Activation of the μ-opioid receptor by alicyclic fentanyl: Changes from high potency full agonists to low potency partial agonists with increasing alicyclic substructure

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Abstract
Fentanyl analogs represent an important group of new psychoactive substances and knowing their efficacy and potency might assist in interpreting observed concentrations. The potency of fentanyl analogs can be estimated from in vitro studies and can be used to establish structure–activity relationships. In this study, recombinant CHO-K1 cells (AequoScreen) expressing the human μ-opioid receptor were used to establish dose–response curves via luminescent analysis for cyclopropyl-, cyclobutyl-, cyclopentyl-, cyclohexyl-, and 2,2,3,3-tetramethylcyclopropylfentanyl (TMCPF), on three separate occasions, using eight different concentrations in an eight-fold serial dilution in triplicates starting at ~60 μM. Fentanyl was used as a full agonist reference while morphine and buprenorphine were included for comparison. Cyclopropylfentanyl (EC50 = 4.3 nM), cyclobutylfentanyl (EC50 = 6.2 nM), and cyclopentylfentanyl (EC50 = 13 nM, efficacy 48%) were full agonists slightly less potent than fentanyl (EC50 = 1.7 nM). Cyclohexylfentanyl (EC50 = 3.1 μM, efficacy 48%) and TMCPF (EC50 = 1.5 μM, efficacy 65%) were partial agonists less potent than morphine (EC50 = 430 nM). Based on the results, cyclopropyl-, cyclobutyl-, and cyclopentylfentanyl would be expected to induce intoxication or cause fatal poisonings at similar concentrations to fentanyl, while the toxic or fatal concentrations of cyclohexylfentanyl and TMCPF would be expected to be much higher.

KEYWORDS
cyclopropylfentanyl, fentanyl analogs, NPS, potency, μ-opioid receptor agonist

1 | INTRODUCTION

Fentanyl analogs, belonging to the opioids, represent an important group of new psychoactive substances (NPS), especially when looking at drug overdoses and the number of deaths.1 Opioids exert their effects by activating the three major types of opioid receptors (μ, δ, and κ).2 However, the primary receptor involved in opioid addiction and the often lethal respiratory depression caused by opioid overdose is the μ-opioid receptor.2,3

In cases of suspected intoxication, knowing the efficacy and potency of the NPS might assist toxicologists and medical examiners in their interpretation. Similarly, predictions of potency based on the structure could be of value in scheduling decisions on new uncharacterized NPS. Potency can be studied in several ways,
including functional studies in laboratory animals or receptor-based studies in vitro. The latter can be divided into receptor binding assays and receptor activity studies. Receptor binding studies measure the affinity of a ligand to the opioid receptor and are suitable for high throughput. However, binding studies cannot differentiate between full and partial agonists, and even an antagonist such as naloxone shows binding affinity. Receptor activation assays instead measure proximal or downstream activation of the receptor, allowing the potency and efficacy values for a certain agonist to be determined, making them more informative than binding assays. Receptor activation can be measured via different assays based on, for example, aequorin, GTPγS binding, cAMP, and β-arrestin recruitment. The interpretation of the data is complicated by the fact that potency is substantially affected by the assay conditions such as the reagent concentration, which reporting system was used, the cell type, and if a human or murine receptor was used, making it difficult to compare the results from different assays.

Alicyclic fentanyls share their overall chemical structure with fentanyl, except for the substitution of the propionamide with amides with increasingly large cyclic structures containing either a cyclopropyl-, cyclobutyl-, cyclopentyl-, cyclohexyl-, or 2,2,3,3-tetramethylcyclopropyl ring (see Figure 1). In 2017, cyclopropylfentanyl was reported in 59 deaths in Sweden. Cyclopropylfentanyl and 2,2,3,3-tetramethylcyclopropylfentanyl (TMCPF) have also been encountered as NPS in Sweden. Similarly, cyclopropylfentanyl findings have been reported in the USA and Switzerland. Information about the pharmacokinetics of alicyclic fentanyls is limited but we know that they are metabolized differently in biological systems.

