EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1440

IN VITRO APPROACH TO EVALUATING OPIOID RECEPTOR SUBTYPE SPECIFICITY

Michael G. Feasel
Theodore S. Moran
Andrew J. Walz

RESEARCH AND TECHNOLOGY DIRECTORATE

March 2017

Approved for public release; distribution unlimited.

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-03-2017	Final	Mar 2016 – Oct 2016

4. TITLE AND SUBTITLE
In Vitro Approach to Evaluating Opioid Receptor Subtype Specificity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
R.0018390.4.2.6

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Feasel, Michael G.; Moran, Theodore S.; and Walz, Andrew J.

5d. PROJECT NUMBER
40471002

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, ECBC, ATTN: RDCB-DRT-O, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1440

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
ECBC, ATTN: FY16 Section 219 Seedling Program, APG, MD 21010-5424

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Reported potencies of opioid compounds were derived from in vitro, in vivo, and ex vivo methods in various model species for all the experiments that were conducted. In this study, a human receptor-expressing cell system was used to measure the potency and efficacy of carfentanil (a known ultra-potent opioid) and 4-chloro-*N*-[(2*Z*)-1-[2-(4-nitrophenyl)ethyl]piperidin-2-ylidene]benzene-1-sulfonamide (W-18, a suspect opioid that has received much attention as a public health concern). This system demonstrated the ease and efficiency of using a set of cell-based tools to screen for opioid activity and specificity for future compounds of interest and suspect or unknown opioid or opiate compounds. Carfentanil was more specific for the μ-opioid receptor (MOR) than previously thought, but its low median effective concentration indicated that it could have off-target effects at physiologically relevant concentrations, which could potentially lead to toxicity. W-18 was inactive at all four human receptor subtypes (δ-opioid receptor, κ-opioid receptor, MOR; and opioid-like receptor 1), which refutes reports that W-18 is an ultra-potent opioid (10,000 times more potent than morphine). Although this study does not account for reported toxicity, it does rule out the opioid system as the culprit receptor.

15. SUBJECT TERMS
Carfentanil; Opioid analgesics; Fentanyl; Potency; Pharmacology; Opiate analgesics; Efficacy; Forskolin; Opioid-like receptor 1 (OLR1); δ-Opioid receptor (DOR); κ-Opioid receptor (KOR); μ-Opioid receptor (MOR); 4-chloro-*N*-[(2*Z*)-1-[2-(4-nitrophenyl)ethyl]piperidin-2-ylidene]benzene-1-sulfonamide (W-18)

16. SECURITY CLASSIFICATION OF: UNCLASSIFIED			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	26	19b. TELEPHONE NUMBER *(include area code)* (410) 436-7545

Blank

PREFACE

The work described in this report was authorized under project no. 40471002. The work was started in March 2016 and completed in October 2016.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Dr. Way Fountain and the Section 219 Seedling Program Office (U.S. Army Edgewood Chemical Biological Center [ECBC], Aberdeen Proving Ground, MD) for their forward thinking in supporting all 219 programs and for choosing to fund and support this program, in particular.
- Drs. Alison Myska and Sara Peacock-Clark (Defense Threat Reduction Agency, Fort Belvoir, VA) for their support of this program.
- Drs. Christopher Whalley and Robert Kristovich (ECBC) for their continuous support and enthusiasm for this program.

Blank

CONTENTS

FIGURES

TABLES

IN VITRO APPROACH TO EVALUATING OPIOID RECEPTOR SUBTYPE SPECIFICITY

1. INTRODUCTION

1.1 Opioid Background

Opioid and opiate analgesics are popular clinical treatments for acute and chronic pain; however, they are also drugs of abuse. The wide use and variety of applications (on- and off-label) have led to much interest in the toxicology and pharmacology of these compounds, specifically the congeners for which no preclinical or clinical data exist.

Fentanyl is the prototype for the synthetic class of opioid analgesics. However, since its inception, more potent derivatives have been synthesized in an effort to increase its therapeutic effect and minimize its adverse effect potential. This has come in the form of creating highly specific and potent agonists to the μ-opioid receptor (MOR); the receptor subtype is responsible for the profound analgesia and euphoria associated with acute pain relief, muscular rigidity, respiratory depression, and apnea.[1,2]

Carfentanil was synthesized not long after fentanyl and was immediately recognized for its potency with regard to its ability to relieve pain as measured by the rat-tail withdrawal (RTW) assay.[3] Because carfentanil has never been marketed as a human clinical drug, very little pharmacological or toxicological data have been collected since its synthesis. Carfentanil had a successful, albeit short-lived role, as a veterinary sedative; it was used in large animal sedation and takedown.[4] Since then, carfentanil has been replaced by other ultra-potent opioids, such as buprenorphine and etorphine. In addition, carfentanil has been reported to be a specific agonist or activator at the MOR.[5]

Carfentanil toxicity has been of interest to the chemical defense community since its reported use to quell a hostage situation in 2002, known as the Moscow theatre hostage crisis.[6] However, public health concerns revolve around the illicit sale and distribution of carfentanil in heroin and fentanyl markets, which contribute to the highest levels of opioid-related overdoses seen in history.[7] In 2016, media and county officials in Ohio reported eight cases of overdose deaths directly caused by the inclusion of carfentanil in heroin and fentanyl tablets.[8] The Ohio incident coincided with a dramatic nationwide spike in opioid-related emergency room visits and deaths.

