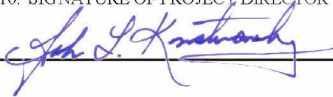|	
Document Title:	**Synthesis, Spectroscopy and Pharmacology of Emerging Synthetic Opioids**
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CATEGORICAL/DISCRETIONARY ASSISTANCE PROGRESS REPORT

The information provided will be used by the grantor agency to monitor grantee cash flow to ensure proper use of Federal funds. No further monies or other benefits may be paid out under this program unless this report is completed and filed as required by existing law and regulations (Uniform Administrative Requirements for Grants and Cooperative Agreements — 28 CFR, Part 66, Common Rule, and OMB Circular A-110).

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Keck Graduate Institute	2016-R2-CX-0059	7 Revision

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Synthesis, Spectroscopy and Pharmacology of Emerging Synthetic Opioids	$618,790	☐ REGULAR ☑ SPECIAL REQUEST ☐ FINAL REPORT

9. NAME AND TITLE OF PROJECT DIRECTOR	10. SIGNATURE OF PROJECT DIRECTOR	11. DATE OF REPORT
John L. Krstenansky, Professor	*[signature]*	9/27/2019

12. COMMENCE REPORT HERE (Continue on plain paper)

See attached Revision of Special Draft Final Summary Overview

13. CERTIFICATION BY GRANTEE (Official designated)

Kirsten Torguson
Digitally signed by Kirsten Torguson
Date: 2019.09.27 13:17:06 -07'00'

14. DATE

OJP FORM 4587/1

PURPOSE

The purpose of the project was to provide spectroscopic and pharmacological information relevant to law enforcement on two related series of synthetic opioids, AH-7921 and U-47700 and a variety of analogues based on their core structures. An authentic series of analogs were provided from chemical synthesis and were characterized by NMR, HRMS, GC-MS, IR, and Raman. These data will be delivered in the final report of the grant, submitted for inclusion to the SWGDRUG database, made accessible at the request of forensic labs, and disseminated in posters and publications. Finally, these analogs were tested for opioid agonist action using a cell line expressing the human μ-opiate receptor (hMOR). These data provide evidence as to which of these analogs possess opiate activity and are liable for abuse and subject to properly being defined as analogs of the scheduled substances.

PROJECT DESIGN AND METHODS

AIM 1: Use combinatorial approaches with commercially available starting materials to create both reported and novel analogs within each of the series.

AH-7921 & related analogs were synthesized using the methods in the original Allen & Hanbury's patents. U-47700 and related analogs were synthesized using the methods in the Upjohn patents. Since, U-47700 is chiral, we synthesized the individual enantiomers separately rather than simply using racemic material. While this does not affect spectra, we found the pharmacological differences to be significantly different. Additionally, since AH-7921 and U-47700 have a common ethylenediamine core that differs in how that core is attached to a cyclohexane ring, we also synthesized some ethylenediamine analogs that were fused to rings

in different ways. This was done to determine how easily one could modify the core structures and still have opioid activity.

AIM 2: Generation of a stable clonal OPRM1 expressing cell line and determination of rank order of potencies for signaling via Gα$_i$.

We developed versatile lenti-viral expression system that enabled us to rapidly develop stable OPRM1-expressing cells in virtually any type of cell line or in vivo. This approach enabled us to express an amino terminal three sequential hemaglutinin antigen (3x HA) -tagged human OPRM1 receptor (aka human μ-opioid receptor; hMOR) in human fibrosarcoma HT1080 cells (EF1α-3xHA-OPRM1-expressing HT1080 cells). We then worked to optimize conditions for screening compounds to assess the functional activity of the cloned human OPRM1 receptor which was validated by assessing for dose responses for well-established prototypic OPRM1 receptor agonists morphine and DAMGO. Two functional activities were assessed to pharmacologically characterize OPRM1 pharmacology: Gα$_i$ activity, which is assessed by quantifying the suppression of forskolin (FSK)-induced cAMP levels across various drug dosages and analogue-induced receptor internalization.

Significant assay optimization was required to 1) get a robust response to agonist (signal/noise ratio) and 2) be able to establish naloxone reversibility for the response from opioid agonists, which is required to determine if alterations in cAMP levels were a result of OPRM1 receptor activity or via non OPRM1 specific activity of the compounds. Finally, to confirm that the assay was able to accurately determine EC$_{50}$ values for known standards we proceeded to run full concentration response curves for parent compounds AH-7921 (EC$_{50}$= 26.49 ± 11.2 nM), U-47700 (EC$_{50}$ = 8.8± 4.9 nM), and morphine (EC$_{50}$ = 39.3 nM). The EC$_{50}$ value

for morphine was almost identical to several published reports, and the EC_{50} values for AH-7921

and U-47700 were well within ranges of analgesic potential reported in the original patents. To

the best of our knowledge, we are the first lab to report EC_{50} values for AH-7921 and U-47700

at cloned human OPRM1 receptors.

