Detection, chemical analysis and pharmacological characterization of dipyanone and other new synthetic opioids related to prescription drugs

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Abstract (223 words)

The emergence of structurally diverse new synthetic opioids (NSOs) has caused the opioid crisis to spiral to new depths. Little information is available about the pharmacology of most novel opioids when they first emerge. Here, using a β-arrestin 2 recruitment assay, we investigated the *in vitro* μ-opioid receptor (MOR) activation potential of dipyanone, desmethylmoramide and acetoxymethylketobemidone (O-AMKD) – recent NSOs that are structurally related to the prescription opioids methadone and ketobemidone. Our findings indicate that dipyanone (EC_{50}=39.9 nM; E_{max}=155% vs. hydromorphone) is about equally active as methadone (EC_{50}=50.3 nM; E_{max}=152%), whereas desmethylmoramide (EC_{50}=1335 nM; E_{max}=126%) is considerably less active. A close structural analogue of ketobemidone (EC_{50}=134 nM; E_{max}=156%) and methylketobemidone (EC_{50}=335 nM; E_{max}=117%), O-AMKD showed a lower potency (EC_{50}=1262 nM) and efficacy (E_{max}=109%). Evaluation of the opioid substitution product buprenorphine and its metabolite norbuprenorphine confirmed the increased *in vitro* efficacy of the latter. In addition to *in vitro* characterization, this report details the first identification and full chemical analysis of dipyanone in a seized powder, as well as a postmortem toxicology case from the United States involving the drug. Dipyanone was quantified in blood (370 ng/mL), in which it was detected alongside other NSOs (e.g., 2-methyl AP-237) and novel benzodiazepines (e.g., flualprazolam). While dipyanone is currently not commonly encountered in forensic samples worldwide, its emergence is worrisome and representative of the dynamic NSO market.

Keywords (4-6)

New synthetic opioids; Dipyanone; Desmethylmoramide; Acetoxymethylketobemidone (O-AMKD); μ-Opioid receptor (MOR); Forensic toxicology

1. Introduction

New psychoactive substances (NPS) continue to add complexity to a rapidly changing, globalized recreational drug market [1]. Among the different NPS subclasses, the group of substances with opioid effects (i.e., new synthetic opioids or NSOs) has largely expanded in recent years. In addition to a rising number of NSOs, of particular concern is the high potency of many analogues, presenting grave danger to people who use drugs and new challenges to clinicians, law enforcement personnel and toxicologists [1, 2].

Until 2018, the majority of NSOs detected each year were fentanyl analogues [3]. However, recent years painted a different picture, with drugs chemically different from fentanyl increasingly gaining traction [4, 5]. While U-series analogues (e.g., U-47700) and 2-benzylbenzimidazoles, also known as nitazenes (e.g., isotonitazene), have taken the lead in number of non-fentanyl-related new analogues, plenty of structurally diverse, somewhat obscure, NSOs have made an appearance on the recreational opioid market in the last decade [1, 6]. Drug manufacturers often seem to find inspiration for "new" synthetic opioids in published scientific and/or patent literature [7]. Many U-series analogues, for example, originate from pain research conducted by the Upjohn company in the 1970s [8, 9]. Nitazene analogues can be traced back further in time to the 1950s, when various analogues were studied by CIBA for their potential as analgesics [10, 11]. Recently published literature, too, is being scoured by illicit drug manufacturers. This was exemplified in 2019 by the emergence of brorphine, a benzimidazolone opioid first described in a 2018 research paper [12–14]. Moreover, some NSOs that have been identified on recreational drug markets are analogues or metabolites of known prescription drugs (e.g., *O*-desmethyltramadol) or are registered as opioid medications in some countries but not in others (e.g., tianeptine) [1].

Methadone (**Figure 1**) (e.g., Dolophine®) is an opioid analgesic included in the World Health Organization's Model List of Essential Medicines [15]. While it can be prescribed as an analgesic for the management of cancer pain, it is most commonly used as a maintenance drug for the treatment of opioid use disorder (OUD) [16, 17]. Methadone is particularly useful in this context owing to long-acting, reportedly full μ-opioid receptor (MOR) agonism. Its long duration of action typically enables the use of one dose to prevent opioid withdrawal symptoms for 24 hours [17]. Another drug that is frequently used for the treatment of OUD is buprenorphine (often in combination with the opioid antagonist naloxone, e.g., in Suboxone®) (**Figure 1**) [17, 18]. Unlike methadone, which has a long plasma half-life, the long duration of action of buprenorphine can be attributed to its extremely high MOR binding affinity [19, 20]. Combined with (*in vitro*) partial agonism at MOR, the unique pharmacological profile of buprenorphine has been shown to lead to full analgesia with a ceiling effect

in terms of respiratory depression [17, 20–23]. A potentially confounding factor when studying the *in vivo* effects of buprenorphine, however, is the presence of the active metabolite norbuprenorphine (**Figure 1**). This major *N*-dealkylated metabolite has been shown to have a higher efficacy than the parent drug [19], potentially contributing to the effects of (orally administered) buprenorphine [19, 23–25].

In 2021, two NSOs structurally related to methadone first appeared on the recreational drug market (**Figure 1**) [1]. The first analogue, dipyanone (*N*-pyrrolidino methadone), can be traced back to the 1940s. In 1946, dipyanone was included in a comparative study of new analgesic drugs by Eli Lilly [26, 27]. Further research by Bockmühl and Ehrhart showed that dipyanone had comparable analgesic activity to methadone [28, 29]. Dipyanone was also studied in 1957 by Janssen and Jageneau, who further confirmed that the drug had a similar analgesic potency as methadone [30]. Unlike methadone and other structurally related opioids (e.g., dipipanone or *N*-piperidino methadone, and phenadoxone or *N*-morpholino methadone) that were eventually included in the 1961 Single Convention on Narcotic Drugs, dipyanone is not currently scheduled on the international level [31]. In September 2021, dipyanone became the subject of a formal notification by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Early Warning System (EWS) on NPS after an identification in Germany. That same month, the drug was also identified in a powder reported by the National Forensic Laboratory in Slovenia [32]. In the United States, dipyanone was detected in seized material received in November 2021 [33]. Almost in parallel with the emergence of dipyanone, a second methadone-related NSO (coined desmethylmoramide) was formally notified by the EMCDDA EWS in November 2021 following an identification in Germany [1]. As its name suggests, desmethylmoramide is closely related to the internationally controlled opioid moramide [31]. Desmethylmoramide was never marketed nor scheduled internationally, yet it is listed as an opioid in the "Oxford Catalogue of Opioids" [34] based on its inclusion in the International Nonproprietary Name (INN) System as coordinated by the World Health Organization (WHO). The original synthesis and pharmacological evaluation of desmethylmoramide can be traced back to the 1950s. Van Proosdij-Hartzema and De Jongh showed that desmethylmoramide was about 3.5 times less potent than methadone [35]. A similar *in vivo* potency ratio between the two structural analogues was reported by Janssen and Jageneau in 1957 [30, 36].

Bearing some structural resemblance to methadone [26], ketobemidone (e.g., Ketorax®) is an opioid analgesic known to have *N*-methyl-D-aspartate (NMDA) antagonist properties [37–39] (**Figure 1**). The drug was originally studied in the 1940s, and was evaluated alongside methadone, dipyanone and the closely related methylketobemidone in a study by Scott *et al.* [26, 27]. In preclinical studies,

ketobemidone was more potent than methylketobemidone and somewhat comparable in analgesic potency to methadone. Unlike ketobemidone, methylketobemidone was never marketed or placed under international control [31], possibly due to its weaker analgesic action in animals and reported strong emetic effect in dogs [27]. Of the different evaluated drugs, only methadone and ketobemidone eventually progressed to clinical trials [27]. The acetoxy ester of methylketobemidone (acetoxymethylketobemidone or O-AMKD) (**Figure 1**) emerged on the recreational drug market in 2020. The drug was formally notified to the EMCDDA EWS by Germany after its first identification in an online-sourced powder [1]. Further information on the availability of O-AMKD on the recreational drug market is limited [12, 40] and, while the drug was patented in 1948 [41], details about its pharmacological effects are lacking. In the context of NPS, it is interesting to note that O-AMKD is a structural isomer of the cathinone methylenedioxypyrovalerone (MDPV) [42].