In 2016, the compound 4-chloro-*N*-[(2*Z*)-1-[2-(4-nitrophenyl)ethyl]piperidin-2-ylidene]benzene-1-sulfonamide (W-18) was targeted as a major public health concern because of the reported seizures of neat W-18 in kilogram quantities between Florida and Canada.[9] This drug compound was synthesized in the 1980s by the University of Alberta in an effort to find a potent opioid analgesic. It is reported to be 10,000 times more potent with regard to pain relief in the mouse phenylquinone writhing assay (median inhibitory concentration [IC_{50}] of 3.7 ng/kg, compared with 38 μg/kg for morphine).[10] However, this assay is nonspecific to opioids and does not characterize the drug's receptor target. In addition, this assay is performed

in the mouse animal model that, although acceptable for therapeutic and analgesic screening, is less than ideal when opioid toxicity is assessed. Rodents have a high tolerance for the drug. Although rodents are suitable for therapeutic assessments, toxicity and lethality assessments should be performed in higher-order species. W-18 was therefore included in this study to assess its opioid activity, or lack thereof. It was also judged on its potency and efficacy with comparison to control compounds and the known ultra-potent opioid carfentanil.

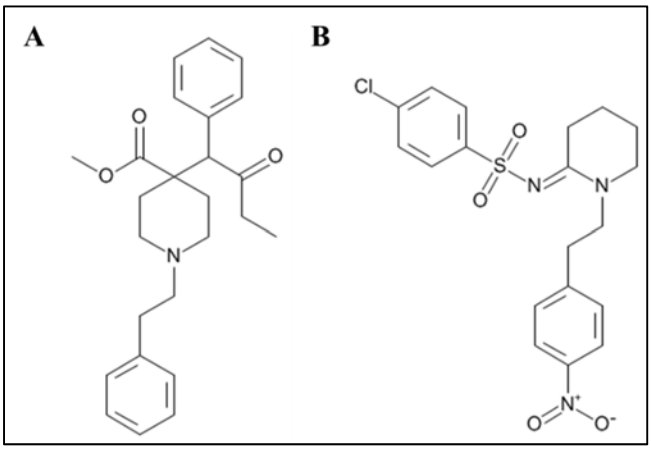

Figure 1. Chemical structures of (A) carfentanil and (B) W-18.

1.2 Previous Methods and Potency Reporting

Dr. Paul Janssen (creator of this class of synthetic opioids) initially used animal models to measure potency. The RTW test (nociception assay) became the standard for measuring the point at which an analgesic sufficiently masked the pain caused by dipping a rat's tail in a hot water bath. This therapeutic value was then compared to an ex vivo model (i.e., the guinea pig ileum [GPI] assay). The GPI is known to be rich in MORs and has historically been the in vitro benchmark for MOR pharmacology,[11–13] which involves incubating GPI tissue in media (with or without drugs) and measuring the electrophysiological output from the tissue (i.e., smooth muscle contraction). When an MOR agonist is present during incubation, the tissue has less electrophysiological activity, and a dose–response curve can be plotted. The decrease of activity in the ileum is reflected at the organismal level as manifestation of opioid-induced constipation, which is a very common side effect of any opioid drug, and for which, there is now a commercial therapeutic.

Other methods have been used to measure opioid potency and efficacy (mostly radioligand binding assays [K_i]). In one method, rat brain homogenate is used to analyze the binding of radiolabeled agonists and the inhibition of binding using antagonists. The other methods are too numerous to list, but all generate some sort of relative potency ranking for how well the drug elicits a response after binding at the MOR, as compared with other drugs. The varying methods of establishing K_i values of opioid drugs has led to wide ranges of K_i-correlated potencies being reported in literature (Figure 1). None of these are wrong, but all should be accompanied with caveats.[14]

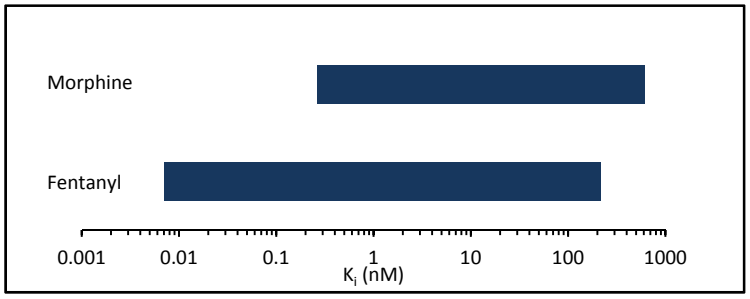

Figure 2. Reported K_i values for morphine[11,12] and fentanyl.[11,13] K_i values correlate with potency and efficacy.[14]

Although a rodent model is useful for the therapeutic effects of opioid analgesics, there is a potential problem with employing one for opioid toxicity testing. A rodent model is generally sufficient for verifying analgesic therapy response; however, it is a poor animal model for toxicity or lethality testing because of an opioid's mechanism of action and symptomology. Species differences in the therapeutic index of opioids are presented in Table 1.

Table 1. Reported Therapeutic and Toxic Responses to Carfentanil[15,16]

Species	Effective Dose (mg/kg)	Effective Endpoint	Lethal Dose (mg/kg)	Therapeutic Index (LD_{50}/ED_{50})*
Rat	0.00032	Analgesia	3.39	10,594
Dog	0.0047	Analgesia	5.0	1,064
Monkey	0.0001	Immobilization	0.001 (estimated)	10

*LD_{50} is median lethal dose and ED_{50} is median effective dose.

Opioid agonists act as central nervous system depressants that slow or stop transmission of signals in nerves involved with pain and nociception (i.e., therapeutic effects), but they also attenuate respiratory drive. Peripheral effects include muscular rigidity, gastrointestinal slowing, and in some cases, cardiac arrhythmia.[17,18] Prolonged or profound depression of respiratory drive leads to apnea and death, if not treated with an opioid antagonist or airway management. Lower-order species, such as rodents, do not succumb to the apneic and hypercapnic effects of opioids as readily as higher order species do. This species-based difference in central response and tolerance to hypercapnia led to an artificially large therapeutic index of these compounds in lower-order species. Therefore, claims that carfentanil is 10,000 times more potent than morphine or 100 times more potent than fentanyl do not necessarily correlate to toxicity but only to the therapeutic response in a less-than-ideal animal model.

