Pharmacological evaluation and forensic case series of *N*-pyrrolidino etonitazene (etonitazepyne), a newly emerging 2-benzylbenzimidazole 'nitazene' synthetic opioid

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Abstract (250 words)

Novel synthetic opioids continue to emerge on recreational drug markets worldwide. In response to legislative bans on fentanyl analogues, non-fentanyl structural templates, such as 2-benzylbenzimidazoles ('nitazenes'), are being exploited to create new μ-opioid receptor (MOR) agonists. Here, we pharmacologically characterize an emerging cyclic analogue of etonitazene, called *N*-pyrrolidino etonitazene (etonitazepyne), using *in vitro* and *in vivo* methods. A series of analytically confirmed fatalities is described to complement preclinical findings. Radioligand binding assays in rat brain tissue revealed that *N*-pyrrolidino etonitazene has high affinity for MOR (Ki=4.09 nM) over δ-opioid (Ki=959 nM) and κ-opioid (Ki=980 nM) receptors. In a MOR-β-arrestin2 activation assay, *N*-pyrrolidino etonitazene displayed high potency (EC_{50}=0.348 nM), similar to etonitazene (EC_{50}=0.360 nM), and largely exceeding the potencies of fentanyl (EC_{50}=14.9 nM) and morphine (EC_{50}=290 nM). When administered s.c. to male Sprague Dawley rats, *N*-pyrrolidino etonitazene induced opioid-like antinociceptive, cataleptic, and thermic effects. Its potency in the hot plate test (ED_{50}=0.0017 mg/kg) was 10-fold and 2,000-fold greater than fentanyl (ED_{50}=0.0209 mg/kg) and morphine (ED_{50}=3.940 mg/kg), respectively. Twenty-one overdose fatalities associated with *N*-pyrrolidino etonitazene were found to contain low blood concentrations of the drug (median=2.2 ng/mL), commonly in the context of polysubstance use. *N*-Pyrrolidino etonitazene was reported as a cause of death in at least two cases, demonstrating toxicity in humans. We demonstrate that *N*-pyrrolidino etonitazene is an extremely potent MOR agonist that is likely to present high risk to users. Continued vigilance is required to identify and characterize emergent 2-benzylbenzimidazoles, and other non-fentanyl opioids, as they appear in the marketplace.

Introduction

Over the past five years, new synthetic opioids have evolved into one of the fastest-growing groups of new psychoactive substances (NPS) worldwide (UNODC 2021). In addition to alarming mortality statistics, the complexity and volatility of the recreational opioid market continue to expand as new drug analogues emerge (UNODC 2020). Fentanyl analogues dominated NPS opioid markets prior to 2019, but different class-wide bans in the United States (U.S.) and China (Bao et al. 2019; 117th Congress 2021) largely halted the production and proliferation of fentanyl-related drugs. In the wake of these bans, the recreational drug market has adapted accordingly with a subsequent shift towards non-fentanyl opioids. Structurally diverse drugs with opioid properties now monopolize NPS opioid markets worldwide, as fentanyl analogues – with the exception of *para*-fluorofentanyl - are rarely observed in forensic casework (UNODC 2020; EMCDDA 2021; Krotulski et al. 2021f).

Each newly emerging opioid poses significant risks to public health due to the propensity for sedation and respiratory depression – the latter being a common and potentially lethal adverse effect that is attributable to activation of the μ-opioid receptor (MOR). Beginning in 2019, the NPS opioid subclass of 2-benzylbenzimidazoles, or 'nitazenes', began increasing in prevalence, becoming drugs of concern due to suspected high potency. Originally reported in the late 1950s by the pharmaceutical company CIBA (Gross and Turrian 1957; Hunger et al. 1957, 1960a, b), this subclass of potent opioids first appeared on the recreational drug market post fentanyl-class scheduling in 2018 with the emergence of isotonitazene (EMCDDA 2020; Blanckaert et al. 2020; Vandeputte et al. 2021a). While etonitazene, the prototypical drug in this series, was sporadically identified in the drug supply between 1966 and 2003 (EMCDDA 2020; Ujváry et al. 2021), it was the emergence of isotonitazene in 2019 (Blanckaert et al. 2020) that really marked the dawn of the current nitazene surge. Isotonitazene quickly rose in popularity (Vandeputte et al. 2021a), contributing to hundreds of fatalities in the U.S. alone (Krotulski et al. 2020a). It dominated the U.S. novel synthetic opioid market for about a year, eventually leading to the drug being placed under Schedule I control by the U.S. Drug Enforcement Administration (DEA) (Vandeputte et al. 2021a). Likely as a result, isotonitazene popularity declined and different nitazene analogues with minor structural modifications started appearing, as a means to circumvent scheduling. In 2020, four out of ten new synthetic

opioids reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) were nitazene analogues; by comparison, only one new fentanyl analogue, isobutyrylfentanyl, was reported that year (EMCDDA 2021). In 2021, three nitazenes were newly reported in Europe. In the U.S., NPS Discovery reported the emergence of 19 new synthetic opioids in 2019 and 2020, of which seven were fentanyl analogues and only one was a nitazene analogue. By contrast, in 2021, nine new synthetic opioids were reported, of which seven were nitazene analogues and none were fentanyl analogues (Krotulski et al. 2021c).

Generally, the nitazene analogues appearing on clandestine markets have differed structurally either in their alkoxy chain (e.g., isoto-, eto-, meto-, proto- and butonitazene), halogen substitution (e.g., flunitazene) and/or the presence of a nitro group on the benzimidazole moiety (e.g., etodesnitazene, metodesnitazene) (**Figure 1**). *N*-Pyrrolidino etonitazene, colloquially referred to as 'etonitazepyne' online, differs from the aforementioned substitution patterns as it is the first 2-benzylbenzimidazole analogue to contain a pyrrolidino ring rather than the *N,N*-diethyl amine configuration. A related nitazene analogue contains a piperidine substitution, and is named *N*-piperidinyl etonitazene or 'etonitazepipne' (Krotulski et al. 2021a) (Vandeputte & Verougstraete *et al.*, submitted). Significantly, and unlike other nitazenes identified thus far, *N*-pyrrolidino etonitazene has never been described in the literature and can thus be defined as the first truly 'novel' NPS opioid of the 2-benzylbenzimidazole subclass.

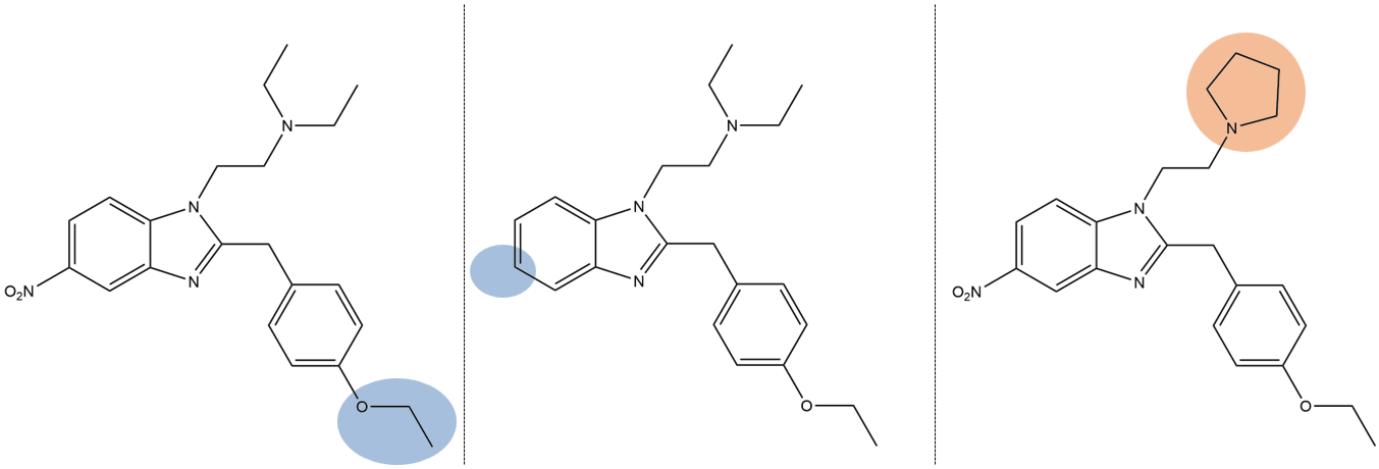


Figure 1. Chemical structures of etonitazene (left), etodesnitazene (middle), and *N*-pyrrolidino etonitazene (right). Highlighted features in etonitazene and etodesnitazene indicate positions that are commonly modified to produce additional nitazene analogues.