Dipyanone, desmethylmoramide and O-AMKD are all structurally related to known prescription opioids, however, little is known about their effects and harm potential as NSOs. To fill this knowledge gap, the current study aimed at evaluating the intrinsic *in vitro* MOR activation potential of these three poorly studied NSOs. To complete the panel, buprenorphine and norbuprenorphine were also evaluated, together with methadone, ketobemidone, fentanyl, morphine, and hydromorphone. In addition to the *in vitro* experiments, this report details the first analytical characterization and identification of dipyanone in drug powder material and in a postmortem blood specimen.

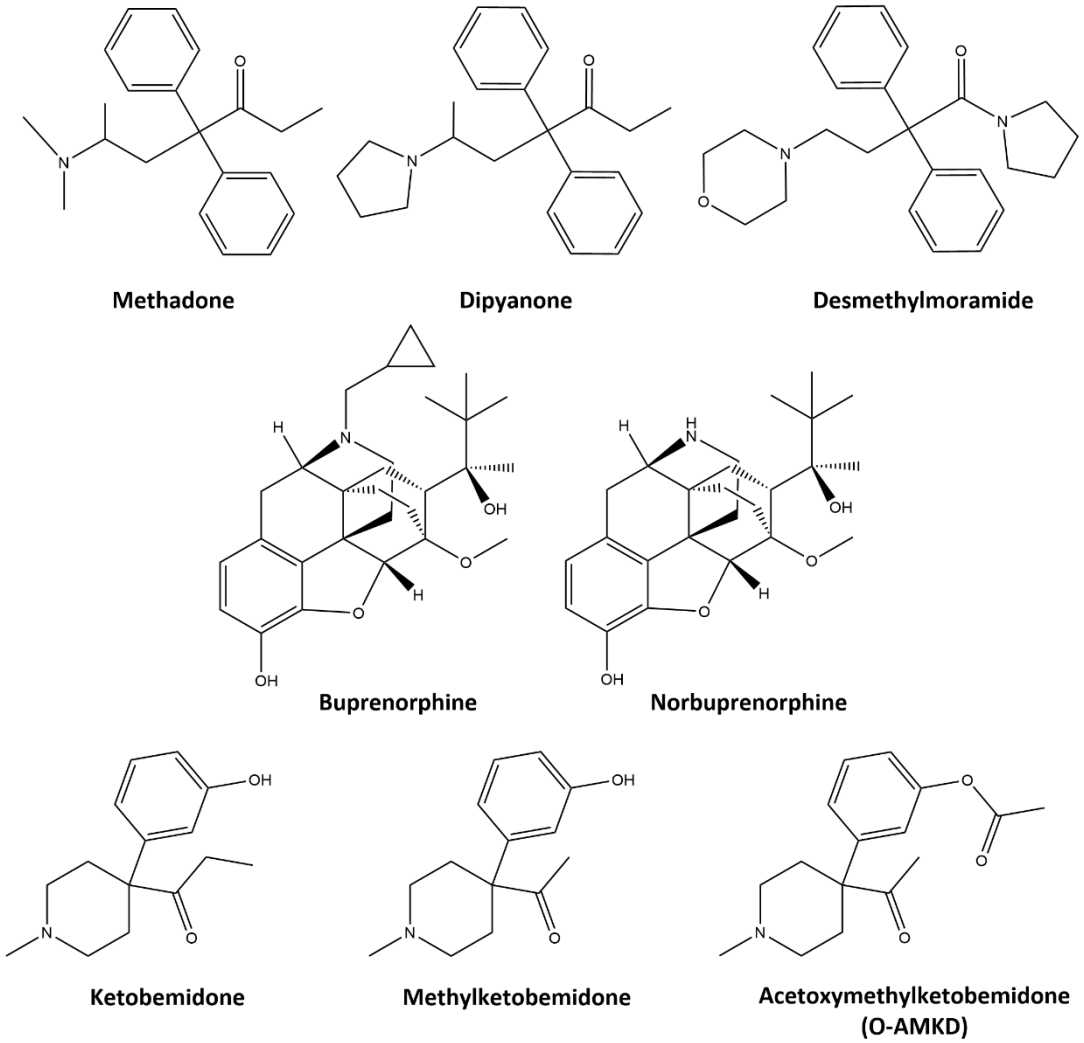


Figure 1 Chemical structures of the different opioids included in the study. Dipyanone (*N*-pyrrolidino methadone), desmethylmoramide, and acetoxymethylketobemidone (O-AMKD) are new synthetic opioids related to the prescription opioids methadone and ketobemidone.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards for morphine, fentanyl, (±)-methadone HCl, buprenorphine HCl, norbuprenorphine, dipyanone HCl, desmethylmoramide, acetoxymethylketobemidone HCl, and methylketobemidone were obtained from Cayman Chemical (Ann Arbor, MI, U.S.). Ketobemidone was from Chiron AS (Trondheim, Norway) and hydromorphone HCl was purchased from Fagron (Nazareth, Belgium). All concentrations reported in this work are expressed as those of the free bases of the compounds. The human embryonic kidney (HEK) 293T cells (passage 20) were kindly gifted by Prof. O. De Wever (Ghent University Hospital, Belgium). Dulbecco's Modified Eagle's Medium (DMEM) (GlutaMAX™), Opti-MEM I Reduced Serum Medium, penicillin-streptomycin (10,000 U/mL and 10,000 µg/mL), and amphotericin B (250 µg/mL) were supplied by Thermo Fisher Scientific (Waltham, MA, U.S.). Fetal bovine serum (FBS) and poly-D-lysine were obtained from Sigma-Aldrich (Darmstadt, Germany). Promega (Madison, WI, U.S.) supplied the Nano-Glo® Live Cell Assay System, containing the Nano-Glo® Live Cell Substrate and Nano-Glo® LCS Dilution Buffer. For toxicology and chemistry testing, standard reference material for dipyanone was purchased from Cayman Chemical as a powder and prepared at a concentration of 1 mg/mL in methanol. Drug-free human blood was purchased from BioIVT (Westbury, NY, U.S.). Sodium borate decahydrate was purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Ethyl acetate, *N*-butyl chloride, liquid chromatograpy-mass spectrometry (LC-MS) grade water and methanol were purchased from Honeywell Chemicals (Charlotte, NC, U.S.). Formic acid was purchased from Thermo Fisher Scientific.

2.2. *In vitro* functional characterization at the µ-opioid receptor

The ability of the test drugs to activate MOR was evaluated using a β-arrestin 2 (βarr2) recruitment assay [43, 44]. In short, activation of human MOR, fused to one part (LargeBiT, LgBiT) of a split nanoluciferase (NanoLuc Binary Technology®, Promega), leads to recruitment of the intracellular protein βarr2, fused to the complementing nanoluciferase subunit (SmallBiT, SmBiT). The presence of G protein-coupled receptor kinase 2 (GRK2) enhances βarr2 recruitment [45]. Upon recruitment of βarr2 to the activated receptor, functional complementation of the nanoluciferase takes place, restoring its enzymatic activity. After addition of the substrate furimazine, a measurable bioluminescent signal is generated.

