Probe Report

Probe project: Selective KOP Receptor agonists

Title: Agonists and Antagonists for the Kappa Opioid Receptor

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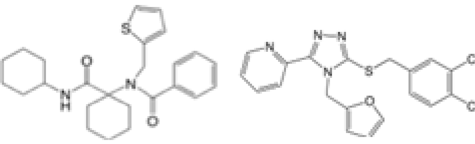
PubChem Summary Bioassay Identifier (AID): 1786

Probe(s) Structure & Characteristics:

This joint KU & Burnham Center Probe Report describes two selective agonists for the kappa (κ) opioid receptor [KOP receptor or KOR] representing two novel scaffolds or chemical series: (1) bisamides exemplified by CID5236771 and (2) triazoles exemplified by CID44601470.



ML139 **ML138**

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]
5236771 *(bisamides)* **ML139**	KOR *κ-opioid receptor*	120 nM IC_{50} SID87218782 AID2284	MOR *μ-opioid receptor*	>32 μM EC_{50} SID87218782 AID2352	> 270X *(Dx/HCS)* >530X *(HCS/HCS)*	KOR Transflour <60 nM EC_{50} [SID87218782, AID2359] MOR Transflour >32,000 nM EC_{50} [SID87218782, AID2352]
			DOR *δ-opioid receptor*	>32 μM EC_{50} SID87218782 AID2370	> 270X *(Dx/HCS)* >530X *(HCS/HCS)*	DOR Transflour >32,000 nM EC_{50} [SID87218782, AID2370]
44601470 *(triazoles)* **ML138**	KOR *κ-opioid receptor*	870 nM IC_{50} SID87334039 AID2284	MOR *μ-opioid receptor*	>32 μM EC_{50} SID87334039 AID2352	> 37X *(Dx/HCS)* >91X *(HCS/HCS)*	KOR Transfluor 350 nM EC_{50} [SID87334039, AID2359] MOR Transfluor >32000 nM EC_{50} [SID87334039, AID2352]
			DOR *δ-opioid receptor*	>32 μM EC_{50} SID87334039 AID2370	> 37X *(Dx/HCS)* >91X *(HCS/HCS)*	DOR Transfluor >32000 nM EC_{50} [SID87334039, AID2370]

Recommendations for the scientific use of this probe:

The probe candidates described in this report, by selectively activating 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 activate or block 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 affords 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 aims of this project are to identify subtype specific small molecule agonists of the human kappa opioid (KOP) receptor. Such agonists may be useful in elucidating different signaling modalities for kappa receptors, but more importantly may be partial agonists that will facilitate the discovery of new molecular scaffolds for kappa antagonists. 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 receptor activation by its endogenous ligand dynorphin-A reduces extracellular dopamine. It has been suggested that stimulation of κ receptors by endogenous opioids like dynorphins should produce an aversive state that can antagonize the effects of other addictive compounds like alcohol, cocaine and nicotine. Moreover, exogenous κ agonists have also been observed to attenuate drug-taking behavior (2-6). Interestingly, kappa opioid antagonists have also been considered for use in treatment of cocaine abuse and appetite suppression (6).

The κ opioid receptors provide desirable substrates for targeted therapy and unraveling pathways responsible for mediating addictive behavior. Kappa opioid agonists offer a means to <u>*modulate*</u> the effects of stimulant drugs. However, there are currently no approved agents or compounds for these purposes (2).

In this probe report, we describe the discovery and optimization of novel agonists for the kappa-opioid (κ-opioid) receptor [KOP receptor or KOR] that is greater than 100-fold

selective over the mu- (μ) [MOP receptor or MOR] and delta- (δ) [DOP receptor or DOR] opioid receptors (as measurable within the limits of solubility). We sent the probe for broad GPCR paneling and these data are discussed below (see **Table 9**). Furthermore, these probes and their analogs represent a novel chemical classes compared to current literature agonists with potentially novel pharmacology that may serve as interesting tools to advance addiction research.

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 and to characterize compound signaling. To demonstrate subtype specific binding in competitive assays a cohort of radiolabeled compounds are available commercially. [^{3}H]-Diprenorphine has been used to demonstrate high affinity and selectivity (K_d = 0.1 nM) toward KOR and MOR. [^{3}H]-U-69,593, [Phenyl-3,4-^{3}H] can be used to demonstrate high affinity and selectivity (Kd = 1 nM) toward KOR. Likewise, [^{3}H]-DAMGO, [Tyrosyl-3,5-^{3}H(N)] is also available for MOR displacement binding and Naltrindole [5`,7`-^{3}H] or [^{3}H]-Enkephalin are available for DOR binding studies. Additionally, these receptors couple through a multiplicity of G proteins and signaling pathways that can be studied by available assays including determination of the changes in cAMP, calcium, and certain phospho-kinase levels in cells.

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 agonists different from current literature probes, with potencies of less than 1 μM for the kappa(κ)-opioid receptor (KOR), but with 100X selectivity against the mu(μ)-opioid receptor (MOR) and 100X selectivity against the delta(δ)-opioid receptor (DOR).

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. Listing of all Assays and AIDs for this project

PubChemBioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
Summary of small molecule agonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Summary]	1786	Agonist	Summary	N/A	N/A
uHTS identification of small molecule agonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Confirmatory]	1777	Agonist	Primary	Cell-based	Luminescence -DiscoveRx β-arrestin & 1536
SAR analysis of small molecule agonists of the kappa opioid receptor via a luminescent beta-arrestin assay [Confirmatory]	2284	Agonist	SAR	Cell-based	Luminescence -DiscoveRx β-arrestin & 1536
HTS Dose response counterscreen for assays utilizing the enzyme, b-galactosidase [Confirmatory]	1966	Agonist	Counterscreen	Biochemical	Luminescence &1536
HTS Image-Based Screen for Selective Agonists of the KOR Receptor [Confirmatory]	2133	Agonist	Alternate	Cell-based	HCS – Transfluor & 384
HTS Image-Based Screen for Agonists of the MOR Receptor [Confirmatory]	2344	Agonist	Selectivity	Cell-based	HCS – Transfluor & 384
HTS Image-Based Screen for Agonists of the	2343	Agonist	Selectivity	Cell-based	HCS –

DOR Receptor [Confirmatory]					Transfluor & 384
SAR analysis of Agonists of the Kappa Opioid Receptor (KOR) using an Image-Based Assay [Confirmatory]	2359	Agonist	Alternate	Cell-based	HCS – Transfluor & 384
SAR Analysis of Agonists of the MOR Receptor using an Image-Based Assay [Confirmatory]	2352	Agonist	Selectivity	Cell-based	HCS – Transfluor & 384
SAR Analysis of Agonists of the DOR Receptor using an Image-Based Assay [Confirmatory]	2370	Agonist	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 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.

The primary screening protocol is described below.

Assay materials:

1) OPRK1 beta-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 25 nL to wells Columns 1-2 should be DMSO only (- Control), Columns 3-4 should contain Dynorphin A (+ Control). Working concentration = 200 μM, FAC = 1 μM and Columns 5-48 contain test compounds (10μM final in well concentration).
2) Immediately following pintool addition, spin plates at 500 rpm for 1 min, return assay

plates to an incubator at 37C
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 0.56, the signal to background (S/B) was 5.02 , signal to noise (S/N) was 33.79 and signal window was 5.03.

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 (EC_{50} < 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 agonist activity in the KOR agonist 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 will be 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 the library in the initial primary screen. Active compounds were then tested in an alternative format for GPCR activation, via the imaging-based KOR High-Content Transfluor Agonist 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: uHTS identification of small molecule agonists of the kappa opioid receptor via a luminescent beta-arrestin assay *(AID* 1777*)*

Assay 2: SAR analysis of small molecule agonists of the kappa opioid receptor via a luminescent beta-arrestin assay *(AID 2284)*

Assay 3: HTS Image-Based Screen for Selective Agonists of the KOR Receptor *(AID 2133)*

Assay 4: SAR analysis of Agonists 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 activation, which might present the opportunity for false positives from the initial primary assay. The enhancement of activity of the β-galactosidase fragment complementation in the primary KOR1 β-Arrestin Assay in the presence of test agent could lead to increased 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*)*

Selectivity assays

The imaging-based MOR and DOR High-Content Transfluor Agonist Assays provide for the determination of KOR receptor selectivity. The probe criteria specifies the necessity of at least 100-fold selectivity against MOR and 10-fold against 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 Agonists of the MOR Receptor *(AID* 2344*)*

