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PNNL-36739

Heracles: Predictive Tools for Opioid Crisis Intervention

m/q Initiative Project Report

June 2024

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U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Abstract

The opioid crisis in the United States is being fueled primarily by fentanyl and its molecular analogs, which can be anywhere from 50 to 1,000 times more potent than morphine. Fentanyl itself is straightforward to synthesize; furthermore, the structure is such that fentanyl's flexible, rotatable side chains are easy to modify to create new analogs. Reference-free computational techniques to predict and identify new fentanyls have the potential to provide a desperately needed preemptive advantage to regulatory stakeholders and toxicologists. The computational pipeline *Heracles* was developed with this preemptive advantage in mind. Heracles has two primary components: 1) the creation of an *in silico* library of putative fentanyl analogs, and 2) a downselection pipeline to prioritize generated fentanyl analogs predicted to be potent and easy to synthesize. Experimental observables were also predicted for prioritized analogs, with validation of the observables begun. Heracles has demonstrated potential to aid in the advancement of reference-free paradigms while providing new tools to first responders and other stakeholders attempting to mitigate the opioid crisis.

Acknowledgments

This research was supported by the *m/q* Initiative under the Laboratory Directed Research and Development (LDRD) Program at Pacific Northwest National Laboratory (PNNL). PNNL is a multi-program national laboratory operated for the U.S. Department of Energy (DOE) by Battelle Memorial Institute under Contract No. DE-AC05-76RL01830.

Contents

1.0 Introduction

Most opioid overdose deaths in the U.S. involve illicitly manufactured fentanyl analogs, which can be up to 10,000 times more potent than morphine (e.g., carfentanil). In addition to the unpredictable potency of illicit fentanyl, a primary challenge facing regulatory agencies is the ease of synthesizing fentanyl analogs that fall outside existing regulatory control listings by making small modifications to the fentanyl backbone. These new analogs further complicate the ability to identify and characterize them in seized or collected samples. For samples obtained after long delays post-exposure, or those containing previously unencountered chemicals (such as new analogs), there are often no available reference standards. Furthermore, the toxicity profiles of these new analogs are unknown, limiting the ability to quickly and effectively treat exposures. In the case of metabolized compounds taken from biomedical samples, it is difficult to obtain useful forensic signatures related to the parent compound's identity, or to determine from which precursors it was derived.

This project—*Heracles*—was an effort to overcome these limitations by generating an *in silico* library of fentanyl analogs and prioritizing those of greatest predicted risk for use in identifying such compounds in degraded and metabolized samples in the absence of readily available references. It is intended as proof-of-principle for this type of computational workflow to enable new capabilities for prediction and recognition of chemicals that may be pursued for nefarious use. The approach is intended to be suitable for samples containing degraded or metabolized compounds. The information produced in developing this library can further inform medical countermeasure development and provide key forensic information on synthesis routes and precursors.

1.1 Computational Pipeline Development & Analysis

The Heracles computational pipeline was developed in five stages. A diagram representation of the full pipeline is depicted in **Figure 1**. The stages can be summarized as follows:

1) Compilation of existing fentanyl analogs into an *in silico* library

2) Enumeration and recombination of *in silico* library analogs to create a new putative fentanyl analog database

3) Structure and property-based analysis of analogs to inform filtering techniques

4) Downselection of putative analog database to hit list of predicted dangerous fentanyl analogs

5) Calculation of experimental observables for hit list compounds

In the first stage, the collection of existing fentanyl analogs involved pulling from publicly available databases including PubChem (https://pubchem.ncbi.nlm.nih.gov/), Cayman Chemical (https://www.caymanchem.com/), and Isomer Design (https://isomerdesign.com/Home/). A total of 61,451 fentanyl analogs—represented in Simplified Molecular Input Line Entry System (SMILES) notation—were compiled following deduplication.