HEK 293T cells stably expressing the MOR-βarr2-GRK2 assay system were routinely cultured in DMEM (GlutaMAX™, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 mg/L amphotericin). The cells were incubated at 37°C in a

humidified atmosphere containing 5% CO_2. On day one, cells were seeded on white, poly-D-lysine-coated 96-well plates at a density of 5 x 10^4 cells/well. The next day, the cells were washed twice with Opti-MEM® I Reduced Serum Medium, and 90 µL Opti-MEM® was added to each well. Twenty-five µL of Nano-Glo® Live Cell reagent (consisting of a 20-fold dilution of Nano-Glo® Live Cell Substrate with Nano-Glo® LCS Dilution buffer) was subsequently added to the wells. Next, the plate was placed into a Tristar^2 LB942 luminometer (Berthold Technologies GmbH & Co., Bad Wildbad, Germany) and luminescence was continuously monitored until stabilization of the signal (10-15 min). After the equilibration, 20 µL of 6.75x concentrated stock solutions in Opti-MEM® (hydromorphone), Opti-MEM®/MeOH (fentanyl, morphine, ketobemidone, methylketobemidone, methadone, buprenorphine, norbuprenorphine, dipyanone, dipyanone powder, desmethylmoramide), or Opti-MEM®/DMSO (O-AMKD) was added, and luminescence was further monitored for approximately 120 min. Appropriate solvent controls were included in each experiment, and all compounds were tested in concentrations ranging from 1 pM to 100 µM. Based on previous studies from our group [6], hydromorphone was used as a reference agonist for normalization. Fentanyl and morphine were included as common reference opioids [6]. Each compound was tested in at least three independent experiments ($n \geq 3$), with duplicates included for each concentration within an experiment. Absolute luminescence signals were corrected for inter-well variation and solvent controls, after which concentration-responses (areas under the curve, AUCs) were normalized to the maximum response of hydromorphone (set at 100%) using GraphPad Prism 9 (San Diego, CA, U.S.). Normalized data from at least three independent experiments were compiled and three-parameter logistic regression was performed (GraphPad Prism 9) to obtain final potency (EC_{50}) and efficacy (E_{max}, relative to hydromorphone) values for each test drug.

2.3. Analytical characterization

2.3.1. Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed as described previously [46]. In short, an Agilent (Santa Clara, CA, U.S.) 5975 series GC/MSD system was used with a Zebron™ Inferno™ ZB-35HT capillary column (15 m x 250 µm x 0.25 µm) combined with an oven temperature program. Electron impact (EI) ionization was used and masses were acquired from 40 to 550 *m/z*, with a threshold setting of 250. The total run time was 15 min.

2.3.2. Liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS)

LC-QTOF-MS analysis was performed as previously described [46]. In brief, a Sciex (Framingham, MA, U.S.) TripleTOF® 5600+ QTOF was coupled with a Shimadzu Nexera

XR ultra-high performance liquid chromatograph (UHPLC), and a Phenomenex® Kinetex C18 column was used (50 mm x 3.0 mm, 2.6 µm) combined with gradient elution. Positive electrospray ionization (ESI+) was used, as well as an IonSpray Voltage Floating (ISVF) of 2,500 eV and a source temperature of 600 °C. Precursor ion masses were acquired by TOF-MS scan from 100 to 510 Da. Precursor ions were filtered using SWATH® acquisition (27 windows) and fragmented using a collision energy spread (35 ± 15 eV). Product ion masses were acquired by MS/MS scan from 50 to 510 Da. The total run time was 15.5 min.

2.3.3. High-performance liquid chromatography-diode array detection (HPLC-DAD)

Reversed-phase separation was performed on a LaChrom HPLC system from Merck-Hitachi (Tokyo, Japan) as previously described [11]. The column oven was set at 30 °C. A mobile phase consisting of (A) 250 mM phosphate buffer, water, and methanol (4:86:10, *v:v*) and (B) 250 mM phosphate buffer, water, and methanol (4:21:75, *v:v*) at a flow rate of 1 mL/min was used. The gradient was as follows: 0.00 min (95% A and 5% B), 8.00 min (75% A and 25% B), 16.00 min (45% A and 55% B), 24.00 min (5% A and 95% B), 29.00 min (5% A and 95% B), and 30.00 min (95% A and 5% B). Detection was done via diode array detection (DAD) [11]. Fifty µL of a 20 µg/mL solution of the dipyanone powder was injected for analysis.

2.3.4. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR)

All one-dimensional and two-dimensional experiments were collected on a 600 MHz Bruker Avance III spectrometer (Billerica, MA, U.S.) operating at 600.1 MHz for proton (^{1}H) and 150.9 MHz for carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy. The probe for acquisition was a 5 mm TXI H{CN} cryoprobe. The temperature for the sample was -1.0 °C. The sample (12 mg) was dissolved in approximately 0.7 mL deuterated methanol (CD_3OD). Chemical shifts were reported in delta (δ) units, parts per million (ppm) relative to tetramethylsilane (TMS). The spectra were referenced to the CHD_2OD peak at 3.30 ppm for ^{1}H and 49.00 ppm for ^{13}C. The ^{1}H-NMR spectrum was acquired with 16 scans, having a spectral window of 12.0 KHz, spectral offset of 3.7 KHz, and 32,768 data points for resolution of 0.37 Hz. The acquisition time was 2.7 s, with a recycle delay of 1.0 s. The ^{13}C-NMR spectrum was collected with 1024 scans, using a spectral window of 36.2 KHz, spectral offset of 15.0 KHz, and 32,768 data points for resolution of 1.1 Hz. The acquisition time was 0.9 s, with a recycle delay of 2.0 s. The double-quantum filtered correlated spectroscopy (DQF-COSY) spectrum was

obtained with 4 scans, with 1024 points in t_2 with 128 t_1 increments using a pulse width of 9.72 µs, with an acquisition time of 0.13 s. The spectral window for COSY was 7.8 KHz for 1H in both dimensions. The multiplicity-edited heteronuclear single quantum coherence (HSQC) spectrum was obtained with 16 scans, with 512 points in t_2 with 256 t_1 increments using a pulse width of 9.72 µs, with an acquisition time of 0.061 s. The spectral window for the HSQC was 8.4 KHz for ^{1}H and 24.9 KHz for ^{13}C. All data processing was performed in SpinWorks 4.2.10 (2019, by Kirk Marat, University of Manitoba, Canada).

2.3.5. Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared (FTIR) spectroscopy was performed using a Thermo Scientific Nicolet iS5 FTIR with a single-bounce iD7 diamond attenuated total reflectance (ATR) accessory. The resolution was 4 cm^{-1} and 32 scans were collected. The spectral range was 4000 to 400 cm^{-1}. The sample was directly applied to the crystal for analysis and the pressure tower was used to ensure consistent contact between the powder and the diamond.

2.4. Toxicological analysis

Comprehensive toxicological analysis was performed at the Center for Forensic Science Research and Education (CFSRE) (Willow Grove, PA, U.S.) for dipyanone and other drugs. Comprehensive drug screening was performed by LC-QTOF-MS using a SCIEX X500R (Framingham, MA, U.S.) with SWATH™ acquisition and a reference library of more than 1,100 recreational drugs, therapeutic agents, and NPS. Targeted data processing for dipyanone was employed using comparison of the acquired analyte to the corresponding reference material, including retention time difference, exact mass, isotope score, and library match.

Quantitation of dipyanone was performed via liquid chromatography tandem quadrupole mass spectrometry (LC-QQQ-MS) using the method of standard addition [47, 48]. An analytical method specific to dipyanone was developed and validated for application to biological specimens that screened positive for the drug.

To determine the concentration of dipyanone, four replicate samples (0.5 mL) were aliquoted and prepared via fortification with dipyanone. Briefly, one sample remained “blank” with no drug standard added, while three samples were “up-spiked” to 1, 10, and 100 ng/mL. Fentanyl-D_5 was used as the internal standard at a final concentration of 10 ng/mL. A single-step, basic liquid-liquid extraction was performed using 1 mL of borax buffer (10 mM, pH 10.4) and 3 mL

of extraction solvent (*N*-butyl chloride, ethyl acetate, 70:30 *v:v*). The samples were dried under air and reconstituted in 50:50 initial chromatographic conditions. Analysis was conducted using a Waters TQ-S micro LC-QQQ-MS (Milford, MA, U.S.). Chromatographic separation was achieved using gradient elution with 0.1% formic acid in water (MPA) and 0.1% formic acid in methanol (MPB) over an Agilent InfinityLab Poroshell 120 EC-C18 (3.0 x 150 mm, 2.7 µm) analytical column. The chromatographic gradient was as follows: 60A:40B initial, 5A:95B at 3 min with a 1.5-min hold, 60A:40B at 4.6 min with a 0.4-min hold, with a final run time of 5 min. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The column temperature was 60 °C. Following positive electrospray ionization (ESI+), multiple reaction monitoring was used for mass filtration and detection. Three transitions (*m/z* 336.2 > *m/z* 265.2 [quantification ion], 105.1, and 57.1) were monitored for dipyanone. Analyte-internal standard peak area ratios were plotted against the up-spiked concentrations to determine the concentration of dipyanone in the patient blood sample. A linear regression was used to assess correlation (R^2>0.98) between the data points and the concentration of dipyanone was determined by back-calculation of the absolute value of the x-intercept.

