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First Report on Brorphine: The Next Opioid on the Deadly New Psychoactive Substance Horizon?

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Abstract

New psychoactive substances continue to appear on the drug market. Until recently, new synthetic opioids, which are among the most dangerous new psychoactive substances, primarily encompassed analogs of the potent analgesic fentanyl. Lately, also other new synthetic opioids have increasingly started to surface. This is the first report on the identification and full chemical characterization of brorphine, a novel potent synthetic opioid with a piperidine benzimidazolone structure. A powder, identified as brorphine, was obtained from a patient seeking medical help for detoxification. Brorphine was also found in a serum sample of the patient. Liquid chromatography–high-resolution mass spectrometry (LC–HRMS) identified an exact mass of *m/z* 400.1020 and 402.1005 for the compound, corresponding to both bromine isotopes. Further chemical characterization was performed by gas chromatography–mass spectrometry, liquid chromatography–diode array detection and Fourier-transform infrared spectroscopy analyses. Finally, the structure was confirmed by performing ^{1}H-NMR and ^{13}C-NMR spectroscopy. *In vitro* biological activity of brorphine was determined by a cell-based μ-opioid receptor activation assay, resulting in an EC_{50} of 30.9 nM (13.5 ng/mL) and an E_{max} of 209% relative to hydromorphone, confirming the high potency and efficacy of this compound. In a serum sample of the patient, brorphine and a hydroxy-metabolite were found using the LC–HRMS screening method. The presence of opioid activity in the serum was also confirmed via the activity-based opioid screening assay. The occurrence of brorphine is yet another example of how the illicit drug market is continuously evolving in an attempt to escape international legislation. Its high potency poses a serious and imminent health threat for any user.

Introduction

New psychoactive substances (NPS) intend to mimic the (pharmacological) effects of known prescription/controlled medications or illicit drugs and are sold as (allegedly) legal alternatives. By the end of 2019, 731 NPS had been notified to the European Early Warning System (1, 2) and 950 to the United Nations Office on Drugs
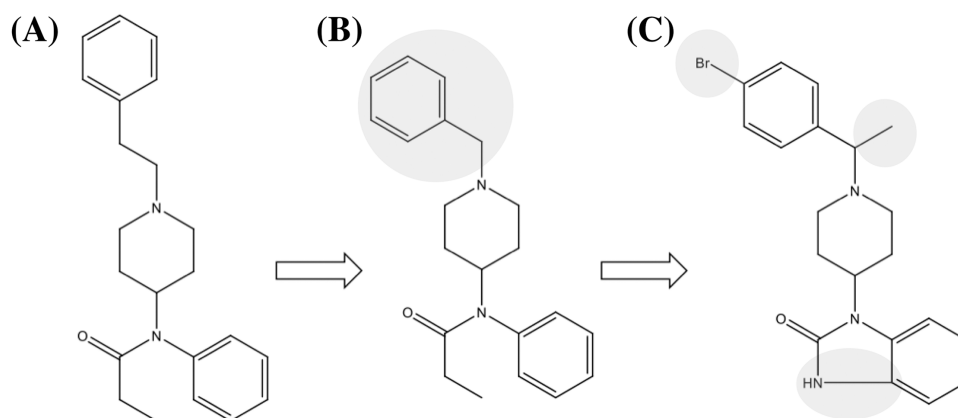
Figure 1. Structural similarities between (A) fentanyl, (B) benzylfentanyl and (C) the molecule discussed in this report, brorphine (1-(1-(1-(4-bromophenyl)ethyl)piperidin-4-yl)-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one).

and Crime (UNODC) (3). Although the rate of appearance of NPS detections in Europe seems to slow down, approximately 50 new substances are still reported annually. As NPS are typically relatively cheap, easily available over the internet and often very potent, many health problems are attributable to their use (1, 2). NPS were involved in about 9.1% of the drug-related hospital emergency presentations in Europe during the period 2014–2017. Although cathinones and synthetic cannabinoids were the most prevalent, new synthetic opioids have also made their entry in Europe (4).

A few years ago, new synthetic opioids primarily encompassed analogs of the potent analgesic fentanyl, the latter having been associated with tens of thousands of deaths in the United States during the past few years (5–7). Whilst fentanyl, as well as its analogs, remains present on the illicit market, the introduction of legislation scheduling these substances has resulted in a marked decline in the number of new "fentalogs" (8). Coinciding with the decrease in the number of new fentanyl-related substances, other new synthetic opioids have started to surface (9), including 2F-viminol, 2-Me-AP-237 and isotonitazene (10), the latter having been associated with several hundreds of deaths in just a few months in the United States (11, 12).

Brorphine [1-(1-(1-(4-bromophenyl)ethyl)piperidin-4-yl)-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one] is a piperidine benzimidazolone derivative and could be considered a fentanyl analog (Figure 1). However, it is not internationally scheduled and is even outside the scope of generic legislations aiming at covering fentanyl analogs (13, 14). Relatively little is known about this novel NPS opioid. Already back in 1967, the opioid activity of piperidine benzimidazolones was reported by Janssen (15). The synthesis of a structural analog of brorphine, where *p*-bromophenyl is substituted for an *o*-chlorophenyl group, was reported in the 1990s, in an attempt to develop an opioid-receptor-like 1 antagonist. Despite its high binding affinity, this compound lacked selectivity and antagonistic activity (16). Recently, the synthesis and *in vitro* pharmacological characterization of brorphine were reported by Kennedy et al. in the search for μ-opioid receptor (MOR) ligands biased toward G protein signaling (17). Biased agonism at MOR has received quite some interest, given the reported—but currently heavily questioned—association of β-arrestin signaling with adverse events like respiratory depression and G protein signaling with the desired (analgesic) effects of opioids (18–20). Interestingly, in the report of Kennedy et al., brorphine was among the most potent compounds, with a potency exceeding that of morphine (17). Both the *N*-1-phenylethyl substituent and the bromo group in

para position were important for the high potency. Using two distinct assays, monitoring either G protein signaling or β-arrestin coupling, brorphine was found to be a full agonist, showing some bias toward G protein signaling. Although no *in vivo* data are available for brorphine itself, intraperitoneal injection of several piperidine benzimidazolone derivatives demonstrated that this class of compounds is capable of crossing the blood–brain barrier (17, 21).