N-Pyrrolidino etonitazene was formally notified in Europe to the EMCDDA Early Warning System (EWS) on NPS in February 2021. In the U.S., the drug was first reported by NPS Discovery in May 2021. Following routine monitoring of the drug market, Blanckaert *et al.* obtained a yellowish powder claimed to contain 'etonitazepyne' from an online vendor. Analysis of the powder resulted in the first identification and full chemical characterization of *N*-pyrrolidino etonitazene (Blanckaert et al. 2021). In the U.S., the first encounter involving *N*-pyrrolidino etonitazene came from a death investigation case from West Virginia, indicating the new opioid may have begun circulating in the recreational drug supply at least as early as March 2021 (Krotulski et al. 2021g). In the months that followed, *N*-pyrrolidino etonitazene was identified in eight postmortem cases in the U.S. collected between January and April 2021, leading to the release of a public health alert aimed to warn public health and safety stakeholders about the potential for widespread harm caused by this novel synthetic opioid (Krotulski et al. 2021d). Further underscoring the dangers of consumption of *N*-pyrrolidino etonitazene, severe toxicity involving the drug was reported in the United Kingdom in July 2021 (Pucci et al. 2021). In December 2021, the U.S. DEA issued a notice of intent to temporarily place *N*-pyrrolidino etonitazene, together with six other nitazenes, in Schedule I of the Controlled Substances Act (DEA 2021).

While *N*-pyrrolidino etonitazene has been linked to severe intoxications and fatalities (Krotulski et al. 2021d; Pucci et al. 2021), unlike other newly emerging NPS, the pharmacology and toxicology of this novel opioid have never been characterized in the scientific literature. Therefore, in this paper, we aimed to bridge existing knowledge gaps by describing the mechanism of action of *N*-pyrrolidino etonitazene as a novel opioid of concern. At the *in vitro* level, we studied μ- (MOR), δ- (DOR) and κ- (KOR) opioid receptor affinity as well as MOR activation potential – the latter to gain greater insight into drug action at the main molecular target implicated in most clinically relevant opioid effects (Williams et al. 2013). Furthermore, we aimed to link *in vitro* data to the *in vivo* pharmacodynamics of *N*-pyrrolidino etonitazene via evaluation of hot plate latency, catalepsy, and body temperature changes in rats. Medicolegal death investigation cases in the U.S. and Canada involving fatalities associated with ingestion of *N*-pyrrolidino etonitazene were also examined, including the results of comprehensive toxicology testing. The details of these cases are summarized and discussed.

Materials & Methods

Chemicals and reagents

The reference standard for *N*-pyrrolidino etonitazene was obtained from Cayman Chemical (Ann Arbor, Michigan, U.S.) and used for radioligand binding, *in vitro* experiments, *in vivo* experiments and toxicology testing. Two sourced powders (i.e., non-certified reference materials) containing *N*-pyrrolidino etonitazene were also evaluated: powder 1 was purchased from an online vendor by the group of Dr. E. Deconinck (Blanckaert et al. 2021), whereas powder 2 was received at the Ghent University Laboratory of Toxicology in the framework of routine forensic toxicology practices. Etonitazene (1 mg/mL in ACN) was obtained from Chiron AS (Trondheim, Norway). Fentanyl and hydromorphone for cell-based assays were purchased as neat powders from Cayman Chemical (Ann Arbor, Michigan, U.S.) and Fagron (Nazareth, Belgium), respectively. Morphine sulfate (morphine) and fentanyl citrate (fentanyl) for radioligand binding assays and *in vivo* studies were generously provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (NDSP, Rockville, MD, U.S.). Dulbecco's Modified Eagle's Medium (DMEM, GlutaMAX™), Opti-MEM I Reduced Serum Medium, penicillin/streptomycin (10000 IU/mL and 10000 µg/mL), and amphotericin B (250 µg/mL) were purchased from Thermo Fisher Scientific (Pittsburgh, Pennsylvania, U.S.). Poly-D-lysine and fetal bovine serum (FBS) were from Sigma-Aldrich (Overijse, Belgium). The Nano-Glo Live Cell Reagent and the corresponding Nano-Glo LCS Dilution Buffer were procured from Promega (Madison, Wisconsin, U.S.). All materials and reagents used for liquid chromatography mass spectrometry (LC-MS) analyses were of LC-MS grade purity. Solvents were purchased from Honeywell Chemicals (Charlotte, NC, U.S.). Ammonium formate (99%) was purchased from Alfa Aesar (Ward Hill, MA, U.S.). Formic acid ampoules (1 mL) were purchased from Thermo Fisher Scientific (Waltham, MA, U.S.). Sodium borate decahydrate was purchased from Millipore Sigma (Burlington, MA, U.S.). Human whole blood, preserved with sodium fluoride and potassium oxalate, was purchased from BioIVT (Westbury, NY, U.S.). Fentanyl-D5 was purchased from Cerilliant Corporation (Round Rock, TX, U.S.).

Radioligand binding in rat brain tissue

Opioid receptor binding assays were carried out as described previously (Truver et al. 2020), with minor modifications. Briefly, whole rat brains minus cerebellum (BioIVT, Westbury, NY, U.S.) were thawed on ice, homogenized in 50 mM Tris HCl at pH 7.5 using a Kinematica

Polytron (setting 6 for 20 sec), and centrifuged at 30,000 g for 15 min at 4° C. The supernatant was discarded, and the pellet was resuspended in fresh buffer and spun again at 30,000 g for 15 min. The pellet was resuspended to yield 100 mg/mL wet weight. Ligand binding experiments were conducted in polypropylene tubes containing 300 µL Tris buffer and 100 µL of tissue suspension for 1 h at room temperature. Radioligands were used at 1 nM final concentration. Specifically, [^{3}H]DAMGO, [^{3}H]DADLE, and [^{3}H]U69,593 (all from Perkin Elmer Life Sciences, Waltham, MA, U.S.) were used to label MOR, DOR, and KOR, respectively. Non-specific binding was determined in the presence of 10 µM naloxone in all cases. Stock solutions of 10 mM morphine, fentanyl, and *N*-pyrrolidino etonitazene were prepared in 100% DMSO and stored at -80°C. On the day of an experiment, aliquots of stock solution were diluted in 50 mM Tris buffer to yield the appropriate concentrations for binding assays. Incubations were terminated by rapid vacuum filtration over Whatman GF/B filters using a cell harvester (Brandel Instruments, Gaithersburg, MD, U.S.). Filters were washed three times with ice cold buffer, transferred to 24-well plates, and Cytoscint (MP Biomedicals, Irvine, CA, U.S.) was added. Radioactivity was counted the following day using a Perkin Elmer MicroBeta2 liquid scintillation counter. Raw cpm data were normalized to percent of radioligand bound, and Ki values were determined using nonlinear regression analysis (GraphPad Prism, San Diego, CA, U.S.).

Determination of in vitro *µ-opioid receptor activation potential*

Cell culture

Human embryonic kidney (HEK) 293T cells, kindly gifted by Prof. Dr. O. De Wever (Ghent University Hospital, Belgium), were previously modified to stably express the MOR-βarr2-GRK2 assay system (cfr. *infra*) and were routinely maintained in DMEM (GlutaMAX™, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 mg/L amphotericin B) in a humidified atmosphere at 37°C and 5% CO_2. Cells were used in passage 8-12, and the stability of the cell line was routinely monitored by means of flow cytometric analysis of coexpressed markers (Vasudevan et al. 2020).

NanoBiT® MOR-β-arrestin 2 recruitment assay

A previously reported cell-based recruitment assay (Cannaert et al. 2019; Vasudevan et al. 2020) was used to determine the intrinsic MOR activation potential of *N*-pyrrolidino etonitazene (reference standard and two sourced powders, cfr. *supra*). Morphine, fentanyl,

and etonitazene were included as comparators. Hydromorphone was used as a reference compound to facilitate comparability with earlier studies (Vasudevan et al. 2020; Vandeputte et al. 2021b). Briefly, the assay is based on the functional complementation of a split nanoluciferase enzyme (NanoLuc Binary Technology®, Promega). In the cells, activation of human MOR, fused to one subunit of the nanoluciferase, leads to the intracellular recruitment of β-arrestin 2 (βarr2) (coexpressed with G protein-coupled receptor kinase 2, GRK2), fused to the complementing subunit. Close proximity of the two subunits subsequently allows functional complementation of the nanoluciferase, restoring its enzymatic activity. Upon addition of the substrate furimazine, a measurable bioluminescent signal is generated.

One day prior to the assay, cells expressing the MOR-βarr2-GRK2 system were seeded on poly-D-lysine coated 96-well plates at 5 x 10^4 cells/well. On the second day, cells were washed twice with OptiMEM I Reduced Serum Medium, and 90 µL OptiMEM I was added to the wells. Nano-Glo Live Cell Reagent was subsequently prepared by 20-fold dilution of Nano-Glo Live Cell Substrate with Nano-Glo LCS Dilution Buffer, and 25 µL was added per well. The plate was then placed into a TriStar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co., Bad Wildbad, Germany), and luminescence was continuously monitored for an initial equilibration period of 10-15 minutes to allow stabilization of the signal. Next, 20 µL of a 6.75-fold concentrated stock solution of each opioid (in OptiMEM, OptiMEM/MeOH or OptiMEM/ACN) was added to the cells and luminescence was monitored for 2 hours. All compounds were tested in concentrations between 1 pM and 100 µM, and appropriate solvent controls were included on all plates. Each compound was tested in at least 3 independent experiments ($n \geq 3$), with duplicates included for each concentration within an experiment.