1.3 Standardizing the Method

A standardized method was employed in this study for MOR activity screening. This method uses Chinese hamster ovary (CHO) cells transfected with the human MOR (hMOR) gene. These cells contain all G_i α subunit ($G_{\alpha i}$)-associated proteins that are associated with the human G-protein coupled receptor (GPCR). Upon agonism at the GPCR, 3',5'-cyclic adenosine monophosphate (cAMP) levels are suppressed, which normally occurs in $G_{\alpha i}$ functioning. This is because activation of the GPCR inhibits adenylyl cyclase (AC), the protein enzyme responsible

for cAMP production. The dose-dependent change in cAMP production can be measured using a commercial off-the-shelf assay kit. The kit functions through competition between a europium (Eu)-labeled cAMP molecule and endogenous cAMP in the cell for binding to cAMP-specific monoclonal antibodies (mAbs) labeled with a ULight dye (PerkinElmer, Waltham, MA).

The assay induces cAMP production with a compound called forskolin and then inhibits that induction with the opioid of interest. Forskolin is known to activate AC and induce cAMP production. In cells where there is an abundance of cAMP, the mAb–dye complex binds to the free cAMP and dissociates it from the Eu–cAMP complex, and the inductions with forskolin result in a decrease in fluorescence resonance energy transfer (FRET). The cAMP levels in the cells decrease when the cells are incubated with an opioid and AC is inhibited. This increases the binding of Eu–cAMP to the ULight–mAb complex, which results in an increased FRET signal. This signal is dose-dependent and increases with opioid concentration.

By investigating only opioid activity using a human receptor model in an in vitro system, the relative potency of opioid compounds can be compared. This eliminates variations in interspecies differences, tissue culturing methods, and interanimal tissue differences. The activity and efficacy of these compounds are truly compared under similar circumstances.

2. METHODS

2.1 Chemicals

A LANCE cAMP 10,000-assay point kit and 384-well ProxiPlate Plus microplates were purchased from PerkinElmer. The LANCE kit consisted of a cAMP standard (50 µM), an Eu–cAMP tracer (ULight-anti-cAMP), a cAMP detection buffer, and a bovine serum albumin stabilizer. Carfentanil was synthesized at the U.S. Army Edgewood Chemical Biological Center, and its purity was verified using carbon-13 NMR and proton NMR.[19] W-18 was procured from Cayman Chemical (Ann Arbor, MI). The δ-opioid receptor (DOR), κ-opioid receptor (KOR), MOR, and opioid-like receptor 1 (OLR1) were purchased from Tocris Bioscience (Park Ellisville, MO). Selective agonists, such as [D-Pen^2,D-Pen^5]enkephalin (DPDPE); (±)-U-50488 hydrochloride; [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO); and nociception were also purchased from Tocris Bioscience. Hank's balanced salt solution (1×), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (1 M), Versene solution, and Geneticin reagent were procured from Life Technologies (Grand Island, NY). Dimethyl sulphoxide (DMSO), 3-isobutyl-1-methylxanthine, and forskolin were procured from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate-buffered saline and Ham's F-12 media were procured from HyClone Laboratories, Inc. (Logan, UT). Fetal bovine serum was procured from Mediatech, Inc. (Manassas, VA).

2.2 Cell Lines

ValiScreen CHO-K1 cells expressing the hMOR (ES-542-C) gene were purchased from PerkinElmer. ChanTest CHO-K1 cells expressing human δ-opioid (hDOR; CT6607), human ƙ-opioid (hKOR; CT6606), and human OLR1 (hOLR1; CT6604) receptors

were purchased from Charles River Discovery (Cleveland, OH). The cells were kept frozen in liquid nitrogen storage (vapor phase) until they were cultured and grown in accordance with PerkinElmer product literature. Cell cultures were split when they reached ~60–80% confluency, and no cells were used past passage 10. The cells were used for opioid assay when they met the requirements described in the product literature (i.e., 60–80% confluency). Cellular solutions used in plating were counted in duplicate on a Countess II hemocytometer (ThermoFisher Scientific, Halethorpe, MD) before use.

2.3 Incubation and Standard Solutions

Standard solutions (10 mM) of carfentanil and W-18 were made in DMSO and stored in a freezer at 4 °F until use. Standard solutions of specific agonists were made in sterile water in the following concentrations:

- DAMGO: 1.95 mM,
- DPDPE: 1.55 mM,
- (±)-U-50488 hydrochloride: 11.55 mM, and
- nociception: 1.1 mM.

Working solutions (500 μM) of carfentanil and the four specific agonists were prepared immediately before the assay was performed. Stimulation buffer, forskolin dilutions, and cAMP standards were made in accordance with the PerkinElmer LANCE Ultra cAMP assay protocol immediately before the assay was performed.

2.4 Assay Protocol

Assay development was also performed in accordance with the protocols set out in the LANCE Ultra cAMP assay development guidelines. PerkinElmer 384-well ProxiPlate microplates were used for all the assays in the following dimensions:

- plate height: 14.4 mm,
- well diameter: 3.15 mm, and
- well volume: 25 μL.

All the plates were read on a Molecular Devices (Sunnyvale, CA) SpectraMax i3× plate reader with an HTRF (Cisbio Bioassays, Bedford, MD) cartridge installed in time-resolved FRET endpoint mode with the following exposure parameters:

- measurement 1: excitation 340 nm, emission 615 nm;
- measurement 2: excitation 340 nm, emission 665 nm;
- plate type: 384-well ProxiPlate Plus white, height 14.4 mm;
- shake: off;
- read order: row;
- read height optimizer: on;
- integration time: 0.5 ms;
- excitation time: 0.05 ms;

- number of pulses: 5;
- measurement delay: 0.03 ms;
- read from: top; and
- read height:
    - MOR: 7.40 mm,
    - DOR: 7.40 mm,
    - KOR: 7.22 mm, and
    - OLR1: 7.22 mm.