Brorphine has been mentioned on drug fora for a while and has been reported to circulate in the American Midwest since the second half of 2019 (22), although no formal reports on its identification (either as a powder or in authentic cases) are available. At the time of writing, we are not aware of any brorphine cases that were reported to the UNODC Early Warning Advisory or the European Monitoring Centre for Drugs and Drug Addiction. Therefore, in this article, we report on the identification and full chemical characterization of brorphine, sourced online and used by a patient querying admission for detoxification. In addition, besides the unequivocal identification of brorphine in serum samples from this patient, its MOR agonistic activity was demonstrated, both as a powder and in the authentic patient sample.

Case History

The patient, who provided written informed consent, was a 24-year-old male who presented with withdrawal symptoms to the emergency department, querying an admission for detoxification. His complaints consisted of generalized pain, situated mainly in the chest, abdomen and muscles. He had a normal blood pressure with a tachycardia of 114/minute, oxygen saturation of 98% and a temperature of 37.3°C. He presented with confusion and bradyphrenia, generalized weakness and cramping. On physical examination, he was clammy and sweaty. A CT scan of the brain, chest X-ray and ECG were all normal. Apart from a slightly elevated gamma-glutamyltransferase of 111 U/L, there were no remarkable abnormalities in the blood lab results. Anamnesis revealed that he had a medical history of opioid misuse with the use of, among others, the synthetic opioids 2-Me-AP-237 and intravenous O-acetylmethylketobemidone (a derivative of the synthetic opioid ketobemidone). He had an episode of abstinence of one year, up to six months before presentation. One to two months before presentation, he started using brorphine orally four times a day, as well as etizolam orally (last use of etizolam was a few days before

presentation). He reported that the use of brorphine resulted in a pretty strong effect, similar to oxycodone or fentanyl, with a resulting "high" that lasted quite long, and the development of tolerance. He also reported to suffer from quite intense cravings after waking up, with "cold turkey" more intense than heroin. His prescribed medications consisted of sertraline, mirtazapine, methylphenidate and enoxaparin. He reported no alcohol use.

Methods

Materials

All reagents used during the analyses were at least of high-performance liquid chromatography (HPLC) grade. For nuclear magnetic resonance (NMR) analysis, deuterated dimethyl sulfoxide (DMSO-d_6, 99.8%) was purchased from Eurisotop (Saint-Aubin, France). Brorphine HCl (5 mg) reference standard ($\geq$98% purity) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Hydromorphone was purchased as hydrochloride salt from Fagron (Nazareth, Belgium). Fentanyl was obtained from LGC Chemicals (Wesel, Germany). Dulbecco's modified Eagle's medium (GlutaMAX™), Opti-MEM® I reduced serum medium, penicillin-streptomycin (5,000 U/mL) and amphotericin B (250 μg/mL) were supplied by Thermo Fisher Scientific (Pittsburgh, PA, USA). Fetal bovine serum (FBS) and poly-D-lysine were obtained from Sigma Aldrich (Overijse, Belgium). The Nano-Glo® Live Cell Assay system (containing the Nano-Glo® Live Cell Substrate and Nano-Glo® LCS Dilution Buffer) was procured from Promega (Madison, WI, USA).

Description of the powder and sample preparation

The powder was obtained from the patient described earlier. As he wanted to go into treatment, he delivered several unopened powder sachets to the emergency physician. A larger, transparent resealable plastic sachet (itself originally packed in an aluminum pouch) contained two smaller powder sachets, one labeled as "Brorphine", and the other as "Etizolam". These were obtained from a Dutch online marketplace, only accessible by invitation, and had been shipped by a courier service to the patient's home. Both powder sachets were labeled as "research chemical powder" and "not for human consumption" (Supplementary Figure 1). Noteworthy is that the original aluminum pouch also contained two A4 sheets of paper, one being a disclaimer from the supplier that the product is for laboratory research purposes only, and the other being a material safety data sheet for etizolam.

The sample labeled as etizolam was indeed confirmed to contain etizolam. This thienodiazepine derivative was the most frequently seized benzodiazepine in Europe in 2017. It is particularly notorious in Scotland, where it is often sold as a "street" benzodiazepine [1] and was implicated in 57% of all drug-related deaths in 2018 [23]. Etizolam will not be discussed further in this report.

The brorphine powder was white and homogenous, as verified by evaluating five independent samplings (CV < 5.3%). The weight of the brorphine sachet, including the powder, was 1.378 g. For all chromatographic analyses, 3.7 mg of this powder was dissolved in 3.7 mL methanol as stock solution and stored at −20°C until analysis. For NMR analysis, 7.5 mg of the powder was dissolved in 0.75 mL DMSO-d_6. For the determination of biological activity at MOR, 3.93 mg of the powder was dissolved in 0.77 mL methanol and further diluted for experiments.

Patient samples

Two serum samples, taken approximately 60 hours apart, were available for (toxicological) analysis. The first sample was collected at the emergency department and the second during hospitalization in the psychiatric ward.

For liquid chromatography–high-resolution mass spectrometry (LC–HRMS) screening and quantitative brorphine analysis, 50 μL sample and 50 μL of internal standard solution (trimipramine-d_3 500 ng/mL in water, routinely applied in our lab—no brorphine isotope-labeled standard was available) were added to a tube and mixed. After addition of 200 μL cold methanol/acetonitrile 50/50, samples were stored for 30 minutes at −20°C, subsequently shaken for 5 minutes and centrifuged for 5 minutes at 16,162g. One hundred microliters of the supernatant was transferred to HPLC vials, and 10 μL was injected onto the ultra-high-performance liquid chromatography (UHPLC) column.

