)	13.1	>32	>32
18	44620908	87544138	0435696	S	97.3	4-Br	H		>20 (n=4)	>19	>32	>32
19	44620901	87544139	0435697	S	94.6	4-OMe	H		18.9 (n=4)	10.8	12.6	>32
20	44620906	87544140	0435698	S	98.9	4-Br	H		19.6 (n=4)	4.8	8.5	>32

* S = Synthesized P = purchased ND = not determined

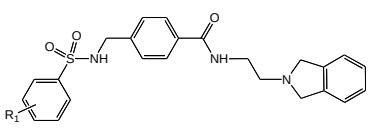
***Probe CID03342390 has 2 SIDs: SID87218794 (ChemDiv); SID87544125 (KU)*

Table does not include 23 additional synthesized compounds (w/ purities all > 94%) with R_1 = 2-Methyl, 4-Methyl, 4-methoxy & 4-Bromo; R_2 = methyl, ethyl, benzyl, i-propyl & sec-butyl; R_3 = H, 4-methyl, 4-bromo & 4-methoxy substituents, as these are still being tested. However, the current probe from these limited SAR exceeds the probe criteria.

Underlined italicized cmpd # = analog submitted

Interestingly, the constrained analog CID44601478 (entry **7** in **Table 3B**) showed no significant (in fact showed negative) inhibition at the higher test concentrations. Based on this observation, these compounds (entries **2**-**5**) were screened for KOR agonism, and CID44601476 alone of the four compounds confirmed as an apparent weak partial agonist. These limited structure-activity relationship studies suggest that both the tether length as well as the substitution on the left hand aromatic ring and right hand nitrogen may be critical to maintain potency and selectivity. Some investigators have suggested that weak partial agonists may also prove of value in the study and potential treatment of polydrug addiction, so these compounds may retain some interest beyond the present probe report. However, their evaluation in this context is beyond the scope of this MLPCN project.

Table 3B: SAR Analysis for Selective κ–opioid receptor antagonists of the sulfonamide sub-scaffold 2

SAR Analysis for KOR Selective Scaffolds (Medicinal Chemistry & Cheminformatics Analysis)		**Potency (μM)** Ave. ± *S.E.M.* (stdv/sqrt (n)) (n = replicates)

#	CID	SID	BCCG MLS-	*	Purity *(%)*	R1	KOR *DRx (n=4)*	KOR *HCS (n=2)*	MOR *HCS (n=2)*	DOR *HCS (n=2)*
21	44601478	87334053	0435603	S	>99.0	2,4,6-Me	>20	>32	>32	13.5
22	44608035	87457828	0435641	S	95.2	3-Me	>20	>32	>32	>32
23	44608036	87457831	0435644	S	94.8	4-Et	>20	>32	>32	>32
24	44620897	87544126	0435684	S	98.1	4-Me	>20	>32	>32	>32
25	44620899	87544130	0435688	S	96.4	4-OMe	>20	>32	>32	>32
26	44620898	87544133	0435691	S	95.3	4-Br	>20	>32	>32	>32

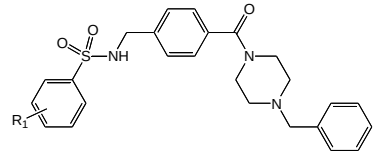
* S = Synthesized P = purchased ND = not determined

Table does not include 6 additional synthesized compounds (w/ purities all > 94%) with R_1 = 2-ethyl, 2-methyl, 4-methyl, 4-methoxy and 4-bromo ring substituents, as these are still being tested. However, the current probe from this limited SAR exceeds the probe criteria.

Underlined italicized cmpd # = analog submitted

Table 3C shows the results of side chain constraint between the carboxylic acid terminus of the aromatic ring centroid of this series and the terminal aromatic substituent in this direction. As seen, this change entirely deprived these compounds of KOR binding activity and therefore this SAR direction was not pursued further.

Table 3C: SAR Analysis for Selective κ–opioid receptor antagonists of the sulfonamide sub-scaffold 3

SAR Analysis for KOR Selective Scaffolds (Medicinal Chemistry & Cheminformatics Analysis)		**Potency (μM)** Ave. ± *S.E.M.* (stdv/sqrt (n)) (n = replicates)

#	CID	SID	BCCG MLS-	*	Purity *(%)*	R1	KOR *DRx (n=3)*	KOR *HCS (n=2)*	MOR *HCS (n=2)*	DOR *HCS (n=2)*
27	9550286	87218766	0071544	P	ND	H	>10	>32	>32	>32
28	9551230	87218786	0075741	P	ND	4-Me	>10	>32	>32	>32
29	20854208	87218785	0364353	P	ND	4-MeO	>10	>32	>32	>32

* S = Synthesized P = purchased ** CH_2 instead of NH ***isoindoline ND = not determined

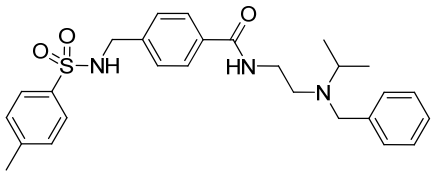
Underlined italicized cmpd # = analog submitted

4. Probe

a. Chemical name of probe compound

N-[2-[benzyl(propan-2-yl)amino]ethyl]-4-[[(4-methylphenyl) sulfonyl-amino]methyl]benzamide **[ML140]**

