**Alkoxy chain length governs the potency of 2-benzylbenzimidazole ‘nitazene’ opioids associated with human overdose**

Grant C. Glatfelter1\*, Marthe M. Vandeputte2, Li Chen3, Donna Walther1, Meng-Hua M. Tsai3, Lei Shi3, Christophe P. Stove2, and Michael H. Baumann1

1Designer Drug Research Unit, National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD, USA

2Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

3Computational Chemistry and Molecular Biophysics Section, National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD, USA

\*Corresponding author

Grant Glatfelter, Ph.D.

Designer Drug Research Unit (DDRU)

National Institute on Drug Abuse (NIDA), Intramural Research Program (IRP)

333 Cassell Drive, Suite 4400

Baltimore, MD 21224

grant.glatfelter@nih.gov

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**ABSTRACT**

Novel synthetic opioids (NSOs) are emerging in recreational drug markets worldwide. In particular, 2-benzylbenzimidazole ‘nitazene’ compounds are problematic NSOs associated with serious clinical consequences, including fatal respiratory depression. Evidence from *in vitro* studies shows that alkoxy chain length can influence the potency of nitazenes at the mu-opioid receptor (MOR). However, structure-activity relationships (SARs) of nitazenes for inducing opioid-like effects in animal models are not well understood compared to relevant opioids contributing to the ongoing opioid crisis (e.g., fentanyl). Here, we examined the *in vitro* and *in vivo* effects of nitazene analogues with varying alkoxy chain lengths (i.e., metonitazene, etonitazene, isotonitazene, protonitazene, and butonitazene) as compared to reference opioids (i.e., morphine and fentanyl). Nitazene analogues displayed nanomolar affinities for MOR in rat brain membranes and picomolar potencies to activate MOR in transfected cells. All compounds induced opioid-like effects on locomotor activity, hot plate latency, and body temperature in mice, and alkoxy chain length markedly influenced potency. Etonitazene, with an ethyl chain, was the most potent analogue in MOR functional assays (EC50=30 pM, *E*max=103%) and across all *in vivo* endpoints (ED50=3 -12 μg/kg). *In vivo* SARs revealed that ethyl, isopropyl, and propyl chains engendered higher potencies than fentanyl, whereas methyl and butyl analogues were less potent. MOR functional potencies, but not MOR affinities, were positively correlated with *in vivo* potencies to induce opioid effects. Overall, our data show that certain nitazene NSOs are more potent than fentanyl as MOR agonists in mice, highlighting concerns regarding the high potential for overdose in humans who are exposed to these compounds.

**INTRODUCTION**

Novel synthetic opioids (NSOs) pose increasing risks to global health and safety (Prekupec et al. 2017). In the United States (US), the misuse of opioids is responsible for staggering mortality statistics, with about two-thirds of recent drug overdose fatalities involving illegally manufactured opioids (O'Donnell et al. 2021; RAND Corporation 2022). Fentanyl is the main driving force behind the US opioid crisis, but a number of highly potent NSOs have appeared in the US, Europe, and elsewhere (Abdulrahim and Bowden-Jones 2022; UNODC 2020a). For example, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported more than seventy new opioid compounds on recreational drug markets since 2009 (EMCDDA 2022b). While fentanyl analogues dominated the NSO market prior to 2018, the recent legislative bans on fentanyl and its analogues (Bao et al. 2019; United States Congress 2020) have fostered the emergence of opioids based on diverse non-fentanyl templates (e.g., cyclohexylbenzamides, thiambutenes, cinnamylpiperazines, 2-benzylbenzimidazoles, benzimidazolones) (Hasegawa et al. 2022; Papsun et al. 2022; UNODC 2020a; b). One particular group of non-fentanyl NSOs is the 2-benzylbenzimidazole opioids, also known as “nitazenes” (EMCDDA 2022b; Papsun et al. 2022; Vandeputte et al. 2021a). Importantly, *in vitro* findings show that certain nitazene analogues are more potent than fentanyl in activating the mu-opioid receptor (MOR), the principal site of action mediating therapeutic and adverse opioids effects (De Luca et al. 2022; Ujváry et al. 2021; Vandeputte et al. 2021b; Vandeputte et al. 2022c).