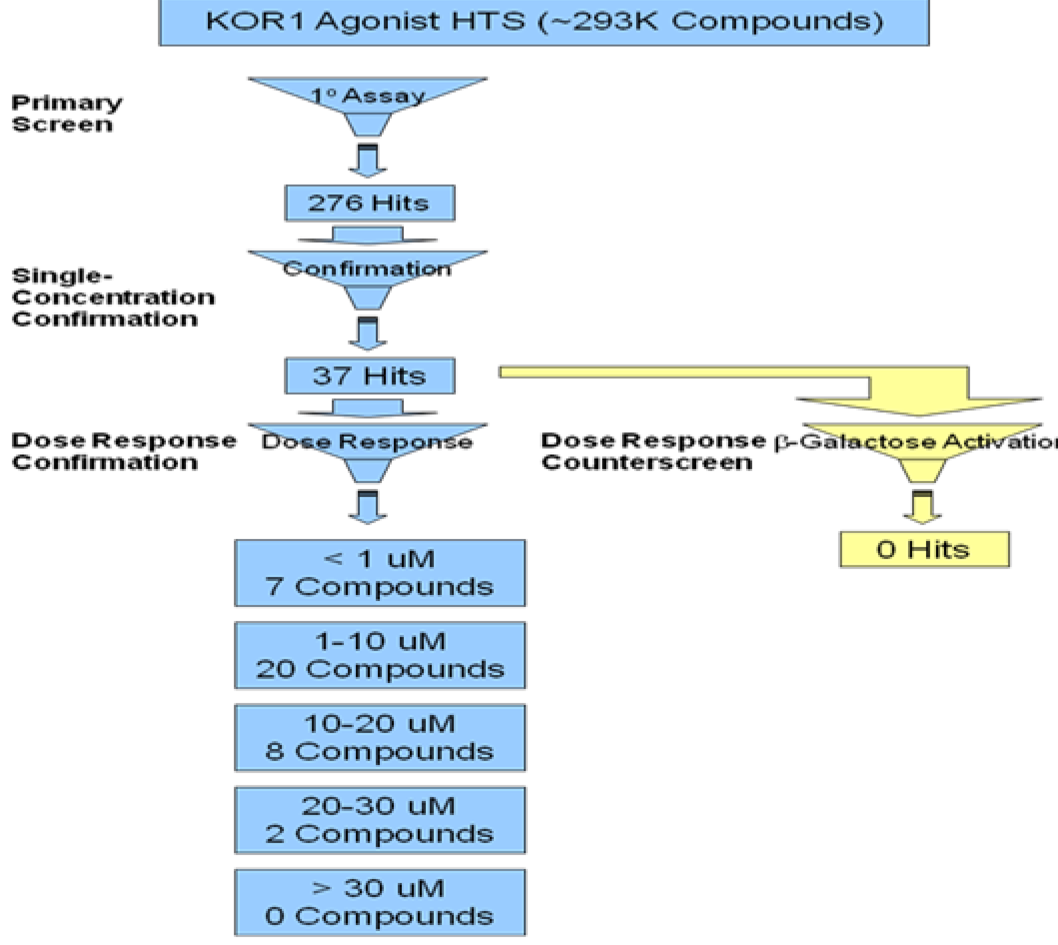
Assay 2: HTS Image-Based Screen for Agonists of the DOR Receptor *(AID 2343)*

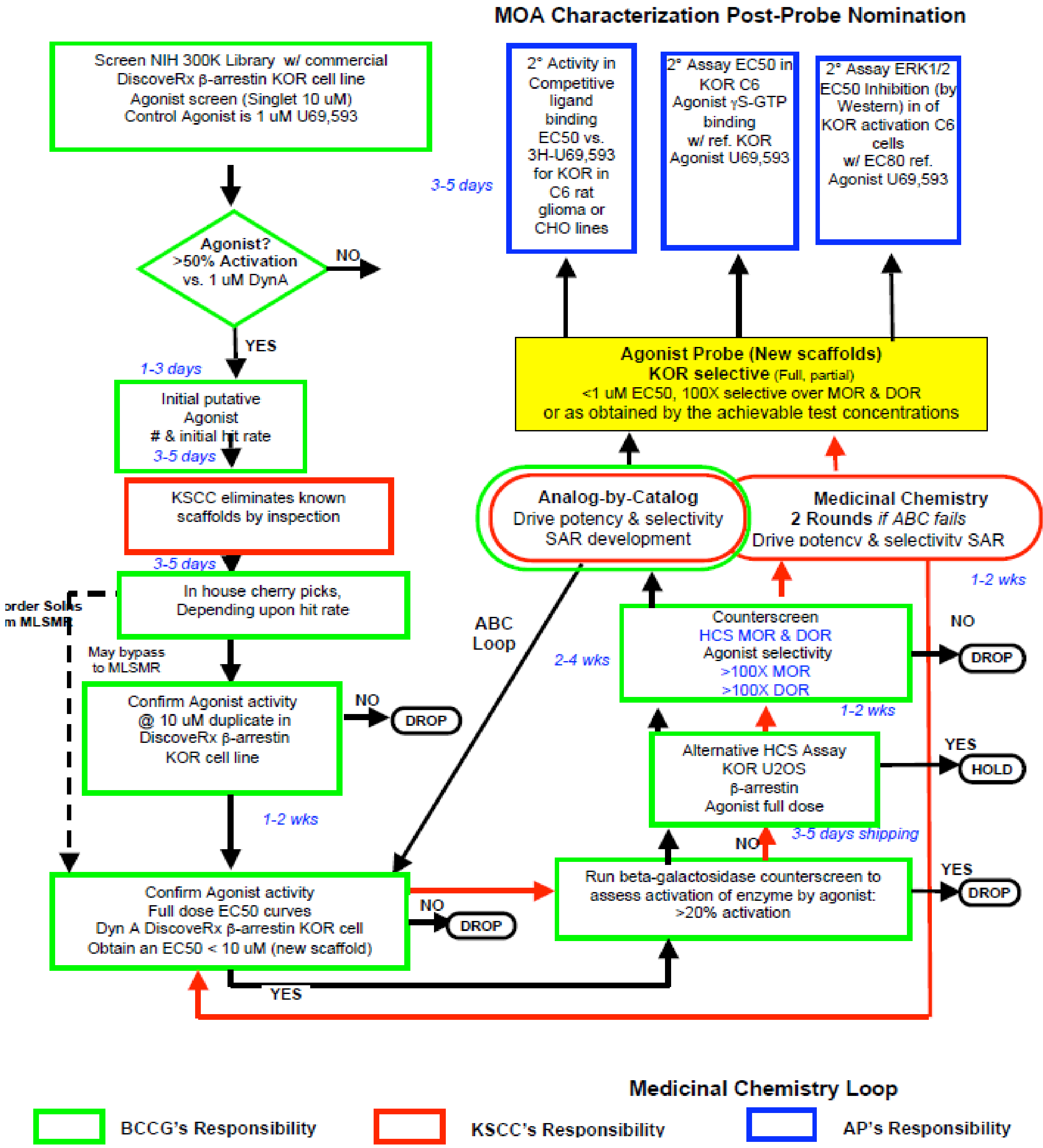
Assay 3: SAR Analysis of Agonists of the MOR Receptor using an Image-Based Assay *(AID 2352)*

Assay 4: SAR Analysis of Agonists of the DOR Receptor using an Image-Based Assay (AID 2370)

3. Center Summary of Results

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





A library of approximately 290,000 compounds was tested in the KOR1 DiscoveRx β-Arrestin primary screen. Upon data analysis, 292 hits with activity >50% at a single concentration point of 10 μM were identified. Liquid samples were then ordered through DPI and 276 compounds were received.

The compound solutions resupplied by the MLSMR were first confirmed in 10 μM single-point duplicate in the KOR1 DiscoveRx β-Arrestin primary assay. Of these, 37 compounds were confirmed to have at least 50% activity at a 10 μM assay concentration. The confirmed compounds were further tested in dose response in the KOR1 DiscoveRx β-Arrestin primary assay to obtain EC50 values and were also tested in a β-galactosidase Counterscreen assay to assess the possibility that these compounds might somehow activate the enzyme. All

compounds were confirmed as active in the KOR1 β-Arrestin dose response assay and none of the compounds were found to have activated β-galactosidase.

The active, confirmed compounds were then tested in the KOR1 High-Content Transfluor Agonist assay for further confirmation, then in the MOR and DOR High-Content Transfluor Agonist 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, 30 structures were ordered though commercial vendors. From these experiments, a diverse SAR emerged and several compounds were discovered that met our established probe criteria.

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

Probe Optimization

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

The screening of the MLPCN compound collection at the Sanford-Burnham Center for Chemical Genomics identified several promising chemotypes (Figure 1) with potencies of the lead exemplar noted from solution reconfirmation. This probe report describes the optimization of probes from the first two chemotypes, bisamides (series 1) and triazoles (series 2). The third series did not yield sufficiently compelling SAR and was set aside.

Series 1	Series 2	Series 3
5 hit compounds		
EC_{50} = < 0.5 uM	EC_{50} = 1.86 uM	EC_{50} = 8.73 μM

Figure 1

Cheminformatics and Medicinal Chemistry Analysis: Probe optimization/SAR

Bisamide series: Compound **1** CID5236771 (MLS-0254632) (entry 1, **Table 3**) was identified through a high-throughput screening campaign on 284,076 compounds as an active and selective scaffold. After confirmation of the initial results, the hit-to-probe process was initiated by both analog-by-catalog approach and collaborative medicinal chemistry effort with KSCC. Probe candidate CID5236771 (MLS-0254632, entry 1) and analogs (entries 2- 9) were obtained from commercial sources. Several additional analogs were prepared using a protocol similar to the route shown in section 4h (entries 10 – 14 in **Table 3**). The structure-activity data are presented **Table 3** below:

Table 3: SAR Analysis for Selective κ–opioid receptor agonist for the Bisamide Scaffold

SAR Analysis for KOR Selective Scaffolds (Medicinal Chemistry & Cheminformatics Analysis)

