

The fentanyl-specific antibody FenAb024 can shield against carfentanil effects

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ABSTRACT

The surge in opioid-related deaths, driven predominantly by fentanyl and its synthetic derivatives, has become a critical public health concern, which is particularly evident in the United States. While the situation is less severe in Europe, the European Monitoring Centre for Drugs and Drug Addiction reports a rise in drug overdose deaths, with emerging concerns about the impact of fentanyl-related molecules. Synthetic opioids, initially designed for medical use, have infiltrated illicit markets due to their low production costs and high potency, with carfentanil posing additional threats, including potential chemical weaponization. Existing overdose mitigation heavily relies on naloxone, requiring timely intervention and caregiver presence, while therapeutic prevention strategies face many access challenges. To provide an additional treatment option, we propose the use of a fentanyl-specific monoclonal antibody (mAb), as a non-opioid method of prophylaxis against fentanyl and carfentanil. This mAb shows protection from opioid effects in a pre-clinical murine model. mAbs could emerge as a versatile countermeasure in civilian and biodefense settings, offering a novel approach to combat opioid-associated mortality.

Introduction

Fentanyl and its many synthetic fentanyl derivatives are potent opioid molecules that, when overdosed, cause respiratory depression-driven mortality. The latest US overdose mortality statistics published by the CDC (including provisional data available for analysis prior to Sept. 3 2023) reveal that the number of deaths per 12-month period is expected to reach 110,469 (Ahmad et al., 2023). The majority of these overdose cases involved synthetic opioids, with fentanyl driving the majority of fatal overdoses. In some US states (particularly the Pacific Northwest), the death rate continues to climb at a staggering pace, prompting the White House to officially designate the week of Aug. 27nd

to Sept. 2nd as Overdose Awareness Week (whitehouse.gov). In association with this proclamation, the White House announced increases in spending on youth education, drug abuse recovery/support programs, substance use disorder (SUD)-focused emergency medical services, etc. Altogether, approximately 500 million USD is being added to the already extensive efforts to reduce the mortality rate, highlighting the nationwide need for novel intervention strategies.

The current situation in the rest of the world is less frightening, although some trends are still alarming enough to warrant consideration. Canada is reporting an increase incidence in overdose deaths since 2020 (Centre on Substance Use, 2021). The 2023 report from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA,

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2023) suggests that the number of reported annual drug overdose deaths has risen to approximately 6500 in the European Union, including Turkey and Norway. These numerical values are very likely underestimated, given complications associated with reporting such statistics and might seem inconsequential when compared to the impact of the opioid crisis in the US; however, they have robustly increased relative to 10 years ago (emcdda.europa.eu). Further, the fentanyl class of opioids has not been a strong driver of EU-wide overdose deaths, although there are well-supported concerns that fentanyl-related molecules may begin causing bigger problems in Ireland and certain Baltic nations in the near future, noting that Estonia has been a historical epicenter for fentanyl overdose in Europe (Uusküla et al., 2020).

Synthetic opioids have been effectively employed in medical settings as analgesics (e.g., during and post-surgery, child birth) and remain in use today. However, the low production costs associated with their synthesis and their extraordinary potencies have propelled them into the illicit-use market. Opioids function by binding to receptors, known as mu opioid receptors (MORs), as well as kappa and delta opioid receptors (KOR and DOR), that modulate different neural and physiological functions (Al-Hasani and Bruchas, 2011). Several more potent fentanyl derivatives, most notably carfentanil which displays a longer half-life and a 50–100-fold higher binding affinity to MORs (K_d fentanyl = 1.2 nM, while K_d carfentanil = 0.024 nM) (Maguire et al., 1992), pose threats to both individuals and more broadly from a chemical weaponization perspective. Indeed, carfentanil has likely already been deployed as a chemical weapon in the past (Riches et al., 2012) and is recognized as a potential chemical threat by e.g., the NIH CounterAct Program and the DEA. Overdose mitigation strategies that would be applicable to both individual consumers and those who may find themselves in danger of exposure to weaponized opioids would be ideal.