The synthesis of 2-benzylbenzimidazole opioids can be traced back to the late 1950s, when this chemical scaffold was investigated for its morphine-like properties (Gross and Turrian 1957; Hunger et al. 1957; 1960a; b; c; 1961; Rossi et al. 1960a; b; Ujváry et al. 2021). The most potent compound, etonitazene, exhibits much higher antinociceptive potency than morphine in a mouse tail-flick assay (Gross and Turrian 1957). None of the nitazene opioids were ever approved for clinical use (Ujváry et al. 2021), but based on their high potencies, the recreational misuse of these compounds was predicted (Shulgin 1975). While only a few reports of etonitazene misuse were ever recorded (Brandenberger 1974; Morris 2009; Reavy 2003; Sorokin 1999a; b; Ujváry et al. 2021), isotonitazene was identified as a problematic NSO in 2019 (Blanckaert et al. 2020; EMCDDA 2020; Krotulski et al. 2019; Mueller et al. 2021; Papsun et al. 2022; Vandeputte et al. 2021a). Isotonitazene exposures spread rapidly among individuals who use opioids, contributing to drug-related accidents and fatalities (EMCDDA 2020; Krotulski et al. 2020; Mueller et al. 2021; Shover et al. 2021). With the gradual decline of isotonitazene popularity (Vandeputte et al. 2021a), various other nitazenes emerged, and a total of ten are currently being monitored on the European drug market (EMCDDA 2022b; UNODC 2023). Recognizing the high harm potential of nitazene opioids, various international drug control laws were implemented to halt further distribution (DEA 2021; UNODC 2021; 2022a).

The rapid proliferation of nitazene analogues on recreational drug markets, combined with an increasing number of case reports associated with their use, has sparked new interest in the pharmacology of these compounds. Recently, various research groups have characterized the mechanism of action of nitazenes at MOR *in vitro* (De Luca et al. 2022; Kanamori et al. 2023; Malcolm et al. 2023; Vandeputte et al. 2022a; Vandeputte et al. 2021b; Vandeputte et al. 2022c). However, recent research assessing relationships between *in vitro* findings and *in vivo* effects is limited. While early mouse studies evaluated the antinociceptive effects of many nitazenes in comparison to morphine, a systematic comparison of *in vitro* and *in vivo* effects of a set of structurally related nitazene analogues, especially compared to fentanyl, is warranted. To address this knowledge gap, the present study examined structure-activity relationships (SARs) of nitazenes for inducingopioid-like effects in mice, and related these effects to *in vitro* binding affinities and functional potencies at MOR. Specifically, we compared the opioid-like effects produced by five nitazene analogues differing in alkoxy chain length (i.e., methyl, ethyl, isopropyl, propyl, and butyl) as compared to the reference opioid compounds morphine and fentanyl (see **Figure 1**).

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**Figure 1.** Structures of 2-benzylbenzimidazole 'nitazene' opioids differing in alkoxy chain length (red).

**MATERIALS AND METHODS**

*Drugs and reagents*

Metonitazene citrate, etonitazene HCl, isotonitazene HCl, protonitazene HCl, and butonitazene free base were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fentanyl HCl and morphine sulphate were provided by the NIDA Drug Supply Program (Rockville, MD, USA). All other chemicals and reagents were obtained from Millipore-Sigma (St. Louis, MO, USA) unless otherwise noted. For *in vitro* experiments, compounds were dissolved in 100% DMSO to yield 1 mM stock solutions, which were subsequently diluted in assay buffer. *In vitro* drug concentrations are expressed as the free base. For mouse behavioral studies, most drugs (metonitazene, etonitazene, isotonitazene, protonitazene, fentanyl) were dissolved in 10% DMSO:90% saline, while morphine was dissolved in saline. Butonitazene was dissolved in 5% Tween 80:5% DMSO:90% saline. All drugs for mouse experiments were administered as the weight of the salt, except for butonitazene which was administered as free base, at a volume of 0.01 mL/g body weight.

*Competition binding for opioid receptors*

Opioid receptor binding assays were carried out in rat brain tissue as described (Truong et al. 2017; Truver et al. 2020; Vandeputte et al. 2022c). Further experimental details can be found in **Supporting Information**.

*MOR forskolin-stimulated cAMP functional assay*

Forskolin-stimulated cyclic AMP (cAMP) accumulation was determined using the CISBIO Homogenous Time-Resolved Fluorescence (HTRF) cAMP-Gi kit (PerkinElmer), in FLP-FRT-HEK cells stably expressing human MOR as previously described (Cai et al. 2019). Detailed description of methods can be found in **Supporting Information**.

*Animals and housing*

C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 7-8 weeks of age, group-housed for one to two weeks with *ad libitum* access to food and water throughout, and maintained in a standard 12-hour light-dark cycle (lights on at 7 am) in vivarium facilities at the National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD, USA. These facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and all procedures were approved by the Animal Care and Use Committee of the NIDA IRP.