Potency (µM) Ave. ± *S.E.M.* (n = replicates)

n value in column refers to KOR DRx replicates

#	CID	SID	BCCG MLS-	*	R1	R2	R3	X	R4	N	KOR *DRx*	KOR *HCS (n=2)*	MOR *HCS (n=2)*	DOR *HCS (n=2)*
1	5236771	87218782	0254632	P	C_6H_{11}	C_5H_{10}		S	2-pyridyl	3	0.12 *±0.01*	<0.06	>32	>32
2	4014737	87218783	0211381	P	C_6H_{11}	C_5H_{10}		S	2-pyrazinyl	3	0.44 *±0.06*	0.14	>32	>32
3	3950926	87218781	0075520	P	C_6H_{11}	C_5H_{10}		O	2-pyridyl	3	0.82 *±0.04*	0.13	>32	>32
4	1282817	87218798	0425703	P	*p*-Br-C_6H_4	C_5H_{10}		O	2-pyridyl	3	0.42 *±0.02*	0.10	>32	>32
5	4579040	87218797	0079438	P	C_5H_9	C_5H_{10}		O	2-pyrazinyl	3	>10	>32	>32	>32
6	1297443	87218767	0024680	P	2,6-dimethyl-C_6H_4	C_4H_8		O	2-pyridyl	3	4.05 *±0.10*	1.11	>32	>32
7	3950572	87218780	0237946	P	C_5H_9	Me	Et	S	2-pyridyl	3	2.07 *±0.17*	0.81	>32	>32
8	3242935	87218796	0007493	P	C_6H_{11}	Me	Et	O	2-pyrazinyl	3	3.71 *±0.19*	1.36	>32	>32
9	3964693	87218795	0080731	P	C_6H_{11}	Me	Et	O	2-pyridyl	3	0.65 *±0.03*	0.17	>32	>32
10	1458208	85787073	0435478	S	C_6H_{11}	H	H	S	2-pyridyl	2	>20	ND	ND	ND
11	44483222	85787075	0435480	S	C_6H_{11}	H	H	S	3-pyridyl	2	>20	ND	ND	ND
12	44483224	85787074	0435479	S	C_7H_{13}	H	H	S	2-pyridyl	2	>20	ND	ND	ND
13	44483221	85787077	0435482	S	C_7H_{13}	H	H	S	3-pyridyl	2	>20	ND	ND	ND
14	44483223	85787076	0435481	S	MeO**	H	H	S	3-pyridyl	2	>20	ND	ND	ND

* S = Synthesized P = purchased ND = not determined TBA = to be assigned
** methyl ester instead of methyl amide
Bold underlined compound = probe submitted
Underlined italicized cmpd # = analog submitted

SAR Analyses for CID5236771 (bisamide series): The above data set illustrate several emerging SAR trends for this chemotype. Foremost, the requirement for substitution on the methylene bridge between amides is demonstrated from the inactive glycine-derived analogs CID1458208 (MLS-0435478), CID44483222 (MLS-0435480), CID44483224 (MLS-0435479), CID44483221 (MLS-0435482) and CID44483223 (MLS-0435481) (entries **10-14**). While the necessity for alkyl substitution is clear, the ideal composition of the R^2/R^3 groups has not been completely optimized for this chemotype The exact nature of the side group on the secondary amide on the left hand side of the general structure appears to have a less dramatic effect on potency than the R^2/R^3 substitution. While a range of hydrophobic groups afforded highly potent analogs, the most potent analog was obtained when R^1 was cyclohexyl (CID5236771 (MLS-0254632), entry **1**). The single case where a direct comparison is possible suggests the thiophene-containing side chain to be advantageous over the furan-containing side chain (CID5236771 and CID3950926, entries **1** and **3**). Finally, a clear advantage was observed for the 2-pyridyl amides over the 2-

pyrazinyl amides, as exemplified by CID5236771 compared to CID4014737 (entries **1** and **2**) compared to CID3242935 (entries **9** and **8**).

Triazole series: The triazole (Series 2) chemotype originally contained four compounds with potency around 2 µM and one example at 6.7 µM. This scaffold was pursued in a short and intense analog campaign to increase potency by at least two fold. The synthesis and purchase of a total of 43 analog compounds provided not only a compound exceeding the necessary increase in potency, but also less potent compounds possessing super agonist efficacies (Emax >100%): cmps **1** - **3**, **5** - **11** in **Table 4** below, which also details the SAR:

Table 4: SAR Analysis for Selective κ–opioid receptor agonist for the Triazole Scaffold

SAR Analysis for KOR Selective Scaffolds (Medicinal Chemistry & Cheminformatics Analysis)								Potency (µM) Ave. ± *S.E.M.* (stdv/sqrt (n)) (n = replicates) *n value in column refers to KOR DRx replicates*					
#	**CID**	**SID**	**BCCG MLS-**	*****	**Purity** *(%)*	**X**	**R**	**n**	**Emax** *(%Act)*	**KOR** *DRx*	**KOR** *HCS (n=2)*	**MOR** *HCS (n=2)*	**DOR** *HCS (n=2)*
1	44601470	87334039	0435589	S	>99	O	3,4-dichloro	3	~140%	0.87 ±*0.06*	0.347	>32	>32
2	2562032	87334040	0435590	S	>99	O	4-methoxy	6	~150%	7.70 ±*1.47*	12.4	>32	>32
3	44601474	87334041	0435591	S	>99	O	4-methyl	5	~150%	6.20 ±*1.26*	5.21	>32	>32
4	44601466	87334042	0435592	S	>99	O	H	8	*Emax not reached*	>17.90	>32	>32	>32
5	44601467	87334043	0435593	S	>99	S	H	6	~150%	13.85 ±*1.18*	23.5	>32	>32
6	44601473	87334044	0435594	S	>99	S	4-methyl	8	~170%	3.59 ±*0.30*	2.38	>32	>32
7	44601472	87334045	0435595	S	>99	S	4-bromo	8	~185%	1.85 ±*0.11*	1.10	>32	>32
8	44601471	87334046	0435596	S	>99	S	4-methoxy	8	~130%	9.62 ±*1.32*	5.98	>32	>32
9	44601468	87334047	0435597	S	93.1	S	2,4-difluro	8	~155%	6.74 ±*0.97*	5.68	>32	>32
10	44601469	87334048	0435598	S	>99	S	2,4-dichloro	8	~120%	2.27 ±*0.46*	8.11	>32	>32
11	44601475	87334049	0435599	S	>99	S	3,4-dichloro	8	~135%	0.73 ±*0.11*	0.43	>32	>32
12	662944	87218751	0058739	P	ND	O	2,4-dichloro	3	~100%	1.86 ±*0.07*	0.93	>32	>32
13	663290	87218759	0058731	P	ND	O	4-bromo	3	~100%	1.92 ±*0.05*	1.36	>32	>32
14	1982054	4260946	0003965	P	ND	O	styryl**	3	~100%	2.22 ±*0.10*	1.43	>32	>32
15	2482316	17388459	0107456	P	ND	O	4-*t*-butyl	3	~100%	2.01 ±*0.07*	0.60	>32	>32
16	662263	860989	0058735	P	ND	O	3-chloro	3	~100%	6.76 ±*0.01*	3.63	>32	>32

* S = Synthesized P = purchased ** a styrene substituent instead of the substituted phenyl substituent
ND = not determined

Table does not include 31 additional synthesized compounds (w/ purities all > 94%) with X=S or O and R = 4-nitro, 4-cyano, 4-trifluormethyl, 4-methoxycarboxylate, 4-acetoxy, 4-i-propyl, 2-methyl, 3-methoxy, 2-methyl-3-nitro, 3,5-difluoro, 2,6-difluoro, 2,4,6-trimethyl, 4-fluoro-2-trifloromethyl, 4-chloro-3-trifloromethyl, and 2-chloro substituents, as these are still being tested. However, the current probe from this limited SAR exceeds the probe criteria.

Bold underlined compound = probe submitted *Underlined italicized cmpd #* = analog submitted

While a limited selection of analogs for the series 2 chemotype are commercially available, limited substitution on the phenyl ring and no available thiophene-containing analogs led us to adopt an entirely synthetic approach. Beginning with the appropriate isothiocyanate and 2-picolynyl hydrazide, we synthesized the 1,2,4-triazole-3-thione scaffolds in two steps and excellent yields (77-82% overall yields) without chromatographic separations (section 4 h). The coupling with a wide range of benzyl halides proceeded smoothly in acetone facilitated by K_2CO_3 to furnish the 43 analogs described in **Table 4.**

In conjunction with canonical SAR substitution strategies (7,8), our approach toward the optimization of the Series 2 compounds benefited from the observation in the Series 1 work that in some cases a switch from the furan to thiophene moiety afforded at least a two-fold increase in potency. For example see: CID5236771 (MLS-0254632)/CID3964693 (MLS-0080731) (earlier section). For this chemotype, the thiophene and furan moieties afforded roughly equipotent analogs within the experimental standard deviation. A more productive approach was varying the substitution on the phenyl ring, producing the two most potent analogs CID44601470 (MLS-0435589) and CID44601475 (MLS-0435599) (entries 1 and 11), both bearing a 3,4-dichlorophenyl moiety. Other phenyl ring substitution also bore interesting results not in potency, but in the E_{max} values. Six compounds (entries **2**, **3**, **5, 6**, **7** and **9** in **Table 4**) synthesized displayed 150% or greater activation at 20 μM. This unexpected (and to our knowledge unprecedented) super agonistic activity provides a strong rationale for further explorations into this chemotype.