Data analysis

Relative light units were corrected for inter-well variability before calculation of the area under the curve (AUC) of each obtained time-luminescence profile (Pottie et al. 2020b). Solvent correction was performed by subtraction of the mean AUC value of the corresponding blank (OptiMEM, OptiMEM/MeOH or OptiMEM/ACN). GraphPad Prism 9 software (San Diego, CA, U.S.) was used to subsequently generate concentration-response curves via three-parameter logistic regression. In line with previous studies (Vasudevan et al. 2020; Vandeputte et al. 2021b), data were then normalized to the maximum response of hydromorphone (arbitrarily set at 100%). As in previous protocols (Pottie et al. 2020a; Vandeputte et al. 2021b),

it was defined upfront that normalized AUC values from the highest concentration(s) of each compound were excluded in the case of a reduction of 20% or more compared to the AUC of the next dilution. Outlier testing was performed using the standard Grubbs' test, leading to the identification and subsequent exclusion of 3 outliers within the total set of 454 data points (0.66%). Normalized AUC values from all experiments were then combined to obtain a final concentration-response curve for each compound, allowing calculation of potency (EC_{50}) and efficacy (E_{max}, relative to hydromorphone) values.

Pharmacodynamic experiments

Animals and surgery

Male Sprague-Dawley rats (300-400 g), purchased from Envigo (Frederick, MD, U.S.), were group-housed under conditions of controlled temperature (22 ± 2 °C) and humidity (45 ± 5%), with ad libitum access to food and water. Lights were on from 7:00 AM to 7:00 PM. The NIDA Intramural Research Program (IRP), Animal Care and Use Committee approved the animal experiments, and all procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Vivarium facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were designed to minimize the number of animals included in the study. Rats were anesthetized with isoflurane using a drop jar which contained a gauze pad saturated with 2 mL of isoflurane, placed beneath a wire mesh floor. Once anesthetized, each rat received a subcutaneous (s.c.) temperature transponder (model IPTT-300, Bio Medic Data Systems, Seaford, DE, U.S.) to allow for the non-invasive measurement of body temperature (Bergh et al. 2021). The temperature transponder emits radio frequency signals that are received by a compatible hand-held reader system (DAS-7006/7r, Bio Medic Data Systems). Transponders are cylindrical in shape, 14 x 2 mm, and were implanted on the back via a pre-packaged sterile guide needle. Rats were single-housed post-operatively and given at least 1 week to recover.

Experimental procedures

On the day of an experiment, rats were brought into the laboratory in their home cages and allowed 1 h to acclimate. Groups of rats (*n* = 6 per dose group) received s.c. injections of vehicle (1 mL/kg, 10% DMSO in saline) or *N*-pyrrolidino etonitazene (0.0003 - 0.010 mg/kg) on the lower back between the hips and were returned to their home cages. Each rat was tested twice in separate experimental sessions, with at least three days of washout between

experiments, and doses were randomly assigned. Pharmacodynamic endpoints including catalepsy score, body temperature, and hot plate latency, were determined prior to injection and at 15, 30, 60, 120, and 240 min post-injection (Bergh et al. 2021). At each timepoint, behavior was observed for 1 min by an experienced rater, and catalepsy was scored based on three overt symptoms: immobility, flattened body posture, and splayed limbs. Each symptom was scored as either 1=absent or 2=present, and catalepsy scores at each time point were summed, yielding a minimum score of 3 and a maximum score of 6. Next, body temperature was measured using a handheld reader sensitive to signals emitted by the surgically implanted transponder. We chose to examine hypothermia as a representative adverse effect of opioid treatment, since body temperature is a convenient physiological measure that decreases in parallel with opioid-induced bradycardia and respiratory depression (Wong et al. 2017). Finally, rats were placed on a hot plate analgesia meter (IITC Life Sciences, Woodland Hills, CA, U.S.) set at 52°C. Rats remained on the hot plate until they exhibited hind paw licking in response to the heat stimulus, and were then returned to their home cages. Time spent on the hot plate was recorded using a timer triggered by a foot pedal. A 45 sec cut-off was employed to prevent tissue damage. Separate groups of rats (*n* = 6 per dose group) received morphine (1.0 - 30 mg/kg, s.c.), fentanyl (0.003 - 0.10 mg/kg, s.c.), or saline vehicle (1 mL/kg, s.c.), and experiments proceeded as described above. Morphine and fentanyl were used as opioid standards, as a means to compare the dose-response effects produced by *N*-pyrrolidino etonitazene.

Data analysis

Pharmacodynamic findings were analyzed using GraphPad Prism 8.2. Raw time-course data for hot plate latency and catalepsy score were normalized to percent maximum possible effect (%MPE), using the following equation:

$$\frac{experimental\ measure - baseline\ measure}{maximum\ possible\ response - baseline\ measure} \times 100$$

The maximum possible response for hot plate latency was 45 sec, whereas the maximal response for catalepsy score was 6. Raw time-course data for body temperature were normalized to change from baseline, Δ temperature in °C, for each rat. Normalized time-course data were analyzed by two-factor (dose x time) ANOVA followed by Tukey's post hoc

test to determine effects of drug doses at each time point. Mean hot plate latency and catalepsy score over the first 60 min were used to construct dose-response relationships, which were analyzed by non-linear regression (response stimulation, normalized response) to determine ED_{50} (potency) values.

Comprehensive toxicological analysis

Initial drug screening

Forensic toxicology drug screening was performed at NMS Labs (Horsham, PA, U.S.) and the Center for Forensic Science Research and Education (CFSRE, Willow Grove, PA, U.S.). Routine and comprehensive toxicology screening (0.5 mL) were performed for the presence of illicit, therapeutic, and novel synthetic drugs by NMS Labs (n > 300) and the CFSRE (n > 950). At NMS Labs, analysis was performed by liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) using an Agilent Technologies 6230 (Santa Clara, CA, U.S.). At the CFSRE, analysis was performed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) using a SCIEX TripleTOF® 5600+ (Framingham, MA, U.S.). Cases screening positive for *N*-pyrrolidino etonitazene were reflexed on to quantitative confirmation with medical examiner approval, only if access to samples and appropriate volume remained – not all cases screening positive were available for quantitative confirmatory testing. The laboratory workflow used has been described by Krotulski *et al.* for confirmation of isotonitazene, brorphine, and metonitazene (Krotulski et al. 2020a, b, 2021e).

Targeted data processing for *N*-pyrrolidino etonitazene was employed. Chemical information for *N*-pyrrolidino etonitazene was programmed into the software, including name, formula ($C_{22}H_{26}N_4O_3$), exact protonated mass (395.2075 Da), and retention time (NMS: 5.18 min, CFSRE: 6.48 min) based on the analysis of standard reference material. Presumptive positive results were filtered based on previously established and validated criteria for mass error, retention time difference, isotope score, and library match (CFSRE only). All data generated during drug screening were manually reviewed by a toxicologist. Manual review included evaluation of isotope distribution, peak shape, and chromatographic features.

Quantitation of N*-pyrrolidino etonitazene*

Quantitation of *N*-pyrrolidino etonitazene in biological samples was performed via liquid chromatography tandem quadrupole mass spectrometry (LC-QQQ-MS) using a standard addition approach (Krotulski et al. 2020a, 2021b; Papsun et al. 2021). A sensitive and specific analytical method was developed and validated for specialized use in this scenario.

To determine the concentration of *N*-pyrrolidino etonitazene, four replicate samples (0.5 mL) were aliquoted and prepared via fortification with *N*-pyrrolidino etonitazene. Briefly, one sample remained "blank" with no drug standard added, while three samples were "up-spiked" to 0.2, 2, and 20 ng/mL of *N*-pyrrolidino etonitazene in matrix. Fentanyl-D5 was used as the internal standard and was added to all samples at a final concentration of 40 ng/mL. A liquid-liquid extraction (LLE) was performed to removed unwanted matrix components; the procedure involved 1 mL of borax buffer (0.1 M, pH 10.4) and 3 mL of extraction solvent (1-chlorobutane, ethyl acetate; 70:30, v:v). Following drying and redissolving in 200 µL 50:50 A:B, analysis was performed using a Waters Xevo TQ-S micro LC-QQQ-MS (Milford, MA, U.S.). Chromatographic separation was achieved using gradient elution with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) over an Agilent InfinityLab Poroshell 120 EC-C18 (3.0 x 100 mm, 2.7 µm) analytical column. The flow rate was 0.4 mL/min. The injection volume was 5 µL. The column temperature was 30 °C. Following positive electrospray ionization (ESI+), multiple reaction monitoring (MRM) was used for mass filtration and detection. Three transitions were monitored for *N*-pyrrolidino etonitazene to increase identification specificity (395.2 > 135.1, 107.0, and 98.0, the quantification ion).