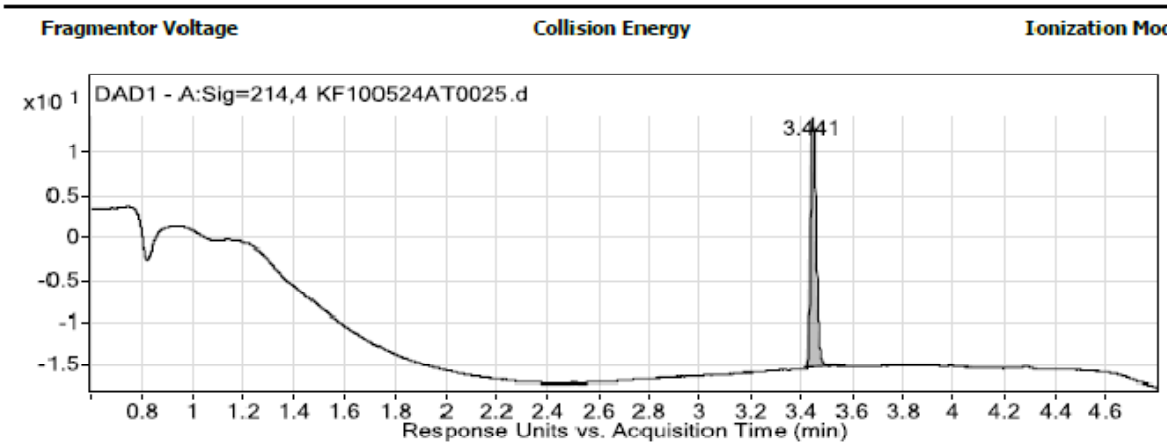
b. Probe chemical structure including stereochemistry if known



c. Structural Verification Information of probe SID

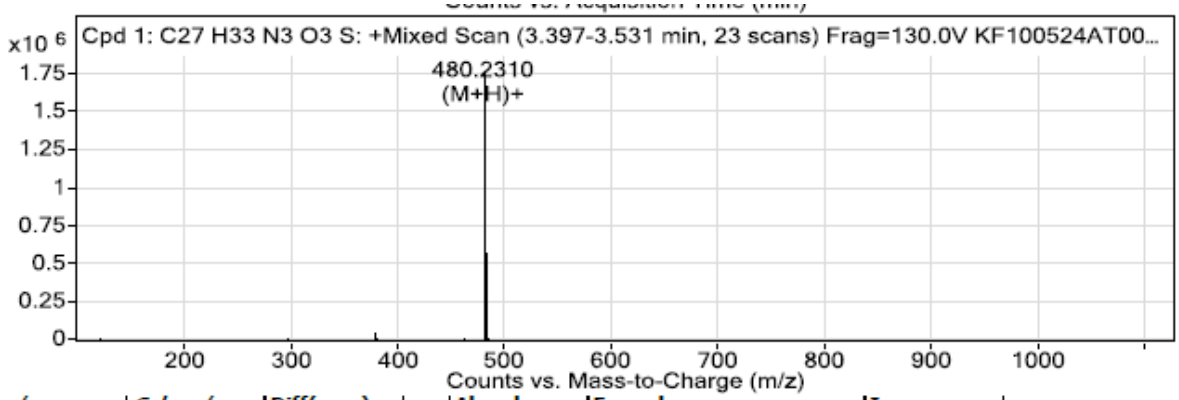
The probe SID is 87544125

Purity: >98% (HPLC)
Mass Spec: HRMS (ESI) *m/z* calcd for $C_{27}H_{33}N_3O_3S$ ([M+H]$^+$), 480.2315, found 480.2316.



