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Intramuscular ENA-001 Combined With Low-Dose Naloxone Versus Intranasal Naloxone for Reduction of Cardiac Arrest Rate in a Simulated Fentanyl-Xylazine Overdose

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Abstract

Overdose fatalities are commonly caused by fentanyl combined with non-opioid depressants such as xylazine. We performed in silico modeling to evaluate the ability of an opioid-receptor-agnostic respiratory stimulant, the BK_{Ca}-blocker ENA-001, combined with low-dose naloxone, both administered intramuscularly (IM) versus intranasally (IN) naloxone, to reduce hypoxia and ischemic cardiac arrest. We additionally simulated fentanyl/propofol as a secondary model of polysubstance overdose. We extended a published pharmacokinetic/pharmacodynamic model describing the effects of fentanyl on ventilatory control with blood-gas kinetics and their reversal by naloxone by including opioid-receptor-independent components: respiratory depression from either xylazine or propofol, and respiratory stimulation by ENA-001.

We simulated no rescue, 4 mg IN naloxone, and 120 mg IM ENA-001 + 0.3 mg IM naloxone, following an overdose with 0.2 mg fentanyl combined with either 12 mg xylazine (primary endpoint) or 64 mg propofol (secondary endpoint). Treatments were administered as a single dose or as three consecutive doses at two-minute intervals. Outcomes were PO_2 nadir, duration of PO_2 < 50 mmHg, apnea duration, and cardiac arrest rate. In fentanyl/xylazine overdose, cardiac arrest rate decreased from 74% (no rescue) to 50% (three consecutive doses of IN naloxone) and 36% (three consecutive doses of IM ENA-001/naloxone; $p < 0.001$ versus IN naloxone and no rescue). The combined treatment resulted in higher PO_2 levels, shorter hypoxia and apnea duration ($p < 0.001$). Similar findings were observed following single-dose reversal and in simulated fentanyl/propofol overdose. In simulated mixed opioid/non-opioid overdose, combining an IM opioid-receptor-independent respiratory stimulant with low-dose naloxone resulted in lower cardiac arrest rates than 4 mg IN naloxone alone. These findings support further studies on ENA-001 combined with naloxone as a rescue strategy for polysubstance overdose where naloxone will cause incomplete reversal. These findings are model-derived and hypothesis-generating.

Categories: Pharmacology, Emergency Medicine, Medical Simulation
Keywords: cardiac arrest, ena-001, fentanyl, naloxone, opioid overdose, pharmacokinetic-pharmacodynamic simulation, respiratory stimulant, simulation model, xylazine

Introduction

In the US, despite the widespread availability of Narcan (4 mg intranasal naloxone), drug overdose remains a major cause of death, particularly in polysubstance overdoses involving fentanyl and non-opioid centrally acting depressants [1-4]. One such depressant is xylazine, an $alpha_2$-adrenoceptor agonist, licensed only for veterinary use [2-4]. The cause of death is respiratory depression causing hypoxia, hypercapnia, cardiac dysrhythmias, and ultimately cardiorespiratory collapse [2-4]. When an opioid is involved in the polysubstance overdose, the competitive opioid receptor antagonist naloxone reverses the opioid component but not the non-opioid component [3,4].

Several non-opioid respiratory stimulants are currently being studied for reversal of opioid- and non-opioid-induced respiratory depression [5]. Among these, the best-studied experimental drug is ENA-001 (previously known as GAL-021), which is a so-called agnostic respiratory stimulant that, through blockade of large-conductance Ca^{2+}- and voltage-activated potassium (BK_{Ca})-channels, expressed on the surface of type I

glomus cells of the carotid bodies (the peripheral chemoreceptors), induces ventilatory stimulation [6,7]. Agnostic respiratory stimulants do not affect the primary cause of respiratory depression. The increase in peripheral chemoreceptor drive by ENA-001 occurs independently of central respiratory depression and its cause.

Directly testing the effects of ENA-001 and naloxone in severe polysubstance overdose is difficult in humans, especially when outcomes include profound hypoxia and cardiac arrest. In silico modelling offers an alternative for evaluating rescue in simulated overdose scenarios [8]. In the current study, we used earlier published pharmacokinetic/pharmacodynamic (PK/PD) models to simulate the effect of reversal by intranasal (IN) naloxone (4 mg) and intramuscular (IM) ENA-001/low-dose naloxone combination [8-10]. We simulated two overdose scenarios: fentanyl combined with xylazine as the primary scenario and fentanyl combined with propofol as a secondary, confirmatory scenario and mechanistic comparison. Simulation endpoints were PO_2 nadir, duration of PO_2 < 50 mmHg, apnea duration, and cardiac arrest rate, similar to the approach used previously by the US Food and Drug Administration (FDA) to evaluate IM naloxone and resuscitation strategies following high-dose fentanyl overdose [8].

Our simulation scenario focuses on the pharmacological rescue of an overdose victim. In real-world settings, additional interventions, particularly airway management and ventilatory support, should be employed upon discovery of an overdose victim and are likely to further improve the chances of recovery.

