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IN VITRO SCREENING OF OPIOID ANTAGONIST EFFECTIVENESS

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Opioid agonists such as fentanyl and its congeners are health risks to members of the U.S. Department of Defense and the general public. Traditionally, opioid overdoses are reversed with an opioid antagonist naloxone (Narcan). However, naloxone is reported to be less and less effective at treating overdoses of more potent and new psychoactive substances. One such substance, acrylfentanyl, has been reported to be an "irreversible binder" of the opioid receptor and thus, Narcan resistant. This study used in vitro methods to test and confirm the nature of ligand-receptor interaction by competing the μ -opioid receptor with a co-administration of acrylfentanyl and naloxone. Reversibility of acrylfentanyl was achieved at naloxone concentrations comparable to those of the control compound.

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PREFACE

The work described in this report was authorized under the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) 219 Seedling Program and Defense Threat Reduction Agency (DTRA; Fort Belvoir, VA) project number CB3281. This work was started in March 2017 and completed in September 2017.

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IN VITRO SCREENING OF OPIOID ANTAGONIST EFFECTIVENESS

1. INTRODUCTION

Opioid overdoses have increased from 2010 to 2016 at an unprecedented rate. In 2016 alone, deaths related to fentanyl increased 540% over those seen in 2013.¹ Illicit drug overdoses and deaths have increased as well, but not to the same degree. In Maryland, there was a 67% increase in drug overdoses, which is second only to that of Delaware (71%).² Perhaps, the increase in deaths due to overdose of synthetic opioids (excluding methadone) is of the greatest concern. Synthetic opioids like fentanyl are becoming more popular, and illicit drug suppliers have been incorporating more and more potent and toxic analogues into these supplies. Carfentanil, the ultrapotent, large animal tranquilizer, has been reported to be responsible for deaths in Ohio, Maryland, and Philadelphia.³ As attention is focused on limiting the number of pharmaceuticals in circulation, illicit drug manufacturers are able to skirt federal drug scheduling by synthesizing fentanyl analogues that are not listed under any controlled substance laws. These compounds, termed “new psychoactive substances” (NPSs), have little-to-no pharmacological or toxicological data and nearly always, no human-use data. This makes attributing drug overdoses very difficult for forensic toxicologists because these NPSs evade the standard toxicological screens used for attribution. NPSs also make clinicians’ and emergency medical personnel’s jobs difficult in that traditional therapeutics for opioid overdoses do not work as efficaciously as they have with known psychoactive compounds like heroin or morphine.

In this study, we used an in vitro method to assess the binding interactions of opioid compounds. We also used one novel NPS (acrylfentanyl) to test whether the reported irreversible opioid-receptor binders were truly irreversible or whether they were resistant to the effects of naloxone through other means (i.e., potency, distribution, or other pharmacological properties). Fentanyl (Figure 1A) was used in this study as the benchmark compound for reference, and acrylfentanyl (Figure 1B) was used because it has been reported to be an irreversible opioid agonist and is Narcan resistant.

Acrylfentanyl was first synthesized in 1982 as part of an effort to synthesize an affinity label of the opioid-receptor macromolecular complex.⁴ It was hoped that one or more of the structures generated as part of the 1982 study would achieve irreversible binding to the receptor for use in pharmacological studies in vivo. In the radioligand binding study, performed as a subpart of this study, acrylfentanyl demonstrated higher potency than fentanyl, from which we concluded that it would be suitable for affinity-labeling studies. However, during the binding affinity studies, it was concluded that acrylfentanyl did not exhibit irreversible binding to the opioid receptor. Although it is not at all curious as to why illicit drug manufacturers would include acrylfentanyl in their inventories, the important question is why have reports of the irreversible nature of this drug compound circulated as rumor.