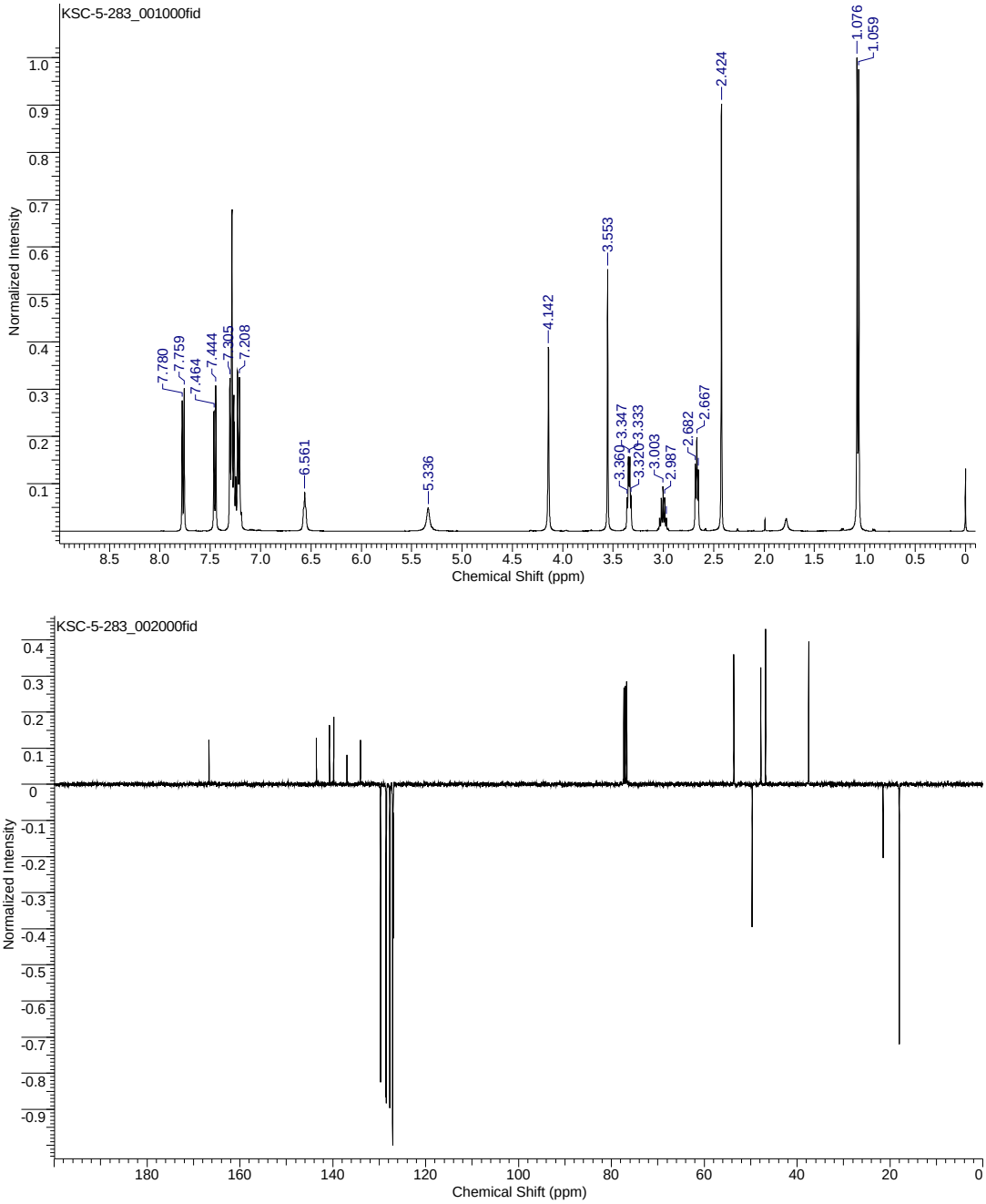
User Chromatogram Peak List

Peak #	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	3.441	29.37	100	46.87	100	100	0.025



m/z	*Calc m/z*	Diff(ppm)	z	Abund	Formula	Ion
480.231	480.2315	-1.12		1769633	C27 H34 N3 O3 S	(M+H)+
480.5104				113896		
480.6709				19308		
481.2339	481.2346	-1.46		577918	C27 H34 N3 O3 S	(M+H)+
481.5132				38024		
481.6766				8362		
482.233	482.2329	0.29		178787	C27 H34 N3 O3 S	(M+H)+
482.5107				10749		
483.2333	483.2337	-0.68		34945	C27 H34 N3 O3 S	(M+H)+
502.2126	502.2135	-1.7	1	132	C27 H33 N3 Na O3 S	(M+Na)+

NMR Purity: >95% pure (^{1}H-NMR): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.07 (d, *J* = 6.8 Hz, 6 H), 2.42 (s, 3 H), 2.67 (t, *J* = 6.0 Hz, 2 H), 3.00 (sept, *J* = 2.8 Hz, 1 H), 3.34 (q, *J* = 5.2 Hz, 2 H), 3.55 (s, 2 H), 4.14 (s, 2 H), 5.34 (br s, 1 H), 6.56 (br s, 1 H), 7.21-7.31 (complex, 9 H), 7.45 (d, *J* = 8.0 Hz, 2 H), 7.77 (d, *J* = 8.4 Hz, 2 H). ^{13}C (100 MHz, $CDCl_3$, APT pulse sequence) δ d (CH, CH_3) 18.0, 21.5, 49.7, 126.9, 127.0, 127.1, 127.7, 128.4, 128.5, 129.7; u (C, CH_2) 37.5, 46.8, 47.8, 53.6, 134.0, 136.9, 139.8, 140.7, 143.5, 166.6.



d. PubChem CID (corresponding to the SID)

PubChem CID is 3342390

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

This probe is commercially available in milligram quantities from Chem Div (catalog # K788-2192), Aurora Screening Library (catalog # kcd-576427) and AKOS Screening Library (catalog # AKG-K788-2192). Nonetheless, KSCC deposited 50 mg of newly synthesized material with the MLSMR (DPI).

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

Table 4. Probe and analog submission for Kappa Opioid Antagonists

Probe /Analog	MLS_ID	CID	SID	Source (vendor or BCCG syn)	Amt (mg)	Date ordered/ Submitted
Probe ML140	MLS002699332	03342390	87544125	KU syn	50	02/26/2010
Analog 1	MLS002699335	9551230	87218786	KU syn	20	02/26/2010
Analog 2	MLS002699336	20854208	87218785	KU syn	20	02/26/2010
Analog 3	MLS002699334	9550286	87218766	KU syn	20	02/26/2010
Analog 4	MLS002699333	20854195	87218784	KU syn	20	02/26/2010
Analog 5	MLS002699337	44601476	87334050	KU syn	20	02/26/2010

g. Describe mode of action for biological activity of probe

This series of antagonists appear to selectively inhibit the activation the kappa opioid receptor in both the primary assay and the HCS image based assay, with a good translation between these two assays. This series of compounds exhibit 25 to 40-fold selectivity over both the mu- and delta- opioid receptors as antagonists as determined by the primary DiscoveRx and follow-up HCS Transfluor assay.