For the activity-based assay, the sample preparation was kept maximally similar to the LC–HRMS protocol. Here, 200 μL serum was mixed with 800 μL cold methanol/acetonitrile 50/50. After centrifugation, the supernatant was transferred to a glass tube and evaporated at room temperature under a gentle stream of nitrogen (Zymark Turbovap, Zymark Ltd, Cheshire, UK). The evaporated extracts were then reconstituted in 100 μL OptiMEM® I reduced serum medium, and 20 μL was used in the bio-assay. In a second set of experiments, a standard curve for brorphine was generated by spiking blank serum with different concentrations of the brorphine reference standard (0.1–100,000 ng/mL).

LC–HRMS analysis

Both the powder and the patient samples were screened with the same LC–HRMS method. The chromatographic separation was performed using an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Accucore phenyl-hexyl column (100 mm length × 2.1-mm i.d., 2.6 μm particle size, Thermo Fisher Scientific) at 40°C. A gradient of mobile phase A (2 mM ammonium formate, 0.1% formic acid (FA) in water) and B (0.1% FA in methanol) with a flow rate of 0.5 mL/min was used for elution of the compounds. Ten microliters of extract of the serum samples or of a 1/100 dilution of the stock solution in methanol was injected on the column. The HRMS system was a Q Exactive (Thermo Fisher Scientific) with an electrospray ionization source used in positive/negative switching ionization mode with a full scan range of 70–1000 m/z. The full scan resolution was 35,000 (full width at half maximum, FWHM) and the automatic gain control (AGC) target was set at 1e6 and the maximum injection time at 120 msec. For screening, high-energy collisional dissociation experiments were performed on compounds that matched the inclusion list [24] and on the five most intense precursor ions using data-dependent acquisition (DDA). Other settings for DDA were a resolution of 17,500 FWHM, AGC target of 1e5 with a maximum injection of 50 msec and an isolation window of 2.0 m/z [24].

A high-resolution accurate mass detection method was developed for the quantification of brorphine using targeted selected ion monitoring on the same Q Exactive system. A measuring window of 5 ppm (with an offset of 1 ppm) was set around the exact monoisotopic mass of 400.1020 m/z (and that of the internal standard) with a mass resolution of 70,000 FWHM. The AGC target was set at 5e4 and the maximum injection time at 200 msec. The same UHPLC conditions as in the screening method were used. Calibration curves ($1/x^2$ weighting) were linear between 1 and 100 ng/mL, and the

lower limit of quantification was set at 1 ng/mL. The method was validated in terms of precision, accuracy, recovery and matrix effects. Within- and between-run precision and within- and between-run biases over all quality control levels were <14.5%. For details on the method validation, see Supplementary Data.

Data were analyzed with Tracefinder 3.3 and FreeStyle 1.1 software (Thermo Fisher Scientific).

GC–MS analysis

One microliter of a one-fifth dilution of the stock solution in a ToxiVial type A (Interchim, Montluçon, France) was injected on a GC-2010 system coupled with a gas chromatography–mass spectrometry (GC–MS) QP2010 Plus mass spectrometer (Shimadzu, Kyoto, Japan). Split injections were performed at an injection temperature of 270°C with helium as carrier gas at a constant flow rate of 0.72 mL/min. A 12 m × 0.20-mm i.d. × 0.33 μm HP-5ms column was used (Agilent, Santa Clara, CA, USA). The temperature program started at 100°C for 3 minutes, was ramped at 30°C/min to 310°C, which was held for another 5 minutes, resulting in a total run time of 15 minutes. The ion source temperature and interface temperature were set at 200°C and 220°C, respectively. The MS operated in scanning mode with a scanning range of 40–550 m/z and an ionization energy of 70 eV.

UHPLC–DAD analysis

The chromatographic separation was achieved on an Acquity UPLC^{TM} system using an Acquity UPLC^{TM} BEH C18 column (100 mm length × 2.1 mm i.d., 1.7 μm particle size), fitted with a Vanguard Acquity BEH C18 guard column (5 mm length × 2.1 mm i.d., 2.6 μm particle size) (Waters, Milford, MA, USA), kept at 35°C. Elution was achieved isocratically using 0.1% FA in water/0.1% FA in acetonitrile 65/35% v/v as mobile phase. Ten microliters of a one-tenth dilution of the stock solution was injected on the column. Detection was done by diode array detection (DAD), monitoring wavelengths between 190 and 400 nm with a slit of 0.5 μm and resolution of 1.2 nm.

FT-IR spectroscopy analysis

The powder was analyzed using Fourier-transform infrared (FT-IR) spectroscopy in reflectance mode on a Spectrum 2 photometer (Perkin Elmer, Waltham, MA, USA). A small amount of the powder was deposited directly on the crystal without any prior sample preparation. The IR spectrum was recorded in the range 4000–400 cm^{-1} with a spectral resolution of 4 cm^{-1}. Spectral data were obtained using the Spectrum Quant software (Perkin Elmer).

NMR analysis

NMR measurements were performed on a Bruker Avance Neo® 400 MHz spectrometer at 25°C. Chemical shifts (δ) are reported in parts per million, and spectra are referenced to the residual solvent peak signal. Coupling constants are given in Hertz.