Overdose by both carfentanil and fentanyl can be reversed using naloxone, provided that a caregiver is present at the time. Naloxone is on the World Health Organization's list of essential medicines, and indeed has saved countless lives. However, overdose reversal is inherently reliant on access to the molecule at the time of need, the presence of a caregiver, and fast action. In contrast, therapeutic overdose prevention strategies avoid these complications as long as the individual is amenable to the approach. Currently, the standard therapeutic prevention strategy is buprenorphine, a MOR partial agonist that is prescribed (Diversion Control Division, 2022) in order to limit drug cravings and withdrawal symptoms. However, the molecule is indeed an opioid itself, which can limit patient access: only an estimated 57% of US pharmacies carry buprenorphine (Weiner et al., 2023), and prescription access itself remains a hurdle, in part due to distribution regulations. Additionally, buprenorphine prophylaxis often relies on consistent self-administration given that the *in vivo* half-life of even this long-lasting opioid is only on the order of days; although once-monthly injectable formulations and an implantable formulation with a 6-month duration also now exist.

We and others have proposed immunotherapeutics as a novel countermeasure to deploy in this evolving crisis (Ban et al., 2021; Bremer et al., 2016; Raleigh et al., 2019; Triller et al., 2023). Prophylactic vaccines or passively delivered mAb have the capacity to provide long-term protection against overdose with no foreseeable opioid-related regulatory or distribution issues. Conceptually, the antibodies would trap the drug within the bloodstream, preventing or limiting its redistribution to the opioid receptors in the brain. This concept is now experimentally supported by several publications (Baehr et al., 2020; Raleigh et al., 2019; Smith et al., 2019; Triller et al., 2023). For isolation of these anti-fentanyl mAbs, others have utilized chemical crosslinkers to conjugate fentanyl to established antigenic protein carriers such as the tetanus toxoid (TT) (Smith et al., 2019) protein or keyhole limpet hemocyanin (KLH) (Raleigh et al., 2019) in order to facilitate recognition of the small molecule antigen as well as recruit T helper cells. We have taken an alternative approach by immunizing mice with our *Trypanosoma brucei* based platform called VSG-immunogen array by sortase tagging or VAST (Triller et al., 2023). VAST is

hypothesized to act as a uniquely combined T cell-independent and T cell-dependent strategy with an extreme epitope focusing capacity in order to elicit strong immune responses against these types of small molecules, facilitating unique humoral immune responses (Gkeka et al., 2023). After isolating and characterizing several mAbs for affinity and specificity, we have identified a lead candidate, based on its very high affinity (low picomolar) for fentanyl via bio-layer interferometry (BLI) studies (Triller et al., 2023). Briefly, all fentanyl antibodies described in the aforementioned manuscript have shown nanomolar or sub-nanomolar affinities and similar crystal structures, with the fentanyl molecule trapped in a deep pocket. *In vivo* experiments in mice demonstrated antibody-mediated protection against fentanyl effects for a subset of the mAbs (Triller et al., 2023). By comparing antibodies with different affinities, it was observed that higher-affinity antibodies, like FenAb208 (similar to FenAb024), provided better protection against fentanyl compared to lower-affinity antibodies in both prophylactic and therapeutic scenarios. In follow up studies, we have assessed more broadly the stability and broader utility of the mAbs, eventually identifying a lead development candidate. Here, we describe this lead mAb (FenAb024) and its capacity to mitigate both fentanyl and carfentanil effects in a pre-clinical murine model. A humanized and stability-optimized version of FenAb024 therefore has the potential to prevent fentanyl and carfentanil-associated mortality both in civilian and biodefense settings.

Materials and methods

Mouse strains

For the experiments described in this manuscript, healthy, female, wildtype C57BL/6 J mice, aged 6–8 weeks at the time of the experiment (Janvier) were used. Mice were kept in the Interdisciplinary Neuro-behavioral Core (INBC) (Heidelberg, Germany). All animal experiments were performed according to the institutional and governmental regulations under the German Animal Protection Law (§8 Tierschutzgesetz), and were approved by the Regierungspräsidium, Karlsruhe, Germany, under the protocol number 35-9185.81/G-285/18.

Experimental design of the passive immunizations

C57BL/6 J wildtype mice were passively immunized by intravenous (i.v.) injection of the monoclonal antibody FenAb024 in 100 μ l of PBS, while control mice were injected with PBS only. Two hours later, mice were injected subcutaneously (s.c.) with either fentanyl (100 or 400 μ g/kg) or carfentanil (5 or 10 μ g/kg) and were subjected to the antinociception and movement tracking assays as described below, until the effects of the opioid diminished.