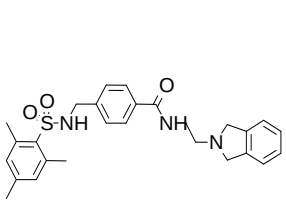
The probe, CID3342390 [ML140] was submitted to the Psychoactive Drug Screening Program at the University of North Carolina (PDSP, Bryan Roth, PI). This program provided screening of the probe against GPCR Panels of 42 CNS-based receptors and the results are summarized in **Table 5** below. While 11 receptor profiles are still pending, the probe showed some moderate (310 – 5400 nM) activities against 5HT5a, 5HT7, α2B, α2C, D3, H2, and M5. The activities against the KOR, MOR and DOR were 40.3, 305.6, and 1,088 nM, respectively. These yield selectivity values of 7.6-fold (MOR/KOR) and 27-fold (DOR/KOR) in the PDSP assays which compare less favorably with those obtained by our DiscoveRx (>40-fold for MOR & DOR) and HCS (>25-fold for MOR & DOR) assays. (*see "**Probe(s) Structure & Characteristics:**"* table on p.1). Although not required by the CPDP and outside the scope of the MLPCN project, the potency of the probe compound relative to other CNS receptors is solid and provides an attractive starting point for post-probe optimization.

Is should be noted the that the nanomolar binding K_is of CID3342390 (40.3 nM) for KOR are more potent (21 to 33-fold) than those determined by either functional assay (see table on p. 1) for CID3342390 (850 nM DiscoveRx; 1310 nM HCS). Corrrelation of the functional EC_{50} data to equilibrium ligand binding values (K_i) is not straightforward, but these data further confirm that the chemotype exemplified by the present probe constitutes a highly potent new scaffold for exploring KOR biology.

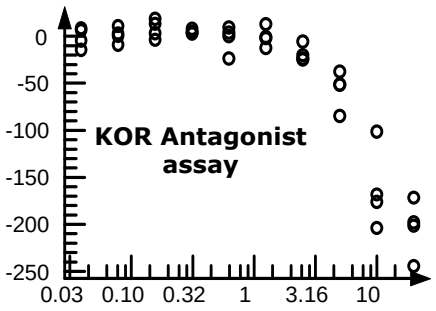
Table 5. Receptor Paneling of KOP agonist [ML140] CID3342390 through the PDSP							
Receptor	**K_i** (nM)	**Receptor**	**K_i** (nM)	**Receptor**	**K_i** (nM)	**Receptor**	**K_i** (nM)
5ht1a		Alpha1A		D1		KOR	40.3
5ht1b		Alpha1B		D2		M1	
5ht1d		Alpha1D		D3	1,234	M2	
5ht1e		Alpha2A		D4		M3	
5ht2a		Alpha2B	1,451	D5		M4	
5ht2b	592	Alpha2C	957	DAT		M5	310.6
5ht2c		Beta1		DOR	1,088	MOR	305.6
5ht3		Beta2		H1		NET	
5ht5a	5,442	Beta3		H2	4,162	SERT	
5ht6		BZP Rat Brain Site		H3		Sigma 1	
5ht7	1,990			H4			
	Green: K_i > 10,000 or 1^{ary} screen missed				Orange: 2^{ndary} assay pending		

We believe that the mode of action of the probe antagonist is the reversible binding at the KOR site occupied by opiates on the basis of the antagonist assay paradigms. (G protein coupling, ERK activation and beta-arrestin recruitment) in which, agonist-stimulation is blocked by increasing doses of the probe. KOR antagonists are of interest because they may find utility in the treatment of drug abuse, depression, and chronic pain. Three of the most potent and selective of the KOR antagonists, NorBNI, GNTI, and JDTic have been recognized for their long acting properties at the KOR that may be associated with JNK activation. While they have common structural features their differences are sufficient to cloud the SAR that underlies this long acting physiological behavior that can be blocked by reversible nonselective opioid antagonists. It is therefore important to determine how this selective, sub micromolar probe functions at the KOR with respect to NorBNI, GNTI, and JDTic.

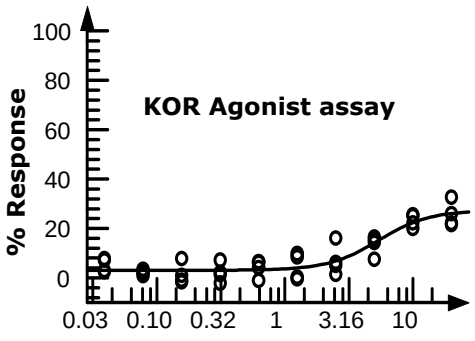
Interestingly, in the β-arrestin assay, one of the weakly active (IC50>20 μM) analog compounds, CID20968607 exhibited only partial inhibition (40-50% I) at the highest tested concentration (data not shown). Another compound CID44601478 exhibited dose-dependent inverse inhibition (see dose response curves below) at the higher concentrations, and when this compound was tested as an agonist, it appeared to behave as a moderately potent (0.6 μM EC50) partial agonist (~20% Emax). CID44601478 did not antagonize U69,593-stimulated G protein coupling nor ERK activation (data not shown), yet it may be of interest to evaluate the intrinsic efficacy of this compound in each assay to determine if it demonstrates a signaling bias towards the β-arrestin pathway. Although confirmation of this is beyond the purview of the MLPCN (and is being pursued as of the submission of the present probe report), such characteristics, if they do confirm, will represent a potentially important new property set for KOR agents.