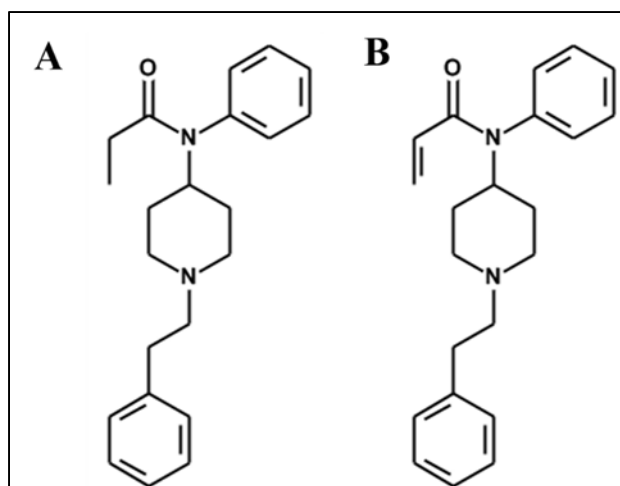


Figure 1. Chemical structures of (A) fentanyl and (B) acrylfentanyl.

Nevertheless, in the current study, we aimed to confirm the reversible nature of acrylfentanyl and to report the higher potency of acrylfentanyl in comparison with fentanyl. This was achieved by establishing a median effective concentration (EC_{50}) for acrylfentanyl and generating median inhibitory concentrations (IC_{50}) for fentanyl and acrylfentanyl after competition with naloxone to assess the reversibility of ligand–receptor interaction. However, we ultimately worked towards establishing a capability at U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) that previously did not exist. This new capability could not only be used to assess the potency of a pharmaceutical at its target receptor but could also be used to assess the binding interaction of the compound (i.e., reversibility), which has major implications in pharmacology, symptomology, and toxicology.

2. METHODOLOGY

2.1 Chemicals

The following list shows the materials and chemicals that were used in this study together with their manufacturers or vendors:

- The Lance cAMP (cyclic adenosine monophosphate, a second messenger) 10,000 assay point kit and 384-Proxiplates were purchased from PerkinElmer, Inc. (Waltham, MA). The Lance kit consisted of 50 μ M cAMP standard; Eu-cAMP tracer, ULIGHT-anti-cAMP; cAMP-detection buffer; and bovine serum albumin stabilizer.
- Fentanyl citrate was procured from Mallinckrodt Pharmaceuticals (St. Louis, MO).
- Acrylfentanyl HCl was procured from Cayman Chemical (Ann Arbor, MI).

- μ -opioid receptor (MOR) selective agonist [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO) was purchased from Tocris Bioscience (Park Ellisville, MO).
- Hank's balanced salt solution 1 $\times$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 1M, Versene Solution, and Geneticin were procured from Life Technologies (Grand Island, NY).
- Dimethyl sulfoxide (DMSO), 3-isobutyl-1-methylxanthine, Forskolin, and naloxone were procured from Sigma-Aldrich Corporation (St. Louis, MO).
- Dulbecco's phosphate-buffered saline modified buffer 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 Line

ValiScreen CHO-K1 cells expressing human MOR (ES-542-C) were purchased from PerkinElmer, Inc. The cells were kept frozen in liquid nitrogen storage (vapor phase) until they were cultured. The cells were grown in accordance with product literature provided by PerkinElmer. The cell cultures were split when they reached ~60–80% confluency, and no cells were used past passage 10. Cells were used for opioid assay only when they met the requirements described in the product literature (i.e., 60–80% confluency). Before use, cellular solutions used in plating were counted on a Vi-CELL XR hemocytometer (Beckman Coulter Life Sciences; Indianapolis, IN). The cells were plated at a concentration of 2.0×10^5 cells/mL, as optimized in previous studies.

2.3 Incubation and Standard Solutions

Standard solutions of fentanyl, acrylfentanyl, and naloxone (10 mM) were made in DMSO and stored until use in a freezer at 4 °F. A standard solution of DAMGO (a synthetic opioid peptide with high μ -opioid-receptor specificity; 1.95 mM) was made in sterile water. Working solutions of fentanyl, acrylfentanyl, naloxone, and DAMGO (500 μ M) were prepared in fresh stimulation buffer immediately before the assay was performed. Stimulation buffer, forskolin dilutions, and cAMP standards were made, as needed, in accordance with the Lance Ultra cAMP assay protocol immediately before the assay was performed.