*Transponder implants and assessment of opioid-like effects in mice*

One week prior to behavioral testing, subcutaneous (s.c.) temperature transponders (14 x 2 mm, model IPTT-300, Bio Medic Data Systems, Inc., Seaford, DE, USA) were surgically implanted on the back of mice as previously described (Glatfelter et al. 2022a; Glatfelter et al. 2022b; Rudin et al. 2022). Brief anesthesia was accomplished by means of drop-jar exposure to isoflurane. Once implanted, the transponders allowed non-invasive body temperature measurement using a handheld reader. Mice were single-housed post-operatively for the duration of behavioral testing.

On the day of an experiment, mice were transported from the vivarium to the testing facilities in their home cages. After a 1 h acclimation period, mice were weighed, and basal body temperature, hot plate latency, and locomotor activity were measured (timepoint -15 min). The total session length was 90 min, with the first 15 min being used as an acclimation period to the chambers and to collect baseline measures. After 15 min in the locomotor chamber, baseline body temperature and hot plate latency measurements were repeated (timepoint 0 min), after which the animals received s.c. injections of test drugs dissolved in respective vehicles. Post-injection, the animals were quickly returned to the locomotor chamber for continuous monitoring of distance traveled (cm) for 75 min. Body temperature (°C) and hot plate latency (sec) were measured every 15 min over the remaining 75 min of the testing session. Cohorts of 12 mice per drug were tested once every 4 – 7 days for a maximum of 6 weeks, and doses were randomly assigned. Experiments were conducted during the light phase, between 9 am and 5 pm local time.

Hot plate latency was determined as previously described (Glatfelter et al. 2022a) by placing the mice onto a 52°C hot plate (IITC Life Sciences, Woodland Hills, CA, USA). Paw licking, paw shaking or flicking, repeated genitalia licking, or repeated jumping were considered reactions to the heat stimulus, leading to immediate removal from the hot plate. A 45 s cutoff time was used to prevent tissue damage. For the measurement of locomotor activity, mice were placed inside a TruScan locomotor chamber (Coulbourn Instruments, PA, USA) and locomotor activity was continuously monitored throughout the experiment. Changes in body temperature (°C) were monitored via a handheld reader (Bio Medic Data Systems model DAS-7006/7r) that interfaces with the implanted transponder.

*Data analysis and statistics*

Concentration-response curves for competition of nitazenes for MOR, DOR, and KOR binding in rat brain membranes were generated using nonlinear regression, and affinities (Ki values) were determined using previously determined Kd values (one site fit Ki). For the determination of potencies (EC50) in the MOR - cAMP functional assay, data were plotted using a three-parameter nonlinear regression fit and *E*max was set to the maximal effect of DAMGO. For mouse studies, body temperature data for each subject were transformed to temperature change from baseline taken at timepoint zero (temperature ∆). Mean temperature ∆ across all timepoints post injection was used to calculate mean effects and plot dose-response curves. Hot plate latencies were normalized for baseline responsiveness calculated as percent maximum possible effect (%MPE) at each timepoint: (experimental measure – baseline measure) / (maximum possible response – baseline measure) x 100. The maximum possible response was 45 sec. Mean %MPE across all timepoints for the first 60 min post injection was used to plot dose-response curves. Locomotor activity data were measured as distance traveled (cm) and plotted for visual depiction with bell-shaped nonlinear regression fits (top constrained to maximum observed effect and ED50 constrained to > 0).

Drug potencies (ED50) in the mouse studies were determined using the ascending limb of the dose response curves for locomotor activity and the full curves for hot plate latency (%MPE) using four-parameter variable slope fits. Potencies for temperature ∆ were determined using three-parameter nonlinear regression fits of all data points. Mean effects for measures in mouse studies were all compared to vehicle controls using one-way ANOVA with Dunnett’s post hoc test (*p* < 0.05). All graphs were created and statistically analyzed using GraphPad Prism 9 (La Jolla, CA, USA).

**RESULTS**

***Nitazenes are MOR-selective ligands***

Nitazenes with varying alkoxy chain lengths were first tested for opioid receptor binding.All nitazenes fully inhibited [3H]DAMGO binding in rat brain membranes, displaying MOR binding affinities in the low nM range (Ki ~4 – 50 nM), with the rank order of affinities being metonitazene > etonitazene > isotonitazene > protonitazene > butonitazene (**Table 1**). **Figure S1A** depicts the competition of these compounds with [3H]DAMGO for binding at MOR. While there is some overlap in confidence intervals, this rank order suggests that increasing alkoxy chain length reduces binding affinity at MOR. While morphine (Ki = 0.8 nM) had a higher MOR affinity than the nitazenes, fentanyl binding (Ki = 4.8 nM) was comparable to that of metonitazene (Ki = 3.6 nM) and etonitazene (Ki = 6.6 nM). Compounds were also tested in competition binding assays with [3H]DADLE and [3H]U69,593 to determine DOR and KOR affinities (see **Figure S1B - C** and **Table S1**). Nitazenes displayed much weaker affinities for DOR