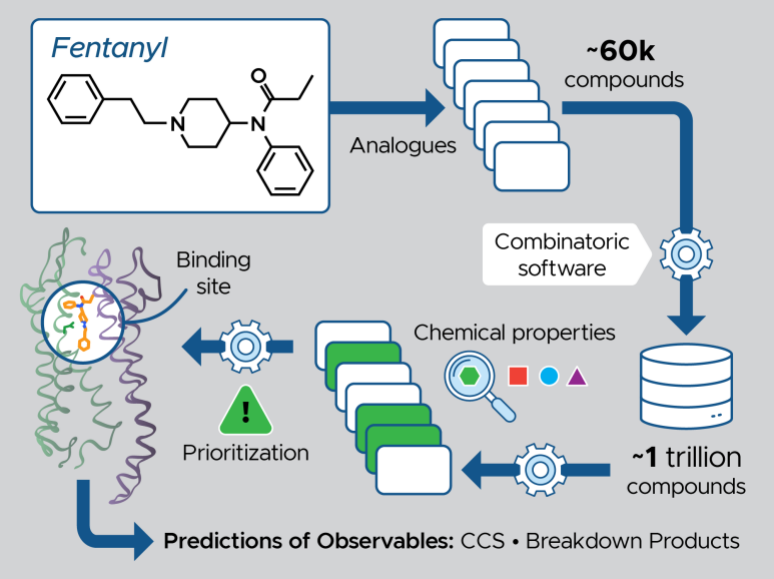


Figure 1. The Heracles computational pipeline involves the following steps: collection of known fentanyl analogs; recombination of knowns to generate massive putative analog set; property- and structure-based assessment and prioritization of analogs; and experimental observable prediction for prioritized fentanyl analogs.

In stage 2, all chemical substructural groups in the *in silico* library had to be identified. In this step, all library compounds were converted to a SMILES Arbitrary Target Specification (SMARTS) representation, which provides a much more straightforward method to identify chemical substructures. To break the analogs into substructures, the fentanyl structural backbone and four substituent groups were first defined as depicted in **Figure 2**. The backbone structure was saved as a SMARTS string that is easily identifiable and contained within all library analogs. At this point, each compound in the fentanyl analog library was decomposed into a backbone and substituent groups. Unique substituent groups were enumerated at each of the four attachment points. As a result, the N-substituent point was found to contain 5,992 unique substructures, the anilide's aromatic point 823, the omega position point 5,249, and the 4-axial substituent point 69.

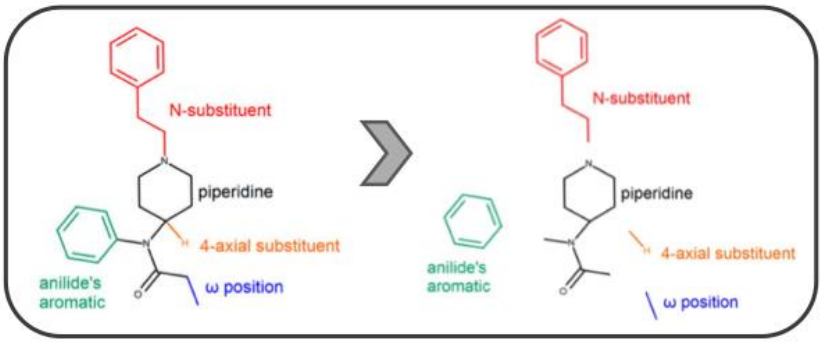


Figure 2. The fentanyl structural backbone, in black, has four primary attachment points for substituent groups, depicted in red, green, orange, and blue.

Given these numbers of unique substructures found within the fentanyl analog library, a back of the envelope calculation indicates that approximately 1.7 trillion new compounds can be formed via recombination of substructures. In reality, not all of these putative structures are physically possible. During the subsequent step of this stage—*in silico* recombination of all substructures at the four attachment points—some structure creation attempts did result in failure. Combinatorics ultimately produced approximately 1 trillion new putative fentanyl analogs. These were converted to SMILES format for ensuing analysis.