Technical Report

The respiratory depression model

Our model is based on earlier published PK/PD models [8-10]. Our model includes a three-compartment fentanyl PK model with a postulated effect-site with hysteresis half-life ($t½k_{e0F}$), a naloxone PK model that includes absorption compartments for IN and IM dosing with IN absorption dependent on the number of naloxone sprays (IN first 2 doses 50% bioavailability, thereafter 18%, IM 100% bioavailability independent of doses; Appendix 1), two transit compartments and three disposition compartments and an effect delay ($t½k_{e0N}$), and O_2 and CO_2 kinetics. The PD part of the power model assumed two additive ventilatory drives [8]:

$$\dot{V}_E(t) = \dot{V}_0 (1 - D_W) + G_0 (1 - D_G)\left(P_{\mathrm{CO}_2}(t) - P_{\mathrm{CO}_2,0}\right)$$

and

$$D_G = D_W = \frac{1}{2}\left(\frac{C_{EF}}{C_{50F}}\right)^{\gamma_F},$$

where $V_E(t)$ is minute ventilation at time t, V_0 is baseline ventilation, D_W is the CO_2-independent drive, G_0 is the baseline gain or slope of the hypercapnic ventilatory response, D_G is the CO_2-dependent drive, $PCO_2(t)$ is the arterial carbon dioxide concentration at time t, $PCO_{2,0}$ is the baseline arterial carbon dioxide concentration, C_{EF} is the fentanyl effect-site concentration, C_{50F} is the fentanyl effect-site concentration causing 50% effect, and γF a power coefficient for fentanyl. Naloxone acts as a competitive antagonist by increasing C_{50F} with

$$C_{50F}(\text{after naloxone}) = C_{50F}\left(1 + \frac{C_{EN}}{IC_{50N}}\right)$$

where C_{EN} is the naloxone effect-site concentration, and IC_{50N} is the naloxone concentration that doubles C_{50F}. We used a power model rather than a sigmoid E_{MAX} model to be able to describe opioid-induced apnea [10].

The model was extended so that the instantaneous depression of ventilatory drive was the sum of an opioid component (fentanyl effect site ± competitive antagonism by naloxone) and a non-opioid component (xylazine or propofol with their own effect sites, without naloxone antagonism). Xylazine and propofol have their own PK (propofol three-compartment model [11] and xylazine two-compartment model) and effect site with distinct potency parameters (Appendix 1). Xylazine disposition had a volume of distribution of 150 L [12] and a clearance of 0.30 L/min, giving a terminal half-life of 6.4 h. These values were derived from the two available human data sets, which report a terminal half-life of 5.8 ± 2.4 h in emergency-department patients presenting after opioid overdose [13], and a median of 12.0 h (range 5.9-20.8 h) in patients testing positive for fentanyl and xylazine [14]. Both values are considerably longer than the 22-50 min observed in animals [12]. No human concentration-effect data for ventilation exist; therefore, the xylazine C_{50} was arbitrarily set at 200 ng/mL and varied from 100 to 300 ng/mL in a dedicated sensitivity analysis. Fentanyl/xylazine and fentanyl/propofol effects on ventilatory drive were somewhat arbitrarily assumed to be additive with

$$ D_G = D_W = \frac{1}{2}\left(\frac{C_{EF}}{C_{50F}}\right)^{\gamma_F} + \frac{1}{2}\left(\frac{C_{EZ}}{C_{50Z}}\right)^{\gamma_Z} $$

where Z is either xylazine or propofol.

ENA-001 was modeled with two parallel first-order absorption compartments and three disposition compartments without effect-site delay. The parameters are those of a non-linear mixed-effects modeling (NONMEM) population analysis of a recently conducted study of intravenous (IV) *versus* IM ENA-001 (ClinicalTrials.gov NCT06967259): 60% of the absorbed dose enters through a faster pathway (absorption half-life 24 min) and 40% through a slower one (half-life 138 min). In this analysis, a single 120 mg IM ENA-001 dose peaks near 580 ng/mL at about 25 min. The ENA-001 effect on ventilation was an additive drive on top of $V_E(t)$, with the ENA-001 potency parameter (C_{25}) describing a 25% increase in ventilation [15]:

$$ \dot{V}_E(t) = \dot{V}_0(1-D_W) + G_0(1-D_G)\left(P_{\mathrm{CO}_2}(t)-P_{\mathrm{CO}_2,0}\right) + \frac{1}{4}\dot{V}_0\left(\frac{C_{ENA}(t)}{C_{25}}\right) $$

where C_{ENA} is the ENA-001 concentration in arterial blood. D_G is capped at 1 so that hypercapnia cannot become an inhibitory rather than an excitatory stimulus. Additionally, since the ENA-001 term is not multiplied by (1 - D), the stimulant's drive persists during apnea.

Finally, cardiac arrest was based on the PO_2 value according to the Mann et al. cardiac arrest rule, *i.e.,* cardiac arrest occurs when PO_2 < 15 mmHg for at least 220 s [8,16]. They based their rule on animal data [17] and assumed cardiac arrest led to the death of the simulated individual. The concept of the cardiac arrest rule is explained in the supplementary information of Ref. [8]. They explain that

> ".... after prolonged hypoxia, cardiac output decreases leading to eventual cardiac arrest, which is most commonly pulseless electrical activity progressing to cardiac arrest. When arterial PO_2 decreases to below 15 mmHg, the model triggers a subsequent precipitous decrease in cardiac output (cardiovascular collapse) after a delay of 220 seconds. The threshold of 15 mmHg is consistent with a threshold observed in dogs below which a precipitous decrease of cardiac output occurs [17]."