The structure–activity relationships for fentanyl analogs have been investigated and reviewed. The binding affinity of cyclopropylfentanyl has been reported as 0.088, 1.2, 0.77, and 2.4 nM in different assays. In addition, Hassani et al reported the binding affinities for cyclobutyl- and cyclopentylfentanyl as 6.2 and 13 nM, respectively. Hassani et al also reported EC50 values from their GTPγS binding assay as 55, 160, and 600 nM for cyclopropyl-, cyclobutyl-, and cyclopentylfentanyl, respectively. The corresponding efficacies were 75%, 61%, and 41% (compared with 10 μM [D-Ala2,
N-Methylphenylglycine (Gly-ol)-enkephalin, DAMGO). This can be compared with an EC50 for cyclopropylfentanyl of 11 nM reported by the Drug Enforcement Administration, also from a GTPγS binding assay. Finally, to study biased agonism, Vasudevan et al. used two similar assays based on the recruitment of a G-protein (mini-Gi) or β-arrestin to study cyclopropyl- and cyclopentylfentanyl, and TMCPF. For the mini-Gi assay they reported EC50 values of 42 and 190 nM, with efficiencies of 280% and 159% (compared with hydromorphone), for cyclopropyl- and cyclopentylfentanyl, respectively. Similarly, for the β-arrestin assay they reported EC50 values of 15 and 180 nM, with efficiencies of 158% and 127% (compared with hydromorphone), for cyclopropyl- and cyclopentylfentanyl, respectively. For TMCPF, no signal was observed in either assay.

Previous literature indicates that both the potency and the efficacy differ between the different alicyclic fentanyl analogs but it is difficult to ascertain how the ring size impact these parameters. Therefore, the aim of this study was to determine the μ-opioid receptor activity induced by a complete series of alicyclic fentanyls and to investigate the structure–activity relationships regarding potency and efficacy.

2 | MATERIALS AND METHODS

2.1 | Drugs and chemicals

Reference standards of cyclopropyl-, cyclobutyl-, cyclo pentyl-, cyclohexyl-, and TMCPF were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The cell culture medium used was DMEM/Ham’s F12 with 15 mM HEPES, L-glutamine, and without phenol red, from Thermo Fisher (Gothenburg, Sweden). Digitonin, ATP, trypsin, and protease-free BSA were from Sigma-Aldrich (Darmstadt, Germany). Fetal bovine serum (FBS) was from Life Technologies, Thermo Fisher (Gothenburg, Sweden). Coelenterazine was from Nanolight Tech (Pinetop, AZ, USA). Stock solutions of 500 μM coelenterazine were prepared in methanol (protected from light), 50 μM digitonin in DMSO, and 10 mM ATP in Milli-Q water and stored at −20°C.

2.2 | Cell lines and cultivation

The receptor activation assay was carried out on AequoScreen recombinant CHO-K1 cell lines purchased from Perkin Elmer (Groningen, the Netherlands) expressing the human μ-opioid receptor (ES-542-AV) and subunit Gα16 coupling receptor activation to an increase in intracellular Ca2+ concentration. The cells also expressed apoaequorin which, when combined with externally added coelenterazine, forms the photoprotein aequorin. When aequorin is exposed to Ca2+, coelenterazine is oxidized with the emission of light. The flash luminescence can easily be read by a plate reader. When combined, the μ-opioid receptor coupled to Gα16 and aequorin provide a convenient model system for measuring receptor activation.

The cell lines were cultured at 37°C in a humidified air atmosphere containing 5% CO2, in Ham’s F12 medium supplemented with 10% FBS and passaged every 3–4 days. The cells were not cultured beyond 30 passages.

2.3 | Dose–response assay

Prior to the dose–response assays, the cells were cultured to a confluence of 70–90% and then trypsinized, centrifuged (150 × g for 5 min at room temperature), and resuspended in pre-warmed assay medium (DMEM/Ham’s F12 without phenol red supplemented with 15 mM HEPES, L-glutamine, and 0.1% protease-free BSA) at a concentration of 3 × 10^5 cells/mL. Coelenterazine was added to the cells to a concentration of 2.5 μM. The cells were gently incubated on a rotating wheel at room temperature for 3 hours while protected from light. The alicyclic fentanyls were prepared in 96-well plates (OptiPlate-96, white opaque microplates from PerkinElmer), in triplicate, at final concentrations of 20,000, 4,000, 800, 160, 32, 6.4, 1.28, 0.256, 0.0512, 0.01024 ng/mL in each well (concentrations after adding the cells). Fentanyl, morphine, and buprenorphine were analyzed in the same concentration range to serve as μ-opioid receptor full- and partial agonist references. As positive controls digitonin (67 μM) and ATP (7 μM) were used. Wells containing cells and medium without drug were used as negative controls. The receptor activation at each drug concentration was determined by dispensing 50 μL of cells into each well (15,000 cells/well) using a Tecan Spark 10 M (Tecan, Switzerland) for the subsequent luminescence reading. The reading protocol was set to 200 luminosity readings, and the cells were added to each well at reading cycle 10 (baseline). Luminescence reading was carried out for ~25 s before moving on to the next well.