SoftMax Pro v.6.5.1 (Molecular Devices) and GraphPad Prism 7.02 (GraphPad Software, Inc.; La Jolla, CA) software were used to acquire and analyze data.

3. RESULTS

3.1 Cell Density Optimization

The cAMP standard curve was generated parallel to the cell density dilutions on the same 384-well plate. Cell density for all future experiments was determined based on the highest signal-to-noise ratio while staying within the 10–90% maximal inhibitory concentration (IC_{10} to IC_{90}) dynamic range of the cAMP standard curve. Cell concentrations were plated at 5000 cells/well (when possible), 3000 cells/well, 1000 cells/well, and 250 cells/well densities and were accomplished by serial dilution. All conditions were performed in triplicate. It was determined that 1000 cells/well met the criteria for cell-density selection in all cell subtypes; this density had comparable signal-to-noise ratio and was within the dynamic range of the linear phase of the cAMP standard curve (Figures 3–5).

Based on the 1000 cells/well density, the experimental forskolin concentration was calculated to be IC_{90} of that density or 3 μM (for all cell subtypes). The IC_{90} was used in these experiments to achieve the highest signal difference between forskolin-activated cells and cells co-stimulated with forskolin and the individual agonists of interest. Therefore, all further experiments were performed with 3 μM forskolin concentrations as the co-administered compound.

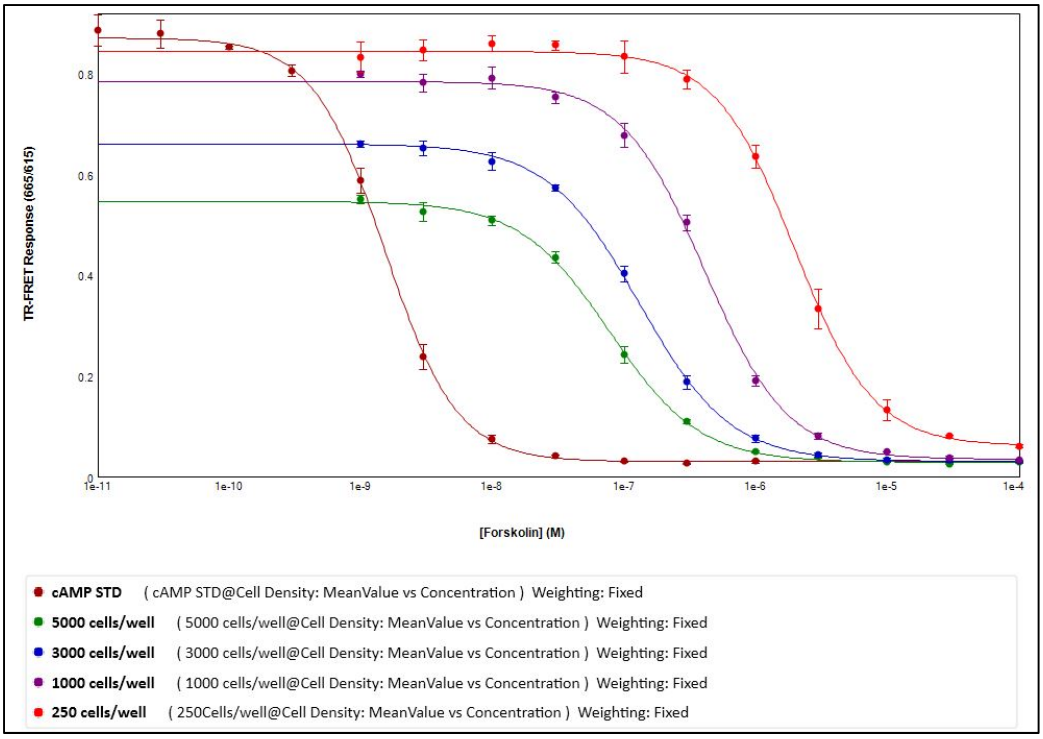

Figure 3. DOR cell density and forskolin optimization experiments. 1000 cells/well is within the linear dynamic range of the instrument.