See Appendix 1 for parameter values used in the simulations.

Simulated overdose scenarios, rescue arms, main and secondary endpoints

Each virtual subject received an intravenous bolus dose of fentanyl (0.2 mg infused over 90 s) combined with either 12 mg xylazine or 64 mg propofol, both given intravenously. These doses were chosen such that the a priori estimated cardiac arrest rates under no-rescue conditions would be 75 ± 3% (± range). Although fentanyl, xylazine, and propofol each produce relatively modest cardiac arrest rates when given alone, their combined effect on the cardiac arrest rates produced an emergent synergistic effect. The combination decreased PO_2 below the 15 mmHg threshold for longer periods of time than either drug alone. Treatment involved three arms: (1) no rescue; (2) 4 mg IN naloxone; and (3) IM simultaneous administration of ENA-001 120 mg combined with 0.3 mg naloxone. All treatments were given 5 min after fentanyl dosing (single administration, only for the fentanyl/xylazine scenario) or 5, 7, and 9 min after fentanyl (three subsequent doses in both scenarios). The naloxone doses used in this study were selected after a separate simulation showing that 0.3 mg IM naloxone gives a similar reduction in cardiac arrest rate as 4 mg IN naloxone following fentanyl/xylazine exposure. The IM ENA-001 dose was chosen because of practical constraints given the current ENA-001 formulation of 30 mg/mL: 120 mg limits the total injection dose to 4 mL. Additionally, we observed a < 3% further decrease in cardiac arrest rates when increasing the dose to 150 mg.

Note that the volume of 4 mL may exceed the recommendations for IM dosing in the deltoid muscle, but may be possible in the gluteus maximus or rectus femoris. We chose three doses, as many overdose victims do get multiple IN naloxone doses. We extrapolated these clinical findings to our simulation scenario and chose three doses to reach adequate ENA-001 and naloxone concentrations in plasma.

Main endpoints were the fentanyl/xylazine cardiac-arrest rate, the arterial PO_2 nadir, the time with arterial PO_2 < 50 mmHg, and apnea duration with apnea defined as VE < 0.5 L/min. Secondary endpoints were the equivalent estimations for the fentanyl/propofol combination.

Simulations and statistical analyses

Each rescue arm was evaluated in a Monte Carlo simulation of 2,000 virtual subjects (1,000 per arm in the

sensitivity analyses) with 30% log-normally distributed between-subject variability on key PK/PD parameters including ENA-001 potency (typical C_{25} was set at 600 ng/mL) [15]. Cardiac-arrest rates are reported ± Wilson 95% confidence intervals and continuous endpoints as means ± 95% confidence intervals. Paired data were compared by McNemar's exact test (arrest data) and paired t-tests (continuous data). Risk differences, relative risks, and model-based numbers needed to treat (with 95% confidence intervals) were obtained from a 2,000-sample paired bootstrap. Sensitivity analyses were performed for ENA-001 characteristics (absorption rate, time of rescue, and potency) on the change in cardiac arrest rates produced by adding ENA-001 120 mg IM to 0.3 mg IM naloxone, and a one-way sensitivity analysis on a range of model inputs based on the combination and the difference between the combination and IN naloxone. Additionally, separate sensitivity analyses were performed on the xylazine potency (xylazine C50 100 to 300 ng/mL for all three treatment arms), the ENA-001 dose (0 to 150 mg), and an analysis of varying the ENA-001 dose and naloxone dose ranges from 0 to 150 mg ± 0.3 mg IM naloxone and from 0 to 0.3 mg IM naloxone ± 120 mg ENA-001, respectively.

Simulations were run in Python (NumPy/SciPy). The Large Language Model Claude Opus 5 (Anthropic, San Francisco, CA) was used to implement the simulation model in Python and assist with the analyses. This study itself is not an artificial-intelligence study; model structure, parameterization, validation, and interpretation were performed by the authors, who take full responsibility for the work.

Simulation results

Main Endpoint: Fentanyl Combined With Xylazine

Single rescue dose. The combination of 0.2 mg fentanyl and 12 mg xylazine, both given intravenously, resulted in a cardiac arrest rate of 74.2% (95% CI 72.3-76.1%) without rescue. A single rescue dose given five min after fentanyl/xylazine reduced the cardiac arrest rate to 52.0% (49.8-54.2%) after IN naloxone and 44.4% (42.2-46.6%) after the combined IM administration of 120 mg ENA-001 and 0.3 mg naloxone (Table *1*). Relative to IN naloxone, the combination raised the PO2 nadir by 4.4 mmHg (4.2-4.7 mmHg), shortened the time with PO2 < 50 mmHg by -0.41 min (-0.47 to -0.34 min) and the apneic period by -0.92 min (-0.97 to -0.86 min), all $p < 0.001$.