Determination of *in vitro* biological activity at MOR

The biological activity of brorphine at MOR was evaluated by means of a previously reported, live cell-based MOR reporter assay (20, 25). The employed assay monitors MOR activation via its interaction with β-arrestin 2 (βarr2) using the NanoLuc Binary Technology® (Promega). Human embryonic kidney 293 T cells stably expressing the MOR reporter system were cultured in Dulbecco's modified Eagle's medium (GlutaMAX™) supplemented with 10%

heat-inactivated FBS, 100 IU/mL penicillin, 100 mg/L streptomycin and 0.25 mg/L amphotericin B at 37°C in a humidified atmosphere containing 5% CO_2. The stability of the cell line was routinely monitored by flow cytometric analysis of co-expressed markers (20). One day prior to the experiments, the cells were seeded on poly-D-lysine coated 96-well plates at 5×10^4 cells/well. After overnight incubation, the cells were washed twice with Opti-MEM® I reduced serum medium to remove remaining traces of FBS. Next, 90 μL Opti-MEM® I and 25 μL Nano-Glo® Live Cell reagent were added to the washed wells. The plate was subsequently placed into a TriStar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). During an initial equilibration period (10–15 minutes), luminescence was continuously monitored until stabilization of the signal. Twenty microliters of each test compound (present as 6.75-fold concentrated stock solutions in Opti-MEM®/MeOH) or, alternatively, of the reconstituted serum extracts (cf. Section Methods—Patient samples) was added per well before continuously monitoring luminescence for 120 minutes. Hydromorphone was used as a reference agonist (10), and appropriate solvent controls were included. The results are presented as mean area under the curve ± standard error of mean (SEM), obtained in three independent experiments ($N = 3$), with duplicates run for each concentration within an experiment. All results were normalized to the maximum response of the reference compound hydromorphone (arbitrarily set at 100%). Curve fitting and statistical analyses were performed using GraphPad Prism 8 software (San Diego, CA, USA). Measures of potency (EC_{50}) and efficacy (E_{max}) were determined via nonlinear regression (four-parameter logistic fit).

Results

LC–HRMS, GC–MS, HPLC–DAD and FT-IR

LC–HRMS analysis of the powder in full scan mode revealed a single peak with a retention time of 5.48 minutes and an exact m/z value of 400.1020. This matched with the exact mass of 400.1019 m/z (mass deviation 0.25 ppm), assigned to single-protonated [M + H] brorphine ($C_{20}H_{23}BrN_3O$) in the HighResNPS library (26) and reported by Kennedy et al. (17). The obtained full scan and product ion spectra are shown in Figures 2A and B. The characteristic bromine isotope pattern is obvious in the full scan spectrum, with obtained precursor ions of 400.1020 and 402.1005 m/z, corresponding to the ^{79}Br and ^{81}Br isotopes, respectively (Figure 2A). The four main product ions had an m/z value of 84.0814 (matching with fragment corresponding to the piperidyl ring (2,3,4,5-tetrahydropyridin-1-ium ion; $C_5H_{10}N^+$)), 104.0625 (the phenylethyl moiety without the bromine atom ($C_8H_8^+$)), 182.9805 (79Br) / 184.9786 (81Br) (the phenylethyl moiety with the bromine atom ($C_8H_8Br^+$)) and 218.1289 (the piperidine benzimidazolone moiety ($C_{12}H_{16}N_3O^+$)) (27). Both the retention time (difference <0.1 min) and the fragment ion spectrum (99.3% fit) matched with those obtained for the reference standard (data not shown).

GC–MS analysis revealed a single peak with a retention time of 12.44 minutes (Figure 2C). A single peak, eluting at 1.05 minutes, and showing two absorption maxima, at 227 and 280 nm, was obtained with LC–DAD analysis (Figure 2D). In both instances, the retention time and MS or DAD spectrum matched those of the analytical standard (data not shown). No impurities (other peaks in the chromatogram) were found via any of the applied chromatographic conditions.
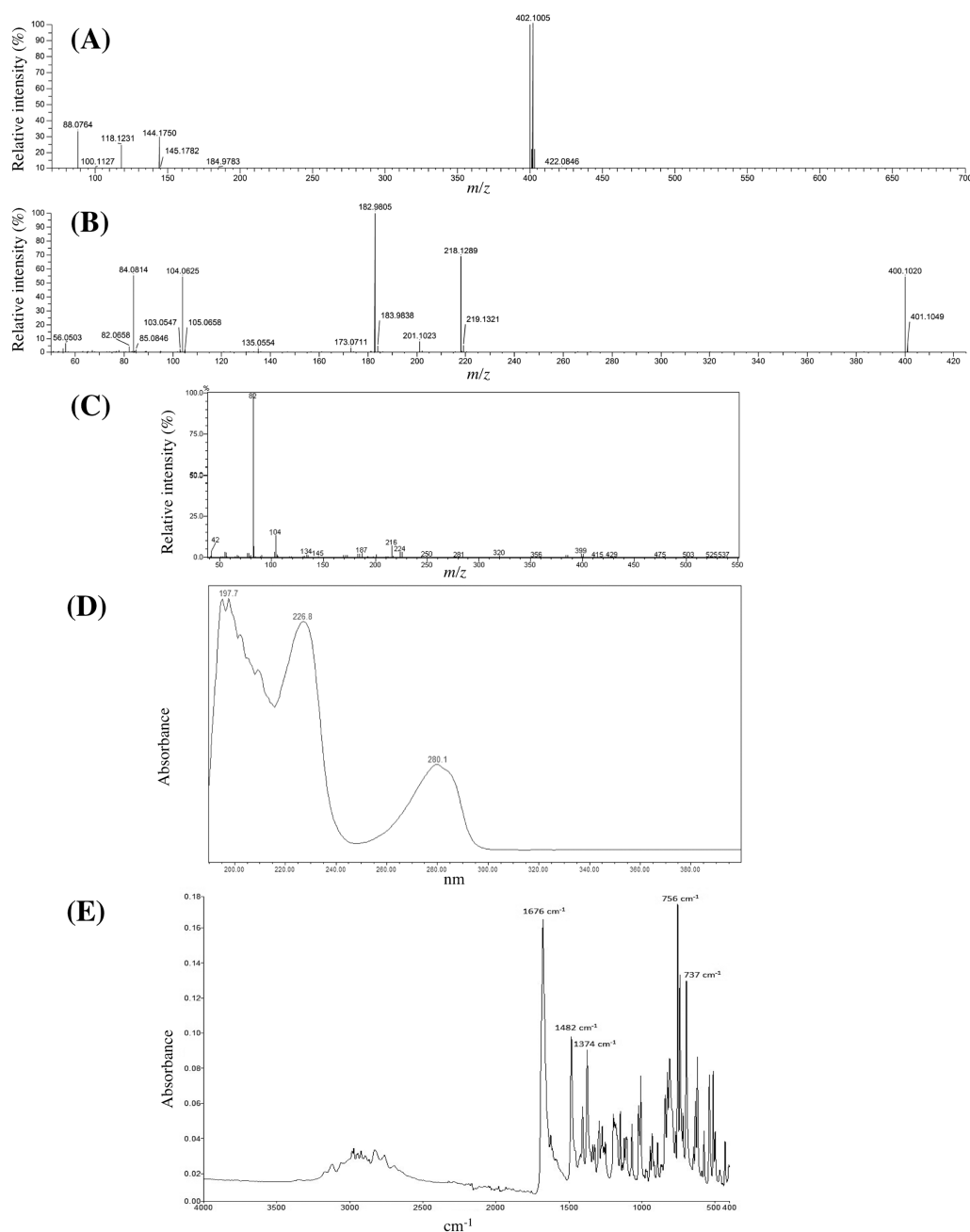
Figure 2. (A) HRMS full scan spectrum; (B) HRMS fragment ion spectrum; (C) GC–MS electron impact ionization spectrum; (D) HPLC–DAD spectrum; (E) FT-IR spectrum of the brorphine powder with indication of the characteristic bands.