To determine the concentration of *N*-pyrrolidino etonitazene in the authentic samples, resulting analyte-internal standard peak area ratios (PAR) were plotted against the up-spike concentration (i.e., 0, 0.2, 2, and 20 ng/mL). A linear regression was used to assess the correlation (R^2 > 0.98) between the data points. The concentration of *N*-pyrrolidino etonitazene was determined by back-calculation of the x-intercept (reported as a positive number).

Qualitative prevalence determination for N-*pyrrolidino etonitazene*

Surveillance of NPS is important for determining the overall impact of a drug prior to availability of confirmatory data from forensic laboratories or medical examiner offices. Surveillance initiatives also allow scientists to determine the extent of drug positivity when

more advanced testing is not available or pursued. Continually acquired LC-TOF-MS high resolution mass spectrometry data from NMS Labs were evaluated to determine the prevalence of *N*-pyrrolidino etonitazene in 2021 by tracking the number of tentative positive identifications each month. Being a large forensic toxicology reference laboratory, NMS Labs has an approximate average yearly case load of more than 100,000 cases including submissions from most states across the U.S. and some territories in Canada. These data were generated from comprehensive toxicological analyses performed on biological specimens submitted in primarily suspected death and impaired driving investigations. The monthly data were used to generate a geographical timeline of *N*-pyrrolidino etonitazene detections. For the purposes of this assessment, one toxicology detection was tallied as one forensic case.

Results

In vitro *opioid receptor affinity and MOR activation potential*

Radioligand binding experiments were performed to evaluate the binding affinity of *N*-pyrrolidino etonitazene at the three main opioid receptors (**Figure 2** and **Table 1**). With a Ki value in the low nanomolar range, the MOR affinity of *N*-pyrrolidino etonitazene was nearly equivalent to that of morphine, and somewhat greater than that of fentanyl. At DOR and KOR, *N*-pyrrolidino etonitazene had weaker effects and displayed lower affinity than morphine or fentanyl. The findings reveal that *N*-pyrrolidino etonitazene has over 200-fold selectivity for MOR over KOR and DOR.

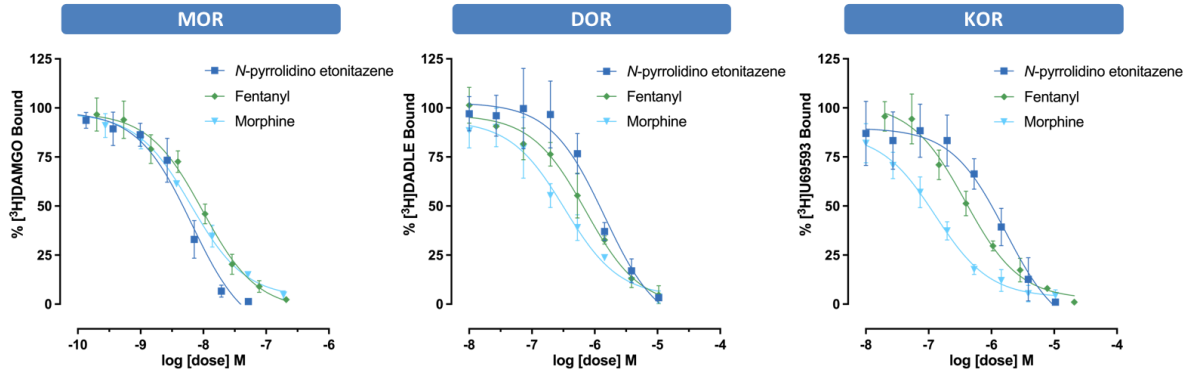


Figure 2. Opioid receptor binding curves for *N*-pyrrolidino etonitazene, fentanyl, and morphine in rat brain tissue. Data are plotted as mean ± standard deviation (SD) for %[^{3}H]DAMGO (MOR), %[^{3}H]DADLE (DOR), or %[^{3}H]U-69,593 (KOR) bound.

Table 1. Opioid receptor affinities at MOR, DOR, and KOR. Ki values (nM) are given as mean ± SD for *n* = 3 experiments performed in triplicate.

Ki values (nM)	MOR [^{3}H]DAMGO	DOR [^{3}H]DADLE	KOR [^{3}H]U-69,593
***N*-Pyrrolidino etonitazene**	4.09 ± 0.63	959 ± 193	980 ± 213
Fentanyl	6.17 ± 0.82	479 ± 76	224 ± 33
Morphine	3.99 ± 0.40	220 ± 41	74.4 ± 11.8

As most clinically relevant opioid effects (i.e., analgesia and respiratory depression) result from MOR activation, this receptor was selected for further evaluation of *in vitro* activation potential (**Figure 3** and **Table 2**). The potency of *N*-pyrrolidino etonitazene exceeded that of morphine and fentanyl by > 800 and > 40 times, respectively. Importantly, the *in vitro* MOR

activation potential of *N*-pyrrolidino etonitazene was comparable to that of etonitazene, previously the most potent 2-benzylbenzimidazole described. In line with values obtained for etonitazene, MOR efficacy of the *N*-pyrrolidino analogue was more than 2 times higher than that of morphine, and comparable to that of fentanyl. Interestingly, the obtained concentration-response curves of both sourced powders overlapped with that of the *N*-pyrrolidino etonitazene reference standard, indicating high purity – for powder 1, this is in line with the extensive analytical characterization, which did not reveal impurities (Blanckaert et al. 2021).

Table 2. Potency (EC_{50}) and efficacy (E_{max}, relative to hydromorphone) values (with 95% confidence intervals) obtained in the MOR activation assay in minimum three independent experiments ($n \geq 3$).

	EC_{50} (nM)	E_{max} (%)
***N*-Pyrrolidino etonitazene (etonitazepyne)**	0.348 (0.137-0.876)	298 (264-333)
Sourced powder #1	0.403 (0.141-1.10)	337 (295-380)
Sourced powder #2	0.419 (0.139-1.20)	320 (279-363)
Morphine	290 (132-668)	115 (106-125)
Fentanyl	14.9 (10.6-21.0)	271 (257-285)
Etonitazene	0.360 (0.128-0.990)	316 (278-355)
Hydromorphone	20.3 (14.1-29.4)	99.9 (94.5-105)