4. Probe(s)

a. Chemical name of probe compound (s) (Chemical IUPAC)

Probe 1 (bisamide - series 1): N-[1-(cyclohexylcarbamoyl)cyclohexyl]-N-(thiophen-2-ylmethyl)pyridine-2-carboxamide **[ML139]**

Probe 2 (triazole – series 2): 2-(5-(3,4-dichlorobenzylthio)-4-(furan-2-ylmethyl)-4H-1,2,4-triazol-3-yl)pyridine **[ML138]**

b. Probe chemical structure(s) including stereochemistry if known

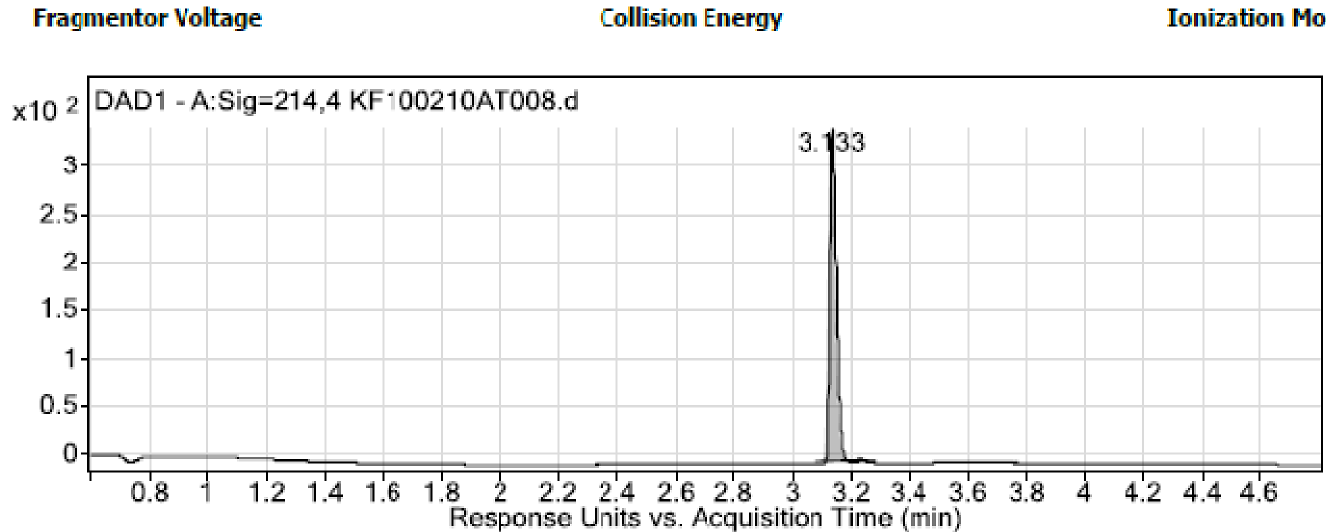
c. Structural Verification Information of probe SID

The probe SIDs are 87218782 (corresponding to CID5236771) and 87334039 (corresponding to CID44601470)

Purity: SID87218782 >98% (HPLC);
SID87334039 >99% (HPLC)

(1) *SID87218782*:

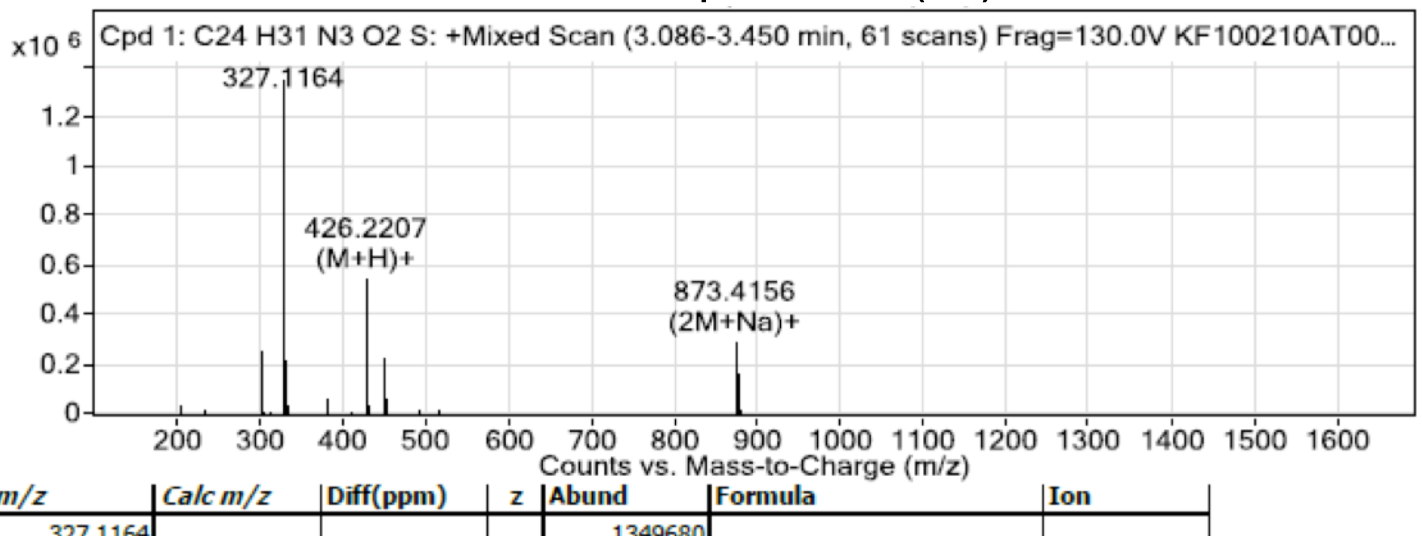
Fragmentor Voltage **Collision Energy** **Ionization Mode**



User Chromatogram Peak List

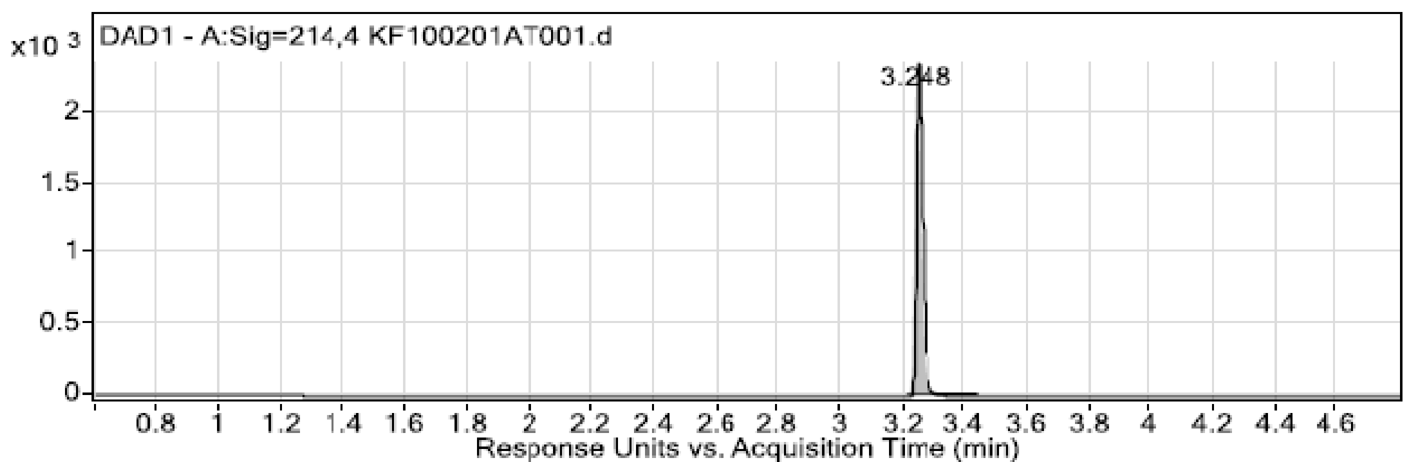
Peak #	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	3.133	341.73	100	517.34	100	98.82	0.024
2	3.228	4.13	1.21	6.17	1.19	1.18	0.022

Counts vs. Acquisition Time (min)



m/z	*Calc m/z*	Diff(ppm)	z	Abund	Formula	Ion
327.1164				1349680		
327.3458				84026		
328.1192				303776		
329.1162				96334		
330.2176				222374		
426.2207	426.221	-0.69	1	557120	C24 H32 N3 O2 S	(M+H)+
427.224	427.224	-0.09	1	158992	C24 H32 N3 O2 S	(M+H)+
448.2027	448.2029	-0.59	1	231651	C24 H31 N3 Na O2 S	(M+Na)+
873.4156	873.4166	-1.18	1	297714	C48 H62 N6 Na O4 S2	(2M+Na)+
874.4188	874.4197	-0.96	1	168813	C48 H62 N6 Na O4 S2	(2M+Na)+

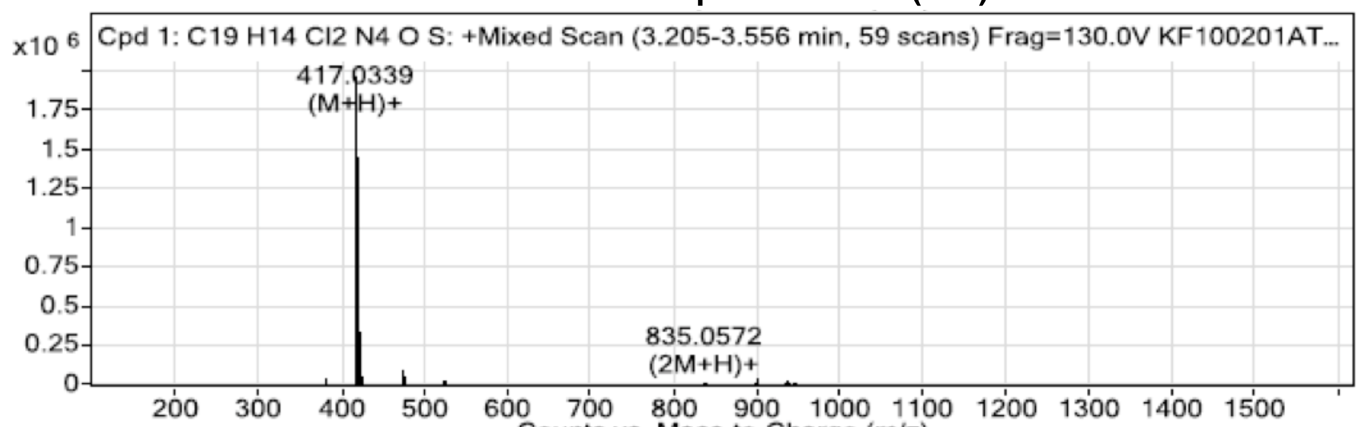
(2) SID87334039:



User Chromatogram Peak List

Peak #	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	3.248	2345.36	100	3513.96	100	100	0.023