CID44601478



[CID44601478] (μM)

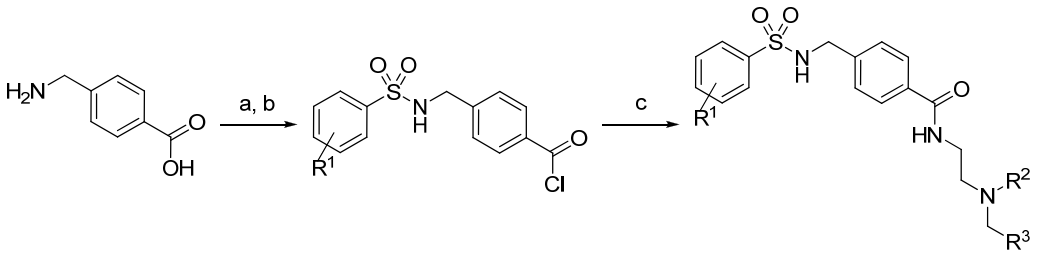


[CID44601478] (μM)

h. Detailed synthetic pathway for making probe

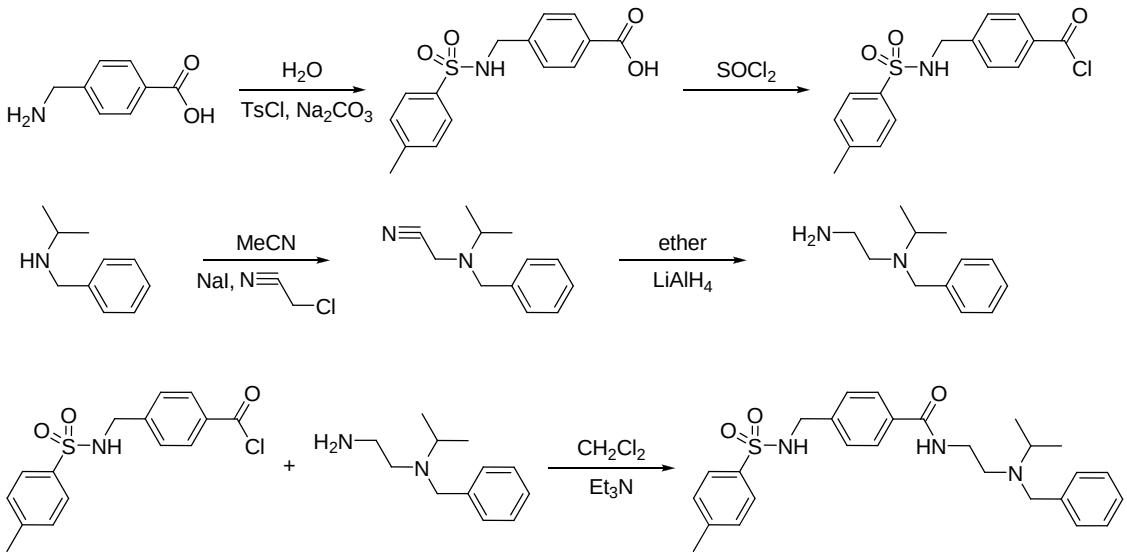
We have developed a convergent, modular synthetic route that provided rapid access to the tentative probe compound as well as the 32 analogs for screening (Scheme 1). The sulfonyl chloride and diamine fragments were coupled together in the presence of triethylamine in CH_2Cl_2. The synthesis of the requisite sulfonamide benzoic acid (1) and diamine fragments (2) were readily achieved using precedented methods.

Scheme 1. Synthetic route for probe molecule and supporting analogs[a]



[a] Condtions: (a) $ArSO_2Cl$, Na_2CO_3, H_2O; (b) $SOCl_2$; (c) $H_2NCH_2CH_2NR^2(CH_2R^3)$, Et_3N, CH_2Cl_2.

Scheme 2. Synthetic route for probe molecule



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

The probe molecule has demonstrated potent antagonist activity in both the primary enzyme complementation assay and the image base orthogonal assay for inhibition of β-arrestin mediated signaling of the kappa opioid receptors, with selectivity over for inhibition of activation of both the mu- and delta opioid receptors.

The criteria for probe as defined in the CPDP Chem Update document filed on October 28, 2009 and revised and refiled on November 21, 2009, and finally refiled after corrections requested by NIH on January 12, 2010 with the NIH PT: a potency for KOR of less than 1 μM, and at least 100-fold selectivity over MOR and 10-fold selectivity over DOR or as

obtained by the achievable test concentrations, (e.g. solubility limited). This current antagonist is a novel scaffold from those in the literature and exceeds the criteria for DOR selectivity, though it formally misses the 100-fold selectivity of against MOR; however, the compounds cannot achieve a higher than 30-40 μM test concentrations without some precipitation, and in most cases the dose-response curve is completely flat so the >32 μM is an under estimate of the true IC50, which is certainly >>32 μM, so well within a factor of 2-3 for an effective >100 –fold selectivity against MOR. The assay provider concurs that even at this *pro forma* lower ~25-40-fold estimate of selectivity the compound should be useful.

In Vitro Pharmacology Profiles of Probe CID03342390 [**ML140**] (*See* **Table 6** *below*). The probe,CID03342390, had poor solubility at all pH's tested.