2.4 Assay Protocol

2.4.1 General Assay Development

Assay development was performed as reported in Section 2.3 and in accordance with the protocols set out in PerkinElmer Lance Ultra cAMP Assay Development Guidelines.^{5,6} PerkinElmer 384-Proxiplates were used for all assays with the following dimensions: plate height 14.4 mm, well diameter 3.15 mm, and well volume 25 μ L. All plates were read on a SpectraMax i3 plate reader (Molecular Devices LLC; Sunnyvale, CA) with a homogeneous

time-resolved fluorescence cartridge installed in the time-resolved fluorescence resonance energy transfer endpoint mode. The reader was set at 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: 7.04 mm.

The data were collected and analyzed in a Molecular Devices SoftMax Pro v.6.5.1.

2.4.2 Acrylfentanyl EC₅₀

Acrylfentanyl was plated as described in Section 2.3 (Figure 2). Dosing was performed in log intervals down each column and always in triplicate. DAMGO was used as the positive control, and the bottom rows of all the test wells served as negative controls because they contained only cells and stimulation buffer. No agonist or cAMP standard was added. The fentanyl and DAMGO tests were not repeated after the initial calculation in this test system.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	cAMP Standards			DAMGO (10 ⁻⁴ -10 ⁻¹⁴ M)			Acrylfentanyl (10 ⁻⁴ -10 ⁻¹⁴ M)																	
B																								
C																								
D																								
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Figure 2. Plate layout used for acrylfentanyl EC₅₀ determination.

2.4.3 Competition Assay and IC₅₀ Generation

For the competition assay, the effective concentration to 90% effect level (EC₉₀) values for DAMGO, fentanyl, and acrylfentanyl were calculated using GraphPad Prism v7.0.2 (GraphPad Software, Inc.; La Jolla, CA) software for each agonist. This single concentration was co-administered to each well and log-dosing of each antagonist was conducted (Figure 3). The single concentration of agonist administered to each test sample was the EC₉₀ value, which is standard practice when a competition assay of this nature is performed as dictated by the assay guidelines.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	cAMP Standards			DAMGO (EC ₉₀) + Naloxone (10 ⁻⁴ -10 ⁻¹⁴ M)			Fentanyl (EC ₉₀) + Naloxone (10 ⁻⁴ -10 ⁻¹⁴ M)			Acrylfentanyl (EC ₉₀) + Naloxone (10 ⁻⁴ -10 ⁻¹⁴ M)														
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Figure 3. Plate layout of agonists at respective EC₉₀ molar concentration and antagonists at log-dosing from 10⁻⁴ to 10⁻¹⁴ M.

3. RESULTS

3.1 Acrylfentanyl Dose–Response

A fentanyl EC₅₀ value had already been generated in this test system and thus, was not repeated.⁶ However, an EC₅₀ test for acrylfentanyl had not previously been conducted so the EC₅₀ value for acrylfentanyl was generated using the same methods used for fentanyl. To properly conduct the competition assay performed with acrylfentanyl, a full dose–response curve had to be generated for each test agent. Potency was assessed for acrylfentanyl, and the EC₅₀, EC₉₀, and efficacy values were calculated (Figure 4). These values were compared to those of fentanyl (Table 1).