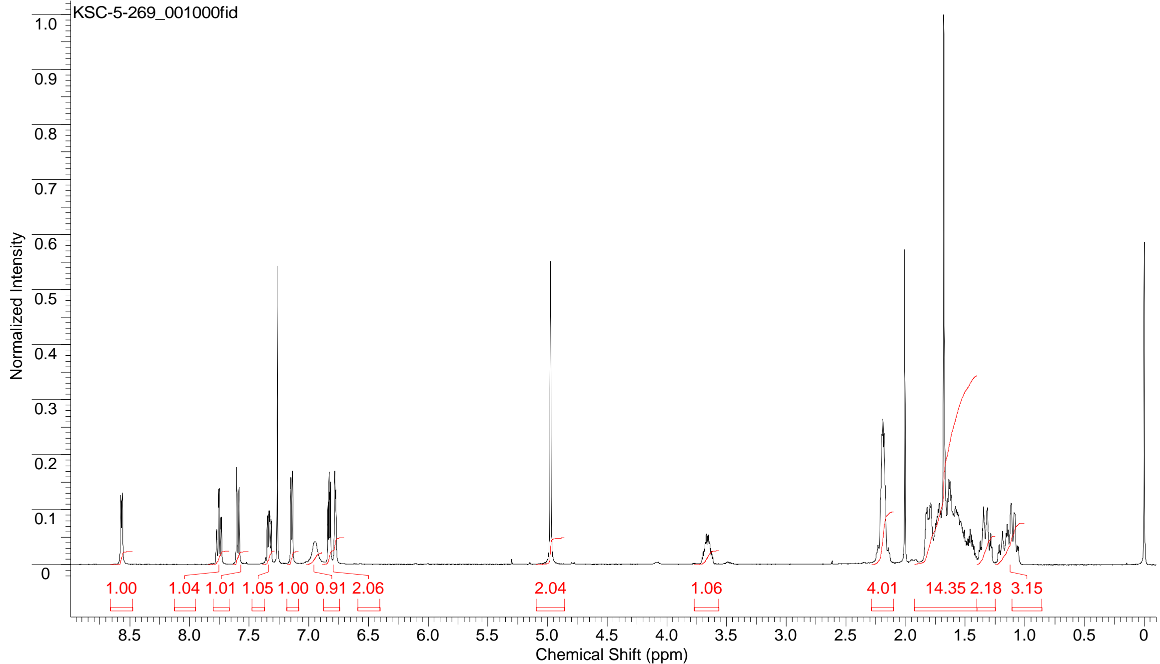
Counts vs. Acquisition Time (min)

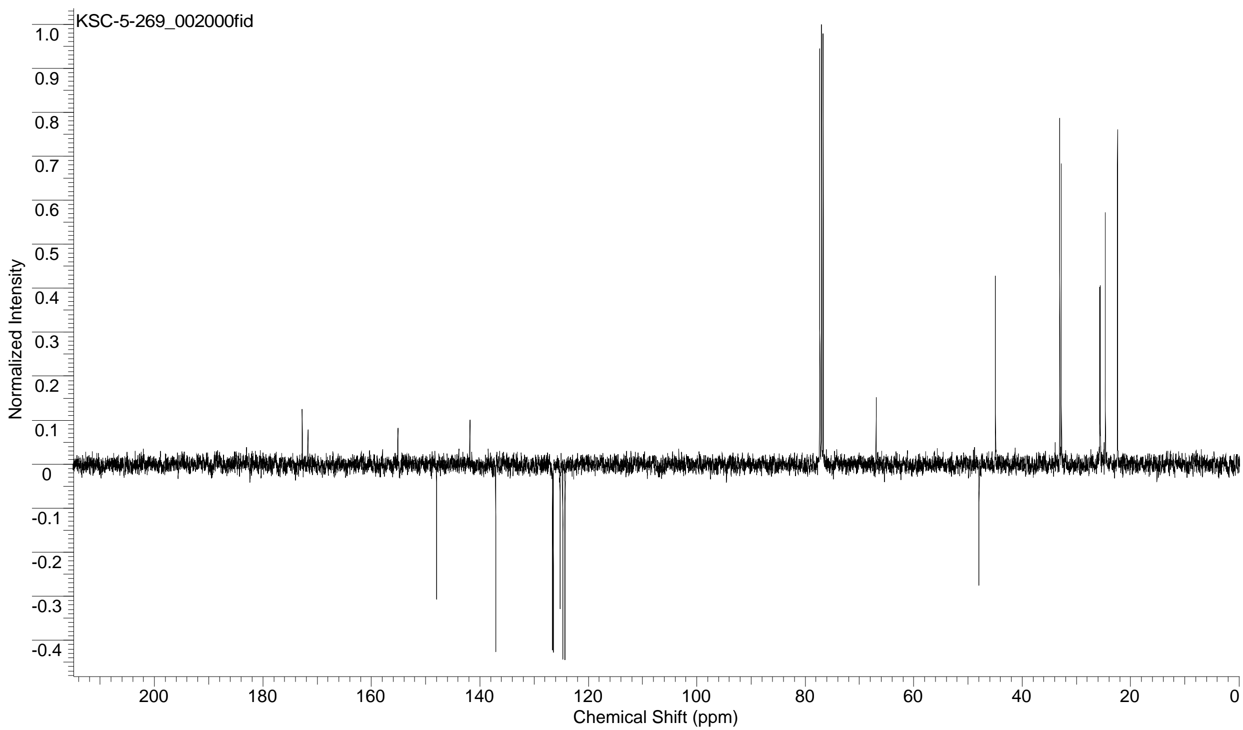


m/z	*Calc m/z*	Diff(ppm)	z	Abund	Formula	Ion
417.0339	417.0338	0.28		1958965	C19 H15 Cl2 N4 O S	(M+H)+
417.2937				124778		
418.0362	418.0367	-1.05		495466	C19 H15 Cl2 N4 O S	(M+H)+
419.031	419.0311	-0.3		1448187	C19 H15 Cl2 N4 O S	(M+H)+
419.297				89142		
420.0333	420.0338	-0.98		344427	C19 H15 Cl2 N4 O S	(M+H)+
421.0284	421.0287	-0.88		319373	C19 H15 Cl2 N4 O S	(M+H)+
422.0311	422.0309	0.45		67492	C19 H15 Cl2 N4 O S	(M+H)+
439.0153	439.0158	-0.99	1	232	C19 H14 Cl2 N4 Na O S	(M+Na)+
833.0596	833.0604	-0.85	1	16374	C38 H29 Cl4 N8 O2 S2	(2M+H)+

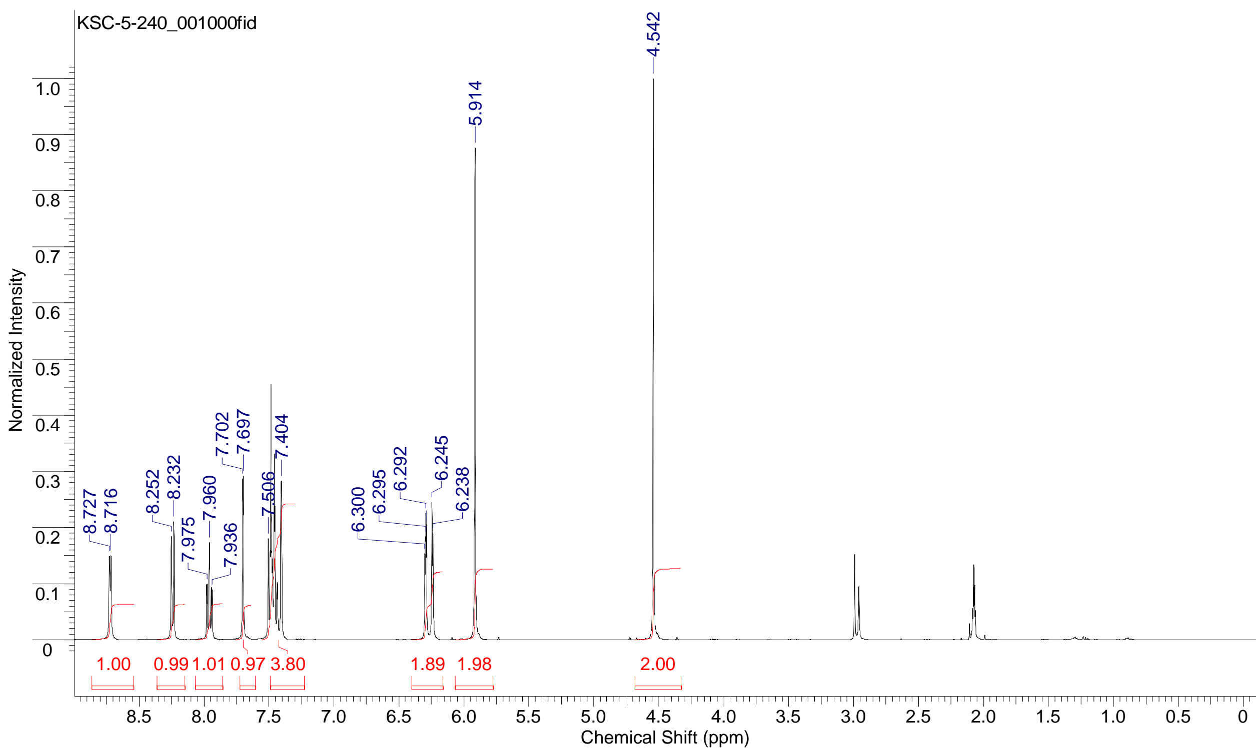
Mass Spec: SID87218782: HRMS (ESI) *m/z* calcd for $C_{24}H_{32}N_3O_2S$ ($[M+H]^+$), 426.2215, found 426.2207.
SID87334039: HRMS (ESI) *m/z* calcd for $C_{19}H_{15}Cl_2N_4OS$ ($[M+H]^+$), 417.0338, found 417.0339.