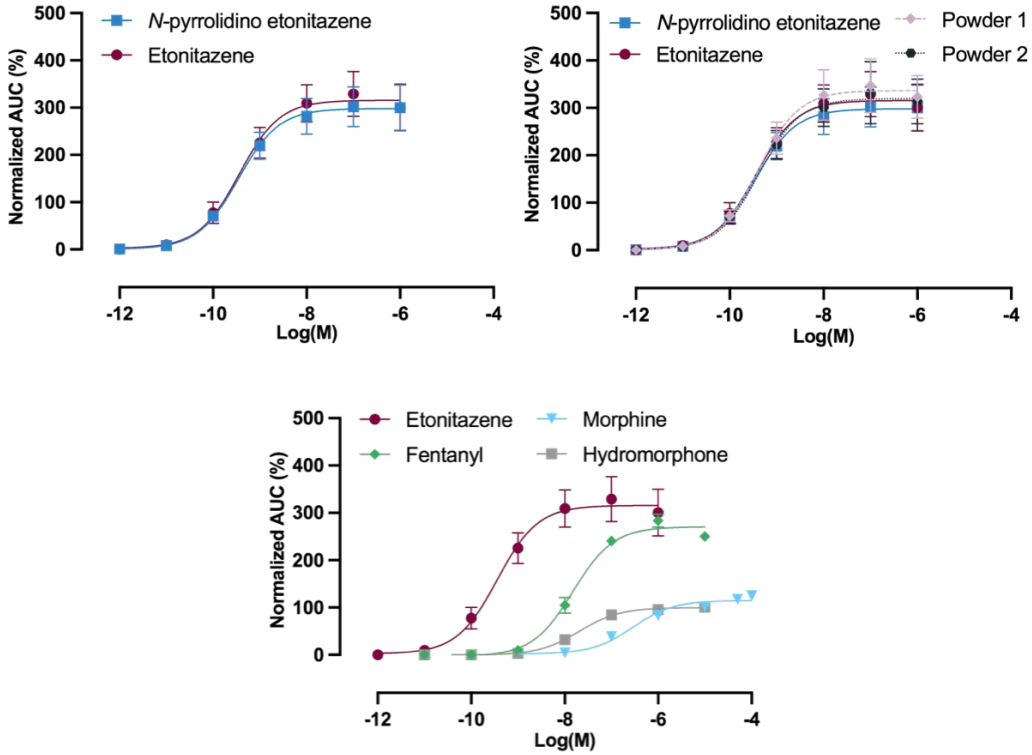


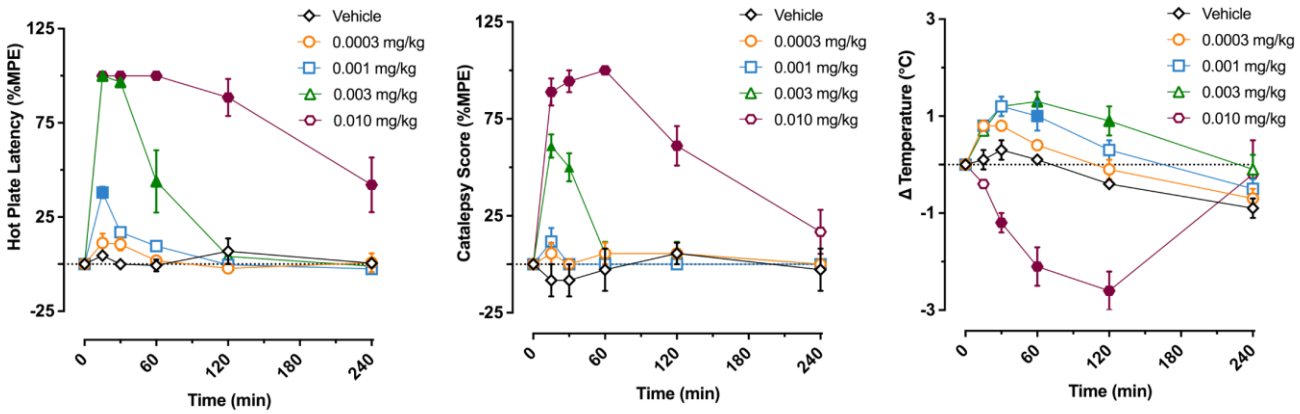
Figure 3. μ-Opioid receptor (MOR) activation profiles ($n \geq 3$) obtained for *N*-pyrrolidino etonitazene and two independently sourced powders containing *N*-pyrrolidino etonitazene. Curves for morphine, fentanyl, etonitazene, and hydromorphone are shown for comparison. Data are presented as mean MOR activation (expressed as area under the curve, AUC) ± SEM and are normalized to the maximum response of hydromorphone.

Pharmacodynamic effects of N-*pyrrolidino etonitazene*

The pharmacodynamic effects induced by *N*-pyrrolidino etonitazene are shown in **Figure 4A**. Two-way ANOVA revealed a significant effect of *N*-pyrrolidino etonitazene dose on hot plate latency ($F[4,145] = 194.1$, $p < 0.0001$), catalepsy score ($F[4, 145] = 119.0$, $p < 0.0001$) and body temperature ($F[4,145] = 52.06$, $p < 0.0001$). Dose-dependent increases in hot plate latency were observed for rats treated with *N*-pyrrolidino etonitazene, indicating an antinociceptive effect. Post-hoc tests revealed that rats treated with the lowest dose of *N*-pyrrolidino etonitazene (0.0003 mg/kg) showed no statistically significant change in hot plate latency compared to the control group. Significant differences in latency were seen at 15 minutes for rats receiving 0.001 mg/kg of *N*-pyrrolidino etonitazene, whereas higher doses of 0.003 and 0.01 mg/kg produced more sustained increases in hot plate latency. The maximum cut-off latency of 45 s was reached in the groups receiving the two highest doses. Nonlinear

regression analysis of the mean hot plate responses over 60 min indicated an ED_{50} value of 0.00168 mg/kg for *N*-pyrrolidino etonitazene (**Figure 4B**). Clear signs of dose-dependent catalepsy - as indicated by immobility, flattened body posture, and splayed limbs - were seen at the two highest doses after 15 and 30 minutes. In line with the hot plate latency assay, rats receiving 0.01 mg/kg *N*-pyrrolidino etonitazene showed the most sustained effects, with a significant catalepsy score until 120 min post-injection. The ED_{50} value to induce catalepsy was 0.00354 mg/kg, about two times weaker than the antinociceptive potency. The 0.001 and 0.003 mg/kg doses of *N*-pyrrolidino etonitazene induced small, albeit significant, increases in body temperature at 60 min post-injection, whereas a pronounced and sustained (30-120 min) drop in body temperature was observed after the highest dose of 0.01 mg/kg. Both fentanyl and morphine produced significant time-dependent effects on hot plate latency, catalepsy scores, and body temperature, which were analogous to the profile of effects produced by *N*-pyrrolidino etonitazene (**Supplementary Figures 1 and 2**). The ED_{50} values for fentanyl- and morphine-induced antinociception were 0.0209 and 3.940 mg/kg, respectively (**Figure 4B**). Thus, our data demonstrate that the antinociceptive potency of *N*-pyrrolidino etonitazene is approximately 10-fold greater than that of fentanyl and 2,000-fold greater than that of morphine.

A.



B.

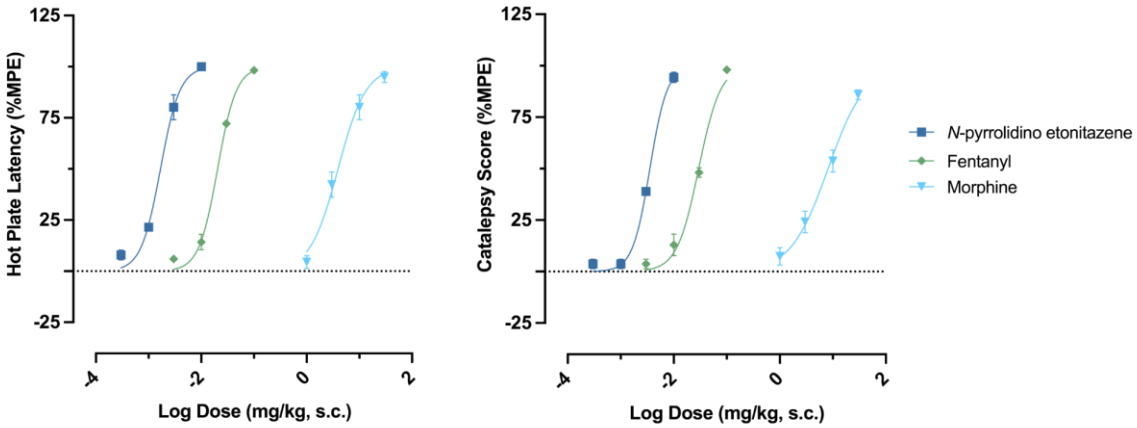


Figure 4. A. Time course of pharmacodynamic effects induced by subcutaneous 10% DMSO (vehicle, 1 mL/kg) or *N*-pyrrolidino etonitazene (0.0003, 0.001, 0.003, 0.010 mg/kg) in male rats. Hot plate latency, catalepsy score, and body temperature are depicted as mean ± SEM for n = 6 rats per dose group. Hot plate latency and catalepsy scores are presented as a percent of maximum possible effect (%MPE), whereas temperature data are expressed as change from baseline (Δ temperature in °C). Filled symbols indicate significant differences as compared to the vehicle-treated group at a given time point ($p < 0.05$, Tukey's). **B.** Dose-response curves obtained from nonlinear regression analysis (response stimulation normalized slope) of the mean hot plate responses and catalepsy scores (expressed as % MPE) over the first 60 min for *N*-pyrrolidino etonitazene, fentanyl, and morphine (n = 6 rats per dose group). Error bars represent SEM.

Toxicology results, case histories, and prevalence of* N-*pyrrolidino etonitazene

N-Pyrrolidino etonitazene was confirmed in 21 blood samples and one urine sample from a total of 21 postmortem cases collected between January and October 2021. Quantitative testing could be performed on samples from 15 cases. A summary of case histories, demographic information and analytical findings is shown in **Table 3.** The mean (± standard deviation) concentration of *N*-pyrrolidino etonitazene in blood was 2.5 ± 1.9 ng/mL, median 2.2 ng/mL, and range 0.3 to 8.3 ng/mL (excluding one case at 25 ng/mL). *N*-Pyrrolidino etonitazene was quantified in one urine sample at a concentration of 1.5 ng/mL.

N-Pyrrolidino etonitazene was detected in 21 cases, of which 17 were from nine U.S. states (**Figure 5**) (8 cases from West Virginia, two cases from Florida, and single cases from Colorado, Kentucky, Minnesota, New Jersey, New York, Pennsylvania, and Tennessee). Four cases from Canada originated from British Columbia. Overwhelmingly, the majority of decedents were male (*n* = 17) *vs.* female (*n* = 3), and one sex was not reported. Decedent ages ranged from 16 to 61 years, with a mean (± standard deviation) age of 41 (± 13). For cases with available history, the information available prior to or after death was consistent with what has been observed for other synthetic opioids (e.g., history of opioid use, drug powder and/or paraphernalia, depressed psychoactive behavior, found unresponsive, etc.).

Of the 21 cases, *N*-pyrrolidino etonitazene was found in combination with fentanyl in 57% of cases (*n* = 12), with methamphetamine in 57% of cases (*n* = 12), and with NPS benzodiazepines in 52% of cases (*n* = 11). Interestingly, a large variety of NPS benzodiazepines was found in combination with *N*-pyrrolidino etonitazene, including flualprazolam, etizolam, flubromazepam, clonazolam, and desalkylflurazepam. Other NPS found concurrently were protonitazene (opioid), 2-methyl AP-237 (opioid), alpha-PHP/alpha-PiHP (stimulant), 3Cl-PCP (hallucinogen), and para-fluorofentanyl (opioid). A significant finding, *N*-pyrrolidino etonitazene was the only opioid found in 33% of cases (*n* = 7).