Fentanyl and carfentanil

Fentanyl was purchased as Fentadon from Dechra Veterinary Products Deutschland GmbH (#1903801, 78.5 μ g/mL Fentanylcitrate). Carfentanil was purchased from Cayman Chemical (#59708-52-0, 100 μ g/mL in methanol). Both were diluted accordingly in NaCl.

FenAb024 expression and purification

Initially, the antibody was selected and cloned after B cell repertoire analysis from fentanyl-immunized splenocytes (Triller et al., 2023). FenAb024 was then produced as an IgG with a mouse VDJ and a human constant region in Human Embryonic Kidney (HEK) 293 F cells (ThermoFisher, #R79007) by co-transfection of equal amounts of purified plasmids encoding the heavy and the light chain (Tiller et al., 2008). HEKs were kept in FreeStyle™ 293 Expression Medium (ThermoFisher, #12338018) and were transfected at cell densities between 8.0×10^5 to 1.0×10^6 cells/mL using FreeStyle™ MAX Reagent (ThermoFisher,

#16447100) or jetOPTIMUS DNA Transfection Reagent (Polyplus, #101000051); both plasmids were diluted accordingly in OptiPRO™ SFM (ThermoFisher, #12309050). Cells were incubated at 37 °C and 5% CO₂ on an orbital shaker at 120 rpm for 6 days. Supernatants were then collected and passed through a gravity chromatography column containing 1 mL of Pierce™ Protein G Agarose resin (ThermoFisher, #20397). The column was washed with 20 mL of wash buffer (150 mM NaCl, 50 mM Tris, pH 7.4) and eluted in 1 mL fractions with elution buffer (150 mM NaCl, 50 mM Glycine, pH 2.8) prior to neutralization with neutralization buffer (1 M Tris, pH 8.0). Samples were analyzed by SDS-PAGE.

FenAb024 stability measurements with Prometheus Panta

The thermal stability of FenAb024 was measured by Prometheus Panta (NanoTemper) using the nanoDSF feature embedded in the machine. The protein was solubilized in purification buffer (50 mM Tris pH 8.0, 150 mM NaCl, 50 mM Glycine). The 350/330 nm ratio was measured as the temperature increased from 20 °C to 100 °C. The T_{onset} (temperature when the unfolding starts) for FenAb024 was determined to be 64 °C, and the T_m (melting temperature, when 50% of the protein is unfolded) 72 °C.

Sandwich ELISA

Antibody levels were determined by sandwich ELISA. First, purified anti-human IgG Fc antibody (Biolegend, #410701) was coated on 96-well ELISA plates (Greiner-Bio-One, #655001) at 2 µg/mL overnight at 4 °C. Plates were blocked for 1.5 h at RT with 4% BSA in PBS. Coated plates were incubated with the serum for 1.5 h at RT. As a control, purified FenAb024 was used in a serial 1:2 dilution at a starting concentration of 0.01 µg/µL. Bound antibodies were detected by goat anti-human IgG secondary antibody coupled to horseradish peroxidase (HRP) (Dianova, #109-035-098) diluted 1:5000 in PBS with 1% BSA, which was then resolved using an ABTS substrate solution complemented with H₂O₂ (Roche). The optical density at 405 nm was determined using a microplate reader (Tecan) after 40 min.

Competition ELISA

Relative affinity was determined using a competition ELISA method. 96-well plates (Costar polystyrene high-binding plates (Corning)) were coated with fentanyl conjugated to BSA at a concentration of 50 ng/mL in a 50 mM carbonate buffer, pH 9.6 (Sigma) overnight. After blocking the plates with 1% gelatin for 1 h and washing with PBS-T, free drug was introduced onto the plates as a competitor. Subsequently, the plates were incubated with HIS-tagged Fab at a concentration of 20 ng/mL for 2 h, followed by washing and an overnight incubation with Penta-His-biotin conjugate at a dilution of 1:5000 (Qiagen). Addition of Streptavidin-HRP at a ratio of 1:5000 (ThermoFisher) was followed by a 1 h incubation and subsequent washing. The Fab bound to the plates was then measured using SigmaFAST OPD substrate (Sigma) and quantitated by absorbance at 492 nm using a microplate reader (Tecan). The fentanyl-hapten structure employed in this assay is further described in (Triller et al., 2023).