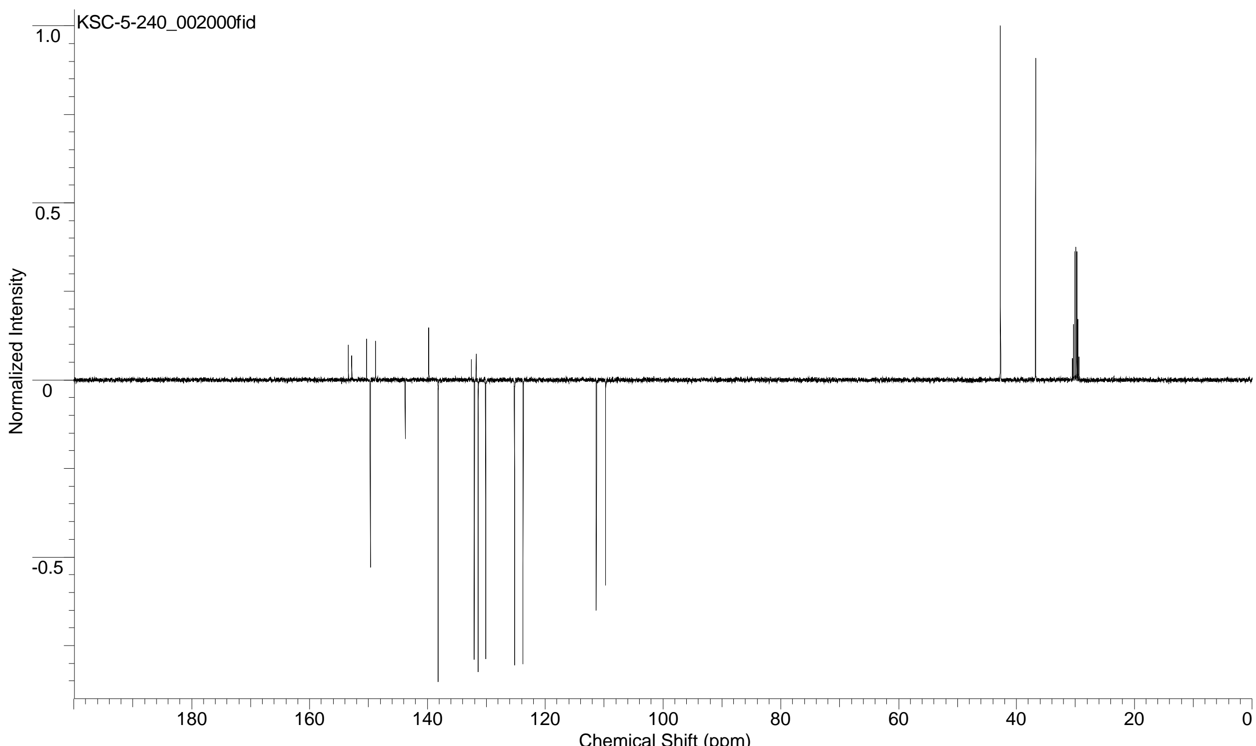
NMR Purity: *(1)* *SID87218782*: >95% (^{1}H-NMR): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.06-1.22 (m, 3 H), 1.28-1.38 (m, 2 H), 1.42-1.83 (complex, 11 H), 2.14-2.26 (m, 4 H), 3.62-3.71 (m, 1 H), 4.98 (s, 2 H), 6.78 (d, *J* = 2.8 Hz, 1 H), 6.83 (t, *J* = 4.4 Hz, 1 H), 6.95 (br s, 1 H), 7.14 (d, *J* = 5.2 Hz, 1 H), 7.33 (dd, *J* = 0.8, 4.8 Hz, 1 H), 7.59 (d, *J* = 8.0 Hz, 1 H), 7.75 (dt, *J* = 1.6, 7.6 Hz, 1 H), 8.57 (d, *J* = 4.8 Hz, 1 H). ^{13}C (100 MHz, $CDCl_3$, APT pulse sequence) δ d (CH, CH_3) 48.0, 124.3, 124.7, 125.2, 126.5, 126.6, 137.1, 148.0; u (C, CH_2) 22.4, 24.7, 25.6, 25.7, 32.8, 33.1, 44.9, 66.9, 141.8, 155.1, 171.7, 172.7.





(2) <u>SID87334039</u>: >90% pure. ^{1}H NMR (acetone-d6) δ 4.54 (s, 2 H), 5.91 (s, 2 H), 6.24 (d, *J* = 2.8 Hz, 1 H), 6.29 (dd, *J* = 2.0, 3.2 Hz, 1 H), 7.40-7.51 (complex, 4 H), 7.70 (d, *J* = 2.0 Hz, 1 H), 7.96 (dt, *J* = 1.6, 7.6 Hz, 1 H), 8.24 (d, *J* = 8.0 Hz, 1 H), 8.72 (d, *J* = 4.4 Hz, 1 H); ^{13}C NMR (acetone-d6, APT pulse sequence) δ d (CH, CH_3) 109.7, 111.3, 123.7, 125.2, 130.1, 131.4, 132.0, 138.2, 143.8, 149.7; u (C, CH_2) 36.7, 42.7, 131.7, 132.5, 139.7, 148.8, 150.3, 152.8, 153.4; (neat) 1701, 1589, 1463, 1446 cm^{-1}





d. PubChem CID(s) (corresponding to the SID)

PubChem CIDs are CID5236771 (corresponding to SID87218782) and CID44601470 (corresponding to SID87334039)

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

(1) The bisamide probe, CID5236771 is commercially available in milligram quantities from Chem Div (catalog # C094-1951), Aurora Screening Library (catalog # kcd-303814), Ambinter Screening Collection (catalog # STK142410), New Chemistry Horizons Laboratories Screening Library (catalog # NCHSC1-21611), Princeton Gold Collection I (catalog # OSSK_529711) and AKOS Screening Library (catalog # AKG-C094-1951). However, KSCC is depositing 50 mg of newly synthesized material with DPI.

(2) The triazole probe, CID44601470 is not commercially available. However, KU SCC has deposited 50 mg of synthesized material with the MLSMR.

f. **Provide MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection:** *(see Table 5)*

Table 5. Probe and Analog Submissions to the MLMR (BioFocus DPI)							
Probe 1: Bisamide Probe - CID5236771							
Probe /Analog	**MLS_ID (BCCG)**	**MLS_ID (MLSMR)**	**CID**	**SID**	**Source (vendor or KU syn)**	**Amt (mg)**	**Date ordered/ submitted**
Probe ML139	MLS-0254632	MLS002699338	5236771	87218782	KU syn	50	02/26/2010
Analog 1	MLS-0080731	MLS002703296	3964693	87218795	KU syn	20	02/26/2010
Analog 2	MLS-0425703	MLS002699340	1282817	87218798	KU syn	20	02/26/2010
Analog 3	MLS-0075520	MLS002699341	3950926	87218781	Chem Div	20	02/26/2010
Analog 4	MLS-0211381	MLS002699342	4014737	87218783	Chem Div	20	02/26/2010
Analog 5	MLS-0237946	MLS002703295	3950572	87218780	KU syn	20	02/26/2010

Probe 2: Triazole Probe - CID44601470							
Probe /Analog	**MLS_ID (BCCG)**	**MLS_ID (MLSMR)**	**CID**	**SID**	**Source (vendor or KU syn)**	**Amt (mg)**	**Date ordered/ submitted**
Probe ML138	MLS-0435589	MLS002699344	44601470	87334039	KU syn	50	02/26/2010
Analog 1	MLS-0058739	MLS002699345	662944	87218751	KU syn	20	02/26/2010
Analog 2	MLS-0058731	MLS002699346	663290	87218759	KU syn	20	02/26/2010
Analog 3	MLS-0435598	MLS002699347	44601469	87334048	KU syn	20	02/26/2010
Analog 4	MLS-0435599	MLS002699348	44601475	87334049	KU syn	20	02/26/2010
Analog 5	MLS-0435594	MLS002699349	44601473	87334044	KU syn	20	02/26/2010

g. Describe mode of action for biological activity of probe

Both probes were submitted to Bryan Roth's Psychoactive Drug Screening Program (PDSP) GPCR Panels and the results are summarized in **Table 6** below. The first bisamide probe CID5236771 [ML139] was indeed very clean with no activity < 10,000 nM except for the KOP, MOP and DOP receptors, with values of 0.79, 671.1, and 1,315 nM, respectively. These yield selectivity values of 850-fold (MOR/KOR) and 1,660-fold (DOR/KOR) in the PDSP assays which compare favorably with those obtained by our DiscoveRx (>270-fold for MOR & DOR) and HCS (>530-fold for MOR & DOR) assays (*see "**Probe(s) Structure & Characteristics:**"* table on p.1).
The second triazole probe CID44601470 [ML138] was not as "clean" and showed some weak activity (1200 – 9600 nM K_is) against 5HT2b, 5HT5a, α2A, α2B, b3, D1, and D5 receptors, in addition to stronger activities against the KOR, MOR and DOR receptors, with values of 0.23, 773.3, and 484.1 nM, respectively. In light of this potent binding at the KOR, even the most potent binding to other receptors in this panel translates to an impressive selectivity of greater than 5,000 fold. These yield selectivity values of 2,100-fold (MOR/KOR) and 3,360-fold (DOR/KOR) in the PDSP assays which compare much more favorably with those obtained by our DiscoveRx (>37-fold for MOR & DOR) and HCS (>91-fold for MOR & DOR) assays.