3. Results

3.1. *In vitro* functional characterization at the μ-opioid receptor

The intrinsic MOR activation potential of the different opioids was assessed using a βarr2 recruitment assay. All compounds were capable of activating MOR, with potencies ranging from 1.35 nM (buprenorphine) to 1.33 μM (desmethylmoramide) (**Table 1**). Dipyanone (EC_{50} = 39.9 nM) was the only NSO with a higher potency than morphine (EC_{50} = 142 nM), and none of the newly tested NSOs were more potent than fentanyl (EC_{50} = 9.35 nM). With the exception of buprenorphine, a partial agonist (E_{max} = 23.2%) compared to hydromorphone, the efficacy of the test drugs was comparable to or exceeding that of hydromorphone (range 98.6-162%).

Table 1 Overview of the potency (EC_{50}) and efficacy (% relative to hydromorphone) values of the different test compounds, fentanyl, and morphine, as obtained in the MOR-βarr2-GRK2 recruitment assay. 95% confidence intervals are given between parentheses.

	EC_{50} (nM)	**E_{max} (%)**
Methadone	50.3 (38.0-66.0)	152 (145-159)
Dipyanone	39.9 (28.2-56.1)	155 (147-163)
Desmethylmoramide	1335 (910-1963)	126 (116-137)
Buprenorphine	1.35 (0.842-2.24)	23.2 (21.8-24.6)
Norbuprenorphine	2.94 (1.97-4.41)	162 (152-171)
Ketobemidone	134 (89.7-204)	156 (146-165)
Methylketobemidone	335 (246-454)	117 (111-123)
O-AMKD	1262 (852-1873)	109 (101-118)
Fentanyl	*9.35 (6.33-13.8)*	*146 (138-155)*
Morphine	*142 (85.4-239)*	*98.6 (91.9-105)*
Hydromorphone	*25.8 (18.0-37.2)*	*100 (94.6-106)*

With EC_{50} values in the low nM range, buprenorphine (EC_{50} = 1.35 nM) and norbuprenorphine (EC_{50} = 2.94 nM) were the most potent compounds of the panel. While comparable in terms of potency, norbuprenorphine (E_{max} = 162%) was about 7 times more efficacious than buprenorphine (E_{max} = 23.2%) in activating MOR. Methadone (EC_{50} = 50.3 nM; E_{max} = 152%) was an order of magnitude less potent than (nor)buprenorphine, and about equally efficacious as norbuprenorphine (**Figure 2**).

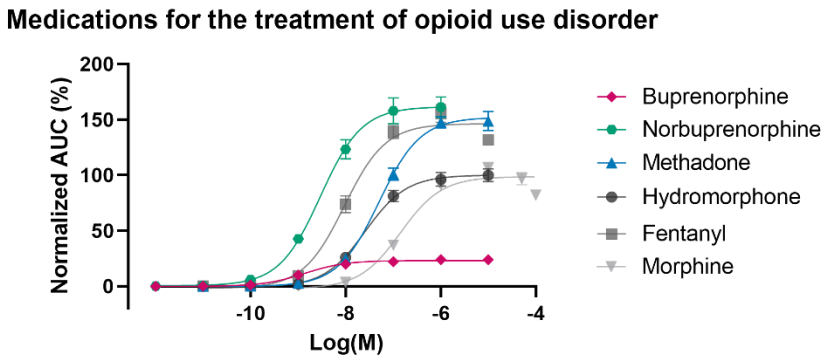


Figure 2 Concentration-response curves obtained in the MOR activation assay for buprenorphine, norbuprenorphine, methadone, and different reference opioids. Data are shown as mean receptor activation ± standard error of the mean (SEM), normalized to the maximum response of hydromorphone (100%). AUC, area under the curve.

Dipyanone (EC_{50} = 39.9 nM; E_{max} = 155%) and methadone showed a comparable opioid activity (potency and efficacy) in the employed *in vitro* assay. Desmethylmoramide, on the other hand, was considerably less potent and somewhat less efficacious (EC_{50} = 1335 nM; E_{max} = 126%) (**Figure 3, panel A**). The concentration-response curve of a seized dipyanone powder showed an almost perfect overlap with that of the dipyanone reference standard (**Figure 3**, **panel B**), indicating a high purity of the seized powder, as confirmed via chemical analysis (see **3.2**).

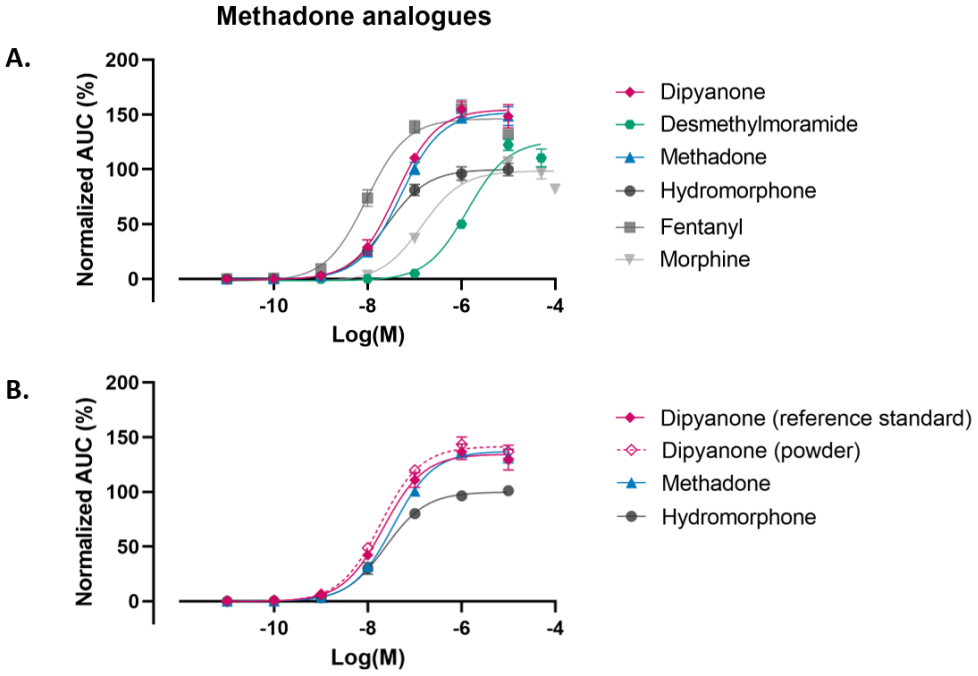


Figure 3 Concentration-response curves obtained in the MOR activation assay for different methadone analogues. **Panel A**: Dipyanone, desmethylmoramide, methadone, and different reference opioids. **Panel B**: Comparison of the receptor activation potential of the seized dipyanone powder and the dipyanone reference standard. Data are shown as mean MOR activation ± standard error of the mean (SEM), normalized to the maximum response of hydromorphone (100%). AUC, area under the curve.

Among the different ketobemidone analogues, ketobemidone (EC_{50} = 134 nM; E_{max} = 156%) itself was the most potent and most efficacious compound, followed by methylketobemidone (EC_{50} = 335 nM; E_{max} = 117%). O-AMKD (EC_{50} = 1262 nM; E_{max} = 109%) was about ten times less potent and had an efficacy of about 70% of that of ketobemidone (**Figure 4**).