Antinociception assay (hot plate)

The hot plate assay was used to test analgesia, as previously described (Cox and Weinstock, 1964). Baseline nociception was assessed by placing the mice on a hot plate at 54 °C and timing the latency to a reaction (in seconds) such as jumping or flicking or licking their paws. The test was aborted after 10 s for fentanyl or 60 s for carfentanil (unless a faster reaction was observed). After opioid injection, the effect of fentanyl or carfentanil was measured as latency to response (seconds), as well as the percentage maximum possible effect (%MPE); the latter, was

calculated as ((post-test latency – baseline)/(maximum latency (10 s or 60 s) – baseline)) × 100. The 60 s cutoff for carfentanil is based on what is usually used in the literature for similar studies (mostly with rats, however) (Raleigh et al., 2019; Smith et al., 2019), while the lower threshold of 10 s was chosen for fentanyl studies because intoxicated control mice reacted more rapidly with fentanyl compared to carfentanil in our studies. Further, 10–20 s cutoffs are also common in hot plate or hot water bath assays with mice (Guillemyn et al., 2012; Keyhanfar et al., 2013; Schmidt et al., 2010; Triller et al., 2023; Watanabe et al., 2020). No values higher than 60 s were tested to prevent heat-related injury and avoid tissue damage to the animal (Yamamoto et al., 2002). This is a precautionary measure to ensure the well-being of the animals undergoing the test. Experiments were conducted using a blinded observational method of collecting data.

Straub tail reaction

The Straub tail reaction, which is frequently combined with stereotypical walking behavior, is a dorsiflexion of the tail that is frequently almost vertical to the orientation of the body or curls back over the animal (Bilbey et al., 1960). This effect is a response to opiates in mice, mediated by activation of the opioid receptor (Aceto et al., 1969; Zarrindast et al., 2001). The phenomenon was recorded as present (S +), absent (S -) or an in-between state of the tail being half up (S +/-).

LABORAS

The automated laboratory animal behavior observation registration and analysis system (LABORAS) was used to monitor the movement of the mice. The system utilizes a home cage environment, it is non-invasive and able to track a wide range of genuine behaviors, such as resting, locomotion, traveled distance, climbing, grooming, eating and drinking, without the need for human observers. It measures the vibrations created by the movement of an animal and converts them into behaviors and tracking data using pattern recognition and signal analysis technology. The software can detect each behavior by its distinct signature of signal characteristics, as well as extract the position and speed of the animal.

In the experiments described in this manuscript, mice were individually placed in a LABORAS cage and provided with food and water. Only a subset of mice from the hot plate assay (Fig. 3B) were assessed in the LABORAS system due to technical limitations in the assay system. A baseline measurement of 15 min was performed for each mouse before the start of the experiment, followed by 15 min sessions after opioid injections. For each session, the software tracked the movement of the animals depicted in heat maps. These data are representative of several independent trials.

Preparation of serum samples and LC-MS/MS analysis

Sample extraction

The serum extraction process employed liquid-liquid extraction (LLE) conducted at room temperature. For the preparation of calibrators and quality controls, 100 µL of human serum was appropriately spiked with fentanyl. A sample volume of 100 µL was also utilized for the analysis of mouse sera, with lower-volume samples supplemented with human serum. Subsequently, 10 µL of an internal standard solution (fentanyl-d₅, final concentration 50 ng/mL; carfentanil-d₁₀, final concentration 5 ng/mL), 50 µL of a 5% ammonia solution, and 700 µL of ethyl acetate were added to all samples. The resulting mixtures were thoroughly vortexed and centrifuged at 14,000 rpm for 10 minutes. Following centrifugation, 600 µL of the respective supernatant was transferred to a glass vial and dried at 40 °C under a nitrogen stream until complete dryness was achieved. Finally, the dried samples were reconstituted in 50 µL of methanol.

LC-MS/MS conditions

A volume of 5 μL of the sample was injected onto a reversed-phase Restek (Bad Homburg vor der Höhe, Germany) Raptor Biphenyl column (5 μm , 150 \times 2.1 mm). The LC-MS/MS system comprised an API 4000 tandem mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems SCIEX, Darmstadt, Germany) and an Agilent Technologies 1100/1200 series HPLC system (Waldbronn, Germany) featuring a G1312A Binary Pump, G1310A Isocratic Pump, and G1313 Autosampler. Gradient elution was executed with a blend of mobile phase A (2 mM ammonium formate buffer containing 0.1% formic acid, pH 3.2) and mobile phase B (methanol containing 0.1% formic acid) according to the following profile: 0.0 min, 20% mobile phase B; 0.0–6.0 min, 20% \rightarrow 100% mobile phase B; 6.0–9.0 min, 100% mobile phase B; 9.0 min, 100% \rightarrow 20% mobile phase B; 9.0–12.0 min, 20% mobile phase B. The flow rate was maintained at a constant 0.4 mL/min, and the total run time was 12 min. Ionization was achieved using electrospray in the positive ionization mode (ESI+). For fentanyl detection, the instrument settings included collision-activated dissociation (CAD)