FT-IR analysis of the sample using attenuated total reflectance revealed main absorbance peaks at 1676, 1482, 1374, 756 and 737 cm^{-1} (Figure 2E). The IR spectrum did not match any entry in an in-house library.

NMR analysis
^{1}H-NMR analysis was in complete agreement with published values (Figure 3A) (17). ^{13}C-NMR analysis was performed for full structural confirmation (Figure 3B). ^{1}H-^{13}C heteronuclear single quantum coherence was performed to aid in structural assignment (Supplementary Figure 2).

Determination of *in vitro* biological activity at MOR
The brorphine reference standard gave rise to a concentration-dependent response in the MOR activation bio-assay (Figure 4), allowing derivation of potency (EC_{50}) and efficacy (E_{max}) data, the latter relative to hydromorphone (Table I). The obtained data show that, whereas brorphine is comparable to hydromorphone (the reference compound used in this assay) in terms of potency, it is about twice as efficacious in activating MOR (E_{max} 191–230%).

Interestingly, the activation profiles of the sourced powder and the brorphine reference material showed an almost perfectoverlap
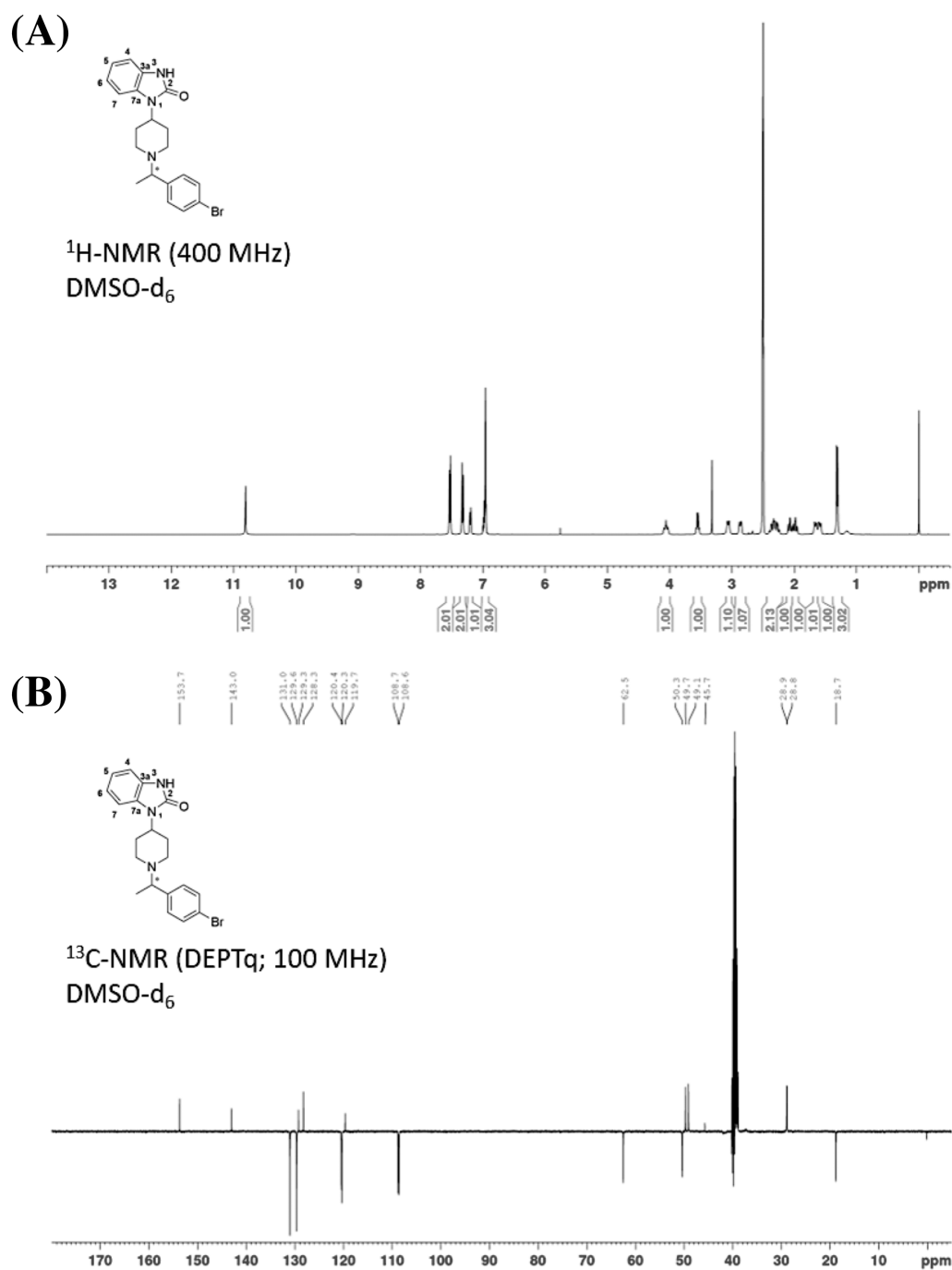
(A)