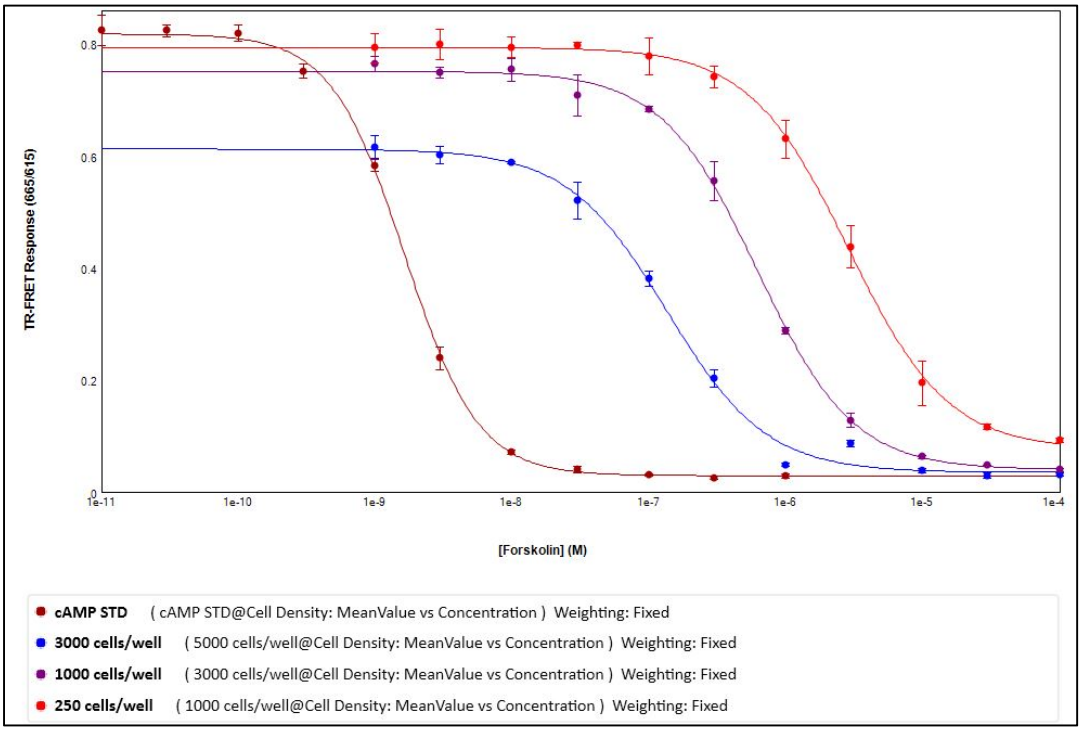

Figure 4. KOR cell density and forskolin optimization experiments. 1000 cells/well is within the linear dynamic range of the instrument.

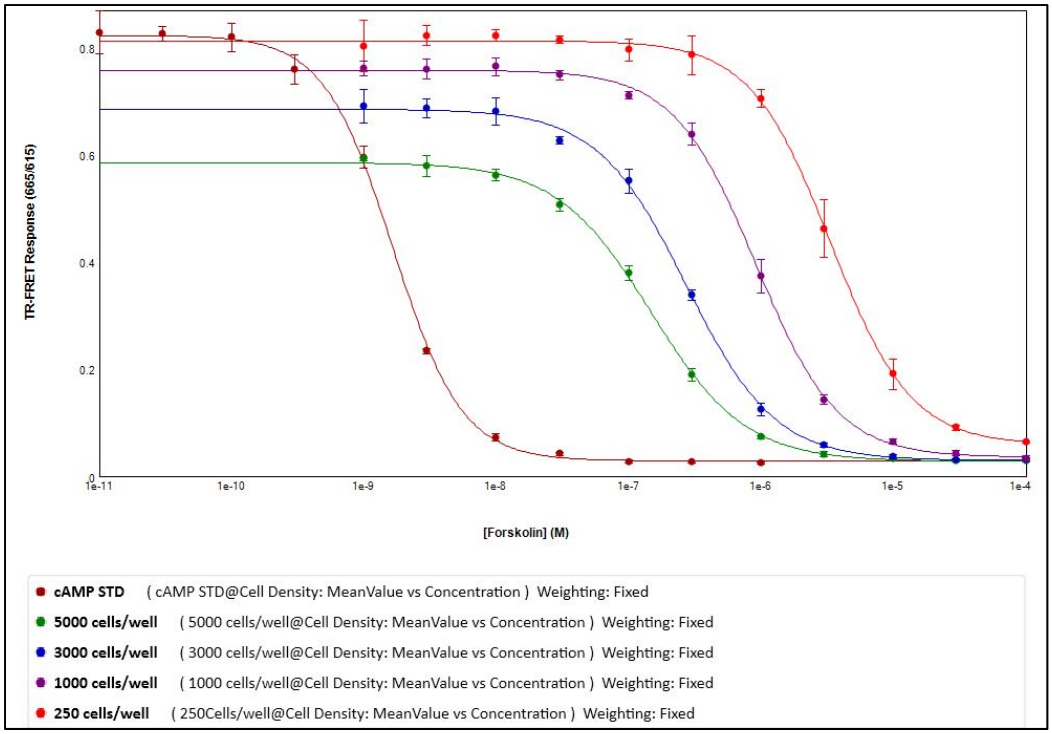

Figure 5. OLR1 cell density and forskolin optimization experiments. 1000 cells/well is within the linear dynamic range of the instrument.

3.2 Carfentanil and W-18 Receptor Specificity and Potency

Efficacy and potency were assessed for each compound at the individual receptor subtypes (Figures 6–9). In fact, carfentanil was found to have very high selectivity at the MOR in comparison with the other three subtypes. In addition, W-18 was found to have no activity across the four human receptor subtypes that were tested in this study at any physiologically relevant concentration. A comparison of the median effective concentration (EC_{50}) values for both compounds and all four receptor subtypes is shown in Table 2.

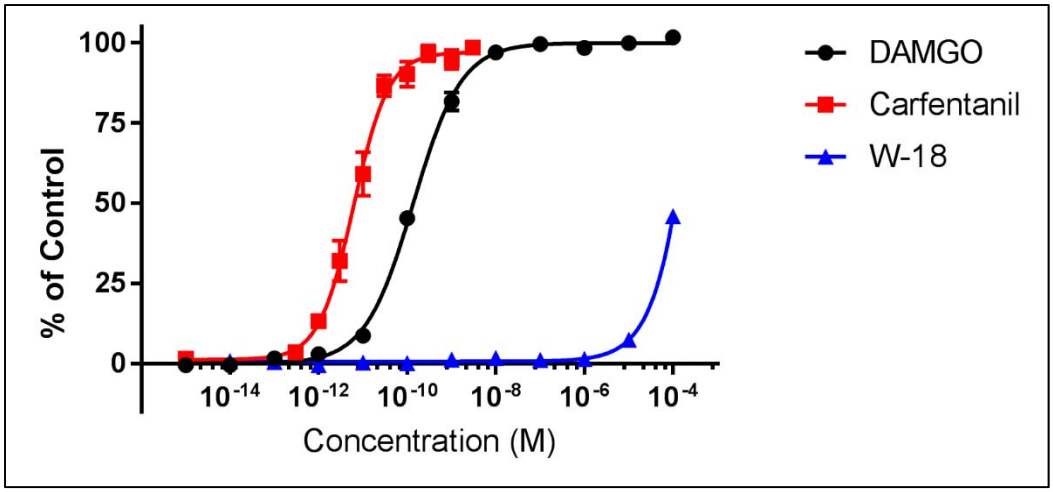

Figure 6. Dose–response curve for hMOR expressing CHO-K1 cells. Control compound was DAMGO. Note that carfentanil is a more potent agonist than the control. W-18 is inactive at the hMOR.