The experiments for each substance were repeated on three different days comprising 6 days of analysis. Fentanyl was included as a reference every day and the data set for fentanyl contains seven experiments, including two from the same day.

2.4 | Data analysis

Luminescence data from each well were summarized over the total reading time and blank measurements were subtracted. The response signals were normalized to the digitonin signal for each plate and then normalized to the plateau signal of fentanyl (average of top two concentrations analyzed in the same experiment), denoted as 100% activity.

The EC50 values and efficacy with 95% confidence intervals (profile likelihood) and curve fittings (non-linear fit, three parameters, bottom constrained to 0%) were calculated using all data points (n = 72, fentanyl n = 168) using GraphPad Prism version 8.3.0 for Windows (GraphPad Software, La Jolla, CA, USA). The efficacy (derived as the top value from the linear regression) of all fentanyl analogs was compared with that of the full agonist fentanyl (5 comparisons, n = 3; fentanyl n = 7) using a one-way ANOVA with
Efficacy and potency of alicyclic fentanyls. Adjusted

| TABLE 1 | Efficacy of alicyclic fentanyls. Adjusted P values are given for the difference in efficacy compared with fentanyl and values < 0.05 were considered significant. For potency, all differences are significant except for between cyclopropyl- and cyclobutylfentanyl. TMCPF, 2,2,3,3-tetramethylcyclopropylfentanyl |
|---------------------------------|---------------------------------|---------------------------------|
|                                 | Efficacy | Potency |
|                                 | % of ref | 95% CI (profile likelihood) | Adj. P values | LogEC50 | EC50 nM | 95% CI (profile likelihood) |
| Fentanyl                        | 97      | 94–100  | Ref | –8.76 | 1.7 | 1.4–2.2 |
| Morphine                        | 103     | 98–108  | Ctrl | –6.37 | 430 | 330–550 |
| Buprenorphine                   | 74      | 71–78   | Ctrl | –6.49 | 320 | 250–410 |
| Cyclopropylfentanyl             | 97      | 91–103  | NS. | –8.37 | 4.3 | 2.5–7.1 |
| Cyclobutylfentanyl              | 91      | 87–94   | NS. | –8.20 | 6.2 | 4.7–8.2 |
| Cyclopropylfentanyl             | 92      | 89–95   | NS. | –7.88 | 13 | 10–17 |
| TMCPF                           | 65      | 61–68   | < 0.0001 | –5.82 | 1500 | 1200–1900 |
| Cyclohexylfentanyl              | 48      | 45–51   | < 0.0001 | –5.50 | 3100 | 2400–4100 |
slow receptor dissociation reported for buprenorphine.\cite{27} In a flash assay, such as the one used in this study, with read times of a few seconds it is possible that the slow dissociation does not impact the assay in the same way as in cAMP and GTP\(\gamma\)S binding assays with incubation times of an hour or more.

The five alicyclic fentanyls can be divided into two distinct groups. The three smaller analogs, cyclopropyl-, cyclobutyl-, and cyclopentylfentanyl were all full agonists with potencies similar to fentanyl, while cyclohexylfentanyl and TMCPF behaved like partial agonists of similar efficacy to buprenorphine but with lower potency. It was remarkable how suddenly the agonist behavior changed when going from a cyclopentyl ring to a cyclohexyl ring, and it is postulated that this might be due to steric hindrance at the binding site.

Except for cyclohexylfentanyl, the alicyclic fentanyl analogs have been studied before, although not together.\cite{5,12,23,24} As there is considerable variability between the different assays the results were compared as fold changes from the EC\textsubscript{50} value and the efficacy of fentanyl. In our study, cyclopropylfentanyl was a full agonist 2.5-fold less potent than fentanyl. In other studies the EC\textsubscript{50} varied from 2.9-fold more potent to 1.7-fold less potent than fentanyl with efficacies between 84% and 107% compared with fentanyl.\cite{5,12,23,24} Cyclobutylfentanyl was a full agonist 3.1-fold less potent than fentanyl in this study, while in the study by Hassanien et al\cite{25} it was 5.0-fold less potent with an efficacy of 69% compared with fentanyl. Similarly, in our study cyclopentylfentanyl was a full agonist 7.6-fold less potent than fentanyl, while in other studies it was 2.8 to 19-fold less potent with an efficacy of 46–80% compared with fentanyl.\cite{12,23,24} Finally, TMCPF behaved as a partial agonist (65% efficacy) 880-fold less potent than fentanyl in our study and Vasudevan et al\cite{12} were unable to obtain a signal with either the mini-Gi or the \(\beta\)-arrestin assay. In general our EC\textsubscript{50}-values compared with fentanyl are similar to those reported in previous studies, even though cyclopropylfentanyl appears somewhat less potent than previously reported. That said, when looking at the efficacies, cyclobutyl- and cyclopentylfentanyl appear to be full agonists in our study while they have previously been reported as partial agonists.