at 9 L/min, curtain gas at 25 L/min, nebulizer gas (GS1) at 15 L/min, heater gas (GS2) at 15 L/min, ion spray voltage at 4200 V, and temperature at 500°C. To achieve sensitive mass spectrometric detection, the multiple reaction mode (MRM) was employed. Qualification and quantification were conducted by monitoring the following transitions: fentanyl 337.2–188.3, secondary 337.2105.2, fentanyl-d5 342.3–188.3, secondary 342.3–105.2, carfentanil 395.21–335.20, secondary 395.21–246.3, carfentanil-d5 400.3–246.2, secondary 400.3–340.3. Analyst Software 1.7.2 (Applied Biosystems SCIEX, Darmstadt, Germany) was utilized for peak integration and subsequent evaluation and quantification.

Quantification and statistical analysis

Statistical analysis for Figs. 2B, D, 3B, D and SFigure 1 were performed in GraphPad Prism (v9.3.1). For Figs. 2B and 3D the unpaired t test with Welch's correction was performed, as equal variances could not be assumed. For Fig. 2D and SFigure 1B the unpaired t test was used.

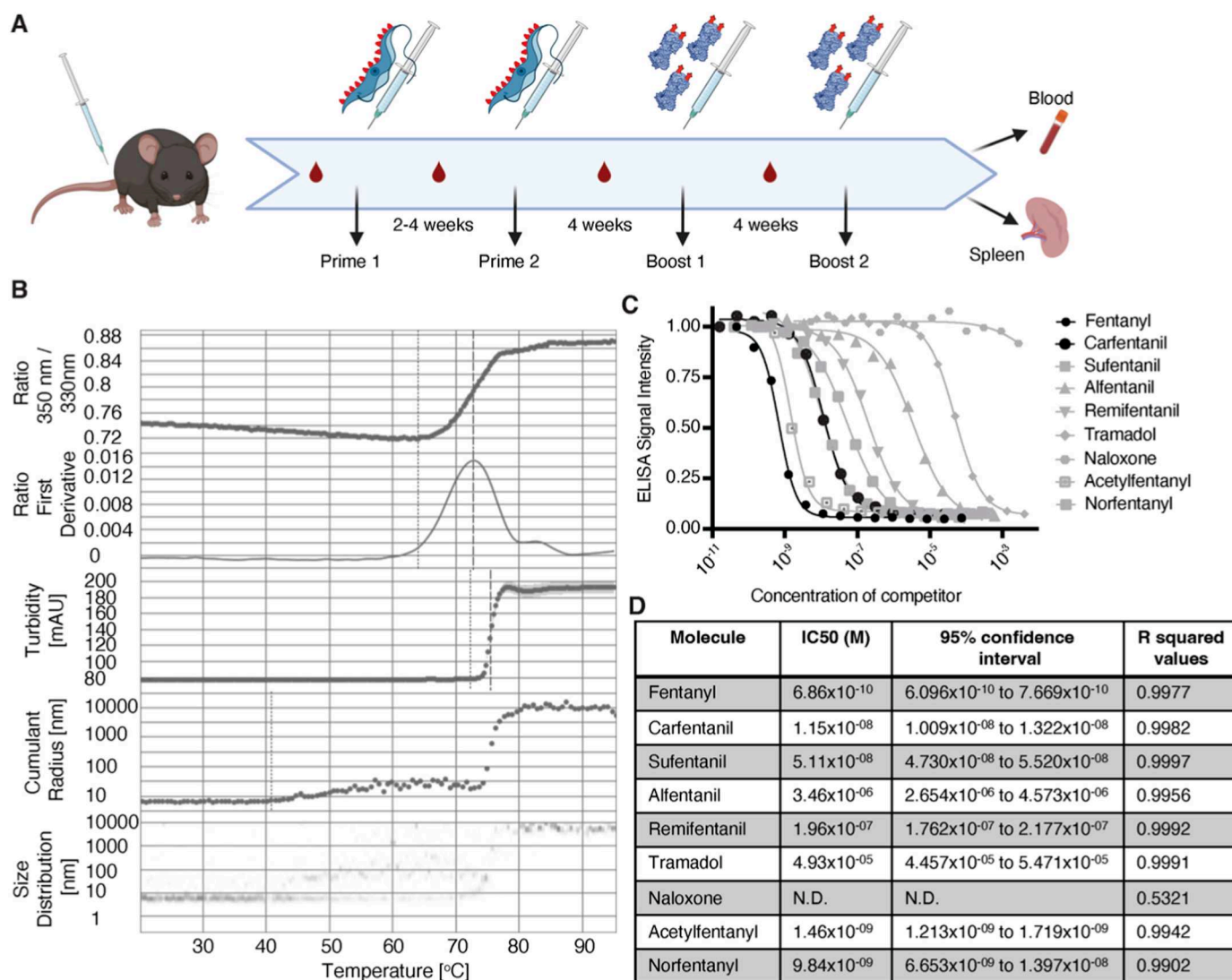


Fig. 1. The VAST platform elicits stable antibodies against fentanyl and carfentanil. (A) Schematic of the VAST platform pipeline. Mice are immunized using whole sortagged trypanosomes for the two primes and the sortagged soluble VSG protein for the boosts. Intermediate bleeds to check titers are illustrated as red drops. (B) Thermal stability of FenAb024, measured with the Prometheus Panta (NanoTemper) using nanoDFS. The T_{onset} was determined to be 64 °C, and the T_m 72 °C. (C) Competition ELISA data generated using fentanyl-coated plates probed with Fab024 (a Fab version of the FenAb024), in the presence or absence of fentanyl (smaller black circles) and various competitors. The mAb can bind to more potent fentanyl derivative molecules such as acetylfentanyl (gray smaller squares), alfentanil (gray smaller triangles), and Carfentanil (larger X-marked circles). (D) IC50 values in molar (M), 95% confidence interval and R squared values for the data plotted in (C). N.D. stands for “not detected”.