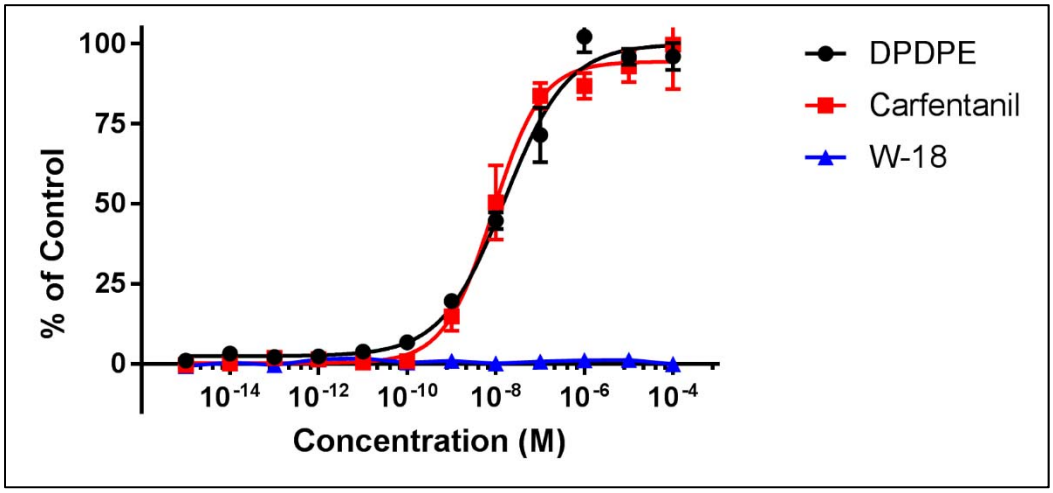

Figure 7. Dose–response curve for hDOR expressing CHO-K1 cells. Carfentanil has similar efficacy but less potency than the control compound DPDPE. W-18 is inactive at the hDOR.

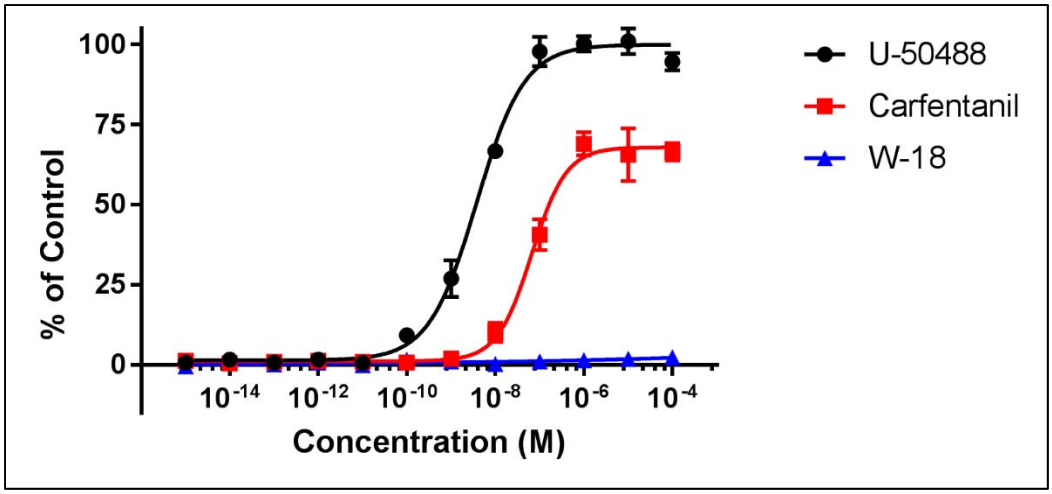

Figure 8. Dose–response of hKOR expressing CHO-K1 cells. Carfentanil shows less efficacy and less potency for this receptor subtype. W-18 was inactive at the hKOR.

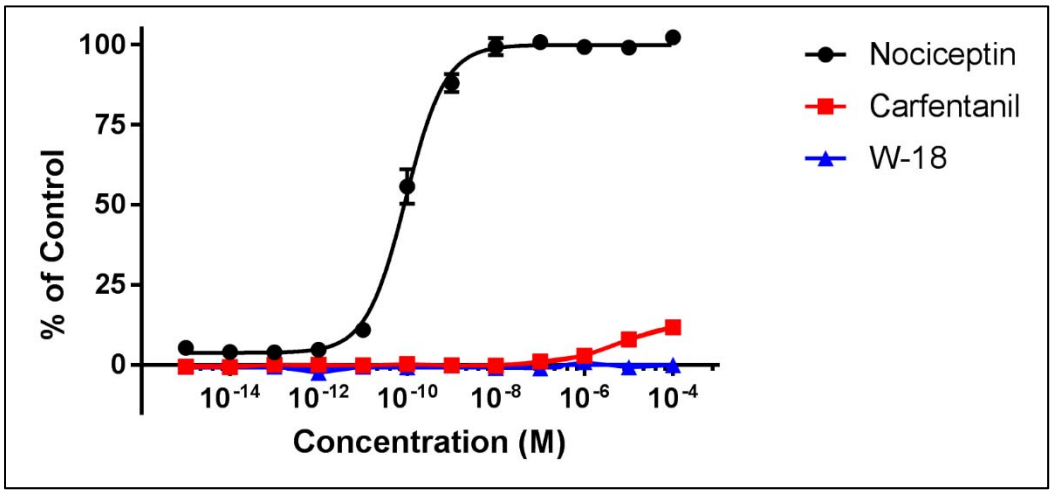

Figure 9. Dose–response of hOLR1 expressing CHO-K1 cells. Carfentanil and W-18 did not show any activity for this receptor subtype.