Cardiac arrest rates and ventilatory outcomes				
Treatment arm	***Cardiac arrest (%) (95% CI)***	***Arterial PO_2 nadir (mmHg) (95% CI)***	***Arterial PO_2 < 50 mmHg (min) (95% CI)***	***Apnea duration (min) (95% CI)***
Fentanyl/xylazine overdose treated with a single rescue dose 5 min after fentanyl/xylazine				
No rescue	74.2 (72.3–76.1)	9.6 (8.9–10.4)	16.0 (15.7– 16.3)	7.44 (7.11– 7.76)
IN naloxone	52.0 (49.8–54.2)[1]	15.1 (14.2– 15.9)	9.95 (9.70– 10.19)	3.69 (3.49– 3.90)
IM ENA-001 + IM naloxone	44.4 (42.2–46.6)[2]	19.5 (18.6– 20.5)	9.54 (9.25– 9.83)	2.78 (2.59– 2.96)
Fentanyl/xylazine overdose treated with three rescue doses at 5, 7, and 9 min after fentanyl/xylazine				
No rescue	74.2 (72.3–76.1)	9.6 (8.9–10.4)	16.0 (15.7– 16.3)	7.44 (7.11– 7.76)
IN naloxone	49.6 (47.4–51.8)[3]	15.2 (14.3– 16.1)	9.24 (9.01– 9.46)	3.40 (3.21– 3.59)
IM ENA-001 + IM naloxone	36.4 (34.3–38.5)[4]	20.3 (19.4– 21.3)	7.30 (7.10– 7.51)	2.00 (1.88– 2.12)
Fentanyl/propofol overdose treated with three rescue doses at 5, 7, and 9 min after fentanyl/propofol				
No rescue	70.7 (68.7–72.7)	2.1 (1.8–2.3)	9.23 (9.16– 9.31)	5.31 (5.24– 5.39)
IN naloxone	58.0 (55.8–60.1)[5]	2.3 (2.0–2.5)	7.84 (7.80– 7.89)	5.04 (4.97– 5.10)
IM ENA-001 + IM naloxone	46.9 (44.8–49.1)[6]	2.7 (2.5–3.0)	7.32 (7.27– 7.36)	4.79 (4.73– 4.84)

TABLE 1: Simulations outcomes of the reversal of fentanyl/xylazine and fentanyl/propofol overdose

IM ENA-001 + IM naloxone is 120 mg IM ENA-001 + 0.3 mg IM naloxone; IN naloxone is 4 mg IN naloxone. 1) Single-dose IN naloxone versus no rescue: RD - 22.3% (-24.0 to -20.4%), RR 0.70 (0.68-0.72), NNT 4.5 (4.2-4.9), p < 0.001. 2) Single-dose combination versus no rescue: RD -29.9% (95% CI -31.8 to -27.7%), RR 0.60 (0.57-0.62), NNT 3.4 (3.1-3.6), p < 0.001; combination versus IN naloxone: RD -7.6% (-8.8 to -6.5%), RR 0.85 (0.83-0.87), NNT 13.2 (11.4-15.4), p < 0.001. 3) Triple-dose IN naloxone versus no rescue: RD -24.7% (-26.5 to -22.8%), RR 0.67 (0.64-0.69), NNT 4.1 (3.8-4.4), p < 0.001. 4) Triple-dose combination versus no rescue: RD -37.9% (-40.0 to -35.7%), RR 0.49 (0.46-0.52), NNT 2.6 (2.5-2.8), p < 0.001; combination versus IN naloxone: RD -13.2% (-14.7 to -11.8%), RR 0.73 (0.71-0.76), NNT 7.6 (6.8-8.5), p < 0.001. 5) Fentanyl/propofol, triple-dose IN naloxone versus no rescue: RD -12.7% (-15.6 to -9.8%), RR 0.82 (0.78-0.86), NNT 7.9 (6.4-10.2), p < 0.001. 6) Fentanyl/propofol, triple-dose combination versus no rescue: RD -23.8% (-25.6 to -21.9%), RR 0.66 (0.64-0.69), NNT 4.2 (3.9-4.6), p < 0.001; combination versus IN naloxone: RD -11.0% (-12.4 to -9.6%), RR 0.81 (0.79-0.83), NNT 9.1 (8.1-10.4), p < 0.001. CI: confidence interval; IN: intranasal; IM: intramuscular; RD: risk difference; RR: relative risk; NNT: number needed to treat.

Administration of three consecutive reversal doses at t = 5, 7 and 9 min after fentanyl/xylazine further reduced the cardiac arrest rate from 74.2% (no rescue) to 49.6% (47.4-51.8%) with IN naloxone, and 36.4% (34.3-38.5%) with the ENA-001/naloxone combination (Table *1*). Relative to IN naloxone, the combination gave a risk reduction of -13.2% (-14.7 to -11.8%), a relative risk of 0.73 (0.71-0.76) and an NNT of 7.6 (6.8-8.5; p < 0.001), and the combination also increased the PO2 nadir by 5.1 mmHg (4.9-5.4 mmHg), changed hypoxia duration by -1.9 min (-2.0 to -1.9 min) and changed the apneic period by -1.4 min (-1.5 to -1.3 min), all p < 0.001. If we disentangle the combination, the two components contribute comparably: given alone, ENA-001 reduced the cardiac arrest rate to 56% and 0.3 mg IM naloxone to 48%, against 36% for the two together.

A sensitivity analysis on the effect of adding IM 120 mg ENA-001 to 0.3 mg IM naloxone is shown in Figure *1*. The protective effect increases with an increase in the proportion of the faster ENA-001 absorption pathway; potency and timing had similar effect sizes. A C_{25} of 300 ng/mL gives a 21% benefit; rescue dosing starting at 3 min after fentanyl gives -16% and at 10 min 2% benefit.