(B)

Figure 3. (A) ^{1}H-NMR spectrum (400 MHz, DMSO-d$_6$) δ: 1.31 (d, $J = 6.7$ Hz, 3H, CH$_3$), 1.59 (d, $J = 11.2$ Hz, 1H, H-3$_{pip}$/H-5$_{pip}$), 1.66 (d, $J = 11.5$ Hz, 1H, H-3$_{pip}$/H-5$_{pip}$), 1.98 (t, $J = 11.6$ Hz, 1H, H-2$_{pip}$/H-6$_{pip}$), 2.07 (t, $J = 11.7$ Hz, 1H, H-2$_{pip}$/H-6$_{pip}$), 2.24–2.40 (m, 2H, H-3$_{pip}$/H-5$_{pip}$), 2.87 (d, $J = 10.4$ Hz, 1H, H-2$_{pip}$/H-6$_{pip}$), 3.06 (d, $J = 10.4$ Hz, 1H, H-2$_{pip}$/H-6$_{pip}$), 3.55 (q, $J = 6.7$ Hz, 1H, NCHPhe), 4.06 (tt, $J = 12.2$ Hz, 4.1 Hz, 1H, H-4$_{pip}$), 6.93–7.01 (m, 3H, H-5$_{Benzimid}$, H-6$_{Benzimid}$, H-7$_{Benzimid}$), 7.20 (d, $J = 6.5$ Hz, 1H, H-4$_{Benzimid}$), 7.33 (d, $J = 8.6$ Hz, 2H, H-2$_{Phe}$, H-6$_{Phe}$), 7.53 (d, $J = 8.6$ Hz, 2H, H-3$_{Phe}$, H-5$_{Phe}$), 10.81 (s, 1H, NH); (B) ^{13}C-NMR spectrum (100 MHz, DMSO-d$_6$) δ: 18.7 (CH$_3$), 28.85 (C-3$_{pip}$/C-5$_{pip}$), 28.90 (C-3$_{pip}$/C-5$_{pip}$), 49.1 (C-2$_{pip}$/C-6$_{pip}$), 49.7 (C-2$_{pip}$/C-6$_{pip}$), 50.3 (C-4$_{pip}$), 62.5 (NCHPhe), 108.6 (C-4$_{Benzimid}$), 108.7 (C-7$_{Benzimid}$), 119.7 (C-4$_{PheBr}$),120.3 (C-5$_{Benzimid}$), 120.4 (C-6$_{Benzimid}$), 128.3 (C-3a$_{Benzimid}$), 129.3 (C-7a$_{Benzimid}$), 129.6 (2C, C-2$_{PheBr}$/C-6$_{PheBr}$), 131.0 (2C, C-3$_{PheBr}$/C-5$_{PheBr}$), 143.0 (C-1$_{PheBr}$), 153.7 (C-2).

(Figure 4). This, combined with the analytical data, indicates the high purity of the obtained powder.

Patient samples

Two serum samples were available from the patient, collected approximately 60 hours apart. Toxicological screening by immunoassays and enzymatic methods of the initial serum sample for ethanol, benzodiazepines, opiates and GHB was negative. Antidepressants (sertraline and mirtazapine, known medications of the patient) and an atypical antipsychotic (risperidone) were found in subtherapeutic ranges. In the second sample, trazodone and nordiazepam (administered in the hospital context) were found. In
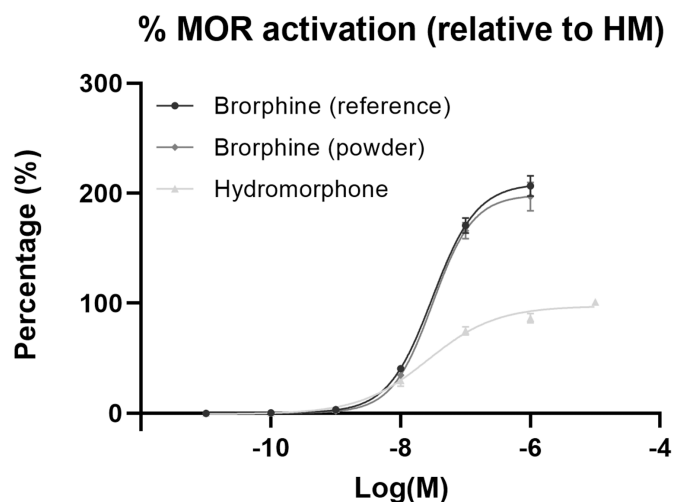
% MOR activation (relative to HM)

Figure 4. Concentration-dependent interaction of MOR with βarr2 upon stimulation with the brorphine reference standard (circles), the obtained brorphine powder (diamonds) and the reference agonist, hydromorphone (HM) (triangles). Data are shown as mean receptor activation $\pm$ SEM ($N = 3$), normalized to the E_{max} of hydromorphone ($=100\%$).

Table I. Values for Potency (EC_{50}) and Efficacy (E_{max}), with 95% Confidence Intervals (CIs), as Obtained in the MOR Activation Assay

	EC_{50} (ng/mL)	EC_{50} (nM)	E_{max} (%)
Hydromorphone	8.38 (4.26–18.9)	26.0 (13.3–58.6)	97.5 (86.9–114)
Brorphine (reference)	13.5 (8.94–20.0)	30.9 (20.5–45.7)	209 (191–230)
Brorphine (powder)	13.5 (7.36–22.3)	–	199 (176–224)

neither of the samples could etizolam, O-acetylmethylketobemidone or 2-Me-AP-237 be detected.