The primary objective guiding the remainder of the computational process was downselecting the trillion generated analogs to a hit list of those predicted to be of highest risk. All analysis undertaken on these analogs was done on supercomputing resources, but the computational time and resource requirements for a 1 trillion-member dataset were still much too high for most algorithms. As such, it was necessary to determine the least computationally expensive techniques to use for analysis and filtering steps beginning in stage 3. To assist in the process, we created a reference set of dangerous fentanyl analogs using the International Narcotics Control Board's (INCB) set of fentanyl-related substances with no known legitimate uses. This set allowed us to establish reference calculated property ranges for over 200 chemical properties among which to search to aid in identifying putative hit list compounds. A set of decoy compounds was also generated using the DUD-E web tool (Mysinger, Carchia and Irwin 2012) for use as a negative control. The combined datasets enabled the use of feature analysis algorithms to determine the relative importance of each property in determining whether a given fentanyl analog is considered dangerous. We could then incorporate high importance properties as a consideration during downselection.

The first downselection step performed in stage 4 was an exact mass filter, as exact mass calculations were within acceptable resource limits for the entire trillion-member generated dataset. In addition, exact mass was one of the high importance properties identified during feature importance analysis. As such, only generated compounds within the INCB reference mass range were downselected for subsequent filtering steps. The other two most important properties according to feature analysis—synthetic accessibility score and predicted blood-brain barrier permeability—further filtered the generated analog set. Compounds remaining after the first three property-based filtered were then accessed with a consensus-based machine learning (ML) model trained on additional calculated chemical properties. Filtering steps along with the approximate number of generated fentanyl analogs remaining following each filter are depicted in **Figure 3**. This downselection process ultimately reduced the set of 1.7 trillion putative analogs to under 2 billion. A molecular docking simulation pipeline using AutoDock Vina (Eberhardt, et al. 2021) was also implemented to interrogate binding potential at the μ-Opioid Receptor (MOR). Our analysis of the pipeline's ability to accurately predict binding affinity showed that while there is a statistically significant different in binding affinity scores overall when comparing known dangerous fentanyls to decoys, individual scores are frequently unreliable. As such, we decided to omit the docking screen from the final filtering steps.

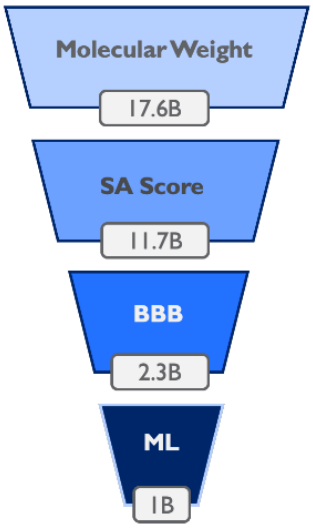


Figure 3. Number of generated putative fentanyl analog compounds remaining after each primary filter was applied. SA Score = synthetic accessibility score; BBB = blood brain barrier; ML = machine learning.

The final stage of the *in silico* portion of the project—stage 5—consisted of predicting experimental observables for downselected hit list analogs to enable their identification in real world scenarios. As this project was funded under the *m/q* Initiative at PNNL, which sought to advance reference-free metabolomics, experimental observables relevant to other projects under this initiative were prioritized. This entailed primarily the prediction of metabolic breakdown products and collision cross section (CCS). The web tool BioTransformer (https://biotransformer.ca) was used for metabolite prediction. Current open access CCS prediction tools—whether density function theory (DFT)- or ML-based—all perform inconsistently across chemical space, and sometimes even across chemical class. With these limitations in mind, we utilized multiple tools for CCS prediction: ISiCLE, a PNNL DFT-based tool (Colby, et al. 2019); DarkChem, a PNNL ML-based tool (Colby, Nunez, et al. 2019), and SigmaCCS, an external ML-based tool (Guo, et al. 2023).

The *in silico* pipeline we developed to assess the risk potential of putative fentanyl analogs performed very well on internal hold-out datasets. However, experimental validation with external data is needed for a robust performance assessment. Given the highly sensitive nature of this project's subject matter, devising an experimental strategy to validate the pipeline proved tricky. Several strategies were begun and abandoned due to unforeseen and insurmountable complications. While we were not able to complete the experimental work in the time and budget allotted, we collected data that we hope will be used in future related work.