Table 2. EC_{50} Values for Each Human Opioid Receptor Subtype

Drug	EC_{50} Values (M)			
	hMOR	hDOR	hKOR	hOLR1
Carfentanil	6.15×10^{-12}	8.55×10^{-9}	6.61×10^{-8}	Inactive
W-18	Inactive			

Based on the EC_{50} values for each of the receptor subtypes, carfentanil showed overwhelming selectivity at the MOR. Carfentanil was 1,390 times more MOR-specific than the DOR and 10,747 times more MOR-specific than the KOR. Even more overwhelming was the fact that carfentanil was at least six orders of magnitude (~4,000,000) times more MOR-specific than the OLR1. However, because of carfentanil's low EC_{50} values at the MOR (carfentanil's intended target receptor), there is potential for DOR and KOR activity at physiologically achievable levels of the drug still falling in the 1–100 nM range. Carfentanil was the only compound that was more potent than the control peptide or compound. Of course, in a controlled setting, carfentanil would likely be dosed to a desired endpoint and MOR-mediated; therefore, off-target agonism of DOR and KOR would be minimal to nonexistent. In a mass administration of carfentanil, as was reported during the Moscow theatre hostage crisis, only the laws of physics control the dosage administered to each casualty. At higher doses, over-dosing of the MOR-toxic mechanisms, combined with an increased likelihood of off-target DOR and KOR mediated pathways, could further complicate symptomology and would likely lead to toxicity.

4. DISCUSSION

Historical animal model work with opioids has shown that effectiveness and toxicity are species-dependent, and that rodents have an artificially large therapeutic index compared with higher-order animals. This is related to the receptor homology and physiology of various species. The study of human receptors in vitro removes all interspecies variability and makes the data more relevant to humans. In addition, by generating EC_{50} values that can be used to relatively compare the potency of suspect or known opioid compounds for the four receptor subtypes, this study highlighted some of the interspecies differences in receptor homology.

Table 3. Comparison of Reported EC_{50} Values with Experimental Values of This Study

Source	EC_{50} Values (nM)			
	MOR	DOR	KOR	OLR1
Cometta-Morini[5]	0.024	3.28	43.1	NA
This study	0.00615	8.55	66.1	Inactive

The method used in this study, which included CHO cells transfected with human receptor genes, demonstrates clear advantages over the methods used in the Cometta-Morini experiments,[5] which used guinea pig whole brains. There was an order of magnitude more sensitivity in the carfentanil activity at hMOR as compared with the guinea pig brain method. The DOR and KOR results were relatively close to the reported values. This could be a contributing factor to the sensitivity of higher-order species to these compounds when compared with sensitivity of smaller species. Other factors could include drug distribution, metabolism, clearance, and absorption differences.

5. CONCLUSIONS

The effort proposed by this seedling project was to assess opioid compounds with regard to their potency for four receptor subtypes in a human model system. This assay used CHO-K1 cells expressing human opioid receptors of all four known subtypes. This method is ideal for assessing opioid potency and efficacy because it is the only system based on the human receptor. This capability can be used to screen suspect opioid compounds for activity and specificity in a safe, rapid, and inexpensive manner. This capability is a useful screening tool for the U.S. Department of Defense and other public health-minded organizations concerned with opioid compounds and their potential pharmacology and toxicology.

We confirmed carfentanil's incredibly high potency in the opioid system. We also reinforced carfentanil specificity at the MOR. Despite this selectivity, carfentanil was used to elicit off-target effects at the other receptor subtypes at physiological concentrations.

For the first time, reports of W-18 activity in the human opioid system are refuted. No other study to date has tested W-18 at the human receptor. Its lack of agonist activity at the receptor leads to many more questions about its toxicity and mechanism of action. We questioned whether it is in fact bioactive and toxic. Further studies need to be conducted to elucidate receptor targets for W-18. However, we can conclude that W-18 does not act either therapeutically or toxicologically through agonism of the opioid receptors.

LITERATURE CITED

1. Ling, G.S.; Spiegel, K.; Nishimura, S.L.; Pasternak, G.W. Dissociation of Morphine's Analgesic and Respiratory Depressant Actions. *Eur. J. Pharmacol.* **1983,** *86* (3–4), 487–488.

2. Shook, J.E.; Watkins, W.D.; Camporesi, E.M. Differential Roles of Opioid Receptors in Respiration, Respiratory Disease, and Opiate-Induced Respiratory Depression. *Am. Rev. Respir. Dis.* **1990,** *142* (4), 895–909.

3. Subramanian, G.; Paterlini, M.G.; Portoghese, P.S.; Ferguson, D.M. Molecular Docking Reveals a Novel Binding Site Model for Fentanyl at the Mu-Opioid Receptor. *J. Med. Chem*. **2000,** *43* (3), 381–391.

4. Cole, A.; Mutlow, A.; Isaza, R.; Carpenter, J.W.; Koch, D.E.; Hunter, R.P.; Dresser, B.L. Pharmacokinetics and Pharmacodynamics of Carfentanil and Naltrexone in Female Common Eland *(Taurotragus oryx). J. Zoo. Wildl. Med.* **2006,** *37* (3), 318–326.

5. Cometta-Morini, C.; Maguire, P.A.; Loew, G.H. Molecular Determinants of Mu Receptor Recognition for the Fentanyl Class of Compounds. *Mol. Pharmacol*. **1992,** *41* (1), 185–196.

6. Riches, J.R.; Read, R.W.; Black, R.M.; Cooper, N.J.; Timperley, C.M. Analysis of Clothing and Urine from Moscow Theatre Siege Casualties Reveals Carfentanil and Remifentanil Use. *J. Anal. Toxicol.* **2012,** *36* (9), 647–656.