When evaluating the prevalence of *N*-pyrrolidino etonitazene in the large cohort of forensic cases submitted to NMS Labs, an increasing trend was observed in the latter months of 2021 (**Figure 5**). In total, 197 cases were determined to be positive for *N*-pyrrolidino etonitazene following LC-TOF-MS analysis. In January 2021, four cases were observed *vs.* November 2021,

when 48 cases were observed. These data suggest that *N*-pyrrolidino etonitazene was still increasing to or possibly reaching peak positivity as of Q4 2021; however, further surveillance into 2022 is warranted. The largest number of positive cases was observed in November 2021.

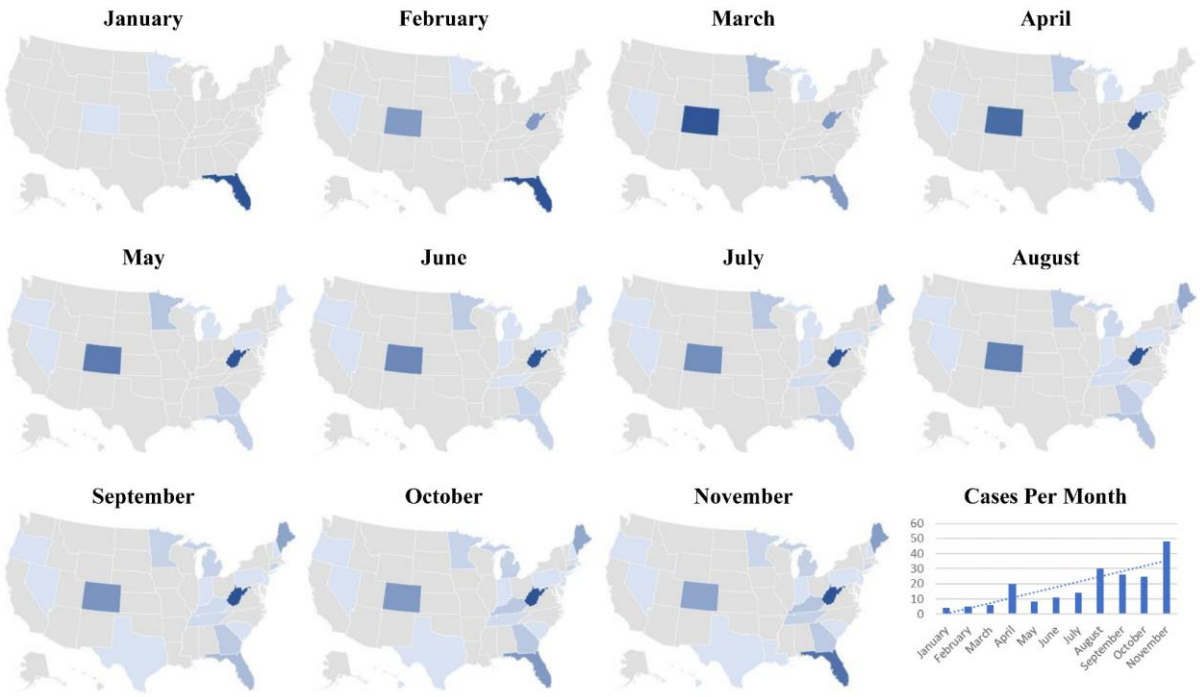


Figure 5. Geographical distribution of *N*-pyrrolidino etonitazene cases over time across the U.S. (nine cases from British Columbia, Canada, are not included). Case tallies are cumulative for each month. Surveillance of *N*-pyrrolidino etonitazene in 2021 shows that the number of cases testing positive increased throughout the year. The prevalence of this drug also increased, which is evident by the broader geographical distribution in the latter part of 2021. The intensity of the color indicates more cases, and the state total is relative to all other states (i.e., the most cases were observed in WV by November).

Table 3. Summary of postmortem forensic toxicology cases positive for *N*-pyrrolidino etonitazene in the U.S. and Canada.

Case #	Collection date (unless noted)	State, Country	Age	Sex	Matrix	Case history (if available)	Manner and Cause of Death	[*N*-Pyrrolidino etonitazene] (ng/mL)	Additional blood toxicology results (ng/mL, if available and unless otherwise noted)
1	1/14/2021	FL, US	53	M	Iliac blood	Decedent found unresponsive in vehicle. Extensive underlying pulmonary and cardiac pathological disease.	COD: Natural	8.3	Caffeine, cotinine, *O*-desmethyltramadol
2	2/25/2021	WV, US	26	M	Subclavian blood	Decedent had a history of drug use, with heroin and snorting as preferred drug and method of use. Individual was found unconscious and unresponsive early in the morning by his girlfriend. A brown powder was found in the decedent's right nostril, believed to be heroin.	MOD: Homicide; COD: Multiple GSWs	2.4	Flualprazolam (6.7), 4-ANPP, cotinine, morphine (7.5), THC (3.6), OH-THC (1.5), THC-COOH (15), amphetamine (52), methamphetamine (99), fentanyl (14), norfentanyl (3.6), xylazine, lidocaine, diphenhydramine, tramadol, quinine, phenethyl-4-ANPP, noscapine
3	3/25/2021 (received)	NY, US	NG	NG	Femoral blood	Not available	Not available	Positive	Etizolam, alpha-hydroxyetizolam, flubromazepam, alpha-PHP/alpha-PiHP, desalkylflurazepam, 2-methyl AP-237
4	3/26/2021	WV, US	44	M	Subclavian blood	Not available	Not available	Positive	Flualprazolam, 4-ANPP, quinine, amphetamine, methamphetamine, fentanyl, norfentanyl, lidocaine, diphenhydramine, 3Cl-PCP, 8-aminoclonazolam

5	4/2/2021 (received)	WV, US	41	M	Postmortem blood	Not available	Not available	Positive	Flualprazolam, lidocaine, naloxone, fentanyl, methamphetamine, lidocaine, quinine,
6	4/2/2021	MN, US	26	M	Femoral blood, Urine	Decedent was found face down in his bedroom. There was a plate with a white powdered substance organized in lines. No needles, tubes or pipes were found. History of marijuana and tobacco use. An internal examination was not performed.	MOD: Accident, COD: Toxic effects of *N*-pyrrolidino etonitazene	Blood: 2.5, Urine: 1.5	Ethanol (0.55 g/L), caffeine, venlafaxine, O-desmethylvenlafaxine (76), THC (9.6), OH-THC (2.6), THC-COOH (33), 7-amino clonazepam
7	4/6/2021	WV, US	30	M	Subclavian blood	Not available	Not available	Positive	Flualprazolam, 4-ANPP, amphetamine, methamphetamine, fentanyl, norfentanyl, diphenhydramine, para-fluoro-4-ANPP, para-fluorofentanyl, quinine
8	4/8/2021	PA, US	28	M	Jugular blood	Not available	Not available	Positive	THC, carboxy-THC, norbuprenorphine, flubromazepam
9	4/9/2021	CO, US	37	M	Femoral blood	Not available	Not available	Positive	Sertraline, desmethylsertraline, alpha-hydroxyetizolam
10	7/7/2021	WV, US	41	M	Subclavian blood	History of "heroin", methamphetamine, cannabis, and alcohol use. Found unresponsive and pronounced dead.	MOD: Accident, COD: Multidrug toxicity	1.7	Ethanol (180 mg/dL), fentanyl (4.4), 4-ANPP, caffeine, naloxone, THC (1.0), amphetamine (18), methamphetamine (210)

11	7/30/2021	BC, Canada	57	M	Subclavian blood	Decedent was found unresponsive on the floor of a sleeping area. The night before, the decedent had gone out drinking with friends. Upon returning home, the individual snorted drugs and was noted to have difficulty standing. Decedent collapsed but could be roused and was assisted to his sleeping area. Approximately 5h later, the individual was pronounced deceased. No drugs or paraphernalia were found at the scene, which the police suspect was cleaned prior to their arrival.	Not available	0.3	Flualprazolam (19), ethanol (1.04 g/L), 4-ANPP, caffeine, amphetamine (240), methamphetamine (1100), fentanyl (130), norfentanyl (62), acetylfentanyl (3.6), phenethyl-4-ANPP
12	8/11/2021	BC, Canada	16	F	Subclavian blood	Decedent with a history of drug use was found unresponsive by boyfriend approximately 6h following a night of what was believed to involve heroin use (IV injection and smoke inhalation). 2 doses of naloxone administered by boyfriend. Numerous used and unused hypodermic needles, burnt tinfoil, and a small empty plastic bag were found on the floor near the decedent.	Not available	0.64	Protonitazene (1.3), 4-ANPP, caffeine, naloxone, amphetamine (51), methamphetamine (110), fentanyl (41), norfentanyl (10), etizolam (9), alpha-hydroxy-etizolam, desalkylflurazepam, 8-aminoclonazolam
13	8/30/2021	WV, US	61	M	Subclavian blood	History of hypertension, cerebrovascular accident, COPD, and back pain from fall. Found unresponsive in bed and pronounced dead.	MOD: Accident, COD: Combined intoxication	3.9	Ethanol (29 mg/dL), amlodipine, caffeine, cotinine, nicotine, quetiapine (1100), venlafaxine (970), O-desmethylvenlafaxine (530), amitriptyline (66), nortriptyline