1.2 Experimental Analysis

cAMP Assays

Opioids are potent drugs that bind to opioid receptors throughout the body, although their primary mechanisms of action are through signaling in neurons. Cell-based assays with neurons are therefore an appealing approach to assess the biological activity of opioids. However,

culturing neuronal cell lines is challenging because mature neurons do not divide. (Gordon, Amini and White 2013) Primary neuronal cell lines can be isolated from tissues and maintained in culture for experiments, but special care must be taken to avoid excitotoxicity, and the inability to propagate these cells for multiple passages limits their availability for large scale experiments. Immortalized neuronal cell lines, such as those derived from tumors, provide increased access to larger numbers of these cells, but such secondary cell lines often display different physiology and morphology from primary neurons and may no longer express key receptors. (LePage, et al. 2005) Nonetheless, literature precedent for MOR activation in secondary cell lines has been previously reported, providing a basis for cell-based assays that can be used to assess biological responses toward fentanyl and fentanyl analogs.

For quantifying the activation of MOR, a G protein-coupled receptor (GPCR), the expression and identity of the G protein to which MOR is coupled must also be known in order to monitor the signaling pathway of interest through a relevant assay, such as ion flux or secondary messenger production. In the literature, we identified the SK-N-SH human neuroblastoma cell line, derived from bone marrow, as endogenously expressing MOR and responsive toward morphine and fentanyl. (Gupta, Devi and Gomes 2011) The HT-29 human colorectal cancer cell line (Nyland, et al. 2007)) has also been reported to express functional MOR.

Fentanyl is a MOR agonist. When fentanyl binds to MOR coupled to $G_{i/o}$ proteins, adenylyl cyclase is inhibited, which decreases levels of adenosine 3′,5′-cyclic monophosphate (cyclic AMP, otherwise known as cAMP) in the cell (**Fig. 4**). (Adhikary and Jaeckel 2022) Assays that quantify cAMP levels in the cell have therefore been widely used to monitor MOR activation. (Wang, et al. 2022) While ion flux is also frequently used to quantify GPCR activation in live cells, these cell responses are transient and occur on a short time scale (seconds), requiring specialized instrumentation such as fluorescent imaging plate reader (FLIPR) technology for reliable quantification.

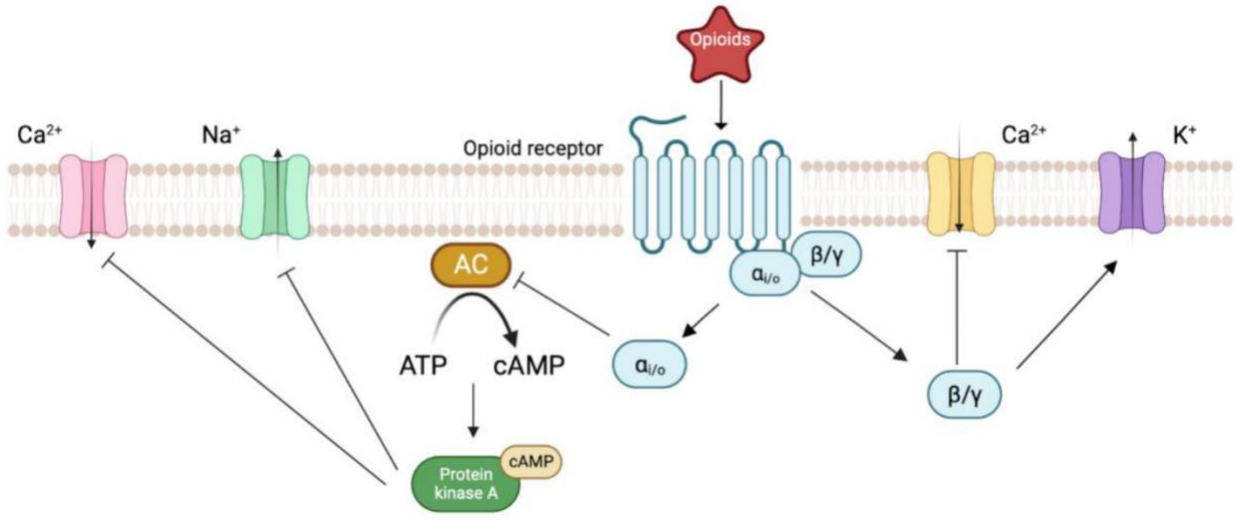


Figure 4. Cell signaling pathways of opioid receptors. Activation of opioid receptors by opioids is mediated by $G_{i/o}$ proteins, which affect ion flux through ion channels and secondary messenger levels through enzymes such as adenylyl cyclase (AC). Figure from (Li, et al. 2023).