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 are measured using UV spectroscopy. In this assay, the probe, CID03342390, has excellent permeability in the PAMPA assay with 3-fold less (though still significant) permeability in the blood brain barrier PAMPA assay.

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. The probe, CID03342390, is highly bound (98.9-99.7%) to both human and mouse plasma.

Plasma Stability is a measure of the stability of small molecules and peptides in plasma and is an important parameter, which strongly can 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. The probe, CID03342390, shows excellent stability (100%) in both human and mouse plasma; however it is rapidly metabolized by microsomes (below).

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. The probe, CID03342390, is rapidly metabolized in either Human or mouse microsomes. It however, does not have significant toxicity to human hepatocytes.

Table 6: Summary of in vitro ADME Properties of Kappa opioid Agonist probe(s)

Probe CID Probe ML# BCCG MLS-#	**Aqueous Solubility** (μg/mL)[a] (@ pH)	**PAMPA Pe** ($x10^{-6}$ cm/s)[b] (@ Donor pH)	**BBB-PAMPA Pe** ($x10^{-6}$ cm/s)[c]	**Plasma Protein Binding (% Bound)**		**Plasma Stability**[d] Human Mouse/	**Hepatic Microsome Stability**[e] Human/ Mouse	**Hepatic Toxicity**[f] LC50 (μM)
				Human 1μM/ 10μM	**Mouse** 1μM/ 10μM			
CID3342390 **ML140** MLS-0239991	0.47 (5.0) 0.12 (6.2) 0.79 (7.4)	>1710 (5.0) >1853 (6.2) >1944 (7.4)	419	99.67/ 99.73	98.90/ 98.92	100/ 100	0.04/ 0.07	>50

[a] in aqueous buffer, pH's 5.0/6.2/7.4
[b] in aqueous buffer; Donor compartment pH's 5.0/6.2/7.4; Acceptor compartment pH 7.4
[c] in aqueous buffer; Donor compartment pH 7.4; Acceptor compartment pH 7.4
[d] % remaining at 3 hr
[e] % remaining at 1 hr
[f] towards Fa2N-4 immortalized human hepatocytes

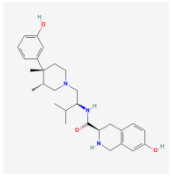
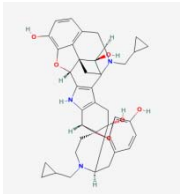
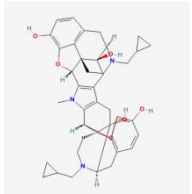
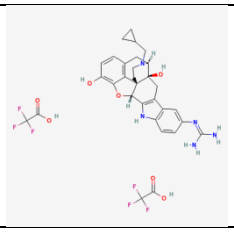
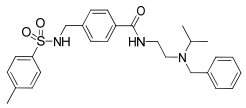
j. A tabular presentation summarizing known probe properties

Table 7. CID:3342390 (MLS0026993322)	
Calculated Property	Value
Molecular Weight	479.63422 [g/mol]
Molecular Formula	$C_{27}H_{33}N_3O_3S$
XLogP3-AA	4.4
H-Bond Donor	2
H-Bond Acceptor	5
Rotatable Bond Count	11
Tautomer Count	2
Exact Mass	479.2243
MonoIsotopic Mass	479.2243
Topological Polar Surface Area	78.5
Heavy Atom Count	34
Formal Charge	0
Complexity	699
Isotope Atom Count	0
Defined Atom StereoCenter Count	0
Undefined Atom StereoCenter Count	0
Defined Bond StereoCenter Count	0
Undefined Bond StereoCenter Count	0
Covalently-Bonded Unit Count	1

5. Comparative data showing probe specificity for target in biologically relevant assays

As described in the CPDP these studies are now on-going in the assay provider's and collaborating laboratories, as post-probe nomination research and we hope to publish jointly in the future. Additionally this probe compound was submitted for broad CNS-centric GPCR paneling at the PDSP (see **Table 6** above) and were both shown to be fairly selective to the opioid receptors. Furthermore, the relatively good agreement among the GPCR panel, cell-based beta-arrestin mediated enzyme complementation assay and the more downstream image based beta-arrestin mediated G-protein redistribution with these compounds is notable.

As defined in the CPDP and the initial teleconference calls with the National Institute on Drug Abuse, this probe project was unusual as there are already examples of very potent agonists and antagonists of the kappa-opioid receptors with low nanomolar EC50 and IC50s, that are very selective against the mu- and delta- opioid subtypes. As emphasized during those initial discussions, the overarching purpose was to find new chemical scaffolds that are chemically distinct from the rich literature of known agonists and antagonists as starting points for further synthesis and work by the assay provider and their collaborative chemists. Furthermore, as NIDA is ultimately interested in new antagonists that are short acting. While these animal model studies are out of scope of the MLPCN and indeed would take much more compounds and more time than a probe nomination project, NIDA is was clear that new scaffolds would be a key measure of success. As a comparative measure, the following table summarizes our antagonist probe against the precedent state-of-art probes. We note that we have conservatively put the 850 nM value obtained in the functional DiscoveRx assay into this table, despite the fact that the Ki values shown for the existing agents are probably more appropriately compared to the Ki value of 4.3 nM obtained for the probe by the PDSP.