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| **Ligand** | **Affinity, potency, & efficacy *in vitro*** | **Potency in mouse studies** |
| **MOR****[3H]DAMGO****Binding** | **MOR****Gi – cAMP** | **Temperature Δ****(°C)** | **Locomotor activity (distance cm)** | **Antinociception** **(% MPE)** |
| **Ki (nM)**  | **EC50 (nM)** | **ED50 (mg/kg)**  | **ED50 (mg/kg)**  | **ED50 (mg/kg)**  |
| Metonitazene | 3.6[1.9 – 6.2] | 0.09[0.08 – 0.1]*E*max = 105%  | 0.48 [0.23 - 1.33] | 0.11 [0.08 - 0.15] | 0.31 [0.25 - 0.38] |
| Etonitazene | 6.6[4.1 – 10.5] | 0.03[0.02 – 0.04]*E*max = 103% | 0.009 [0.004 - 0.022] | 0.003 [0.002 - 0.005] | 0.012 [0.009 - 0.016] |
| Isotonitazene | 15.5[9.4 – 27.0] | 0.06[0.05 – 0.07]*E*max = 103% | 0.021 [0.009 - 0.047] | 0.009[0.003 - 0.014] | 0.021 [0.018 - 0.024] |
| Protonitazene | 21.5[13.9 – 33.6] | 0.18[0.1 – 0.3]*E*max = 108% | 0.03 [0.01 - 0.07] | 0.016 [0.00 - 0.05] | 0.04 [0.03 - 0.05] |
| Butonitazene | 53.1[31.4 – 98.3] | 0.46[0.3 – 0.7]*E*max = 103% | 1.05 [0.43 - 2.95] | 0.91 [0.00 – 3.49] | 1.41 [1.17 - 1.65] |
| Fentanyl | 4.8[2.6 – 8.9] | 0.09[0.07 – 0.12]*E*max = 98% | 0.09 [0.04 - 0.22] | 0.05 [0.03 - 0.13] | 0.16 [0.13 - 0.18] |
| Morphine | 0.8[0.6 – 1.2] | 1.20[1.0 – 1.4]*E*max = 100% | 12.45[7.84 - 20.22] | 3.30[1.43 – 8.90] | 10.19[8.32 - 12.06] |
| DAMGO | NT | 1.57[1.2 – 2.0]*E*max = 100% | NT | NT | NT |
|  |  |  |  |  |  |

**Table 1.** Results obtained for nitazene analogues and comparator compounds upon evaluation of *in vitro* MOR binding, *in vitro* MOR function, and *in vivo* effects in mice. The inhibition constants from MOR binding assays (Ki), potency values from cAMP accumulation assays (EC50), and *in vivo* assays (ED50) are shown as mean values, with 95% CI in brackets, for *n* = 3 (binding experiments), *n* = 5 (cAMP experiments), or *n* = 4 - 6 (mouse experiments) per drug concentration or drug dose. NT, not tested.

(962 – 4,186 nM) and KOR (513 – 4,313 nM) when compared to their effects at MOR. The DOR/MOR and KOR/MOR affinity ratios shown in **Table S1** also support this by showing that nitazenes, fentanyl, and morphine are about 40 – 700-fold selective for MOR over KOR or DOR.

***Nitazenes inhibit MOR-mediated forskolin-stimulated cAMP***

To assess functional potency and efficacy of nitazenes at MOR, a forskolin-stimulated cAMP assay was utilized. Potency and efficacy measures are listed in **Table 1,** whileconcentration-response curves are shown in **Figure S1D**. DAMGO was used as the reference agonist, which displayed potent inhibition of cAMP formation (EC50 = 1.6 nM, *E*max = 100%). Nitazene compounds displayed sub nM potencies (EC50 = 0.03 – 0.46 nM) with full agonist efficacies (*E*max = 103 – 108%). The rank order of potencies for nitazenes in the cAMP assay was etonitazene > isotonitazene > metonitazene ≥ protonitazene ≥ butonitazene. Relative to DAMGO, nitazenes were ~3 – 50-fold more potent at inhibiting MOR-mediated cAMP formation. Etonitazene, the most potent compound (EC50 = 0.03 nM), displayed approximately 3-fold greater potency than fentanyl and 40-fold greater potency than morphine. In general, the functional potencies of nitazenes for agonist activity at MOR did not agree with the rank order of binding affinities at MOR. Instead, the rank order of MOR functional potencies suggests that nitazenes with ethyl or isopropyl side chains (i.e., etonitazene and isotonitazene) had higher potencies compared to nitazenes with shorter (methyl) or longer (butyl) alkoxy tails. Butonitazene, with the longest alkoxy tail, was the least potent (EC50 = 0.5 nM).