The cAMP-Glo assay uses bioluminescence from luciferase to quant cAMP in cells (**Fig. 5**). cAMP leads to activation of protein kinase A, an enzyme that degrades ATP to ADP. Luciferase, which catalyzes the reaction of luciferin to oxyluciferin to produce light, is dependent upon ATP. Thus, increased levels of cAMP in cells leads to higher protein kinase A activity, which decreases ATP concentrations and reduces light output. In fentanyl-treated, MOR-activated cells, lower amounts of cAMP would be expected, leading to higher luminescence in these cells.

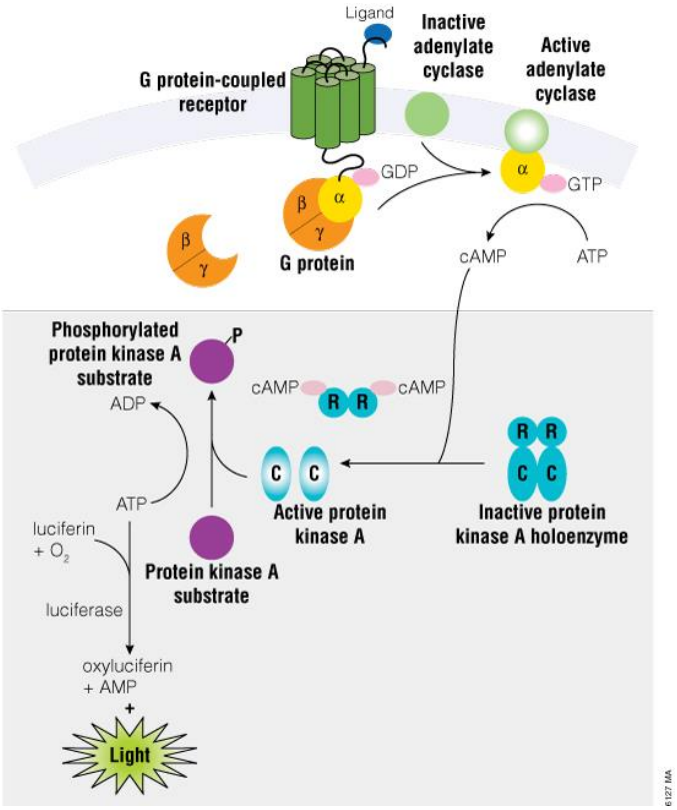


Figure 5. Overview of cAMP-Glo assay, which uses bioluminescence from luciferase to quantify cAMP-induced changes in protein kinase A activity toward ATP.

While initial cAMP-Glo assays in SK-N-SH cells treated with fentanyl hydrochloride suggested this cell line showed MOR activation (**Figure 6**), there was somewhat high error for N = 3 technical replicates. Results in the OHSU HEK-MOR cells and HT-29 were inconsistent. We hypothesized that low levels of MOR expression and/or varying levels of MOR expression across the cell population, was likely a contributing factor to the observed high signal-to-noise for both secondary cell lines endogenously expressing MOR and in the OHSU HEK-MOR cells (personal correspondence). Thus, we opted to pursue human MOR-overexpression in HEK293 cells through lentiviral transduction for more robust quantification of MOR activation. cAMP-Glo assays in the lentiviral transduced HEK-MOR cells showed improved signal-to-noise (**Figure 7**).