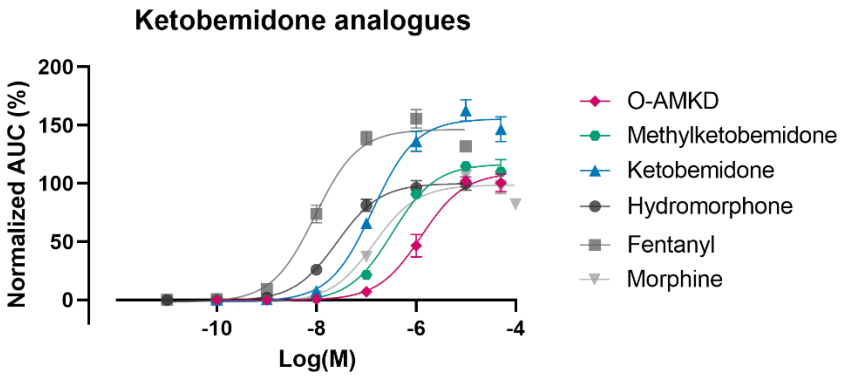


Figure 4 Concentration-response curves obtained in the MOR activation assay for O-AMKD, methylketobemidone, ketobemidone, and different reference opioids. Data are shown as mean receptor activation ± standard error of the mean (SEM), normalized to the maximum response of hydromorphone (100%). AUC, area under the curve.

3.2. Analytical characterization

GC-MS analysis of the drug material resulted in a prominent chromatographic peak at 6.84 min (**Figure 5, panel A**), with no other peaks of interest (except for two internal standards used during analysis). The fragmentation pattern (**Figure 5, panel B**) showed the presence of a base peak at *m/z* 98 and a molecular ion of *m/z* 335. Processing of the powder sample against our in-house library database yielded a positive result for dipyanone. The retention time of the standard was 6.83 min and the MS data was considered to be a high quality match. The *m/z* 98 fragment ion is consistent with the pyrrolidino ring of the structure.

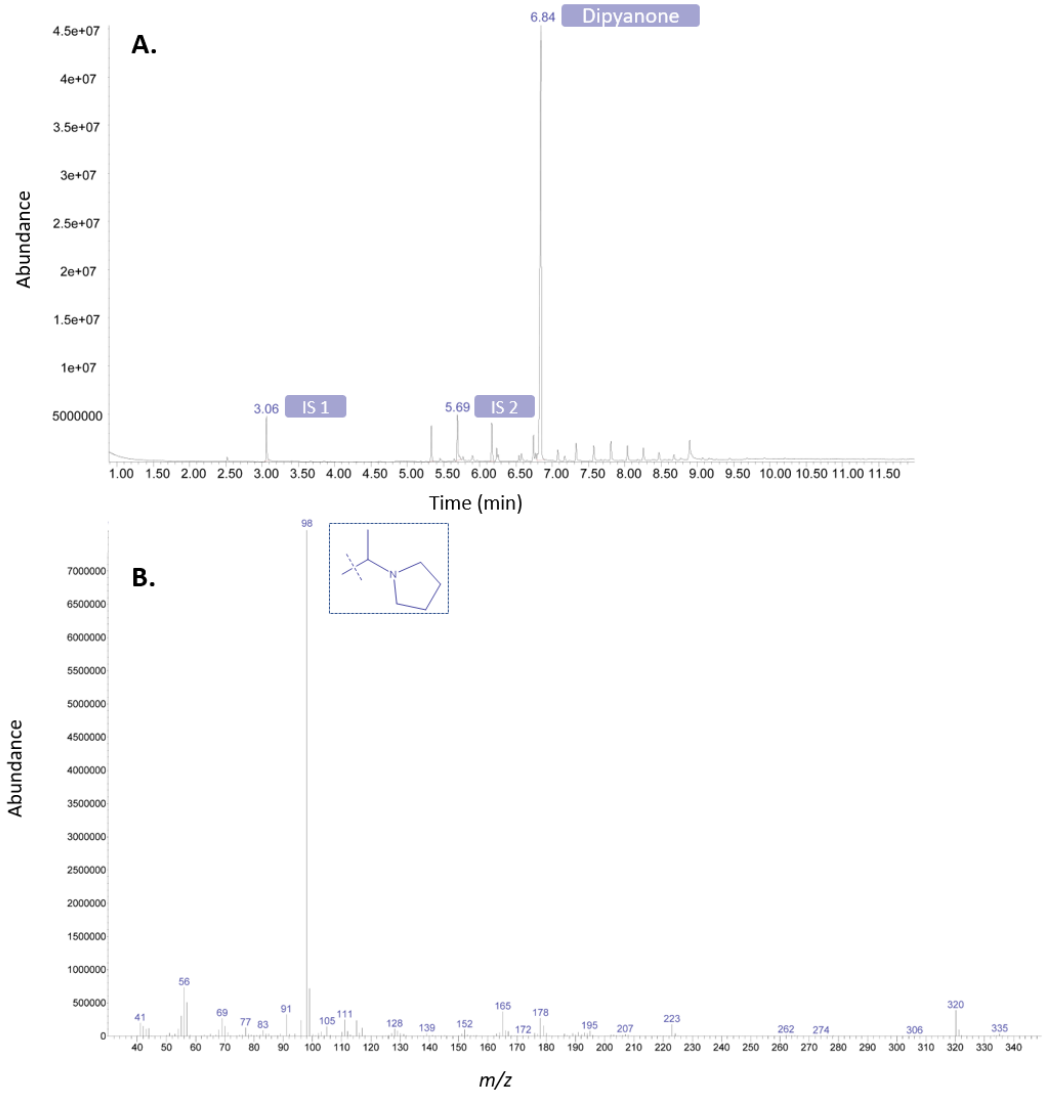


Figure 5 GC-MS data acquired from drug material confirmed to contain dipyanone. **Panel A**: Total ion chromatogram (IS, internal standard). **Panel B**: Mass spectrum of dipyanone.

LC-QTOF-MS analysis of the drug material resulted in a prominent chromatographic peak at 7.50 min (**Figure 6, panel A**). Processing of the sample against our in-house library database yielded a positive result for dipyanone. The TOF-MS data showed the presence of a precursor ion at 336.2330 Da, with no noticeable halogen isotopic contributions (e.g., Cl, Br) (data not shown). The mass error for this identification was 2.2 ppm. The retention time of the standard was 7.60 min. The MS/MS data of the product ion spectrum (**Figure 6, panel B**) was considered to be a high quality match between the drug material and the standard. The 265.1531 Da fragment ion is consistent with the portion of the structure minus the pyrrolidino ring, the 223.1066 Da fragment ion is consistent with the portion of the structure minus the pyrrolidino ring and its connecting three-carbon alkane chain, and the 91.0503 Da fragment ion is consistent with the tropylium ions produced by either phenyl groups.

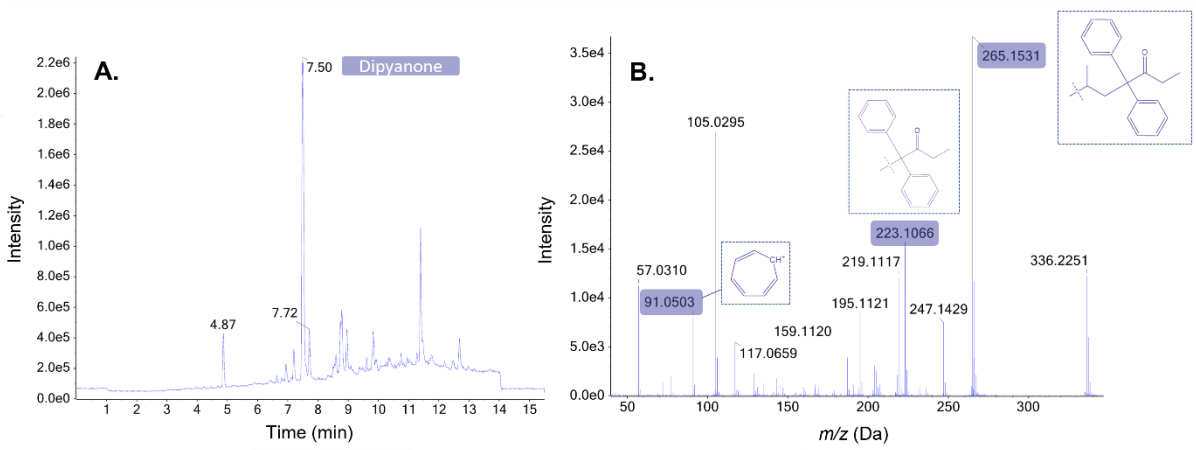


Figure 6 LC-QTOF-MS data acquired from drug material confirmed to contain dipyanone. **Panel A**: Total ion chromatogram. **Panel B**: Product ion mass spectrum (precursor ion at 336.2330 m/z) obtained with a collision energy spread of 35 ± 15 eV.