***Nitazenes induce opioid-like effects in vivo***

Next, we sought to compare the opioid-like effects of nitazenes and standard reference opioid agonists in mice. To this end, the five nitazene compounds and the standard control comparators, morphine and fentanyl, were administered s.c. to mice at various doses (0.001 – 100 mg/kg) to measure acute effects on locomotor activity, body temperature, and hot plate antinociception. **Figure 2** depicts representative time-course plots for the most potent nitazenes, etonitazene (A – C) and isotonitazene (D – F), for all measures. Time-course plots for effects of all other compounds tested in mouse studies can be found in either **Figure S2** (metonitazene, protonitazene, butonitazene)or **Figure S3** (fentanyl and morphine).



**Figure 2.** Time-course of drug effects on hot plate latency, locomotor activity, and body temperature produced by s.c. administration of etonitazene (**A – C**) or isotonitazene (**D – F**) in mice. Latency data are depicted as percent of maximal possible effect (%MPE), locomotor data are depicted as distance traveled (cm), and temperature data are depicted as change from baseline (oC). All values are mean ± SEM for *n* = 4 – 6 mice/dose group.

All compounds produced dose-dependent increases in hot plate latency, hyperlocomotion, and hypothermia (**Figure 3**, **Table S2**, **Table S3**). In contrast to the monophasic dose-response relationships for antinociception and hypothermia, data for locomotor activity displayed inverted U-shaped curves (**Figure 3B**). The potency values for hyperlocomotion, temperature change, and antinociceptive effects are listed in **Table 1**. The relative rank order of potencies across all measures in mouse studies was etonitazene > isotonitazene > protonitazene > fentanyl > metonitazene > butonitazene > morphine. Maximal effects across compounds for antinociception (84-100% MPE), hyperlocomotion (30,000 – 40,000 cm), and hypothermia (-3 °C) were similar. Relative to all other compounds, morphine displayed the lowest maximal effects for hyperlocomotion (~20,000 cm) and the highest maximal temperature reductions (-6 °C). While there was some overlap in confidence intervals with isotonitazene, etonitazene was the most potent compound tested for all measures (ED50 = 0.003 – 0.012 mg/kg), with ~10 – 17-fold higher potencies compared to fentanyl (ED50 = 0.05 – 0.16 mg/kg), and ~850 – 1,400-fold higher potencies compared to morphine (ED50 = 3.30 – 12.45 mg/kg). Isotonitazene (ED50 = 0.009 – 0.021 mg/kg) and protonitazene (ED50 = 0.016 – 0.040 mg/kg) were also generally more potent than both reference opioid agonists, while metonitazene (ED50 = 0.11 – 0.48 mg/kg) and butonitazene (ED50 = 0.91 – 1.41 mg/kg) were more potent than morphine, but not fentanyl.

Threshold effects for etonitazene to produce antinociception-like responses and hypothermia were seen at the 0.01 mg/kg dose (**Figure 2A** and **C**, **Figure 3A** and **C**, **Table S3**), while the threshold dose required for hyperlocomotion was even lower (0.003 mg/kg, **Figure 2B**, **Figure 3B**, **Table S3**). All other compounds required at least 3-fold higher doses compared to etonitazene to produce threshold effects across the same measures in mice. Similar to *in vitro* functional results for MOR activation, the compounds with ethyl and isopropyl substitutions displayed the highest *in vivo* potencies vs. shorter (i.e., methyl) or longer (i.e., butyl) chain substitutions. Overall, the data demonstrate that the potencies for inducing hyperlocomotion were left-shifted compared to potencies for antinociception and temperature change (i.e., opioid-induced hyperlocomotion occurred at lower doses than antinociception and temperature changes).



**Figure 3.** Dose-response curves for hot plate latency (**A**), locomotor activity (**B**), and body temperature changes (**C**) induced by s.c. administration of nitazenes in mice. Data shown are mean effects over the first 60 min of the test sessions. Hot plate latency data are depicted as percent of maximal possible effect (%MPE), locomotor data are depicted as total distance traveled (cm), and temperature data are depicted as change from baseline (oC). All values are mean ± SEM (*n* = 4 – 6).

***MOR functional potencies in vitro predict potencies in vivo***

The present study further sought to assess the relationships between *in vitro* measures and *in vivo* opioid-like effects in mice. To accomplish this, we conducted a Pearson correlation analysis of binding affinity values at MOR (Ki), functional potencies at MOR (EC50), and ED50 potencies from mouse studies by using the values listed in **Table 1**.