On the basis of the anamnesis and with the obtained exact mass and product ions from the powder, the presence of brorphine was confirmed in both available serum samples. For both samples, the retention time of brorphine in the LC–HRMS screening method matched that of the reference standard (i.e., within 0.1 minute). The obtained exact masses of both precursor and fragment ions (taking into account the typical bromine isotope pattern, with m/z 400.1029–402.1000 and 182.9810–184.9784, respectively) matched those of the reference standard [mass deviation < 3 ppm, matching the criterion of 10 ppm (24)] (Supplementary Figures 3A and B). The obtained brorphine concentrations in the first and second serum sample were 69.4 and 7.9 ng/mL, respectively. In addition, a peak with a retention time of 4.79 minutes, an exact mass of 416.0970 m/z and major product ions of 234.1238, 182.9805, 104.0625 and 84.0814 m/z was also found in the first sample, suggesting the presence of a brorphine hydroxy-metabolite, with the OH-group situated on the phenyl ring of the benzimidazolone moiety. Supplementary Figures 4A and B show the obtained HRMS fragment ion spectrum for this metabolite for both the [79]Br isotope and the [81]Br isotope. The fact that, after 60 hours, brorphine was still detectable in the patient's serum, suggests a long half-life. This is in line with the patient's subjective feeling.

The presence of opioid activity in the serum sample collected upon admission of the patient was confirmed by the opioid receptor activation assay: a signal clearly distinct from background was observed (Figure 5). On the one hand, this demonstrates the bio-assay's potential to pick up opioid positive samples without prior knowledge of the compound, which represents a great advantage over other NPS screening methods (e.g., immunoassays). On the

other hand, these data allow some insight into the extent of opioid activity present within the patient's serum sample. More specifically, based on the signals obtained from serum spiked with different concentrations of the brorphine reference standard, it could be derived that in the admission serum sample an activity was present corresponding to a concentration between 10 and 20 ng/mL (23–46 nM) brorphine (Figure 6). The signal in the second serum sample, however, could not be considered unequivocally positive as the bio-assay results were ambiguous and not reproducible. This is not entirely unexpected as this sample was taken 60 hours after admission, and at that time point, no relevant opioid activity is expected to be present anymore. This is also in line with the results from the standard curve generated from spiking serum with different concentrations of brorphine, in which concentrations below 10 ng/mL gave rise to ambiguous results (data not shown). This reliance on the presence of a relevant concentration of a pharmacologically active compound (i.e., a concentration still capable of activating MOR) could be considered a limitation of the activity-based approach.

Discussion

This article reports on the identification and full chemical characterization of an online sourced highly pure powder, identified as brorphine. Brorphine is a novel potent synthetic opioid with a piperidine benzimidazolone structure.

Using a wide array of techniques, including LC–HRMS, GC–MS, LC–DAD, FT-IR and ^{1}H- and ^{13}C-NMR spectroscopy, the powder obtained from a patient was unequivocally identified as brorphine.

The *in vitro* activity of brorphine at MOR was evaluated using a previously reported, live cell-based assay (20, 25). The efficacy of brorphine ($E_{max} = 209\%$) was found to be double that of the
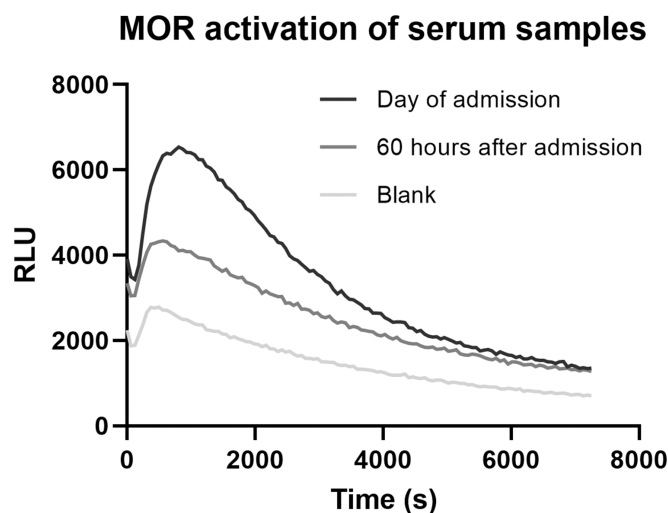
MOR activation of serum samples

Figure 5. Activation profile (relative luciferase units (RLU) in function of time) observed in the MOR activation assay used for activity-based screening for opioids in the patient's serum samples.
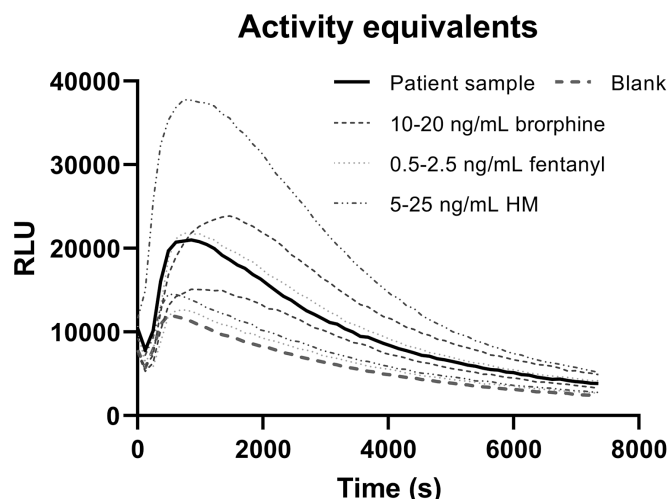
Activity equivalents