									(24), cyclobenzaprine (35), amphetamine (93), methamphetamine (1200)
14	9/1/2021	WV, US	48	M	Subclavian blood	Motor vehicle accident via pedestrian. History of methamphetamine use and COPD.	Pending	1.5	Caffeine, amphetamine (50), methamphetamine (1300)
15	9/12/2021	NJ, US	32	M	Femoral blood	Suspected drug overdose.	Not available	2.5	Flualprazolam 9.5, caffeine, THC-COOH (6.6), trazodone (0.15 mcg/mL)
16	9/17/2021	WV, US	55	M	Subclavian blood	History of IV drug use. Found unresponsive on living room floor and pronounced dead.	MOD: Accident, COD: Combined intoxication	1.9	4-ANPP, caffeine, cotinine, naloxone, quinine, benzoylecgonine (440), morphine (10), 6-MAM (1.2), xylazine (12), THC (0.98), amphetamine (93), methamphetamine (300), fentanyl (25), norfentanyl (1.1)
17	9/22/2021	BC, Canada	28	M	Subclavian blood	Decedent was observed trying to inject himself prior to death, sudden death, suspected drug overdose.	Not available	2.7	Flualprazolam (24), alpha-hydroxyetizolam (2.0), 4-ANPP, caffeine, cotinine, naloxone, desalkylflurazepam (12), THC-COOH (22), THC (1.9), amphetamine (75), methamphetamine (200), fentanyl (120), norfentanyl (18), acetylfentanyl (1.9)
18	9/28/2021	BC, Canada	60	M	Subclavian blood	History of drug use. Drug paraphernalia found at scene.	Not available	25	4-ANPP, caffeine, cotinine, morphine (340), hydromorphone (4.8), fentanyl (97), norfentanyl (17), acetylfentanyl (0.20)

19	9/30/2021	FL, US	53	F	Iliac blood	Individual was a transient living in a motel. Boyfriend woke up and discovered the individual unresponsive and called 911. EMS transported the individual to the ED where she died soon after arrival. History of alcohol use but not a known illicit drug user.	Not available	1.1	Naloxone, 7-amino clonazepam (11), gabapentin (9.0 mcg/mL)
20	10/4/2021	TN, US	46	M	Femoral Blood	History of obesity and "heroin" use.	MOD: Accidental, COD: Combined N-pyrrolidino etonitazene and fentanyl intoxication. Secondary conditions include obesity and cardiomyopathy.	1.7	Fentanyl (2.7), ANPP, Caffeine, Cotinine
21	10/6/2021	KY, US	45	F	Jugular blood	Suspected opioid overdose.	Not available	3.3	Butalbital (1.0 mcg/mL), gabapentin (21 mcg/mL), THC-COOH (13), THC (2.1), ephedrine (16), phenylpropanolamine (75), amphetamine (300), phentermine (900), methamphetamine (3100), fentanyl (30), norfentanyl (0.84), clonazepam (7.1), 7-amino clonazepam (32)

Key: FL – Florida, WV – West Virginia, NY – New York, MN – Minnesota, PA – Pennsylvania, CO – Colorado, US – United States, BC – British Columbia, NJ – New Jersey, KY – Kentucky, TN – Tennessee, M – Male, F – Female, h – hour, MOD – manner of death, COD – cause of death, COPD – chronic obstructive pulmonary disease, GSW – gun shot wound, THC – delta-9-tetrahydrocannabinol, 6-MAM – 6-monoacetylmorphine, EMS – emergency medical services, ED – emergency department

Discussion

N-Pyrrolidino etonitazene, also referred to as 'etonitazepyne' online, has recently emerged on recreational drug markets worldwide (Krotulski et al. 2021d; Blanckaert et al. 2021; Pucci et al. 2021), representing one of the newest additions to the family of 2-benzylbenzimidazole or 'nitazene' opioids. In contrast to other nitazenes that have emerged so far (Ujváry et al. 2021; Vandeputte et al. 2021b) (Vandeputte & Verougstraete *et al.*, manuscript submitted), *N*-pyrrolidino etonitazene cannot be traced back to existing scientific or patent literature. While adverse events and fatalities involving this novel nitazene are increasingly being reported, pharmacological data on *N*-pyrrolidino etonitazene have not previously been reported. In this study, we performed pharmacological characterization of *N*-pyrrolidino etonitazene at the *in vitro* and *in vivo* level to help better understand the risks associated with its increasingly widespread circulation and use.

Taken together, our data indicate that *N*-pyrrolidino etonitazene is a selective and highly potent MOR agonist. While the MOR binding affinity of *N*-pyrrolidino etonitazene was comparable to that of morphine, the use of an *in vitro* MOR activation assay revealed that *N*-pyrrolidino etonitazene is over 800 times more potent and 2.5 times more efficacious than morphine at activating MOR. In addition, *N*-pyrrolidino etonitazene exceeded the *in vitro* potency of fentanyl > 40 times, with comparable efficacy. While these findings clearly indicate the high harm potential of *N*-pyrrolidino etonitazene use, ultimately, *in vivo* effects are largely dependent on blood-brain-barrier penetration, stability, formation of active metabolites, and so on. Hence, to confirm the *in vitro* profile of *N*-pyrrolidino etonitazene as a potent MOR agonist, an evaluation of opioid-related pharmacodynamics in rats was performed. We found that *N*-pyrrolidino etonitazene produces dose-dependent antinociception and catalepsy in rats, with *in vivo* potencies mirroring the trends seen using *in vitro* functional assays. Specifically, *in vivo* potencies for *N*-pyrrolidino etonitazene were in the µg/kg range for both catalepsy and antinociception. In contrast, potencies for morphine were > 2,000 times lower, in the mg/kg range. In line with *in vitro* functional data, the *in vivo* potency of fentanyl was about 10 times lower than that of *N*-pyrrolidino etonitazene in both antinociception and catalepsy assays. In agreement with earlier studies with different opioids (Pöyhiä and Kalso 1992; Taracha et al. 2009; Truver et al. 2020; Bergh et al. 2021), the potency for catalepsy induced by *N*-pyrrolidino etonitazene was slightly right-shifted compared to the effects in the

hot plate assay. As often observed for opioids (Geller et al. 1983; Adler et al. 1988; Rawls and Benamar 2011), the effects of *N*-pyrrolidino etonitazene on body temperature were biphasic: low doses resulted in a small but significant rise in body temperature, whereas higher doses reverted this profile to hypothermia. Similar trends were seen for morphine and fentanyl at the different dose levels evaluated. For morphine and fentanyl, the lowest body temperature was reached at 30 and 0.1 mg/kg, respectively. For *N*-pyrrolidino etonitazene, the most pronounced decrease in body temperature was observed after 10 μg/kg, indicating that this drug is 3,000-fold and 10-fold more potent than morphine and fentanyl in its ability to produce hypothermia in the rat. Importantly, the dose of *N*-pyrrolidino etonitazene responsible for significant hypothermia was much higher than that needed for antinociception, indicating that adverse opioid effects likely require higher doses of the drug. It should be noted that while hypothermia was used in this study as a proxy for adverse opioid effects, an *in vivo* correlation with such effects (e.g., respiratory depression, bradycardia) requires further investigation (Bergh et al. 2021).

While *N*-pyrrolidino etonitazene itself has not been previously described in literature, some related 2-benzylbenzimidazoles have been studied before. One structural analogue that has an *N*-pyrrolidino group but lacks the nitro-group at the 5-position of the benzimidazole ring (*N*-pyrrolidino etodesnitazene) was described in 1960 (Hunger et al. 1960b). In a mouse tail-flick assay, this drug was found to have an antinociceptive potency 20 times greater than morphine. As it is known that the 5-NO_2 group plays an important role in the opioid activity of 2-benzylbenzimidazoles (Hunger et al. 1960a, b; Ujváry et al. 2021; Vandeputte et al. 2021b), it is not unexpected that *N*-pyrrolidino etonitazene has even higher *in vivo* activity, as shown in this study using a hot plate assay. Seki *et al*. (Seki 1967; Seki and Watanabe 1969) later reported on a similar *N*-pyrrolidino etodesnitazene analogue in which the methylene linker between the two aromatic rings is replaced by a thioether bridge. However, this analogue was only 2-5 times more potent than morphine (Ujváry et al. 2021). Further, a chlorinated analogue of *N*-pyrrolidino etonitazene (*N*-pyrrolidino clonitazene) has been described as a synthetic intermediate (Bucha et al. 2018; Ujváry et al. 2021). Carrying a diethylaminoethyl moiety at the 1-position of the benzimidazole ring, etonitazene is the most closely related – and most widely studied – analogue of *N*-pyrrolidino etonitazene. With an *in vivo* antinociceptive potency exceeding that of morphine 1,000-fold in a tail-flick assay,

etonitazene was previously the most active 2-benzylbenzimidazole opioid described (Gross and Turrian 1957; Hunger et al. 1957, p. 57, 1960a; Ujváry et al. 2021). The data presented herein indicate that *N*-pyrrolidino etonitazene is likely on par with etonitazene in terms of opioid activity. *N*-pyrrolidino etonitazene showed comparable *in vitro* MOR activation potential (potency and efficacy) to etonitazene. Importantly, while etonitazene is internationally controlled under the 1961 Single Convention on Narcotic Drugs (UNODC 1975), *N*-pyrrolidino etonitazene is currently not under international control. Recognizing the potential harm the drug may evoke, the U.S. DEA recently announced temporary placement of *N*-pyrrolidino etonitazene into Schedule I of the CSA (DEA 2021). Other countries have taken (or are taking) similar legislative action – e.g., in Belgium, *N*-pyrrolidino etonitazene was very recently (December 2021) listed as a scheduled substance (Belgisch Staatsblad 2022). It remains to be evaluated whether these scheduling initiatives will be effective in the potential decline in availability of *N*-pyrrolidino etonitazene (Vandeputte et al. 2021a).