**Figure 4.** Relationship between *in vivo* potencies to induce antinociception, locomotor activity, and temperature change as compared to *in vitro* potencies for MOR-mediated inhibition of cAMP accumulation (panels **A** - **C**) or mini-Gi recruitment (panels **D** - **F**). Data represent log-log transformations of *in vivo* and *in vitro* potencies. Green inverted triangle = etonitazene, yellow/gold diamond = isotonitazene, orange square = protonitazene, purple circle = fentanyl, blue triangle = metonitazene, red circle = butonitazene, and grey square = morphine. Results from the mini-Gi assay are taken from previously published findings [13].

MOR binding affinities from rat brain were not correlated with *in vitro* MOR functional potencies, or potencies to induce effects in mice (**Figure S4**). However, the *in vitro* MOR functional potencies in the cAMP assay were significantly and positively correlated with the potencies for all measures from mouse studies (**Figure 4, panels A-C**). In addition, we examined relationships between potencies for opioid-like effects in mice reported here and *in vitro* functional potencies for MOR-mediated recruitment of mini-Gi (**Figure 4, panels D-F**) and -arrestin-2 (**Figure S5**) that were previously published (Vandeputte et al. 2021b). Potencies in these functional assays were also significantly and positively correlated with potencies for effects in mice. Overall, these analyses reveal a strong positive relationship between functional potencies at MOR and potencies to induce opioid-like effects in mice.

**Discussion**

Recreational use of non-fentanyl NSOs is on the rise and associated with increased overdose and life-threatening medical consequences (EMCDDA 2022a; UNODC 2020a; 2022b). 2-Benzylbenzimidazole ‘nitazene’ opioids have recently taken the lead in what is now considered the “post-fentanyl analogue” era (EMCDDA 2022c; Papsun et al. 2022; Vandeputte et al. 2021a). Here we compared the *in vitro* and *in vivo* pharmacological effects of nitazene compounds relative to standard reference opioids. Specifically, we examined the SARs for nitazenes with varying alkoxy chain length in terms of binding affinities at opioid receptors (MOR, DOR, KOR), *in vitro* functional activity at MOR, and opioid-like effects in mice, as compared to fentanyl and morphine. All tested nitazenes were MOR-selective in receptor binding studies and displayed potent MOR agonism in functional assays. The nitazenes produced dose-dependent opioid-like effects in mouse studies. Etonitazene was the most potent analogue *in vivo*, and MOR potencies from *in vitro* pharmacological assays were positively correlated with potencies to induce opioid-like effects in mice. Overall, the results show that several nitazenes are more potent than fentanyl both *in vitro* and *in vivo*, and that alkoxy chain length dictates the observed differences in potencies across assays.

The present study is one of the first to systematically evaluate the binding affinities for a series of nitazene analogues at opioid receptor subtypes. The results show that nitazenes display selective and low nanomolar affinity for MOR (Ki = 3.6 – 53.1 nM) when compared to DOR and KOR. The observed selectivity for MOR aligns with the well-established role of this receptor in mediating various therapeutic (e.g., antinociceptive) and adverse (e.g., respiratory depressant) effects commonly associated with opioids (Williams et al. 2013). The MOR selectivity shown here is also consistent with previous findings examining the opioid receptor binding of etonitazene (Ujváry et al. 2021; Vandeputte et al. 2023), isotonitazene and its metabolite *N-*desethyl isotonitazene (Malcolm et al. 2023; Vandeputte et al. 2023; Walton et al. 2022), metonitazene (Malcolm et al. 2023), and *N*-pyrrolidino etonitazene (Vandeputte et al. 2022a). For etonitazene, various MOR binding affinities have been reported in the literature, with Ki values in the sub-nanomolar range (Ki = 0.00042 – 0.11 nM) (Ujváry et al. 2021). In the study by De Luca et al.MOR binding affinities were determined for isotonitazene (Ki in rat membranes = 0.06 nM; Ki in CHO cells expressing MOR= 0.05 nM) and metonitazene (Ki rat membranes= 0.22 nM; Ki CHO-MOR= 0.23 nM) (De Luca et al. 2022). Another recent study by Malcom et al*.* reported MOR binding affinities for isotonitazene (Ki = 0.490 nM) and metonitazene (Ki = 0.776 nM) in Expi293F cells (Malcolm et al. 2023). It is noteworthy that the shorter methoxy tail of metonitazene was associated with decreased MOR affinity in previously published studies, whereas we found the shorter methoxy tail length increased MOR affinity. The discrepancies in the binding results across studies could be related to differences in the methods used, especially the variability in MOR expression levels across experimental systems.