For Fig. 3B the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test was performed, as equal variances could not be assumed. For SFigure 1 A the Dunnett's multiple comparisons test was performed. Differences were determined as significant when $p < 0.05$.

Results

The VAST platform elicits stable antibodies against fentanyl and carfentanil

Using UV-crosslinked trypanosomes to immunize mice simulates the immunological response elicited by the living parasite. As previously described, we have successfully immunized mice against the opioid fentanyl utilizing a platform, the VAST, based on the aforementioned parasites (Triller et al., 2023). Briefly, mice were primed with either Fent-VAST or control-VAST and subsequently boosted with a 10- to 20-fold higher dose of soluble antigen-conjugated Variant Surface Glycoprotein (VSG), the combination of which drives strong adjuvant-free antigen-specific antibody responses (Triller et al., 2023) (Fig. 1A). Indeed, mice immunized with the Fent-VAST developed robust antibody titers against fentanyl, as a result of specific B-cell memory recall (Triller et al., 2023). We then isolated several mAb candidates from the immunized mice, which displayed high affinity and protection against opioid effects in mice (Triller et al., 2023).

From the fully characterized set of anti-fentanyl monoclonal antibodies (FenAbs) that we extracted from those immunizations, FenAb024 was chosen as the lead candidate initially based on affinity. We then assessed the thermostability of FenAb024 with the Prometheus Panta, observing no aggregation effects within biologically meaningful temperature ranges. The T_{onset} (temperature when the unfolding starts) was determined to be 64 °C, and the T_m (melting temperature, when 50% of the protein is unfolded) 72 °C (Fig. 1B). Additional characterization by competition ELISA demonstrated that these mAbs are specific to fentanyl, but can also bind to more potent fentanyl derivative molecules such as carfentanil (Fig. 1, C and D). We therefore identified a high affinity and highly thermostable development candidate that could hypothetically be used to prevent toxicity from several different clinically relevant opioid compounds.