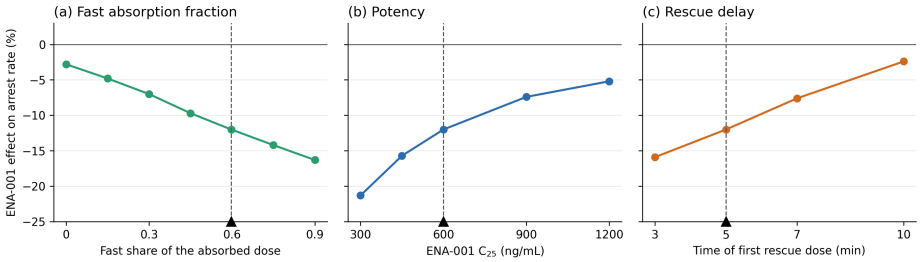


FIGURE 1: Sensitivity analysis on the effect of changes in the faster absorption pathway

(a), ENA-001 potency (b) and time of first rescue dose (c) on changes in cardiac arrest rate produced by ENA-001 120 mg IM combined with 0.3 mg IM naloxone. The triangles and dotted lines give the base values used in the current set of simulations of three rescue doses at 5, 7, and 9 min after fentanyl/xylazine administration.

Next, we performed one-way sensitivity analyses in which individual inputs were varied while all other inputs were kept at their typical values. Figure *2a* shows the effect on the arrest rate in the combined ENA-001/naloxone arm. That rate was most sensitive to xylazine potency, xylazine dose, and rescue timing, which, between them, ranged from 14% to 60%. Figure *2b* shows the benefit of the combination relative to IN naloxone, which is a different analysis: it ranged from 5 to 25% and was most sensitive to ENA-001 potency, between-subject variability, and the naloxone dose in the combination.

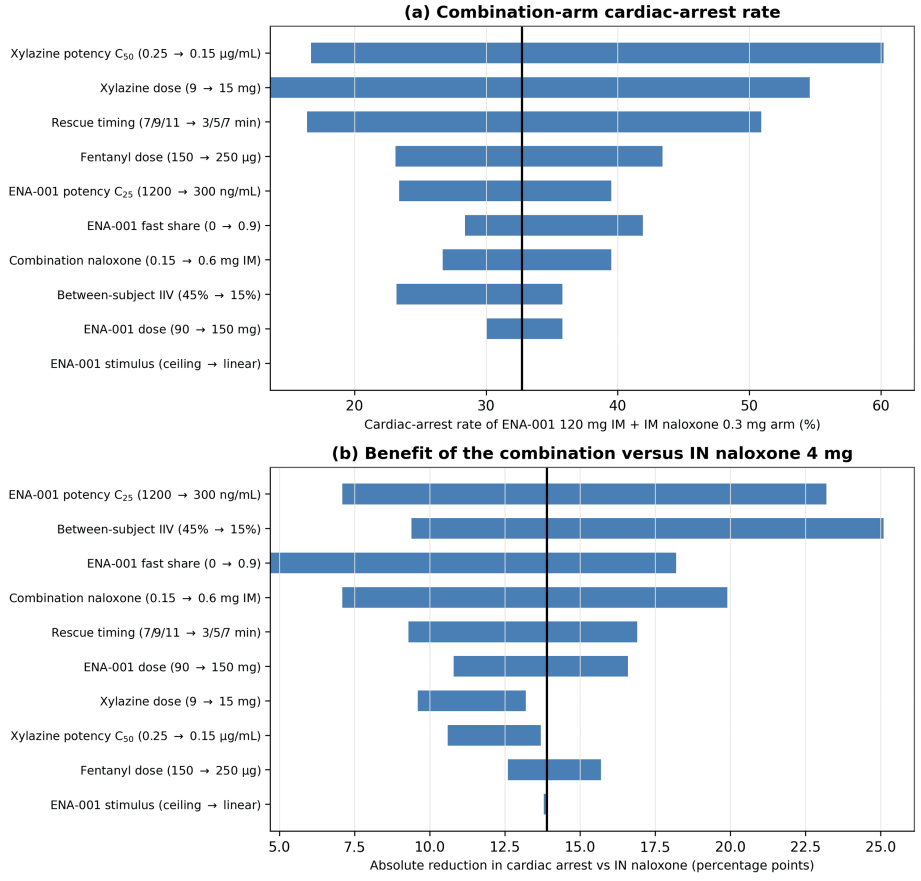


FIGURE 2: One-way sensitivity analyses for a fentanyl/xylazine overdose treated with three doses of the IM combination of ENA-001 120 mg and naloxone 0.3 mg.

(a) Absolute cardiac-arrest risk of the combination; (b) the reduction in cardiac-arrest risk relative to intranasal (IN) naloxone 4 mg. Each bar shows the outcome as the model input is varied across arbitrary ranges; the vertical line marks the baseline estimate, and inputs are ranked by the magnitude of their effect.

Finally, we explored the effect of xylazine potency (Figure 3) on cardiac arrest rates in the three study arms. As expected, lower potency values (higher C_{50} values) are associated with reduced cardiac arrest rates, while the reverse is true for an increase in xylazine potency. Importantly, the observed ranking of the three arms remained unchanged at every value tested, and the advantage of the combination over IN naloxone was present throughout, although it narrowed at both extremes, from 14% at a C50 of 175 ng/mL to 6 to 7% at 100 and 300 ng/mL.