HPLC-DAD analysis revealed a single peak, eluting at 21.96 min, and an absorption maximum at 289.9 nm (**Figure 7**). The wavelength spectrum for dipyanone is reminiscent of that of methadone.

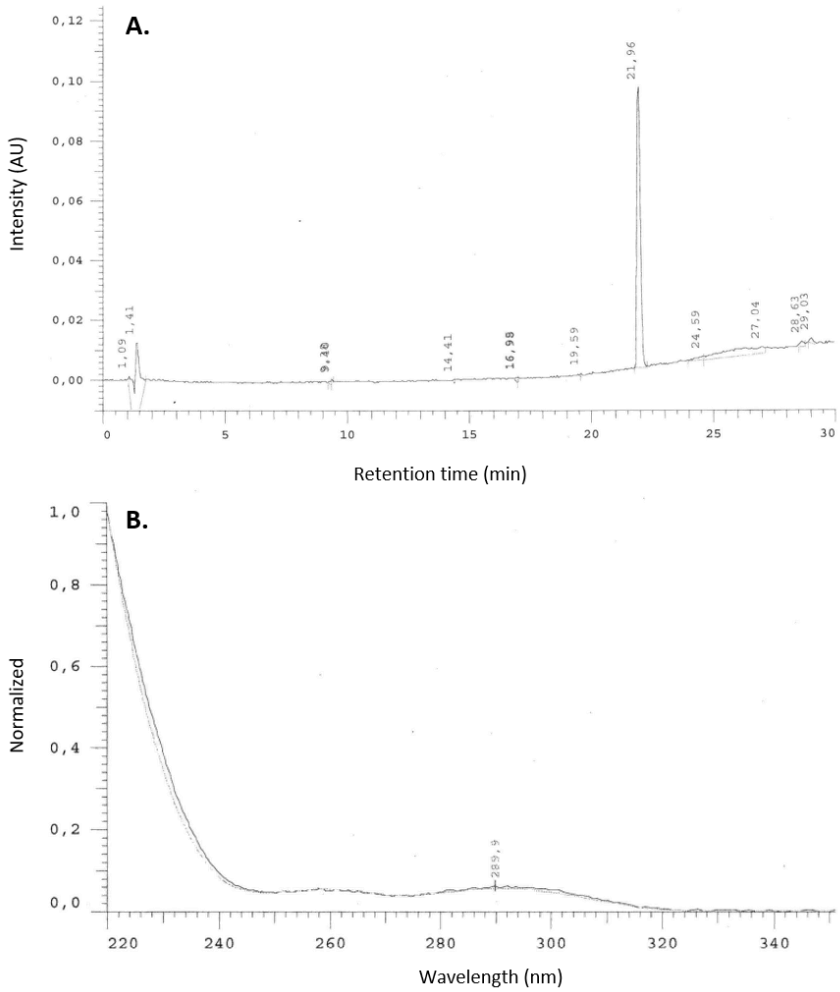


Figure 7 HPLC-DAD data acquired from drug material confirmed to contain dipyanone. **Panel A**: Chromatogram. **Panel B**: Wavelength spectrum.

^{1}H-NMR analysis of the drug material resulted in interpretable spectral peaks (**Figure 8**): δ 7.46 (m, 4H), 7.36-7.41 (m, 4H), 7.23 (d, 2H), 3.51-3.59 (d, 2H), 3.32 (br s, 1H), 3.18 (m, 2H), 3.10 (2, 1H), 2.53 (m, 1H), 2.23 (m, 1H), 2.15 (m, 1H), 2.03-2.08 (br d, 4H), 0.82 (m, 3H), and 0.58 (d, 3H). In addition, also ^{13}C-NMR analysis of the drug material resulted in interpretable spectral peaks (**Figure S1**): δ 213 (1C), δ 141 (2C), δ 129-130 (10C), δ 66 (1C), δ 59 (1C), δ 50-52 (2C), δ 42 (1C), δ 34 (1C), δ 24 (2C), δ 16 (1C), and δ 9 (1C). Additional two-dimensional (e.g., COSY, HSQC) NMR spectra are included in the **Supplementary Material (Figures S2-S3)**. **Table 2** shows the assignment of Hs and Cs in comparison to respective chemical shifts. Assignments were determined based on cross-spectral comparisons. All peaks were accounted for and matched proposed assignments. The results confirm the structure of the molecule in the drug material to be dipyanone.

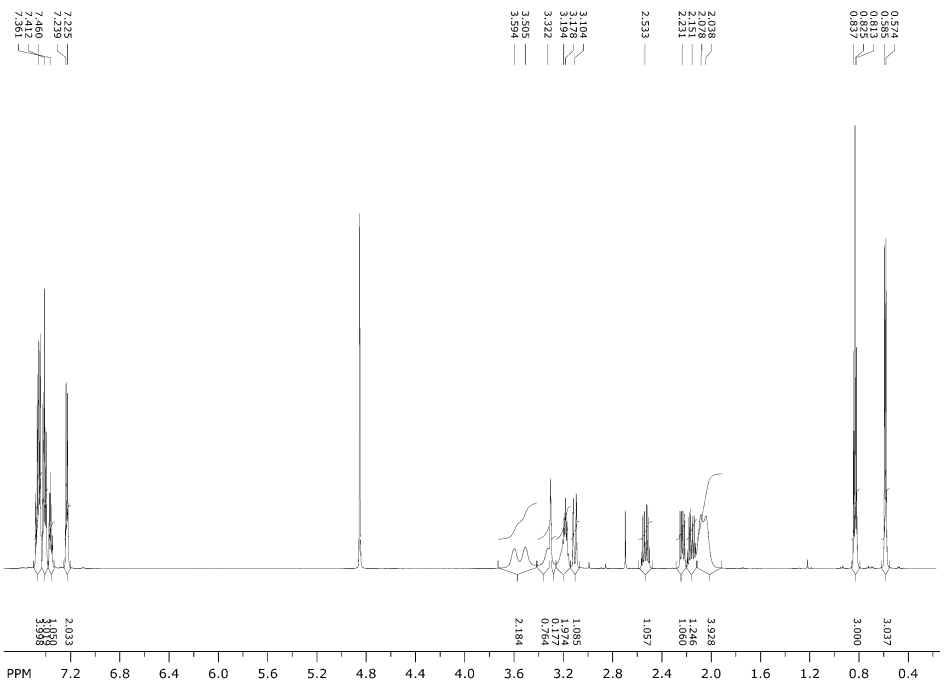
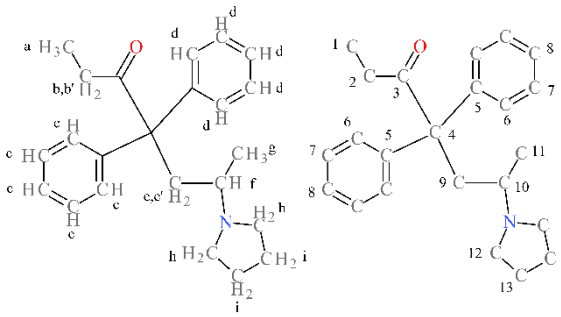


Figure 8 ^{1}H-NMR data acquired from drug material confirmed to contain dipyanone.

Table 2 Interpretation of NMR results compared to the chemical structure of dipyanone.