N-Pyrrolidino etonitazene was identified as a toxicologically significant finding during medicolegal death investigations in the U.S. and Canada where decedents were commonly suspected to have died of an opioid- or drug-related overdose. *N*-Pyrrolidino etonitazene was found both alongside other opioids and without other opioids, demonstrating the potential for this drug to have synergistic opioid/depressant effects and toxicity. Currently available forensic data, paired with the pharmacological data collected as part of this study, lead toxicology experts to believe that *N*-pyrrolidino etonitazene retains opioid toxicity similar to other synthetic opioids and nitazene analogues, with the ability to cause death. In two cases, medical examiners ruled decedent causes of death as the “toxic effects of *N*-pyrrolidino etonitazene” and “combined *N*-pyrrolidino etonitazene and fentanyl intoxication”; in both cases, the manner of death was accidental.

Polydrug use has become a common finding during toxicological evaluation, including across drug classes and countries. Opioids are often found alongside other central nervous system (CNS) depressants, including other opioids or benzodiazepines, and stimulants, most commonly methamphetamine and cocaine. Previous reports have shown that nitazene analogues are commonly found in combination with NPS benzodiazepines, both *in vivo* and within the drug materials themselves, a phenomenon colloquially called “benzo dope”

(Krotulski et al. 2020a, 2021e). In all cases reported here, *N*-pyrrolidino etonitazene was encountered with other drugs in biological samples. Of the different NPS benzodiazepines detected alongside *N*-pyrrolidino etonitazene, flualprazolam was the most commonly encountered – this finding is similar to cases involving isotonitazene in 2019 and 2020. Low ng/mL *N*-pyrrolidino etonitazene concentrations observed in blood for the death investigation cases reported here are in line with the high potency shown using *in vitro* and *in vivo* assessments – generally, as potency increases, the concentration of a drug in a toxicology case decreases, excluding outliers (e.g., suicides, unusually high concentrations) (Fogarty & Vandeputte *et al.*, manuscript resubmitted). All confirmed concentrations were less than 10 ng/mL, stressing the need for increased analytical sensitivity when developing quantitative methods for these new potent nitazene opioids. In comparison to mean (± std. dev.) and median concentrations of isotonitazene (2.2 ± 2.1 ng/mL, 1.8 ng/mL, *n* = 18) and metonitazene (6.3 ± 7.5 ng/mL, 3.8 ng/mL, *n* = 18) in postmortem cases, the mean (± std. dev.) and median concentrations of *N*-pyrrolidino etonitazene (2.5 ± 2.0 ng/mL, 2.4 ng/mL, *n* = 13) indicate that, by crude *in vivo* toxicological assessment, this drug has at least similar to greater potency than both previously prevalent nitazenes, albeit the sample size for *N*-pyrrolidino etonitazene is slightly smaller (Krotulski et al. 2021e). The low blood concentrations of *N*-pyrrolidino etonitazene agree with the *in vitro* findings, as the drug was found to have a MOR activation potency similar to etonitazene. Previously, we showed that both isotonitazene and metonitazene are respectively 2.5 and 12 times less potent than etonitazene *in vitro* (Vandeputte et al. 2021b).

Conclusions

N-Pyrrolidino etonitazene ('etonitazepyne') is a novel synthetic opioid of concern that has been linked to various adverse events and fatalities worldwide. By employing an array of *in vitro* and *in vivo* techniques, we show that *N*-pyrrolidino etonitazene is a selective and extremely potent MOR agonist. With an *in vitro* MOR activation potential comparable to etonitazene, *N*-pyrrolidino etonitazene is among the most active (nitazene) opioids currently circulating on recreational drug markets. Its involvement in 21 postmortem cases in the U.S. and Canada further confirms the high risks associated with consumption of *N*-pyrrolidino etonitazene and its potential to cause death after an overdose.

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expressed in this publication are those of the author(s) and do not necessarily reflect those of the Department of Justice.

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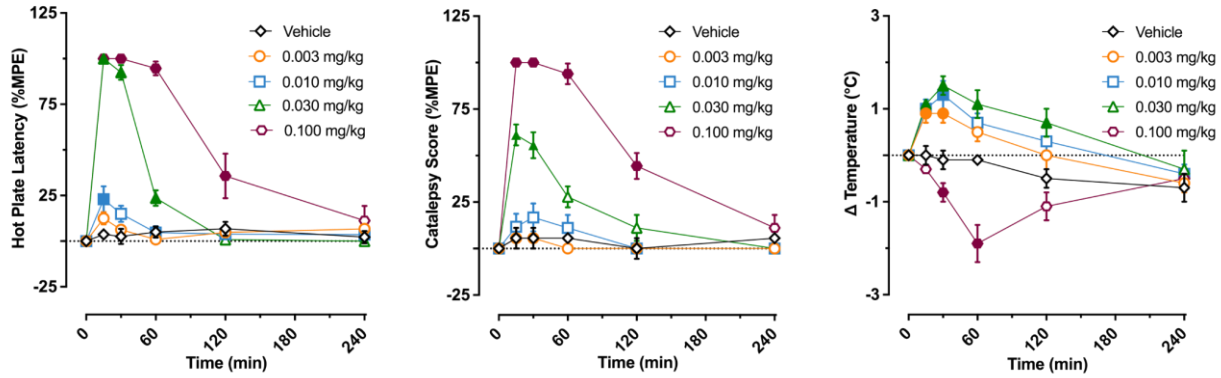
Supplementary Information

A.



B.

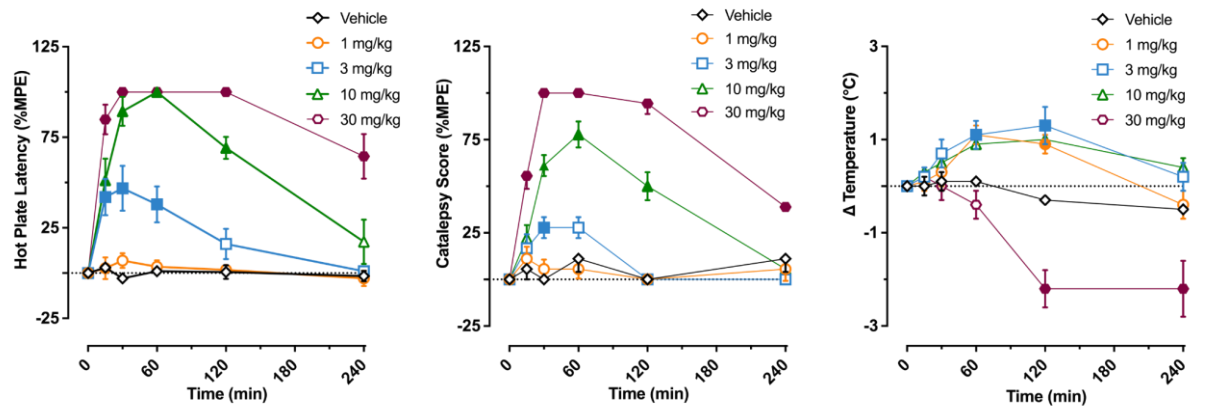


Figure S1. Time course of pharmacodynamic effects induced by subcutaneous saline (vehicle, 1 mL/kg), fentanyl (**A**) (0.003, 0.010, 0.030, 0.10 mg/kg) or morphine (**B**) (1, 3, 10, 30 mg/kg) in male rats. Hot plate latency, catalepsy score, and body temperature are depicted as mean ± SEM for *n* = 6 rats per dose group. Hot plate latency and catalepsy scores are presented as a percent of maximum possible effect (%MPE), whereas temperature data are expressed as change from baseline (Δ temperature in ^{o}C). Filled symbols indicate significant differences as compared to the vehicle-treated group at a given time point ($p < 0.05$, Tukey's).