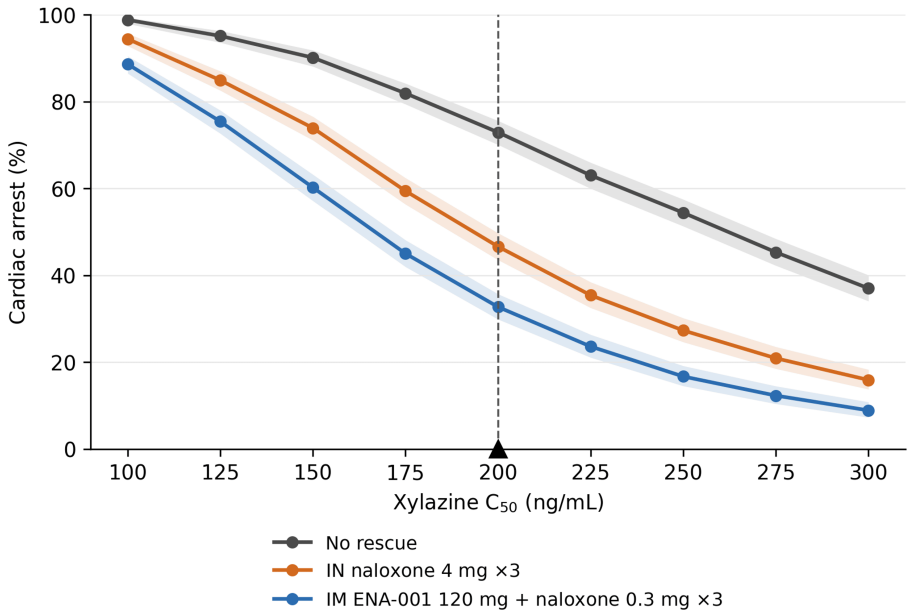


FIGURE 3: Sensitivity analysis on the xylazine potency for a fentanyl/xylazine overdose treated with three rescue doses of ENA-001 120 mg with naloxone 0.3 mg IM, or naloxone 4 mg IN, at 5, 7 and 9 min.

Cardiac-arrest rate in the three treatment arms as the xylazine C_{50} is varied from 100 to 300 ng/mL; shaded areas are Wilson 95% confidence intervals. The triangle and dashed line mark the base value of 200 ng/mL used in the simulations.

Secondary Endpoint: Fentanyl Combined with Propofol

The patterns observed for fentanyl/xylazine were reproduced in the fentanyl/propofol simulations. Without rescue, the cardiac arrest rate was 70.7% (68.7-72.7%), which decreased after three IN naloxone doses to 58.0% (55.8-60.1%) and after three doses of the IM ENA-001/naloxone combination to 46.9% (44.8-49.1%). Relative to IN naloxone, the combination gave a risk reduction of -11.0% (-12.4 to -9.6%; number-needed-to-treat 9.1; $p < 0.001$; Table 1).

Discussion

The carotid bodies (CB) play an important role in the control of breathing and exert approximately 20 to 30% of the total ventilatory drive [18]. They are small organs (1 mm^3) located at the bifurcation of the common carotid arteries and contain oxygen-sensing type I glomus cells, which function as the peripheral chemoreceptors. In addition to changes in arterial PO_2, these cells respond to changes in PCO_2, pH, and other stimuli [19]. Although the precise mechanism of oxygen-sensing within the type I cell remains not fully understood, oxygen-sensitive potassium channels, including BK_{Ca}-channels, are thought to play an important role [19]. Blockade of these channels by hypoxia or specific pharmacological agents causes membrane depolarization, calcium influx, and the subsequent release of excitatory neurotransmitters. These neurotransmitters stimulate the carotid sinus nerve, which projects via the glossopharyngeal nerves to the brainstem respiratory network, thereby increasing ventilation [20]. Several drugs can modulate oxygen-sensitive potassium channels in the CBs, including doxapram, almitrine, and ENA-001 [21]. The use of doxapram and almitrine has been limited by adverse effects. ENA-001 is a novel first-in-class BK_{Ca}-channel blocker that so far seems safe and effective [6,7].

ENA-001 is being developed for several potential indications, including treatment and prevention of drug overdose, prevention and treatment of postoperative respiratory depression, and apnea of prematurity. In

the current simulation study, we focused on its potential role in polysubstance overdose. A respiratory stimulant that is designed to reverse respiratory depression independent of the underlying cause of the depression may be useful in this setting. In this sense, ENA-001 can be considered an "agnostic" respiratory stimulant. Importantly, in case of an opioid-induced respiratory depression, agnostic respiratory stimulants will increase breathing activity without compromising analgesia or causing precipitated withdrawal.

In the current exploratory and hypothesis-generating simulation study, we evaluated the effect of the IM administration of ENA-001 combined with low-dose naloxone in a mixed overdose scenario of fentanyl combined with xylazine. Xylazine is an alpha$_2$-adrenergic drug with profound respiratory depressant properties that is increasingly encountered in combination with illicitly manufactured fentanyl [2-4]. Dexmedetomidine, another alpha$_2$-adrenergic drug, has significant depressant effects on the ventilatory response to hypoxia, suggestive of a common pathway with xylazine [22]. In our simulations, combining xylazine with fentanyl substantially increased cardiac arrest rates.