H Label	^{1}H δ	Labeled Structure	C Label	^{13}C δ
a	0.82		1	9
b	2.53		2	34
b'	2.15		3	213
c	7.23-7.46		4	66
d	7.23-7.46		5	141
e	3.10		6,7,8	129-130
e'	2.23		9	42
f	3.19		10	59
g	0.58		11	16
h	3.17-3.59		12	50-52
i	2.03-2.08		13	24

FTIR analysis of the drug material resulted in a characteristic spectrum (**Figure 9**). Aromatic C-H stretches were observed around 3000 cm^{-1}. The carbonyl moiety of the propionyl group exhibited a 1703 cm^{-1} stretch. The fingerprint region is complex, owing to the more sophisticated nature of the molecule. Overall, these results are further consistent with the drug material containing dipyanone.

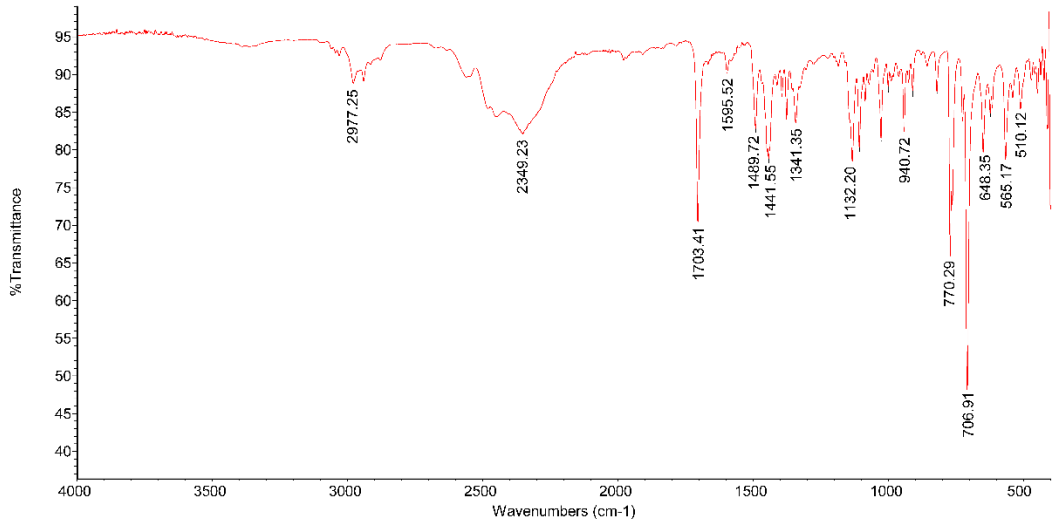


Figure 9 FTIR data acquired from drug material confirmed to contain dipyanone.

3.3. Toxicological analysis

Dipyanone was identified in an authentic blood sample collected in September 2022 in the Unites States. A summary of the case history, demographic information, and analytical findings is shown in **Table 3**. Dipyanone was quantitatively confirmed at a concentration of 370 ng/mL in femoral blood. The drug was found alongside other NSOs (e.g., 2-methyl AP-237) and designer benzodiazepines (e.g., 8-aminoclonazolam, flualprazolam).

Table 3: Summary of a postmortem forensic toxicology case positive for dipyanone.

Collection Date	September 2022
State, Country	Washington, United States
Sex, Age	Male, 30s
Matrix	Femoral Blood
Case History	Individual was found deceased at home during a welfare check. Emergency medical services confirmed death without intervention. Signs of vomiting were apparent. No signs of trauma or foul play. The decedent had a history of depression, anxiety, and sleep apnea. White powder residue and paraphernalia were found alongside the decedent. Presumptive urine screen was positive for methadone, tramadol, and methamphetamine.
Autopsy Findings	Pulmonary congestion, cerebral edema, and hepatic steatosis
Manner and Cause of Death	Accident / Acute mixed drug toxicity (clonazolam, flualprazolam, delorazepam, **dipyanone**, 2-methyl AP237)
[Dipyanone] (ng/mL)	370
Additional blood toxicology results (ng/mL)	2-Methyl AP-237 **(24)**, 8-aminoclonazolam **(7.5)**, flualprazolam **(5.7)**, delorazepam **(6.2)**, bupropion **(100)**, hydroxybupropion **(250)**, *O*-desmethyltramadol **(560)**, chlorpheniramine **(48)**, dextrorphan/levanorphanol **(33)**, dextro/levomethorphan **(450)**, citalopram **(490)**, pseudoephedrine **(180)**

4. Discussion

For millennia, opioids have been extensively studied and prescribed for the treatment of severe pain [29]. The downside of their widespread availability is the broad misuse of opioids, which has culminated in a true overdose crisis in the United States [49]. In addition to the misuse of traditional prescription opioids (e.g., oxycodone), heroin, and illicitly manufactured fentanyl, the last decade has witnessed the increasing emergence of a new generation of synthetic opioids [2, 49, 50]. Often highly potent drugs, NSOs have quickly become a significant cause of mortality and overdose-related fatalities [50]. While great strides have been made in the implementation of (generic) legislations targeting (groups of) NPS opioids, the large body of literature dedicated to opioid drug discovery provides a seemingly endless source of inspiration for "new" opioids to be diverted to recreational drug markets [4, 6, 51]. In many cases, such analogues were only briefly studied in the mid-1900s, and were never marketed due to an unfavorable safety profile or a lack of further research interest and/or funding [7, 10]. As a result, there is typically only limited information available about the risks associated with the unregulated availability of novel synthetic opioids on the recreational drug market. In the current work, we used *in vitro* MOR activation experiments to shed light on the opioid activity of recent NSOs related to the prescription opioids methadone and ketobemidone. Our findings may help mitigate the risks associated with the increasing emergence of NSOs [52].

Dipyanone (*N*-pyrrolidino methadone) and desmethylmoramide can be considered the first NSOs that are structurally related to methadone. Both drugs emerged on recreational drug markets in the second half of 2021 [1, 33]. Our data indicate that dipyanone (EC_{50} = 39.9 nM; E_{max} = 155%) is about equally active as methadone (EC_{50} = 50.3 nM; E_{max} = 152%) in terms of *in vitro* human MOR activation potential. This is in line with the findings of a 1949 study by Bockmühl and Ehrhart, who reported that the antinociceptive activity of both methadone (compound 10820) and dipyanone (compound 10819) was comparable (i.e., about 5-10 times that of pethidine (Demerol®)) after subcutaneous administration in the mouse tail pinch test [28, 29, 53]. Similarly, Janssen and Jageneau [30] reported comparable antinociceptive potencies for methadone (ED_{50} = 5.18 mg/kg) and dipyanone (compound R833; ED_{50} = 6.82 mg/kg) after subcutaneous administration in a mouse hot plate assay. Dipyanone has also been evaluated following peroral administration in humans [26]. Using a thermal radiation technique, the analgesic threshold dose for dipyanone (compound 10819; 1 mg/kg) was half that of methadone (compound 10820; 2 mg/kg) in this study [26]. Conversely, in a study using rats and dogs, the analgesic threshold dose for intraperitoneally administered dipyanone (2 mg/kg in both species) was double that found for methadone (1 mg/kg in both species) [26, 27]. In mice, methadone and dipyanone showed comparable toxicity (LD_{50} ~ 17 mg/kg) [26, 27]. Taken together, while differences in e.g. species, route

of administration, pharmacokinetics and metabolism may impact the eventual in vivo outcome (the study of which was outside the scope of the current research), our *in vitro* results are generally well in line with available literature on dipyanone and methadone, and indicate that both drugs have a similar intrinsic MOR activation potential, with a potency about twice that of morphine [27]. Furthermore, it is interesting to note that dipyanone was also studied alongside ketobemidone (compound 10720) and methylketobemidone (compound 10726) in the work by Scott *et al.* [26, 27]. Finally, our *in vitro* experiments indicate that dipyanone is considerably less potent than fentanyl (EC_{50} = 9.35 nM), but has a comparable efficacy ($E_{max,dipyanone}$ = 155% and $E_{max,fentanyl}$ = 146%). With the caveat that *in vitro* results are not necessarily predictive of *in vivo* effects, the obtained maximum MOR activation potential of dipyanone indicates that high doses of the drug may induce a level of opioid effects comparable to that produced by fentanyl.