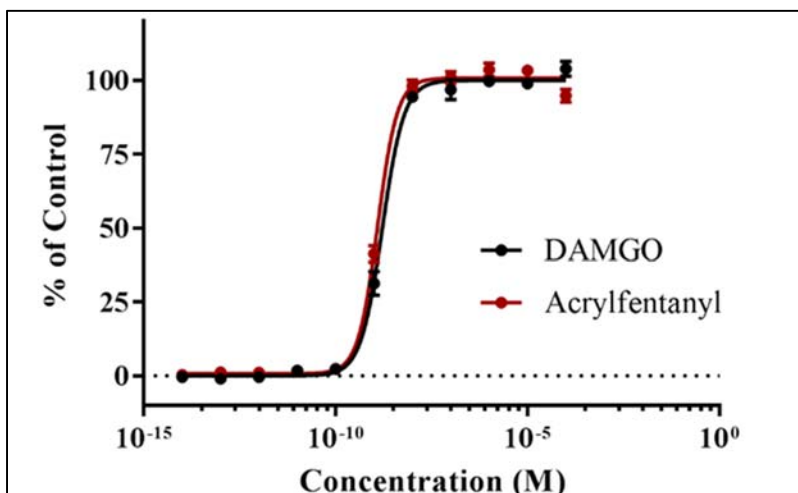


Figure 4. Dose–response curve for acrylfentanyl with control compound DAMGO for reference. Data points are plotted as mean $\pm$ standard error of the mean (SEM).

Table 1. EC₅₀, EC₉₀, and Efficacy Values for Opioid Agonist Compounds

Drug	EC ₅₀ (M)	EC ₉₀ (M)	Efficacy (%)
Fentanyl	5.11 E-10	2.89 E-09	100
Acrylfentanyl	1.26 E-09	4.58 E-09	101
DAMGO	1.66 E-09	6.98 E-09	100

Based on the EC₅₀ calculation as a reflection of potency, acrylfentanyl was calculated as being 0.41 times as potent as fentanyl in regards to its ability to not only bind, but cause a cAMP response in the cells. This is contradictory to the 1982 radioligand binding study, which indicated that acrylfentanyl was 1.14 times more potent than fentanyl based on receptor binding alone.⁴ In this study, we demonstrated that receptor binding does not always indicate potency and that functional assays may be more effective in attempting to translate to physiological responses in vivo.

3.2 Competition Assay

After the EC₅₀ and EC₉₀ values were calculated for fentanyl and acrylfentanyl, the competition assay was performed. Cells were incubated with the EC₉₀ value of fentanyl or acrylfentanyl and log-dosing of naloxone. All other assay steps remained the same. This experimental design was conducted in accordance with the Lance cAMP development guidelines and reflects how much antagonist is required to reverse the EC₉₀ value of an agonist. From the resulting backwards-S dose–response curves, IC₅₀ values were calculated for naloxone against the respective agonist challenge compounds, fentanyl and acrylfentanyl (Figure 5 and Table 2).

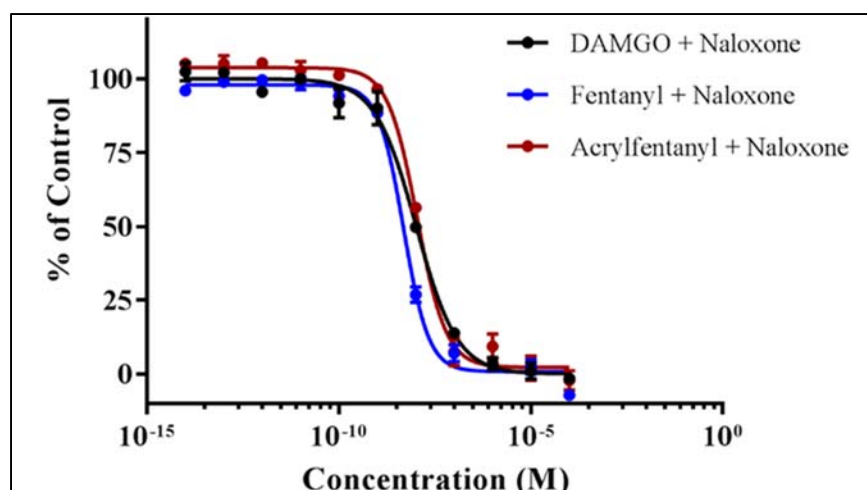


Figure 5. Inhibition dose–response curves for DAMGO (black), fentanyl (blue), and acrylfentanyl (red) with naloxone antagonism. Data points are plotted as mean $\pm$ SEM.

Table 2. IC₅₀ Values for Naloxone when Challenged with EC₉₀ Values of DAMGO, Fentanyl, and Acrylfentanyl

Drug	IC ₅₀ (M)	Efficacy (%)
Fentanyl	4.85 E-09	98
Acrylfentanyl	1.09 E-08	104
DAMGO	1.04 E-08	100

Based on the IC₅₀ values of fentanyl and acrylfentanyl, 2.25 times more naloxone was required to reverse the cAMP inhibition caused by acrylfentanyl binding and receptor agonism.