Using an *in vitro* cAMP-Gi MOR activation assay, we showed that all nitazenes are potent (EC50 = 0.03 – 0.5 nM) and efficacious (Emax = 103 – 108% vs. DAMGO) MOR agonists. Etonitazene, isotonitazene, and protonitazene were more potent than fentanyl (EC50 = 0.10 nM), and all tested nitazenes were more potent than morphine (EC50 = 1.22 nM). While all of the nitazenes had comparable efficacies, the rank order of potencies indicated that either an ethyl or isopropyl side chain was optimal for MOR activation. The *in vitro* SAR trends reported here confirm the findings by Vandeputte et al. (2021b) and Kanamori et al. (2023), who previously reported on the pharmacological characterization of nitazenes with varying alkoxy chain length using different *in vitro* assays. We found that alkoxy chain length differentially impacted MOR binding affinity and functional potency of nitazenes. Recent *in silico* studies suggest that the isopropyl moiety in isotonitazene induces a rearrangement in the MOR extracellular terminal domain that stabilizes the receptor in a closed form, preventing the entrance of other ligands (De Luca et al., 2022). The authors of the *in silico* work hypothesize a link between MOR binding pose and the greater functional potency of isotonitazene vs. metonitazene (De Luca et al. 2022). Our results support existing evidence that MOR binding affinity does not always accurately predict MOR activation *in vitro* and *in vivo* (Baumann et al. 2018; Vandeputte et al. 2022a; Vandeputte et al. 2023; Vandeputte et al. 2022c)*.* On the other hand, a recent study found the rank order of MOR binding affinities and functional potencies were similar for a small group of nitazenes (Malcolm et al. 2023), illustrating that relationships between receptor binding and function can be correlated, especially when test compounds are structural analogues. One possible reason for the disconnect between MOR binding affinities and functional potencies in the present study is the different assay systems employed to measure these endpoints. The MOR binding assays used rat brain tissue preparations, whereas the functional assays used cells transfected with human MOR.

In our mouse experiments, nitazenes produced potent opioid-like effects, with etonitazene, isotonitazene, and protonitazene showing higher potencies (ED50 = 0.003 – 0.021 mg/kg) compared to fentanyl (ED50 = 0.05 – 0.16 mg/kg). Importantly, all of the nitazenes tested here were more potent than morphine (ED50 = 3.30 – 12.45 mg/kg). The potencies of etonitazene and isotonitazene to induce antinociception in hot plate and tail-flick assays have been previously reported (ED50 = 0.016 and 0.020 mg/kg, respectively) in BALB/cByJ mice (Lee et al. 2022). Another group recently reported potencies in the tail-flick assay for isotonitazene, its *N-*desethyl metabolite, and metonitazene (ED50 = 0.011, 0.040, and 0.321 mg/kg, respectively) in C57BL/6J mice (Malcolm et al. 2023). The antinociceptive potency values from these published studies are nearly identical to the potencies for hot plate latency shown here for etonitazene, isotonitazene, and metonitazene (ED50 = 0.012, 0.021, 0.310 mg/kg, respectively). Other investigators examining opioid-like effects of nitazenes in rats have also reported antinociceptive potencies in the µg/kg range (Vandeputte et al. 2022a; Vandeputte et al. 2023; Vandeputte et al. 2022c; Walton et al. 2022). For etonitazene, previous work has reported antinociceptive potencies of 0.0017 mg/kg, 0.005 mg/kg, and 0.003 mg/kg using the mouse phenylquinone abdominal stretching, tail-flick, and hot plate assays, respectively (Aceto et al. 1994; Jacobson 1988; Ujváry et al. 2021).

The results from the current study further revealed a trend of ~2 – 3-fold higher potencies for nitazene-induced hyperlocomotion relative to potencies for antinociception and hypothermia, which was also true for fentanyl and morphine. By contrast, prior work suggested lower potencies for opioid-induced hyperlocomotion relative to potency for antinociception in the mouse warm-water tail-withdrawal test, when testing the effects of fentanyl analogues (Varshneya et al. 2019; 2021). The previous studies examining the effects of fentanyl analogues did not compute potency values for hyperlocomotion, making absolute comparisons to our work difficult. Given that the prior studies used a different strain of mice (Swiss Webster vs. C57BL/6J used here), type of antinociception assay, and class of opioids, there may be strain-, assay-, or drug-related differences in the relative potencies to induce opioid-like effects in rodent models. Importantly, the antinociceptive potencies reported by Varshneya et al. for fentanyl (0.08 mg/kg) and morphine (7.8 mg/kg) are similar to the potencies shown here.