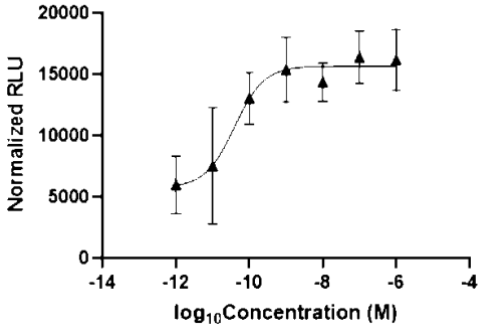


Figure 6. cAMP-Glo assay for response of SK-N-SH cells toward fentanyl hydrochloride. Relative luminescence units (RLU) are normalized to protein content for each well, determined via BCA assay. Error bars represent ± standard deviation. N = 3 (technical replicates).

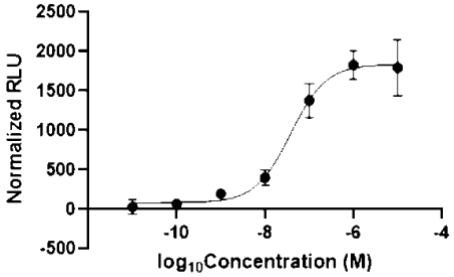
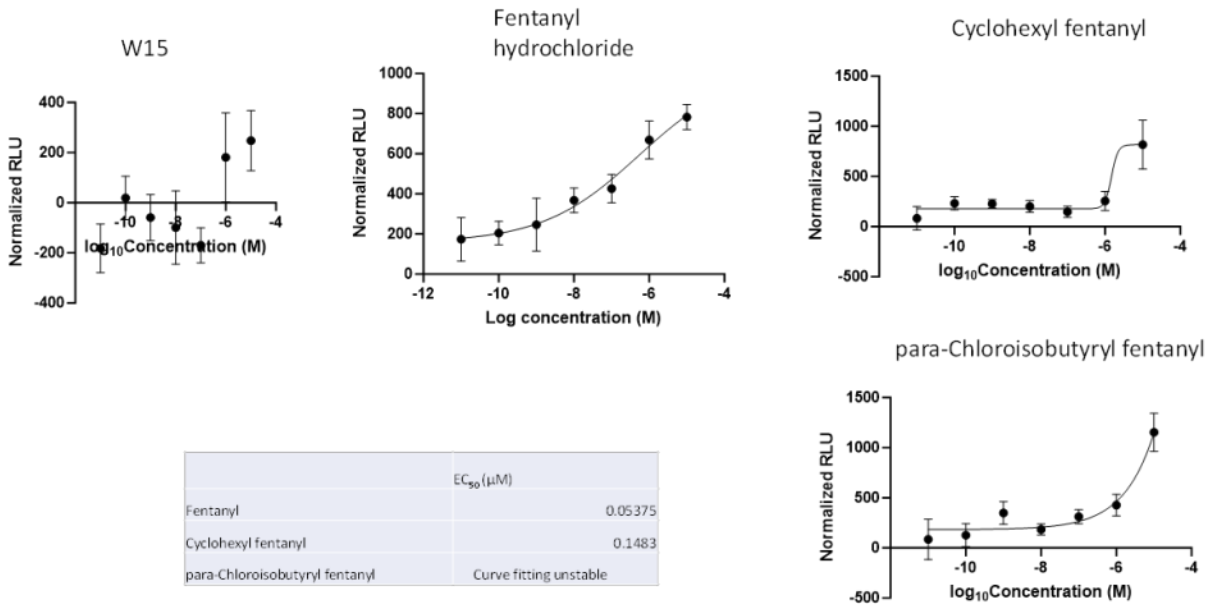


Figure 7. cAMP-Glo assay for response of HEK-MOR overexpressing cells toward fentanyl hydrochloride. Relative luminescence units (RLU) are normalized to protein content for each well, determined via BCA assay. Error bars represent ± standard deviation. N = 3 (technical replicates).

Applying the cAMP-Glo assay to HEK-MOR cells treated with fentanyl hydrochloride and the two fentanyl analogs cyclohexyl fentanyl and para-chloroisobutyryl fentanyl appeared to show some MOR activation (**Figure 8**). Qualitatively, W-15 appeared to be ineffective at MOR stimulation, although these data showed high signal-to-noise. Culturing cells at higher cell passage numbers may also have decreased the overall expression levels of MOR in later experiments compared to initial assays. As the cAMP-Glo assay involves live cell stimulation prior to lysis and analysis and measures second messenger production several steps downstream of MOR activation, the effect of these compounds on other cellular pathways could potentially have an unexpected influence on proteins or signaling molecules that affect the assay readout. Additional experiments to repeat these assays at lower cell passage number and a higher concentration range may provide further insight into the response of these cells toward fentanyl and other compounds of interest.