With the assay established, the synthesized analogs from this project were then tested.

First all analogs were tested at three concentrations (10 nM, 100 nM, and 1 µM). For

compounds demonstrating naloxone (10 µM)-reversible suppression of cAMP levels at the 100

nM dose or lower (compared to FSK only) were then subjected to extended full dose range

screening so that EC_{50} values for these agents could be accurately determined. These

compounds were deemed as having high analgesic and abuse potential.

We also optimized a sensitive assay to determine the internalization properties of

analogues. Receptor internalization has been correlated with biased signaling and tolerance

potential of GPCR agonists. The 3xHA tag enabled us to use antibodies to detect cell surface

expression of the µMOR. Our results uncovered a striking difference in the internalization

capabilities between AH- and U-series analogues such that AH-series analogues trigger

substantially more receptor internalization at maximal dose then the U-series analogues,

suggesting potentially divergent addictive potential of these analogues. Future studies are

currently underway and we will explore this possibility.

AIM 3: Analytical Characterization of Analogs.

NMR spectra were recorded on a Varian 400MR spectrometer (proton frequency

399.765 MHz) equipped with an AutoX DB broadband probe. Pulse sequences, acquisition, and

data processing were accomplished using VnmrJ software (VnmrJ 4.2, Agilent Technologies, Santa Clara, CA). The spectrometer was locked on to D_2O and spectra were acquired at 28°C without spinning. Water suppression suitability studies were carried out using the presaturation (presat), WET (WET1D), and excitation sculpting (water ES) pulse sequences (VnmrJ 4.2, Agilent Technologies, Santa Clara, CA) with automatic suppression of the tallest peak (water at δ 4.86 ppm), an observation pulse of 90° (10.8 μs), a spectral width of 6410.3 Hz, a relaxation time of 30 s, and an acquisition time of 5.112 s. 8 scans were taken. Three replicates were taken for each sample.

HRMS spectra were collected on an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS spectrometer using direct infusion to the nanoelectronspray source. Samples were dissolved in HPLC grade methanol to a final concentration of ~0.01 mg/mL. Spectra was run with 0.1% (v/v) formic acid/ HPLC grade methanol as solvent.

Purity determinations were performed by GC/MS using a Shimadzu GC/MS 2010 SE with an Rtx-5MS column (a DB-5 MS equivalent); 30m x .25mm x .25m. The carrier gas was helium at 1 mL/min, with the injector at 280°C, MSD transfer line at 280°C, and ion source at 200°C. Injection Parameters: Split Ratio = 1:15, 1 μL injected. MS Parameters: Mass scan range: 34-550 amu & Threshold: 100. Acquisition mode: scan. The oven program was as follows: 1) 90°C initial temperature for 2.0 min; 2) Ramp to 300°C at 14°C/min; 3) Hold final temperature for 10.0 min.

Infrared spectroscopy was performed using a Perkin Elmer Spectrum 100 FTIR with ZnSe ATR attachment (1 bounce), Number of scans: 4, Number of background scans: 4, Resolution: 4 cm^{-1}, Sample gain: 8, Aperture: 150.

Raman spectra were recorded using a Rigaku Progeny 1064 nm handheld spectrometer. Instrument parameters: 350 mW power, 1000 ms exposure, 30 scans were averaged, threshold = 0.80.

Optical rotations were taken using a VeeGee C25L Full-Circle Manual Polarimeter (200 mm = 2 dm path length; λ = 589 nm = D-line).

Melting points were performed in triplicate using a calibrated Büchi Melting Point B-545.

DATA ANALYSIS

Spectroscopic data was obtained from each of the instruments in the form of data files that are specific to the manufacturer of the instrument. Since we did not own many of the instruments we used in the study, data processing had to be done using third party software. We purchased ACD Spectrus Workbook software for this purpose, because it can read all formats of data files and has capabilities that aid in the interpretation of the spectra and the ability to link information from different spectra for further aid in assignment and interpretation.

For pharmacological data, all data are presented as mean ± SEM. Each dose treatment from Catchpoint cAMP assay was performed in triplicate and data was analyzed in GraphPad Prism 7 using one-way ANOVA with Dunnett multiple comparison test with forskolin (FSK) only as standard, with $p < 0.05$. The EC_{50} values were obtained from fitting data from individual dose treatment to sigmoidal curves (variable slope). Receptor internalization data from three independent experiments was analyzed in GraphPad Prism 7 using unpaired t-test, with $p < 0.05$.