In addition to the *in vitro* pharmacological characterization of a dipyanone reference standard (purity ≥ 98%), comprehensive chemical and pharmacological characterization was performed on a seized powder that was received in November 2021 [33]. Using various analytical techniques, dipyanone was unequivocally identified in the powder. In addition, while no dedicated purity testing was performed, none of the applied techniques could distinguish the obtained powder from the reference standard. Hence, the analytical characterization together with our *in vitro* results pointed towards a high purity of the powder (i.e., no peaks potentially related to impurities could be identified, and the concentration-response curves for the powder and the analytical reference standard showed an almost perfect overlap).

The NSO dipyanone was detected for the first time in a postmortem toxicology case from Washington state in the United States. Interestingly, the initial presumptive screening of the urine showed positivity for methadone, the presence of which could not be confirmed in blood. Given the close structural similarity between methadone and dipyanone, cross-reactivity in immunoassays is not surprising. Dipyanone was found at a relatively elevated concentration (370 ng/mL) comparative to other synthetic opioids (typically ~1-20 ng/mL) [54], which further solidifies that dipyanone is on the lower potency scale of synthetic opioids, corroborating the *in vitro* data presented in this manuscript. In this case, dipyanone was found alongside other novel drugs, including other synthetic opioids and designer benzodiazepines, showing an example of the drug combination “benzo-dope” that is increasing in prevalence across North America [55]. It is interesting to note that the concentration of dipyanone in this case was considerably larger than that of 2-methyl AP-237 (24 ng/mL), which is also considered a lower potency NSO with an EC_{50} value of 749 nM in the same βarr2 recruitment assay as employed here [56]. Of note, postmortem cases involving 2-methyl AP-237 have been reported to contain

concentrations of the drug exceeding 800 ng/mL [56], hence in this case the relative contribution to the overall opioid effects are anticipated to be limited.

Approximately 30 times less potent and somewhat less efficacious than methadone and dipyanone, desmethylmoramide was one of the least active NSOs of the panel (EC_{50} = 1335 nM; E_{max} = 126%). The drug was ~ 10 times less potent than morphine (EC_{50} = 142 nM; E_{max} = 98.6%), although it did activate the receptor with a somewhat higher efficacy, potentially indicating stronger opioid effects with desmethylmoramide. In 1957, an antinociceptive potency of 17 mg/kg was reported for desmethylmoramide using a tail-flick assay in rats [35]. By comparison, methadone (ED_{50} = 4.8 mg/kg) and morphine (ED_{50} = 7.6 mg/kg) were about 3.5 and 2 times more potent than desmethylmoramide in this study [35]. Subcutaneous administration of desmethylmoramide (compound R530) in a mouse hot plate test resulted in an *in vivo* potency of 13.6 mg/kg, which was approximately 2-2.5 times higher than the potencies of dipyanone (ED_{50} = 6.82 mg/kg) and methadone (ED_{50} = 5.18 mg/kg). Morphine (ED_{50} = 12.0 mg/kg) showed a more comparable potency as desmethylmoramide in this study [30, 36]. Taken together, our *in vitro* findings mirror the trends observed in early *in vivo* experiments and indicate a substantially lower opioid activity for desmethylmoramide as compared to the structural analogues dipyanone and methadone. While more research is needed to confirm these findings in humans, it can be expected that larger doses are needed to obtain significant opioid effects with desmethylmoramide compared to dipyanone, methadone or fentanyl.

O-AMKD emerged on the recreational opioid markets in 2020, approximately one year prior to the first emergence of dipyanone and desmethylmoramide [1]. We found that O-AMKD (EC_{50} = 1262 nM) was approximately 4 times less potent than methylketobemidone (EC_{50} = 335 nM), and 9 times less potent than ketobemidone (EC_{50} = 134 nM). In terms of efficacy, O-AMKD (E_{max} = 109%) and methylketobemidone (E_{max} = 117%) showed a comparable maximum MOR activation, whereas ketobemidone was more efficacious than both structural analogues (E_{max} = 156%). Methylketobemidone was less active than ketobemidone *in vitro*, a finding that reflects early *in vivo* studies in which methylketobemidone (compound 10726) was about 8-16 times less potent than ketobemidone (compound 10720) in rats and dogs [26, 27]. While O-AMKD was included in a CIBA patent from 1949 [41], to the best of our knowledge, the current study represents the only pharmacological evaluation of O-AMKD published to date. Like desmethylmoramide, O-AMKD is not among the most intrinsically harmful NSOs when considering its µM potency and somewhat intermediate efficacy at MOR (comparable to or exceeding that of morphine, but below that of fentanyl) [6]. However, other factors such as off-target effects and/or the formation of active

metabolites (including potential formation of the more active methylketobemidone) might contribute to the eventual *in vivo* toxicity profile of O-AMKD.

To the best of our knowledge, no fatalities linked to O-AMKD or desmethylmoramide have been reported to date, and this report details the first postmortem case involving dipyanone. In this context, different supply and demand factors should be considered, as well as the possibility of toxicology labs not actively screening for these NSOs. Moreover, it can be hypothesized that, compared to e.g. nitazene opioids [11], the overall relatively limited MOR activation potential of the NSOs covered here may explain the (hitherto) apparent lack of popularity of these drugs on the NPS opioid market. Contributing factors impacting a drug's popularity may include e.g. the required amount of drug to be taken (affecting the pricing per dose), the obtained "high" in users, etc. [51]. In addition, as recently hypothesized for the class of cinnamylpiperazine NSOs (e.g., AP-237), the µM *in vitro* potency of desmethylmoramide and O-AMKD suggests a lower risk of overdose *in vivo*, and, consequently, fewer forensic postmortem cases [56]. On the other hand, the moderate opioid activity of NSOs such as dipyanone suggests that these drugs could be used as non-registered alternatives to methadone in an attempt to minimize opioid withdrawal symptoms. Some online forum discussions among opioid NPS users indeed indicate the use of dipyanone and desmethylmoramide for this purpose. Importantly, the use of non-registered drugs for self-treatment without medical supervision brings along a series of risks (e.g., unknown purity of the preparation, pharmacokinetics, interactions with other drugs, etc.).

In the context of opioids used for maintenance treatment, we also performed *in vitro* functional characterization of buprenorphine and its major *N*-dealkylated metabolite norbuprenorphine. Both compounds showed a comparable potency in the low nM range ($EC_{50,bup}$ = 1.35 nM; $EC_{50,nor}$ = 2.94 nM). Notably, buprenorphine was a partial agonist compared to hydromorphone (E_{max} = 23.2%), whereas norbuprenorphine largely exceeded the efficacy of hydromorphone (E_{max} = 162%). A similar trend in efficacy difference between buprenorphine and norbuprenorphine was previously reported using a [^{35}S]GTPγS assay in CHO cells stably expressing the rat MOR [19]. However, in that assay, norbuprenorphine (EC_{50} = 1.5 nM) was less potent than buprenorphine (EC_{50} = 0.08 nM) [19]. The high opioid activity of norbuprenorphine indicates that this metabolite may contribute to the *in vivo* effects of buprenorphine [19, 23, 57].

5. Conclusion

In summary, we investigated the *in vitro* MOR activation potential of NSOs that are structurally related to methadone (dipyanone and desmethylmoramide) and ketobemidone (O-AMKD). While the studied opioids are intrinsically less potent than fentanyl, their uncontrolled availability and unsupervised use

are reasons for concern. The reported identification and quantification of dipyanone in a fatality (370 ng/mL) underscores this warning. This report further details the first identification and full chemical analysis (GC-MS, LC-QTOF-MS, HPLC-DAD, NMR, FTIR) of dipyanone in a seized powder. Careful monitoring is required to detect other (potentially more potent) NSOs related to prescription opioids that may emerge on recreational drug markets.

6. Declarations

Conflict of Interest

The authors have no financial or non-financial interests to disclose.

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