	EC_{50} (μM)
Fentanyl	0.05375
Cyclohexyl fentanyl	0.1483
para-Chloroisobutyryl fentanyl	Curve fitting unstable

Figure 8. cAMP-Glo assay for determining half-maximal effective concentration (EC_{50}) of fentanyl, fentanyl analogs cyclohexyl and para-chloroisobutyryl fentanyl, and a non-fentanyl analog (W-15) in HEK-MOR overexpressing cells. N = 3 (technical replicates).

Metabolite identification

In the final stage of experimental work, we attempted to identify predicted fentanyl analog metabolites in samples originally containing parent analog compounds. While we were unable to complete this analysis in the time given, some raw data were collected. The initial goal was to identify metabolites from the fentanyl analogs para-Chloriosobutyryl Fentanyl, Fentanyl HCL, and Cyclohexyl Fentanyl incubated using human liver microsomes (with W-15, a negative control, as the internal standard after incubation) by LC-IMS-MS. The original methodology utilized a no extraction method, but few metabolite peaks were observed in the resulting chromatograms. We suspected that the metabolite signal was being suppressed by leftover salts from the sample preparation procedure, so we decided to add a cleanup step to remove salts and try again. The chromatograms for the samples with the cleanup step were much richer, and several peaks are observed after subtraction from the controls. **Figure 9** shows examples of the chromatograms for samples subtracted from controls obtained with the cleanup step in positive mode.

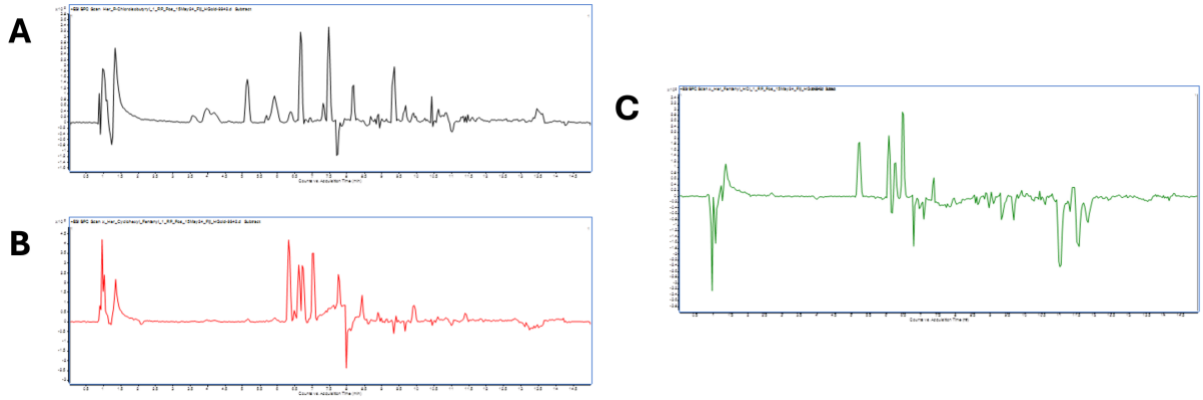


Figure 9. LC-IMS-MS chromatograms for samples containing the parent compounds para-Chloriosobutyryl Fentanyl (A), Cyclohexyl Fentanyl (B), and Fentanyl HCl (C).