Table 8. Comparison of prior art and current probe					
Antagonist Name	JDTic	NorBNI - Norbinaltorphimine	BNI - Binaltorphimine	GNTI – Guanidinyl-naltrindole di-trifluoroacetate	Not Applicable
Chemical Structure					
Ki KOR (nM)	0.02	0.04	0.14	0.04	850
Ki MOR (nM)	2.1 (202X)	16.7 (417X)	1.26 (9X)	36.9 (923X)	>32,000 (> 40X)
Ki DOR (nM)	300 (15,000X)	10.2 (255X)	5.81 (42X)	70 (17500X)	>32,000 (> 40X)
PubChem CID	9956146	5480230	5484197	9853099	3342390
PubChem SIDs:	46052753; 24225384; 14931787	50104378; 49963059; 47959969; 47810970; 26751686; 14937121; 14912769; 11693572; 7980165; 841865	24600619; 51940254; 14863933; 669631	14815182; 17405121; 50106355; 53777665; 24278454	87544125
PMID	11495579	9857089	2839664	10822054	Not applicable

While this probe does meet most of the potency criteria for a probe as agreed to upon in the CDPD, the potency and selectivity remain to be improved further. However, the assay provider is interested in this structure and finds the SAR intriguing. Clearly this sulfonamide probe provides novel scaffolds distinct from BNI, GNTI and JDTic that might be further developed as was the main goal of this project. This scaffold while chemically distinct is reminiscent structurally with regard to the overall length and the presence of derivitizable aromatic rings at both ends. Again, we consider this probe project to be successful in defining a novel chemical scaffold that may well have different and interesting pharmacological properties than the current literature examples.

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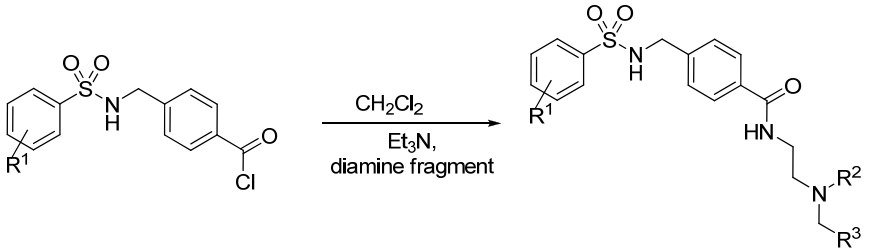
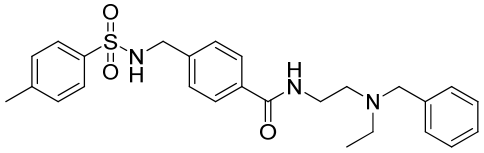
Appendix: Synthetic procedures and compound characterization.

Series 2 Compounds:

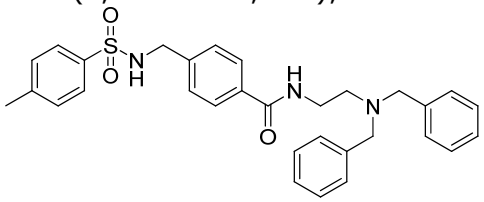
Synthesis of Series 2 Antagonist Compounds:



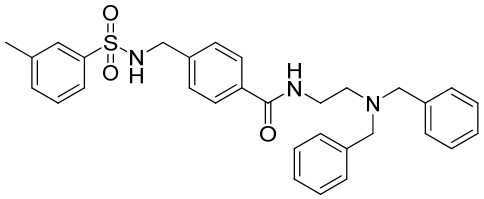
General procedure for coupling of the sulfonamide acid chlorides with the diamine fragments. The acid chloride, Et_3N (2.5 equiv) and the diamine fragment (1 .0 equiv) in CH_2Cl_2 (10 mL/mmol) were stirred at rt for 5 h. Aqueous saturated $NaHCO_3$ (0.5 mL) was added, the solvents removed in vacuo and the residue extracted with CH_2Cl_2 (3 × 2 mL). The combined filtrates were concentrated and purified by mass-directed, reverse phase preparative HPLC to afford the amino amide compounds.



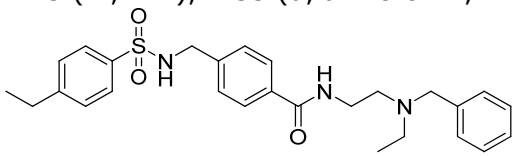
N-(2-(benzyl(ethyl)amino)ethyl)-4-((4-methylphenylsulfonamido)methyl)benzamide (SID **87457826**). ^{1}H NMR ($CDCl_3$) δ 1.13 (t, *J* = 7.2 Hz, 3 H), 2.47 (s, 3 H), 2.64 (q, *J* = 7.2 Hz, 2 H), 2.69 (t, *J* = 6.0, 2 H), 3.46 (dt, *J* = 5.1, 6.0 Hz 2 H), 3.63 (s, 2 H), 4.21 (s, 2 H), 4.84-4.97 (br, 1 H), 6.62-6.69 (br, 1 H), 7.25-7.37 (m, 9 H), 7.59 (d, *J* = 8.4 Hz, 2 H), 7.81 (d, *J* =8.4 Hz, 2 H);