Is should be noted the that the subnanomolar binding K_is of CID5236771 (0.79 nM) and CID44601470 (0.23 nM) for the KOR receptor are the markedly more potent than those determined by either functional assay (see table on p. 1) for CID5236771 (120 nM DiscoveRx; <60 nM HCS) and CID44601470 (870 nM DiscoveRx; 350 nM HCS) and the rank order is inverted. Although corrrelation of the functional EC_{50} data to equilibrium ligand binding values (K_i) is not straightforward, these potencies and selectivites compare very favorably with even the most potent previously reported compounds in the literature and augue favorably for the utility of the presently reported probes or their analogs in the future. For example, the comparative K_i data for salvinorin A (also obtained in the PDSP under analogous conditions), a KOR agonist of extremely high contemporary interest, are 7.4 nM for KOR, 1370 nM for the MOR, and >10,000 for the DOR.

Table 6. Receptor Paneling of KOP agonist Probes 1 [ML139] CID5236771 & 2 [ML138] CID44601470 through the PDSP (Bryan Roth, PI).

Receptor	**Probe** K_i (nM)		**Receptor**	**Probe** K_i (nM)		**Receptor**	**Probe** K_i (nM)	
	ML139	ML138		ML139	ML138		ML139	ML138
5ht1a			Alpha2A		5,525	H1		
5ht1b			Alpha2B		9,601	H2		
5ht1d			Alpha2C			H3		
5ht1e			Beta1			H4		
5ht2a			Beta2			KOR	0.79	0.23
5ht2b		1,237	Beta3		7,312	M1		

5ht2c				BZP Rat Brain Site				M2		
5ht3				D1		1,796		M3		
5ht5a		4,986		D2				M4		
5ht6				D3				M5		
5ht7				D4				MOR	671.1	773.3
Alpha1A				D5		7018		NET		
Alpha1B				DAT				SERT		
Alpha1D				DOR	1,315	484.1		Sigma 1		
	Green: K_i > 10,000 or 1^{ary} screen missed							Orange: 2^{ndary} assay pending		

G protein coupling assays using the ^{35}S-GTPγS binding assay were undertaken to demonstrate single point efficacy for the probes and select derivatives. Full concentration response curves were pursued to determine agonist potencies, which are shown in **Figure 2**. Interestingly, the efficacy and potency of the current compounds in the G protein coupling assay was highly correlative with the observations made in the beta-arrestin translocation assay further reinforcing this assay as a sensitive and selective means to identify novel agonists.

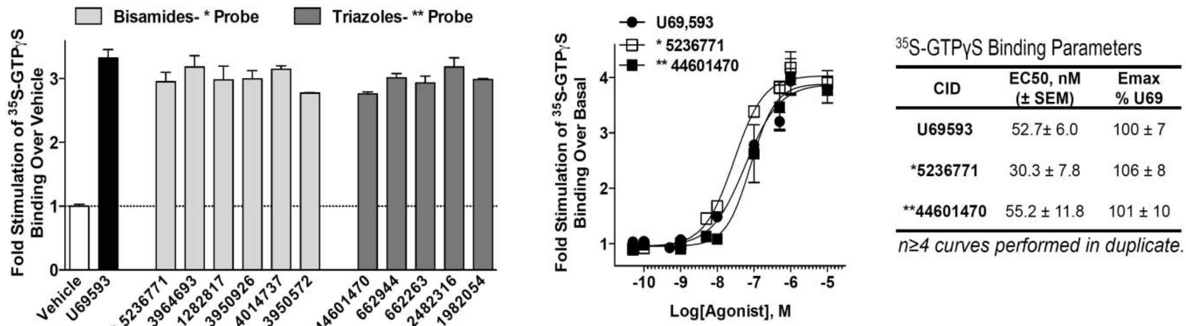


Figure 2. ^{35}S-GTPγS binding assay – Secondary functional assays for KOR agonist probes and selected analogs.

Since agonists can differentially activate GPCRs to engage diverse signaling cascades, a secondary, downstream Map kinase assay (Erk1/2 activation) was used to evaluate agonist activity in **Figure 3**. Single point efficacy curves reveal a tendency for CID44601470 to be more efficacious in this assay, which was confirmed by full concentration response curve analysis.

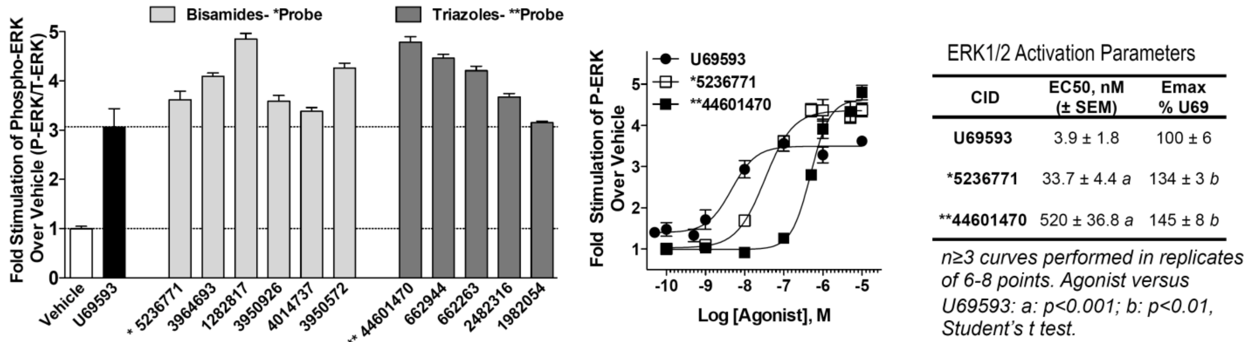


Figure 3. Downstream MAP Kinase Activation - Secondary functional assays for KOR agonist probes and selected analogs.

The probe appears to bind reversibly to the opiate agonist binding site of the receptor on the basis of the beta-arrestin primary and secondary assays. As described in the introduction to the report, the site specificity will be tested through competitive assays with

known KOR ligands. It is clear from these report findings that the probes are able to activate beta-arrestin. It will be of interest in the follow up studies whether they will demonstrate a similar efficacy for G protein signaling, since it is possible that biased agonism towards one or the other pathway produces a distinctive physiological response profile.

h. Detailed synthetic pathway for making probe

Synthesis of Bisamide probe

Bisamide Probe 1: N-(1-(cyclohexylcarbamoyl)cyclohexyl)-N-(thiophen-2-ylmethyl) picolinamide (SID87218782). The carboxylici acid precursor [1-(N-(thiophen-2-ylmethyl) picolinamido)cyclohexanecarboxylic acid] (64 mg, 0.186 mmol) was combined in a Biotage microwave process vial (2-5 mL size) with HOBt (30 mg, 0.223 mmol), DCC (46 mg, 0.223 mmol) and cyclohexylamine (55 mg, 0.558 mmol) in MeCN (2 mL). The reaction was heat at 100 °C for 6 min using microwave irradiation. After cooling, the reaction mixture was adsorbed on Celite and chromatographed on silica to afford the bisamide product SID87218782 (27 mg, 0.063 mmol, 34% yield).

Triazole Probe 2: 2-(5-(3,4-dichlorobenzylthio)-4-(furan-2-ylmethyl)-4H-1,2,4-triazol-3-yl)pyridine (SID87334039). Following the general procedure listed below and silica gel chromatography, furan thione (93 mg, 0.36 mmol) and 3,4-dichlorobenzyl chloride (0.66 mL, 0.43 mmol) afforded the product as an off-white solid (116 mg, 0.278 mmol, 77% yield). R_f = 0.24 (1:1 Hexanes: EtOAc); mp = 99.0-99.5 °C; ^{1}H NMR (acetone-d6) δ 4.54 (s, 2 H), 5.91 (s, 2 H), 6.24 (d, *J* = 2.8 Hz, 1 H), 6.29 (dd, *J* = 2.0, 3.2 Hz, 1 H), 7.40-7.51 (complex, 4 H), 7.70 (d, *J* = 2.0 Hz, 1 H), 7.96 (dt, *J* = 1.6, 7.6 Hz, 1 H), 8.24 (d, *J* = 8.0 Hz, 1 H), 8.72 (d, *J* = 4.4 Hz, 1 H); ^{13}C NMR (acetone-d6) δ d 109.7, 111.3, 123.7, 125.2, 130.1, 131.4, 132.0, 138.2, 143.8, 149.7; u 36.7, 42.7, 131.7, 132.5, 139.7, 148.8, 150.3, 152.8, 153.4; (neat) 1701, 1589, 1463, 1446 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{19}H_{15}Cl_2N_4OS$ ([M+H]$^+$), 417.0344., found 417.0353.

N-(Furan-2-ylmethyl)-2-picolinoylhydrazinecarbothioamide. 2-Picolynyl hydrazide (410 mg, 2.99 mmol) and furfuryl isothiocyanate (416 mg, 2.99 mmol) in MeCN (15 mL) were stirred for 16 h at rt. The reaction mixture was filtered, the precipitate washed with additional MeCN (3 × 10 mL) and dried under vacuum to afford the thioamide as an off-white solid (610 mg, 2.21 mmol, 74% yield), which was used without further purification. IR (neat) 3135, 1673, 1533, 1500, 1465 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{12}H_{13}N_4O_2S$ ($[M+H]^+$), 277.0759, found 277.0761.