Figure 6. "Activity equivalents" plot (relative luciferase units (RLU) in function of time), showing that the opioid activity found in the patient's serum sample is equivalent to a serum concentration between 10–20 ng/mL brorphine, 5–25 ng/mL hydromorphone (HM) or 0.5–2.5 ng/mL fentanyl. The patient sample and the brorphine-spiked serum samples were fully processed (see Materials and Methods). To eliminate potential differences in recovery, HM and fentanyl were spiked post-extraction and corresponding serum concentrations were calculated taking into account the different dilution steps during sample preparation.

reference agonist hydromorphone ($E_{max} = 97.5\%$), indicating potentially higher levels of opioid effects with brorphine. With an EC_{50} value of 30.9 nM (95% confidence interval (CI) 20.5–45.7 nM) (Table I), the potency of this emerging synthetic opioid approaches that of the potent analgesic fentanyl (18.7 nM; 95% CI 15.0–23.3 nM) [20]. Kennedy et al. used a different βarr2 recruitment assay, a commercially available β-galactosidase-fragment complementation assay (PathHunter®, DiscoverX), to evaluate brorphine's βarr2 recruitment potential to human MOR, readily pointing out the high potency of brorphine (EC_{50} 182 ± 42 nM) within their set of tested compounds [17]. The difference in absolute EC_{50} values can be attributed to the different experimental set-ups, hampering easy comparison of the results. The same study also investigated the capability of brorphine to activate G protein signaling using a ^{35}S-GTPγS binding assay in membranes from CHO-K1 cells expressing human MOR (EC_{50} 4.8 ± 0.41 nM). The authors found

that brorphine showed a certain degree of bias toward activating the G protein pathway [17]. However, the findings of the βarr2 recruitment assay reported here, combined with those of a miniGi recruitment assay reported elsewhere [28], do not support this purported bias toward the G protein pathway. More studies—including evaluation *in vivo*—are needed to further clarify this.

Brorphine can be considered a substituted analogue of benzylfentanyl, in which the acyl group is part of a closed ring structure (Figure 1). We previously found that various substituted benzylfentanyls activate MOR to only a very limited extent, with E_{max} values of less than 10% compared to hydromorphone. In stark contrast to brorphine, the potencies of these compounds were in the high nM to μM range [29]. Furthermore, as opposed to these benzylfentanyls, brorphine is currently not covered by generic legislations targeting fentanyl analogues [13, 14], exposing a potentially dangerous legislative loophole.

This is also the first report on the identification of brorphine in an authentic biological sample. Apart from the unequivocal identification of brorphine in two serum samples via LC–HRMS, the use of a MOR activation assay also revealed the presence of opioid activity in one serum sample. The employed assay monitors the *total* MOR activity present in the patient sample, i.e., the activity conferred by brorphine, as well as by its metabolite(s). At this point, there is no information as to whether brorphine metabolites (such as the hydroxy-metabolite found here) have (relevant) opioid activity. The opioid activity scored by the bio-assay indicated a brorphine serum concentration between 10 and 20 ng/mL (23–46 nM), whereas the analytical concentration was 69.4 ng/mL (159 nM). Both serum concentrations lie in the lower (i.e., non-linear) part of the concentration-response curve of brorphine spiked in serum (data not shown), suggesting the applicability of the bio-assay to serve as a semiquantitative tool, as also recently demonstrated for synthetic cannabinoid receptor agonists (30). Another advantage of being able to assess total MOR activity is that this approach also allows to make comparative statements *versus* a reference compound, here hydromorphone. Hence, the activity can be expressed as an "activity equivalent". More specifically, the activity present in the extract of the patient admission sample lay within the range observed for extracts from serum samples spiked with 10–20 ng/mL brorphine (Figure 6, dashed lines). Taking into account the different dilution steps during the sample preparation, this would be equivalent to the activity exerted by roughly 5–25 ng/mL (16–78 nM) hydromorphone or 0.5–2.5 ng/mL (1.5–7.4 nM) fentanyl in serum (Figure 6). Although in the context of opioid users the aspect of potential tolerance greatly hampers unequivocal interpretation, this approach does provide insight into whether the MOR activity present in a patient's sample is high or not. In the current case, we can conclude that a high opioid activity was present. Although *in vitro* pharmacological characterization may lead to some guidance on anticipated potency, the eventual *in vivo* effect is determined by many factors, amongst which bioavailability, intrinsic potency (at distinct signaling pathways), metabolic stability, potential conversion to active metabolites and penetration through the blood brain barrier. In the context of opioid users, the aspect of potential tolerance also plays an important role. The patient from this case had a known recent history of opioid misuse and presented with withdrawal symptoms to the emergency department, meaning that a certain degree of opioid tolerance can be expected. The fact that he was conscious upon presentation at the emergency department, despite having high opioid activity in his blood, is in line with this.

Moreover, the fact that the MOR activation assay was able to find a signal in the patient sample further underscores the potential of activity-based screening. The general toxicology screening was negative, and if there would not have been information about the potential presence of brorphine in the patient's sample, this positivity would undoubtedly have been missed, given the novelty of this compound. In contrast, as long as there is relevant activity present within a sample, activity-based screening will allow to determine the presence of any (known or unknown) opioid, thereby serving as a universal non-biased approach.

In conclusion, this study presents the first identification and full chemical characterization of brorphine, an emerging synthetic opioid with a piperidine benzimidazolone structure. The identification of brorphine has not been previously reported, neither in powder form nor in biological samples. Along with the demonstration that brorphine acts as a strong MOR agonist, the fact that a large amount of powder was obtained, corresponding to many doses, is a cause of great concern. Should use of this compound become more widespread, we consider it likely that new cases, including fatalities, will emerge, as was the case for another recently emerging opioid, isotonitazene (10–12). Very recent data from the US (A. Krotulski, D. Papsun, personal communication) indeed **point** at fatalities with involvement of brorphine. Within the dynamic landscape of NPS, the high potency and efficacy of brorphine once again stress the importance of closely monitoring every new drug on the block.

Supplementary Data

Supplementary data is available at *ANATOX Journal* online.

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