Our analysis used established PK/PD models of respiratory depression coupled to the models of O_2 and CO_2 kinetics and the Mann et al. cardiac arrest rule [8]. The modelling framework has been applied to high-dose fentanyl and its reversal with IM naloxone [8]. For example, simulations performed by the US FDA estimated that 1.63 mg fentanyl would cause 50% cardiac arrest in simulated subjects without rescue, which was reduced to 25% after 2 mg/2 mL IM naloxone. Our base cardiac arrest rate of approximately 74% reflects the emergent interactive effects of fentanyl and xylazine. In contrast to the FDA study, where IM naloxone was dosed one min after reaching a 60% drop in ventilation following fentanyl, we dosed our simulated subjects at least five min after fentanyl. We consider this five-min delay more representative of a community overdose scenario, where immediate treatment seems unlikely. Earlier dosing reduced the cardiac arrest rate further, by 20% when the first dose was at three min, while a delay to seven min raised it by >50% (Figure *1c*). Also, the xylazine respiratory potency (and ENA-001 potency, see below) may play a role here, as in humans the potency remains unknown and may affect arrest rates (Figure *3*); additional studies are needed to address this issue.

The ENA-001 potency for reversal of a mixed drug overdose is unknown. We earlier observed a C_{25} value of about 600 ng/mL for reversal of alfentanil-induced respiratory depression and an approximately 50% lower value for reversal of propofol-induced blunting of the hypoxic ventilatory response [15,23]. Both studies were performed under experimental isocapnic conditions, which differ from real-world poikilocapnic conditions in which variations in PCO_2 influence the ventilatory drive. Further studies are needed to determine the ENA-001 potency under poikilocapnic conditions. The sensitivity analysis gave expected outcomes with greater potency resulting in lower cardiac arrest rates (Figure *1b*).

The magnitude of the reversal effect follows directly from the pharmacokinetics of intramuscular ENA-001. The population analysis identifies two parallel absorption pathways, with about 60% of the absorbed dose taking the faster of the two (ClinicalTrials.gov NCT06967259). Both are slow relative to the timescale of an overdose: the faster pathway has an absorption half-life of 24 min, so that five minutes after a 120 mg IM dose the plasma concentration is only about half of its eventual peak. This is the single most important constraint on what intramuscular ENA-001 can achieve in the first minutes after an overdose, and it is why the benefit in Figure *1a* rises steadily with the portion of dose taking the faster route.

The one-way sensitivity analyses (Figure *2a*) further illustrate the dependence of the absolute cardiac arrest estimates on model assumptions. For the ENA-001/naloxone combination, there were large changes in cardiac arrest rates upon variations in xylazine potency, xylazine dose, and rescue timing. Figure *2b* gives a different outcome: the difference in cardiac arrest rates between the two active treatment arms. The between-treatment difference was particularly sensitive to ENA-001 potency, to the assumed between-subject variability, and to the naloxone dose in the combination, and least sensitive to the shape of the ENA-001 concentration-effect relationship. It is important to realize that the two graphs report on related, complementary outcomes. For example, increasing overdose severity can change the absolute arrest rate (Figure *2a*), but has a smaller effect in the between-treatment comparison (Figure *2b*), because it also shifts the outcome in the IN naloxone arm.

We acknowledge that the xylazine parameters are most uncertain. For the disposition parameters, we relied on two independent human studies that reported data from patients presenting to the emergency department following a fentanyl/xylazine overdose. They observed terminal half-lives of about six and 12 h [13,14], far longer than the 22-50 min observed in animal studies [12]. Neither human study reported a volume of distribution, so we adopted the animal value of 150 L [12] and fitted the clearance to reproduce the observed human half-life. Since there are no human xylazine respiratory studies, the respiratory potency of xylazine is unknown. In a sensitivity analysis, we observed a large effect on cardiac arrest rate when varying the potency from 100 to 300 ng/mL (Figure *3*) under no rescue conditions (from 99 to 37%). The comparison between rescue treatments remains robust, though with similar rankings for the three arms at every potency value tested. Importantly, the simulated combination produces lower cardiac arrest rates than IN naloxone across all parameter values evaluated.

There are several limitations to our modeling exercise. We applied PK/PD models originally developed to describe opioid-induced respiratory depression and its reversal by naloxone [8-10]. The effects of xylazine on ventilatory control are poorly studied and not described by validated PK/PD models. Consequently, the xylazine potency value in this study was approximated and calibrated to the target overdose severity in the absence of human PK/PD data. We mechanistically modeled xylazine (and propofol) as direct suppressors of the ventilatory drives D_G and D_W. We also assumed an additive effect of xylazine (and propofol) on fentanyl-induced respiratory depression; the synergistic effect on cardiac arrest emerged from the nonlinear coupling between ventilation, blood-gas kinetics, and the cardiac arrest rule. Although all of our assumptions may be physiologically plausible, they are model- and scenario-dependent and need validation. Our *in silico* study should therefore be considered exploratory and hypothesis-generating. Considering the uncertainties mentioned above and the fact that the true rescue efficacy in mixed overdoses in the community is unknown, we suggest interpreting the absolute cardiac arrest rates as scenario-specific model outputs rather than absolute estimates of cardiac arrest probabilities in real-world overdose. Still, we argue that the relative rates are a more realistic observation, with improvement in rescue from no rescue to IN naloxone (4 mg) to the IM combination of 120 mg ENA-001 and 0.3 mg naloxone. Finally, apart from hypoxia, xylazine lethality *in vivo* also involves alpha$_2$-mediated bradyarrhythmias and cardiovascular collapse, which are independent of respiratory depression. Because ENA-001 acts peripherally on carotid body chemoreceptors, it will not reverse hemodynamic collapse when such effects occur independent of the hypoxic insult from respiratory depression.