4-(Furan-2-ylmethyl)-3-(pyridin-2-yl)-1H-1,2,4-triazole-5(4H)-thione. To a slurry of the above thioamide (530 mg, 1.92 mmol) in water (25 mL) was added NaOH (4.00 g, 100 mmol). The reaction was heated at reflux for 2 h, the starting thioamide dissolved promptly upon warming. The reaction was cooled to rt, diluted with aqueous HCl (1 N, 20 mL) and acidified to pH = 6 with concentrated HCl. The solid precipitate was filtered, washed with water (2 × 15 mL) and dried under vacuum to afford the thione as a white solid (478 mg, 1.85 mmol, 96% yield), which was used without further purification. IR (neat) 3021, 2894, 2766, 1585, 1550, 1502, 1463 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{12}H_{11}N_4OS$ ($[M+H]^+$), 259.0654, found 259.0642.

Synthesis of Series 2 thiophene scaffold:

MeCN
74%
H_2O, NaOH
reflux

N-(Thiophen-2-ylmethyl)-2-picolinoylhydrazinecarbothioamide. 2-Picolynyl hydrazide (883 mg, 6.44 mmol) and thiophene isothiocyanate (1,000 mg, 6.44 mmol) in MeCN (20 mL) were stirred for 16 h at rt. The reaction mixture was filtered, the precipitate washed with additional MeCN (3 × 10 mL) and dried under vacuum to afford the thioamide as an off-white solid (1,642 mg, 5.62 mmol, 87% yield), which was used without further purification. IR (neat) 3141, 1672, 1527, 1499, 1466 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{12}H_{13}N_4OS_2$ ($[M+H]^+$), 293.0531, found 293.0516.

4-(Thiophene-2-ylmethyl)-3-(pyridin-2-yl)-1H-1,2,4-triazole-5(4H)-thione. To a slurry of the above thioamide (602 mg, 2.06 mmol) in water (25 mL) was added NaOH (4.00 g, 100 mmol). The reaction was heated at reflux for 2 h, the starting thioamide dissolved promptly upon warming. The reaction was cooled to rt, diluted with aqueous HCl (1 N, 20 mL) and acidified to pH = 6 with concentrated HCl. The solid precipitate was filtered, washed with water (2 × 15 mL) and dried under vacuum to afford the thione as a white solid (530 mg, 1.93 mmol, 94% yield), which was used without further purification. IR (neat) 3019, 2896, 1584, 1549, 1501, 1462 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{12}H_{11}N_4S_2$ ($[M+H]^+$), 275.0475, found 275.0412.

K_2CO_3

General procedure for the synthesis of series 2 analogs from thiones and benzyl halides. The thione scaffold (0.1 to 0.3 mmol), K_2CO_3 (2 equiv) and the benzyl halide (1.2 equiv) were combined in acetone (15 mL/mmol substrate) and stirred in a sealed vial. After 15 h, the solvent was removed and the residue washed with CH_2Cl_2 (2 × 3 mL) and filtered. The combined filtrates were evaporated down and either chromatographed on silica or subjected to mass-directed, reverse phase preparative HPLC purification.

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

Both probes are novel chemical scaffolds and have demonstrated potent (< 1μM EC50) agonist activity in both the primary enzyme complementation assay and the image base orthogonal assay for β-arrestin mediated signaling and activation of the kappa opioid receptors, with selectivity over for activation of both the mu- and delta opioid receptors. The bisamide probe, CID5236771, has *exceeded* the criteria for probe as defined in the CPDP Chem Update document filed on October 28, 2009 and revised and refilled on November 21, 2009, and finally refilled after corrections requested by NIH on January 12, 2010 with the NIH PT: KOP receptors of less than 1 μM, and at least 100-fold selective over MOP and DOP receptors or as obtained by the achievable test concentrations, (e.g. solubility limited), regardless of whether this selectivity is calculated between the enzyme complementation/HCS or the HCS/HCS assays.

The triazole probe, CID44601470, formally misses the 100-fold selectivity of against MOR and DOR, though it approaches the desired selectivity when the HCS/HCS ratios are considered. However, all these compounds may not be able to 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 IC_{50}, 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 ~40-fold estimate of selectivity this probe compound should be useful. Furthermore, we note the intriguing result that it is in the triazole scaffold series (**Table 4**) that the apparent super-agonism is experimentally seen, in contrast to the bisamide series (**Table 3**). While we do not currently have any mechanistic understanding of this difference, the clear pharmacologic difference between these two classes of compounds supports nomination of this a bonafide 2nd agonist probe for the kappa-opioid receptor. KU, SBCCG and the assay provider all concur on this point.

In Vitro Pharmacology Profiles of Probes CID5236771 [**ML139**] and CID44601470 [**ML138**] (*See* **Table 7** *below*). Both probes had poor solubility at all pH's tested, with the 2nd triazole probe (CID44601470) the poorest solubility.

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, both probes CID5236771 [**ML139**] and CID44601470 [**ML138**] have excellent permeability. CID5236771 [**ML139**] had moderate brain barrier permeabilty while CID44601470 [**ML138**] had almost 3-fold selectivity in the blood brain 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. CID5236771 [**ML139**] and CID44601470 [**ML138**] are both highly bound (90-99%) 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. Both CID5236771 [**ML139**] and CID44601470 [**ML138**] shows excellent stability (>99%) in both human and mouse plasma.

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. Both CID5236771 [**ML139**] and CID44601470 [**ML138**] are rapidly metabolized in either Human or mouse microsomes. CID5236771 [**ML139**] shows high levels of toxicity toward human hepatocyctes in our assay, whereas CID 44601470 does not.

Table 7: Summary of *in vitro* ADME/T Properties of Kappa Opioid Agonist probe(s)

Probe CID Probe ML#	Aqueous Solubility (μg/mL)[a] (@ pH)	PAMPA Pe (x10^{-6} cm/s)[b] (@ 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			
5236771 [ML139]	5.7 (5.0) 6.6 (6.2) 6.5 (7.4)	908 (5.0) 971 (6.2) 916 (7.4)	90	99.59/ 99.07	94.05/ 90.70	100/ 99.06	0/ 0.63	5.7/6.6/6.5
44601470 [ML138]	<0.1 (5.0) 0.29 (6.2) 0.14 (7.4)	1793 (5.0) 1921 (6.2) 2089 (7.4)	242*	99.75/ 99.80	99.80/ 99.64	100/ 100	0.03/ 0.03	>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's 7.4; Acceptor compartment pH 7.4
[d] % remaining at 3 hr
[e] % remaining at 1 hr
[f] towards Fa2N-4 immortalized human hepatocytes
* Unable to detect any compound at 1 μM

j. A tabular presentation summarizing known probe properties

Table 8. Calculated physical properties of Kappa opioid Agonist probe(s)

Calculated Property	Probe Identity	
	CID5236771 *(bisamide)*	**CID44601470** *(Triazole)*
	MLS-0254632	MLS-0435589
Molecular Weight [g/mol]	425.58684	417.31166
Molecular Formula	$C_{24}H_{31}N_3O_2S$	$C_{19}H_{14}Cl_2N_4OS$
XLogP3-AA	4.6	4.3
H-Bond Donor	1	0
H-Bond Acceptor	3	4
Rotatable Bond Count	6	6
Tautomer Count	2	0
Exact Mass	425.2137	416.026537
MonoIsotopic Mass	425.2137	416.026537
Topological Polar Surface Area	62.3	56.7
Heavy Atom Count	30	27
Formal Charge	0	0
Complexity	587	477
Isotope Atom Count	0	0
Defined Atom StereoCenter Count	0	0
Undefined Atom StereoCenter Count	0	0
Defined Bond StereoCenter Count	0	0
Undefined Bond StereoCenter Count	0	0
Covalently-Bonded Unit Count	1	1

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

As described in the CPDP these further biological 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.

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 many examples of very potent agonists and antagonists of the kappa-opioid receptors with low nanomolar EC50 and IC_{50}s, 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. As a comparative measure, the **Table 9** summarizes our two agonist probes against the precedent state-of-art probes, and clearly these two scaffolds the bis- amides and the triazoles provide novel scaffolds as was the main goal of this project. Clearly potency and selectivity remain to be improved further, however, the SAR helps to define some areas to improve these. The bisamide probe is within the same range of potency as U69,593 a commonly used reference compound, but lacks its exquisite selectivity against MOR and DOR. It also has comparable or slightly better selectivity against MOR and DOR than TRK-820 has. The second triazole probe is a less selective and less potent than the first bisamide probe, however, as pointed out in a previous section, it demonstrates the novel pharmacology of super-agonism as compared to the bisamide probe or most of these literature agonists.

Table 9: Comparative summary of two current probes CID5236771 & CID44601470 to literature examples

Agonist Name	U69,593	U50,488	R-84760	TRK-820 - Nalfurafine	Probe 1 (bisamide)	Probe 2 (triazole)
Chemical Structure						
EC_{50} KOR (nM)	80-190	2.2	0.436	0.025	120	870
EC_{50} MOR (nM)	Ki = 5,286 (2110X)	Ki = 1,095 (2610X)	297 (681X)	3.2 (128X)	>32,000 (> 270X)	>32,000 (> 37X)
EC_{50} DOR (nM)	Ki >10,000 (>1330X)	Ki = 1,772 (4220X)	IC_{50}=1030 (2,360X)	289 (11,600X)	>32,000 (>270X)	>32,000 (> 37X)
PubChem CID	13298444	3036289	133036	6445230	5236771	44601470
PubChem SIDs:	54000015; 33865503	36104498; 11111942; 11111943; 26756547; 14901839; 7980053; 49878458; 10744781; 47574471	23920220; 14781224; 29311044	11969670; 50424931; 14761136; 15829610; 43037501; 786912	87218782	87334039
PMID	17951511	17951511	8001635	15383632	Not Applicable	Not Applicable

Additionally this probe compound was submitted for broad 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.

In conclusion, we consider this probe project to be successful in defining novel chemical scaffold distinct form the current literature examples. Future work beyond the scope of the MLCPN is needed to verify whether the potentially novel pharmacological of these probes will provide added value to their utility.

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