Our study represents the first to assess SARs for acute locomotor effects of nitazenes in mice. Unlike rats, which exhibit robust opioid-induced catalepsy, mice display increased locomotion in response to opioid administration (Santos et al. 2022; Vandeputte et al. 2022a; Vandeputte et al. 2022c). Emerging evidence suggests that opioid-induced locomotion in mice may represent an *in vivo* index of drug efficacy at MOR, whereby full agonists produce maximal stimulation of motor activity but partial agonists do not (Santos et al. 2022). We found that nitazenes produce dose-dependent and efficacious locomotor stimulation in mice, consistent with the *in vitro* findings showing the drugs are fully efficacious agonists at MOR. Interestingly, all of the compounds tested, except for morphine, induced biphasic locomotor responses, characterized by suppression of activity at high doses. The suppressive effect of high-dose opioid administration may be related to the recruitment of various adverse effects, such as motor incoordination (unpublished observations) and respiratory depression (Malcolm et al. 2023). Our mouse studies were not designed to address the adverse effects of high-dose nitazene administration. Nonetheless, future studies should assess the relationships between therapeutic effects (e.g., antinociception) and adverse effects (e.g., respiratory effects) of nitazenes to establish the relative risks of these compounds. Overall, the SAR for nitazenes demonstrated that intermediate chain lengths produce the most potent compounds (i.e., etonitazene and isotonitazene) for hyperlocomotion and other endpoints. Nitazene compounds with shorter (i.e., metonitazene) or longer (i.e., butonitazene) chain lengths display weaker potencies relative to etonitazene and isotonitazene.

As noted above, MOR binding affinities of nitazenes did not correlate with their *in vitro* functional potencies or potencies to produce effects in mice. Instead, strong positive correlations were observed between *in vitro* MOR functional potencies reported here and elsewhere (Vandeputte et al. 2021b) and *in vivo* measures. These findings agree with prior work (Vandeputte et al. 2023) and highlight the potential utility of some *in vitro* functional assays to predict *in vivo* effects of newly emerging opioids (Vandeputte et al. 2022b). The lack of correlation between *in vitro* binding affinities and functional potencies in our study may reflect differential structural requirements to occupy vs. activate MOR, as previously shown for fentanyl (Ricarte et al. 2021; Zhuang et al. 2022). Interestingly, the correlations between *in vitro* functional endpoints and *in vivo* measures observed presently occurred with assays assessing G protein dependent and independent signaling pathways at MOR. Despite the conflicting reports on the role of biased signaling at MOR in some opioid-like *in vivo* effects (Kliewer et al. 2020; Kliewer et al. 2019; Raehal et al. 2005), our data support the idea that opioid-like effects of nitazenes in rodents may be related to multiple MOR-coupled signaling transduction pathways, as suggested by others (Gillis et al. 2020; Malcolm et al. 2023).

While legislative scheduling of nitazenes may have slowed their proliferation and diversification on recreational drug markets, recent trends indicate their continued circulation among people who use opioids (EMCDDA 2022c; Krotulski et al. 2022c; Papsun et al. 2022). Since the first identification of isotonitazene on the European drug market in 2019 (Blanckaert et al. 2020), the NPS opioid supply has contained different nitazene analogues alternating in popularity. Following the initial isotonitazene peak in 2019-2020, a subsequent rise in detections of metonitazene was observed at the end of 2021. This was followed by a more subtle uptick in identifications of *N*-pyrrolidino etonitazene (‘etonitazepyne’) during the last quarter of 2021 (Krotulski et al. 2022c; Papsun et al. 2022). Throughout 2022, various nitazenes remained present on drug recreational markets, without any one analogue clearly taking the lead. Among those, alkoxy chain variants are the most commonly detected in toxicology cases in the U.S. across 2022 (Krotulski et al. 2022a; b; c). When examining the nitazene structural template, it is noteworthy that modifications at only three positions of this template have led to newly emerging analogues (**Figure 1**). Among these analogues, a majority are the alkoxy side chain variants evaluated in the present study. However, further diversification of the nitazene class cannot be excluded. Continuous monitoring of emerging NSO trends and studies to characterize their effects will be necessary to keep up with the ever-changing landscape of the recreational opioid drug market.

**Author Contributions**

D.W., L.C., and M.M.T. conducted *in vitro* competition binding experiments and efficacy experiments. G.C.G. conducted mouseexperiments. G.C.G. and M.M.V. wrote the initial draft of the manuscript. M.H.B., G.C.G., L.S., M.M.V., and C.P.S. designed the experiments, assisted with data interpretation, and critically revised the manuscript.

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**Conflict of Interest Statement**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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**\*\*Tables, Figures, and corresponding legends are placed in the draft where relevant.\*\***