4. DISCUSSION

NPS and the illicit drug acrylfentanyl were reported to be more potent than fentanyl and Narcan resistant by irreversibly binding the opioid receptor. This has been a cause of great concern for law enforcement and public health officials as well as clinicians. In this ECBC study, we analyzed these reports and developed the following standards for opioid research:

- the nature of the ligand–receptor interaction should be tested;
- a poorly studied opioid compound should be assessed to determine whether or not it is, in fact, more potent; and
- reversibility (i.e., affinity of the interaction at the receptor-binding site) should be observed and measured.

This paradigm of experiments assessed acrylfentanyl to be less than half (0.41) times as potent as fentanyl, but calculated an antagonist load of 2.25 times greater than that of fentanyl in order to reverse the effects.

These results demonstrate that potency should not be the sole concern when dealing with a threat compound. Inhibition and reversal data, such as those generated from our competition assay, also indicate the severity of toxicity from compound to compound. If acrylfentanyl had been assessed only for potency, it could have been shrugged off as a less harmful opioid congener. However, that information, combined with the amount of antagonist needed to reverse agonism, shows acrylfentanyl to be more hazardous than initially thought. This also indicates that, although not entirely irreversible when bound to the opioid receptor, acrylfentanyl may require more naloxone or other reversal agent if an acrylfentanyl overdose is suspected.

In addition, this study confirms that data gathered from historical publications that discuss radioligand binding assays may differ from data gathered from functional assays such as ours. According to the 1982 publication on acrylfentanyl, this synthetic drug is 1.4 times more potent than fentanyl, yet we observed a functional EC₅₀ response less than half as potent as fentanyl. This indicates a substantial difference between a bound receptor and an activated receptor. This caveat can signify even more substantial differences when looking at physiological responses. For in vivo studies, it may be more useful to have functional data than binding data because these higher order responses will be manifested only when the receptor is activated and not merely bound.

As it stands, this assay is only capable of measuring the cAMP response of cells to agonism or antagonism. Other cellular responses are known to be manifested by agonism of all G-protein-coupled receptors (GPCRs), including sodium potassium ion flux, calcium flux, and β -arrestin recruitment. These responses can be measured but require different assay kits and instrumentation. By complementing this study with an ion flux or β -arrestin recruitment assay, a complete picture of the GPCR activation and reversal by antagonists of interest as well as receptor bias can be better assessed for each agonist or antagonist tested.

5. CONCLUSIONS

The work performed in this study under the ECBC 219 Seedling program with collaboration from the Defense Threat Reduction Agency (Fort Belvoir, VA) helps the Army and other members of the U.S. Department of Defense gain more insight into the opioid epidemic and the chemical weapons threat that opioids pose (e.g., reported use of opioids in the 2002 Moscow Theatre Siege).⁷ The standards established under this effort can be applied to current toxicological assessment programs. ECBC can gain more projects and programs that can be used to train the Warfighter to deal with an opioid threat compound in the field and domestic arenas.

Future studies should focus on the problem of opioid-toxicity treatment by measuring metabolism of the drug compound and compare that to the metabolic rate of the reversal agent, itself. Metabolic clearance data, when combined with a potency and competition assay, would not only indicate how much more (or less) a reversal agent would be required initially, but also how frequently it would have to be re-administered.

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ACRONYMS AND ABBREVIATIONS

cAMP	cyclic adenosine monophosphate, a second messenger
DAMGO	[D-Ala ² , NMe-Phe ⁴ , Gly-ol ⁵]-enkephalin
DMSO	dimethyl sulfoxide
ECBC	U.S. Army Edgewood Chemical Biological Center
EC ₅₀	median effective concentration
EC ₉₀	effective concentration to 90% effect level
GPCR	G-protein-coupled receptor
IC ₅₀	median inhibitory concentration
MOR	μ-opioid receptor
NPS	new psychoactive substance
SEM	standard error of the mean

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