An important strength of the study is that the ventilatory control model has been validated in various experimental human studies and is based on earlier published models [9,10,23,24]. It is a physiological model of the ventilatory control system that includes a ventilatory controller with a power function for respiratory depression, two distinct ventilatory drives: CO_2-independent and CO_2-dependent, CO_2 and O_2 kinetics, and a rule describing the occurrence of cardiac arrest. It allows negative (opioids, xylazine, propofol) and positive (ENA-001) inputs that mechanistically alter breathing and consequently PO_2 and cardiac output.

Conclusions

In this simulation study, we explored the effect of adding IM ENA-001, an agnostic respiratory stimulant, to low-dose IM naloxone in the reversal of a mixed fentanyl/xylazine overdose. We observed an improvement in cardiac-arrest-free survival rate from 26% (no rescue) to 48-50% (IN naloxone) and 56-64% (IM combination), depending on the dosing regimen. Although the absolute estimates should be considered with care as they are model- and scenario-dependent, the simulations consistently favored the combination over IN naloxone. Our simulations illustrate that in a mixed overdose, naloxone will partly reverse the opioid component, while the agnostic stimulant increases ventilation irrespective of the cause of depression. In this model, the two components contribute comparably, and neither on its own matches the combination. The benefit is limited by the relatively slow absorption of ENA-001, suggesting that formulations or routes providing faster systemic exposure could further improve early rescue. Our findings support further experimental and clinical evaluation of the ENA-001/naloxone combination as a potential rescue strategy for overdoses involving opioid and non-opioid respiratory depressants.

Appendices

Appendix 1

Paramater	Estimate	Unit	Explanation
Fentanyl pharmacokinetics and pharmacodynamics			
V_1 ; V_2 ; V3	8.8 ; 13.5 ; 120	L	Volume of compartments 1, 2 and 3
CL ; Q_2 ; Q_3	0.57 ; 1.44 ; 1.69	L/min	Clearances
$t½k_{e0F}$	4.5	min	Fentanyl blood Effect-site equilibration half-life
C_{50F}	2	ng/mL	Effect-site concentration causing 50% drive depression
γ (fentanyl)	0.5		Power coefficient
IC_{50N}	1.17	ng/mL	Naloxone effect-site concentration that doubles the effective C_{50F}
Naloxone pharmacokinetics			
V_1 ; V_2 ; V_3	34.4 ; 70.2 ; 146	L	Volume of compartments 1, 2 and 3
CL ; Q_2 ; Q_3	3.84 ; 5.11 ; 2.25	L/min	Clearances

k_{TR} IN; IM	0.69 ; 2.31	min^{-1}	Transit rate constant
k_{IN} IN; IM	0.043 ; 0.138	min^{-1}	Absorption rate into central compartment
IN bioavailability 1, 2 ; 3 doses	0.50 ; 0.18	—	
$t½k_{e0N}$	1.1	min	Naloxone blood Effect-site equilibration half-life
Xylazine and propofol pharmacokinetics and pharmacodynamics			
Propofol V_1 ; V_2 ; V_3	4.27 ; 18.9 ; 238	L	Volume of compartments 1, 2 and 3
Propofol CL ; Q_2 ; Q_3	1.89 ; 1.29 ; 0.836	L/min	Clearances
Propofol k_{e0}	0.456	min^{-1}	Effect-site equilibration rate
Propofol C_{50}	1.5	µg/mL	Effect-site concentration for 50% drive depression
Propofol γ	1.4	—	Power coefficient
Xylazine V_1 ; V_2	40 ; 110	L	Volume of compartments 1 and 2
Xylazine CL ; Q_2	0.3 ; 1.6	L/min	Clearances
Xylazine k_{e0}	0.2	min^{-1}	Effect-site equilibration rate
Xylazine C_{50}	0.2	µg/mL	Effect-site concentration for 50% drive depression
Xylazine γ	1.2	—	Power coefficient
ENA-001 pharmacokinetics and pharmacodynamics			
k_a fast ; slow	0.0288 ; 0.00503	min^{-1}	IM absorption rate constant
Fraction fast ; slow	0.60 ; 0.40		Fraction IM ENA-001 taking each absorption route
V_1 ; V_2 ; V_3	31.4 ; 57.6 ; 556	L	Volumes of compartment1, 2 and 3
CL ; Q_2 ; Q_3	51.9 ; 103 ; 44	L/h	Clearances
C_{25}	600	ng/mL	Concentration producing +25% of ventilatory drive
Ventilatory control			
$\dot{V}_0$	10.1	L/min	Baseline minute ventilation
G_0	8	$L \cdot min^{-1} \cdot kPa^{-1}$	Baseline slope of the hypercapnic ventilatory response
$PCO_{2,0}$	4.8 (36)	kPa (mmHg)	Baseline arterial PCO_2

TABLE 2: Parameter values used in the simulations

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Disclosures

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