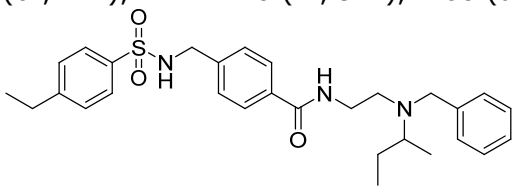
N-(2-(dibenzylamino)ethyl)-4-((4-methylphenylsulfonamido)methyl)benzamide (SID **87457827**). ^{1}HNMR ($CDCl_3$) δ 2.48 (s, 3 H), 2.73 (t, *J* = 6.0 Hz, 2 H), 3.50 (dt, *J* = 4.8, 6.0 Hz, 2 H), 3.65 (s, 4 H), 4.23 (s, 2 H), 4.89 (br, 1 H), 6.45 (br, 1 H), 7.25-7.38 (m, 14 H), 7.54 (d, *J* = 8.4 Hz, 2 H), 7.82 (d, *J* = 8.4 Hz, 2 H);



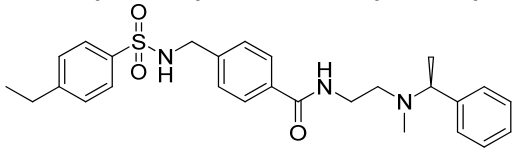
N-(2-(dibenzylamino)ethyl)-4-((3-methylphenylsulfonamido)methyl)benzamide (SID **87457834**). ^{1}HNMR ($CDCl_3$) δ 2.46 (s, 3 H), 2.73 (t, *J* = 5.7 Hz, 2 H), 3.50 (dt, *J* = 5.0, 5.7 Hz, 2 H), 3.65 (s, 4 H), 4.25 (s, 2 H) 6.45 (br, 1 H), 7.24-7.35 (m, 13 H), 7.43-7.45 (m, 2 H), 7.55 (d, *J* = 8.0 Hz, 2 H), 7.72-7.75 (m, 2 H);



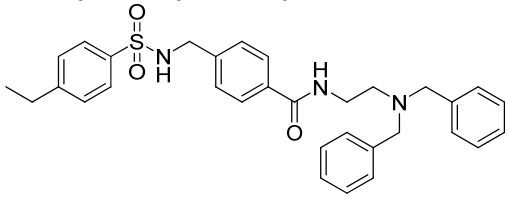
N-(2-(benzyl(ethyl)amino)ethyl)-4-((4-ethylphenylsulfonamido)methyl)benzamide (SID **87457835**). ^{1}HNMR ($CDCl_3$) δ 1.13 (t, *J* = 7.2 Hz, 3 H), 1.30 (t, *J* = 7.5 Hz, 3 H), 2.65 (q, *J* = 7.2 Hz, 2 H), 2.70 (t, *J* = 5.2 Hz, 2 H), 2.76 (q, *J* = 7.5 Hz, 2 H), 3.46 (dt, *J* = 5.2, 6.4 Hz, 2 H), 3.63 (s, 2 H), 4.22 (d, *J* = 6.0 Hz, 2 H), 4.80 (t, *J* = 6.3 Hz, 1 H), 6.66 (br, 1 H), 7.24-7.40 (m, 9 H), 7.60 (d, *J* = 7.2 Hz, 2 H), 7.83 (d, *J* = 8.5 Hz, 2 H);



N-(2-(benzyl(sec-butyl)amino)ethyl)-4-((4-ethylphenylsulfonamido)methyl)benzamide (SID **87457836**). ^{1}HNMR ($CDCl_3$) δ 0.85 (t, *J* = 6.8 Hz, 3 H), 0.97 (d, *J* = 6.0 Hz, 3 H), 1.20 (t, *J* = 7.5 Hz, 3 H), 1.29 (m, 1 H), 1.52 (m, 1 H), 2.55-2.70 (m, 5 H), 3.24 (m, 1 H), 3.28-3.39 (m, 2 H), 3.62 (d, *J* = 14.9 Hz, 1 H), 4.12 (d, *J* = 6.4 Hz, 2 H), 4.69 (br, 1 H), 6.415 (br, 1 H), 7.11-7.31 (m, 9 H), 7.44 (d, *J* = 7.7 Hz, 2H), 7.73 (d, *J* = 7.7 Hz, 2 H);



(R)-4-((4-ethylphenylsulfonamido)methyl)-N-(2-(methyl(1-phenylethyl)amino)ethyl)-benzamide (SID **87457837).** ^{1}HNMR ($CDCl_3$) δ 1.20 (t, J = 7.6 Hz, 3 H), 1.32 (d, J = 6.4 Hz, 3 H), 2.21 (s, 3 H), 2.43 (m, 1 H), 2.53 (m 1 H), 2.66 (q, J = 7.6 Hz, 2 H), 3.36 (m, 2 H), 3.61 (q, J = 6.4 Hz, 1 H), 4.13 (d, J = 6.0 Hz, 2 H), 4.76 (br, 1 H), 6.57 (br, 1 H), 7.10-7.34 (m, 9 H), 7.54 (d, J = 7.6 Hz, 2 H), 7.73 (d, J = 8.2 Hz, 2 H);



N-(2-(dibenzylamino)ethyl)-4-((4-ethylphenylsulfonamido)methyl)benzamide (SID **87457838**). ^{1}HNMR ($CDCl_3$) δ 1.30 (t, J = 7.6 Hz, 3 H), 2.69-2.80 (m, 4 H), 3.50 (dt, J = 4.5, 6.4 Hz, 2 H), 3.65 (s, 4 H), 4.24 (s, 2H), 6.44 (br, 1 H), 7.25-7.35 (m, 13 H), 7.38 (d, J = 7.6 Hz, 2 H), 7.55 (d, J = 7.6 Hz, 2 H), 7.84 (d, J = 7.6 Hz);