Based on the results, cyclopropyl-, cyclobutyl-, and cyclopentylfentanyl would be expected to induce intoxication or cause fatal poisonings at similar concentrations to fentanyl, while the concentrations of cyclohexylfentanyl and TMCPF required to cause intoxication would be expected to be much higher. That said, it is difficult to extrapolate from in vitro receptor activation to potency in vivo as the latter is also affected by important pharmacokinetic factors such as uptake, distribution, plasma binding, blood–brain barrier penetration, metabolism, and elimination. Even though analogs with lower potency and efficacy can still be abused, as illustrated by the high number of fatalities observed with buprenorphine,\cite{29} it is not possible to speculate on how high the concentrations of cyclohexylfentanyl and TMCPF are required to cause lethal respiratory failure and if such doses are forensically relevant. Interestingly, to the best of our knowledge, no deaths have been reported in the scientific literature attributed to these two analogs.

Earlier studies from our group have shown that the main routes of metabolism for alicyclic fentanyls include dealkylation to form normetabolites as well as oxidation of the alicyclic rings. With increasing ring size fewer normetabolites are formed, in favor of oxidation.\cite{15} If the oxidized metabolites maintain their activity while the normetabolites, analogous to norfentanyl, are inactive this could contribute to the observed potency and/or duration of effects of fentanyls with larger cyclic structures in vivo, but further experiments are needed to verify that this is the case.

5. CONCLUSIONS

In our study, using the AequoScreen assay, all alicyclic fentanyl analogs exhibited activity at the \(\mu\)-opioid receptor. Cyclopropyl-, cyclobutyl-, and cyclopentylfentanyl were all full agonists with a similar potency to fentanyl. On the contrary cyclohexylfentanyl and TMCPF were partial analogs of similar efficacy to buprenorphine but with lower potency.

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REFERENCES

1. Kronstrand R, Guerrieri D, Vikingsson S, Wohlfarth A, Green H. Fatal poisonings associated with new psychoactive substances. In: New psychoactive substances. Cham: Springer; 2018:495-541.
2. Prekupec MP, Mansky PA, Baumann MH. Misuse of novel synthetic opioids: a deadly new trend. J Addict Med. 2017;11(4):256-265.
3. Baumann MH, Kopajtic TA, Madras BK. Pharmacological research as a key component in mitigating the opioid overdose crisis. Trends Pharmacol Sci. 2018;39(12):995-998.
4. Higashikawa Y, Suzuki S. Studies on 1-(2-phenethyl)-4-(N-propionylanilino)piperidine (fentanyl) and its related compounds. VI. Structure-analgesic activity relationship for fentanyl, methyl-substituted fentanyls and other analogues. Forensic Toxicol. 2008;26(1):1-5.
5. Lipinski PFJ, Kosson P, Matalinska J, et al. Fentanyl family at the mu-opioid receptor: uniform assessment of binding and computational analysis. Molecules. 2019;24(4):740.
6. Volpe DA, McMahon Tobin GA, Mellon RD, et al. Uniform assessment and ranking of opioid mu receptor binding constants for selected opioid drugs. Regul Toxicol Pharmacol. 2011;59(3):385-390.
7. Liu AM, Ho MK, Wong CS, Chan JH, Pau AH, Wong YH. Galphal(16/z) chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. J Biol Mol Screen. 2003;8(1):39-49.

8. Baumann MH, Majumdar S, Le Rouzic V, et al. Pharmacological characterization of novel synthetic opioids (NSO) found in the recreational drug marketplace. Neuropharmacology. 2018;134(Pt A):101-107.

9. Olson KM, Duron DI, Womer D, Fell R, Streicher JM. Comprehensive molecular pharmacology screening reveals potential new receptor interactions for clinically relevant opioids. PLoS ONE. 2019;14(6):e0217371.

10. Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem. 1997;272(46):28869-28874.

11. Kuo A, Magiera J, Rethwan N, et al. In vitro profiling of opioid ligands using the cAMP formation inhibition assay and the beta-arrestin2 recruitment assay: no two ligands have the same profile. Eur J Pharmacol. 2020;872:172947.

12. Vasudevan L, Vandeputte M, Deventer M, Wouters E, Cannaert A, Stove CP. Assessment of structure-activity relationships and biased agonism at the mu opioid receptor of novel synthetic opioids using a novel, stable bio-assay platform. Biochem Pharmacol. 2020;177:113910.

13. EMCDDA. European Monitoring Centre for Drugs and Drug Addic-tional. Report on the risk assessment of N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]cyclopropanecarboxamide (cyclopropylfentanyl) in the framework of the council decision on new psychoactive substances, risk assessments, publications Office of the European Union, Luxembourg; 2018.

14. Helander A, Backberg M, Signell P, Beck O. Intoxications involving acrylfentanyl and other novel designer fentanyls – results from the Swedish STRIDA project. Clin Toxicol (Phila). 2017;55(6):589-599.

15. Åstrand A, Toreskog A, Watanabe S, Kronstrand R, Green H, Vikingsson S. Correlations between metabolism and structural elements of the alicyclic fentanyl analogs cyclopropyl fentanyl, cyclobutyl fentanyl, cyclopentyl fentanyl, cyclohexyl fentanyl and 2,2,3,3-tetramethylcyclopropyl fentanyl studied by human hepatocytes and LC-QTOF-MS. Arch Toxicol. 2019;93(1):95-106.

16. Drug Enforcement Administration DJ. Schedules of controlled substances: temporary placement of cyclopropyl fentanyl in schedule I. Temporary amendment; temporary scheduling order. Fed Regist. 2018;83(3):469-472.

17. Fogarty MF, Papsun DM, Logan BK. Analysis of fentanyl and 18 novel fentanyl analogs and metabolites by LC-MS-MS, and report of fatalities associated with methoxyacetylfentanyl and cyclopropylfentanyl. J Anal Toxicol. 2018;42(9):592-604.

18. Fagiola M, Hahn T, Avella J. Five postmortem case reports with qualitative analysis of cyclopropylfentanyl by LC-MS-MS. J Anal Toxicol. 2019;43(4):e1-e6.

19. Brockbals L, Staeheli SN, Gentile S, et al. Fatal poisoning involving cyclopropylfentanyl - investigation of time-dependent postmortem redistribution. Forensic Sci Int. 2019;294:80-85.

20. Vardanyan RS, Hruby VJ. Fentanyl-related compounds and derivatives: current status and future prospects for pharmaceutical applications. Future Med Chem. 2014;6(4):385-412.

21. Wilde M, Pichini S, Pacifici R, et al. Metabolic pathways and potencies of new fentanyl analogs. Front Pharmacol. 2019:10:238.

22. Vuckovic S, Prostran M, Ivanovic M, et al. Fentanyl analogs: structure-activity-relationship study. Curr Med Chem. 2009;16(19):2468-2474.

23. Hassani H, Bassman JR, Perrien Naccarato CM, et al. In vitro pharmacology of fentanyl analogs at the human mu opioid receptor and their spectroscopic analysis. Drug Test Anal. 2020;12:1212-1221.

24. Drug Enforcement Administration DJ. Cyclopropyl fentanyl, background information and evaluation of ‘three factor analysis’ (factors 4, 5 and 6) for temporary scheduling – supporting document of schedules of controlled substances: temporary placement of cyclopropyl fentanyl in schedule I. Temporary amendment; temporary scheduling order. Fed Regist. 2018;83(3):469-472.

25. Milligan G, Marshall F, Rees S. G16 as a universal G protein adapter: implications for agonist screening strategies. Trends Pharmacol Sci. 1996;17(7):235-237.

26. Rizzuto R, Simpson AW, Brini M, Pozzan T. Rapid changes of mitochondrial Ca2+ revealed by specifically targeted recombinant aequorin. Nature. 1992;358(6384):325-327.

27. Khanna IK, Pillarisetti S. Buprenorphine – an attractive opioid with underutilized potential in treatment of chronic pain. J Pain Res. 2015;8:859-870.

28. Kriikku P, Hakkinen M, Ojanpera I. High buprenorphine-related mortality is persistent in Finland. Forensic Sci Int. 2018;291:76-82.

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