7. Gladden, M.R.; Martinez, P.; Seth, P. Fentanyl Law Enforcement Submissions and Increases in Synthetic Opioid-Involved Overdose Deaths—27 States, 2013–2014. *MMWR Morb. Mortal. Wkly. Rep.* **2016,** *65* (33), 837–843.

8. Kounang, N. Elephant Tranquilizer to Blame for at Least 8 Ohio Deaths. *CNN Wire* [Online] 6 Sep 2006. http://wtvr.com/2016/09/06/elephant-tranquilizer-to-blame-for-at-least-8-ohio-deaths/ (accessed 2 Mar 2017).

9. Kroll, D. W-18, the High-Potency Research Chemical Making News: What It Is and What It Isn't. *Forbes* [Online] 30 Apr 2016. http://www.forbes.com/sites/davidkroll/2016/04/30/w-18-the-high-potency-research-chemical-making-news-what-it-is-and-what-it-isnt/#7e73c7bb2354 (accessed 2 Mar 2017).

10. Knaus, E.E.; Warren, B.K.; Ondrus, T.A. Analgesic Substituted Piperidylidene-2-sulfon(cyan)amide Derivatives. U.S. Patent 04,468,403, 28 Aug 1984.

11. Brasel, C.M.; Sawyer, G.W.; Stevens, C.W. A Pharmacological Comparison of the Cloned Frog and Human Mu Opioid Receptors Reveals Differences in Opioid Affinity and Function. *Eur. J. Pharmacol*. **2008,** *599* (1–3), 36–43.

12. Chen, J.C.; Smith, E.R.; Cahill, M.; Cohen, R.; Fishman, J.B. The Opioid Receptor Binding of Dezocine, Morphine, Fentanyl, Butorphanol and Nalbuphine. *Life Sci.* **1993,** *52* (4), 389–396.

13. Traynor, J.R.; Nahorski, S.R. Modulation by Mu-Opioid Agonists of Guanosine-5'-O-(3-[35s]thio)triphosphate Binding to Membranes from Human Neuroblastoma SH-SY5Y Cells. *Mol. Pharmacol*. **1995,** *47* (4), 848–854.

14. Volpe, D.A.; McMahon Tobin, G.A.; Mellon, R.D.; Katki, A.G.; Parker, R.J.; Colatsky, T.; Kropp, T.J.; Verbois, S.L. Uniform Assessment and Ranking of Opioid μ Receptor Binding Constants for Selected Opioid Drugs. *Regul. Toxicol. Pharmacol*. **2011,** *59* (3), 385–390.

15. Port, J.D.; Stanley, T.H.; Steffey, E.P.; Pace, N.L.; Henrickson, D.R.; McJames, S.W. Intravenous Carfentanil in the Dog and Rhesus Monkey. *Anesthesiology* **1984,** *61* (3), A378.

16. Feldman, P.L.; James, M.K.; Brackeen, M.F.; Bilotta, J.M.; Schuster, S.V.; Lahey, A.P.; Lutz, M.W.; Johnson, M.R.; Leighton, H.J. Design, Synthesis, and Pharmacological Evaluation of Ultrashort-Acting to Long-Acting Opioid Analgesics. *J. Med. Chem.* **1991,** *34* (7), 2202–2208.

17. Pan, J.B.; Ji, N.; Pan, W.; Hong, R.; Wang, H.; Ji, Z.L. High-Throughput Identification of Off-Targets for the Mechanistic Study of Severe Adverse Drug Reactions Induced by Analgesics. *Toxicol. Appl. Pharmacol*. **2014,** *274* (1), 24–34.

18. Stringer, J.; Welsh, C.; Tommasello, A. Methadone-Associated Q-T Interval Prolongation and Torsades de Pointes. *Am. J. Health Syst. Pharm*. **2009,** *66* (9), 825–833.

19. Walz, A.J.; Hsu, F.-L. Synthesis of 4-Anilinopiperidine Methyl Esters, Intermediates in the Production of Carfentanil, Sufentanil, and Remifentanil. *Tetrahedron Lett.* **2014,** *55* (2), 501–502.

ACRONYMS AND ABBREVIATIONS

AC	adenylyl cyclase
cAMP	3',5'-cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CHO-K1	Chinese hamster ovary ancestral cell line
DOR	δ-opioid receptor
EC_{50}	median effective concentration
ED_{50}	median effective dose
FRET	fluorescence resonance energy transfer
$G_{\alpha i}$	α subunit proteins
GPCR	G-protein coupled receptor
GPI	guinea pig ileum assay
h	human
IC	inhibitory concentration
K_i	inhibitory constant
KOR	κ-opioid receptor
LD_{50}	median lethal dose
mAB	monoclonal antibody
MOR	μ-opioid receptor
OLR1	opioid-like receptor 1
RTW	rat-tail withdrawal assay
W-18	4-chloro-*N*-[(2*Z*)-1-[2-(4-nitrophenyl)ethyl]piperidin-2-ylidene]benzene-1-sulfonamide

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical
Biological Center (ECBC)
RDCB-DRT-O
ATTN: Feasel, M.
Moran, T.
Whalley, C.
RDCB-DRC-C
ATTN: Walz, A.

Defense Threat Reduction Agency
J9-CBS
ATTN: Graziano, A.

Department of Homeland Security
RDCB-PI-CSAC
ATTN: Negron, A.
DHS-S&T-RDP-CSAC
ATTN: Strang, P.

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

Defense Technical Information Center
ATTN: DTIC OA

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.
RDCB-DEM
ATTN: Grodecki, J.


EDGEWOOD CHEMICAL BIOLOGICAL CENTER
A U.S. Army RDECOM Laboratory