PROJECT OUTCOMES AND FINDINGS

Raw GC/MS files for the analogs were provided to Jason Bordelson (DEA Chicago) and are being reviewed by NIST for inclusion in the next version of the SWGDRUG database (http://www.swgdrug.org/ms.htm) Monographs containing all of the spectra for each of the analogs are being created and will be submitted to SWGDRUG to be housed on their website and openly available to forensic researchers. The DEA lab in Chicago additionally requested the corresponding NMR, IR, and HRMS data on the compounds for incorporation into their internal databases. We are still exploring other routes of making the data as widely available to the forensic community as possible.

Our pharmacological studies using an *in vitro* functional assay for human OPRM1 receptors provides a foundation from which effects in humans can be anticipated. These results have provided key fundamental insights on the structure-activity relationships (SAR) of the U- and AH-series analogues and information on their possibility in being abusable opioids. Through our combinatorial approach we provide data on the opioid activity of known and novel analogs whose hMOR activity was previously undocumented in the literature. Through novel substitution of the AH-series we have discovered a new patentable class of opiate agonists. Finally, we have established stereoselectivity of the OPRM1 receptor for the U-series analogues. The *R, R* enantiomer of U-47700 is significantly more potent than the *S, S* enantiomers at hMORs. Recent literature on the pharmacology of U-47700 has only used racemic material that does not provide this crucial information.

Whether these novel compounds have fewer side effects, can provide improved analgesic potential or have differences in abuse potential needs to be further investigated. The

OPRM1 internalization results reported here suggest that U-47700, a structural isomer of AH-7921, triggers sufficiently different receptor conformations that result in similar Gα_i coupling, but divergent agonist mediated internalization rates, implying these two agents may have different pharmacodynamics *in vivo* that warrant further investigation. This work will be the subject of funding applications to the NIH relative to their program announcement concerning emerging psychoactive drugs.

Poster Presentations:

1. John L. Krstenansky, Alexander Zambon, Thomas Hsu, Jayapal Mallareddy, and Lauren Waugh. Conformational Considerations of Ethylenediamine Opioids AH-7921 and U-47700. *70th AAFS annual meeting*, Seattle WA. February 21, 2018. Poster K14.

2. Tom Hsu, Jayapal Mallareddy, Kayla Yoshida, Tim Lee, Edna Kemboi, Emily Park, John Krstenansky, and Alexander Zambon. Assessing the Activity of Synthetic Opioid AH-7921 and U-47700 Analogs in cloned Human Mu-Opioid Receptor Expressing Fibrosarcoma HT-1080 Cells. *Experimental Biology 2018*, San Diego, CA. April 2018.

3. John L. Krstenansky, Alexander C. Zambon, Thomas Hsu, and Jayapal Mallareddy. Ethylenediamine Opioid Analogs, AH-7921 and U-47700 and Their Actions on Cloned Human OPRM1 Receptors. *71st AAFS annual meeting*, Baltimore, MD. February 20, 2019. Poster K32.

Manuscript:

Tom Hsu, Jayapal Reddy Mallareddy, Kayla Yoshida, Vincent Bustamante, Tim Lee, John L. Krstenansky, Alexander C. Zambon, Synthesis and pharmacological characterization of

ethylenediamine synthetic opioids in human μ-opiate receptor 1 (OPRM1) expressing cells. *Pharmacology Research and Perspectives* 7(5) e00511 DOI: 10.1002/prp2.511

Monographs:

Individual monographs are being created for 17 AH-series compounds and 26 U-series compounds for hosting at the SWGDRUG website: http://www.swgdrug.org/monographs.htm

IMPLICATIONS FOR CRIMINAL JUSTICE POLICY AND PRACTICE

These studies demonstrate that for simple analogs of AH-7921 and U-47700, the parent compounds within the series are the most potent opioids. As expected, other closely related analogs also have opioid activity at the human receptor, so can properly be considered analogs of these Schedule I substances and potentially subject to prosecution. While these are less potent, this does not mean that such analogs would not be marketed. Prior experience with emerging synthetic cannabinoids showed that regulation of JWH-018 was quickly followed by a number of analogs including UR-144, which is significantly less potent then JWH-018. Previous to our work, activity of most of these opioid analogs had neither been demonstrated in humans nor used assays relying on the human receptor. Presumably, this could both aid prosecution and provide additional data justifying scheduling decisions.

The availability of the spectral data in the open forensic database SWGDRUG could speed the identification of an otherwise 'unknown' substance in either seized or toxicological samples.