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Appendix A – Methodology

cAMP Assays

SK-N-SH cells were cultured on high glucose Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) + Gibco Antibiotic/Antimycotic + Pen-strep. HT-29 cells were cultured in Roswell Park Memorial Institute (RPMI) media with L-glutamine + 10% FBS + 1% Pen-strep. Stably transfected HEK-MOR cells from Oregon Health Sciences University (OHSU) were generously provided by Dr. Seksiri Arttamangkul. OHSU HEK-MOR cells were cultured in high glucose DMEM + 10% FBS + G418 + Pen-strep. Lentiviral transduced HEK-MOR cells were culturing in high glucose DMEM + 10% FBS + 10 µg/mL puromycin.

cAMP quantification was performed using the bioluminescent cAMP-Glo™ assay (Promega) as recommended by the vendor. Kinase-Glo® Buffer and Kinase-Glo® Substrate were dispensed into single-use aliquots and stored at –20 °C. Buffer and substrate were mixed on the day of each experiment to generate Kinase-Glo® Reagent for the cAMP assay. Induction buffer was prepared from 1X phosphate-buffered saline (PBS) with 500 µM isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Cat. # I7018) and 100 µM 4-(3-butoxy-4-methoxybenzyl) imidazolidone (Ro 20-1724, Sigma Aldrich Cat. # B8279)], at room temperature. The adenylyl cyclase activator forskolin was added to stimulate cAMP production.

Cells were cultured in 96-well plates. Cell media was removed, and cells were washed with 1X PBS. Induction buffer (10 µL) was added to each well, and cells were incubated at 37 °C for 15 min. Induction buffer (10 µL) containing fentanyl at various concentrations and forskolin (25 µM) were added to each well, and cells were incubated at 37 °C for 30 min. Then, cAMP-Glo™ Lysis Buffer (20 µL) was added to each well. The plate was incubated at room temperature for 15 min with agitation. At this point, 4 µL of the reaction mixture was removed and diluted with 96 µL for protein quantitation using a bicinchoninic acid (BCA) assay. To each well, cAMP Detection Solution (40 µL; prepared by adding 2.5 µL of Protein Kinase A to 1.0 mL of cAMP-Glo™ Reaction Buffer) was then added, and samples were mixed by agitating the plate for 30-60 seconds, followed by incubation at room temperature for 20 min. Room temperature Kinase-Glo® Reagent (80 µL) was then added to each well, mixed by agitation for 30-60 seconds, and incubated at room temperature for 10 min. Luminescence was quantified using a plate reader.

LC-IMS-MS

First, samples with no extraction method were prepared. Nine samples were generated by incubating para-Chloriosobutyryl Fentanyl, Fentanyl HCL, and Cyclohexyl Fentanyl using human liver microsomes in triplicate. Nine Controls and nine 0 Time Point Controls were also generated, resulting in a total of 27 samples. These were then incubated using human liver microsomes. A 10µL spike of each of the above (100µg/mL) were added with 10µL of W-15 (100µg/mL) as internal standard after incubation. The 0 Time Point Controls were not incubated but kept on ice. With an incubation volume of 500µL, Para-Chloriosobutyrl and Cyclohexyl were incubated at 37°C for 10 minutes; Fentanyl HCl was incubated for 30 minutes. All samples were quenched with 500µL of ice-cold Methanol, then centrifuged and the entire supernatant removed.

Next samples with no extraction method and a cleanup procedure were prepared. This procedure largely followed the prior one, but with the following adjustments. Firstly, a total of 18

samples were generated in this round (nine incubated samples and nine controls, we decided that the 0 Time Point controls were not necessary for this study). After quenching and centrifugation, the cleanup procedure was performed using Supelco-Supel Swift HLB SPE Columns 30 mg/1mL. Finally, samples were dried down using speedvac and reconstituted with 200 µL 80:20 methanol:dH_2O.

Samples and controls were analyzed by LC-IMS-MS using an Agilent Ultrahigh performance liquid chromatography (UHPLC) 1290 Infinity II system using a Reverse Phase column (Hypersil GOLD, 2.1 × 150 mm, 3 µm particle C18 column (Thermo Scientific)). The UHPLC system was coupled to an Agilent 6560 Ion Mobility quadrupole TOF MS system (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization source (ESI). Samples were analyzed in positive and negative ion modes. Prior to the analysis of the samples, a solvent blank (methanol:water 80:20) was injected to check for contamination, and an instrument standard QC (synthetic mixture of metabolites) was injected to check the instrument performance.

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