The high-affinity fentanyl-specific antibody FenAb024 can shield mice from fentanyl effects

To investigate the capacity of FenAb024 in mitigating the effects of fentanyl, we utilized a preclinical murine behavioral model. FenAb024 was administered intravenously 2 h prior to a 100 µg/kg fentanyl injection, followed by the hot plate antinociception assay 5 min after opioid treatment and the laboratory animal behavior observation registration and analysis system (LABORAS) assessment measured every 30 min in three consecutive 15-min sessions (Fig. 2A). The measurable analgesic response was reduced by approximately 50% of the maximal possible effect (MPE) after the fentanyl challenge in mice passively immunized with 15 mg/kg FenAb024 (Fig. 2B), a dose chosen after evaluating several pilot and published studies (Triller et al., 2023). LABORAS analysis showed a scattered movement pattern at baseline, visualizing the natural, explorative behavior of mice (Fig. 2C). After fentanyl challenge, unprotected mice ran in a circular fashion at the periphery of the cage displaying thigmotaxis as expected, and displayed a higher pace, as demonstrated by the increase in distance traveled relative to baseline (Fig. 2D). This distinct movement pattern after opioid treatment diminished over time back to the natural, explorative behavior. By contrast, mice that received FenAb024 did not exhibit such behavior, but rather rested in a corner of the cage after initial cage exploration (Fig. 2D).

Antibodies protect against the effect of opioids by binding to the drug in the bloodstream, which reduces the presence of freely circulating opioids, preventing their entry into the central nervous system (Crouse

et al., 2022). Therefore, we measured the fentanyl concentration in the serum of mice 2 h after opioid challenge using mass spectrometry (LC-MS/MS), validating that FenAb024 traps fentanyl in the serum (Fig. 2E). The amount of FenAb024 circulating in mouse serum at the time of drug challenge correlated with the fentanyl concentration, showing that higher quantities of circulating mAb trap higher quantities of opioid as expected (Fig. 2E).

FenAb024 can protect mice from carfentanil effects

We then assessed whether this multi-opioid-binding antibody could also protect mice from other opioids, specifically carfentanil. Carfentanil is commonly said to be 100-fold more potent than fentanyl in humans (Smith et al., 2019), although there is a dearth of literature supporting this statement in animal models. Therefore, we first compared the effects of carfentanil and fentanyl in this murine system to identify a carfentanil dose that would provide an appropriate and measurable effect. A 10 µg/kg dose of carfentanil induced an effect that was comparable to an 800 µg/kg dose of fentanyl in the context of these particular assay systems (Fig. 3A), which is indeed in line with published ranges. We then assessed the protective capacity of FenAb024 against 5 and 10 µg/kg carfentanil. Passive immunization with 5 mg/kg FenAb024 led to a more than 50% decrease in %MPE (Fig. 3B). This effect was dose dependent, with 10 mg/kg FenAb024 providing a higher protective capacity of approximately 60% reduced effect. Movement tracking via the LABORAS system showed the distinct circular pattern for all mice directly after carfentanil injection, but a faster normalization in behavior for the FenAb024 administered mice in a dose dependent manner (Fig. 3C). This was supported by quantitation of the total traveled distance (SFigure 1 A). Another way to assess the effect of opioids in rodents, is a phenomenon called the Straub tail reaction. This is the dorsiflexion of the tail resulting in a tail oriented vertically to the body or even curled over the back of the mouse (Straub 1911, cited by Bilbey et al., 1960). In untreated mice, the Straub tail reaction was visible up to 3 h after carfentanil treatment, which was up to 1 h longer than in the FenAb024 administered mice (SFigure 2 A).

To assess the lowest dose of antibody that would provide protection, we challenged the mice with 5 µg/kg carfentanil after 5 mg/kg FenAb024 injection. The analgesic activity was close to baseline for mice receiving FenAb024 with an %MPE of approximately 4% (Fig. 3D). Carfentanil effects were undetectable for the FenAb024 treated mice within 2 h after the challenge in their movement tracking (Fig. 3E). A normal behavior could also be observed for the traveled distance after FenAb024 administration (SFigure 1B). The Straub tail reaction was partially visible for 1 h in mice passively immunized with FenAb024, while the effect was more pronounced and longer-lasting in control mice (SFigure 2B).

Discussion

The synthetic opioid fentanyl is the main driver of the overdose crisis, which is a major public health burden in North America, with rising concerns also in Europe and elsewhere. Several fentanyl derivatives have also been detected in the illicit drug supply, including the substantially more potent derivative carfentanil. The potency of carfentanil raises additional concerns; it is already recognized as a potential chemical weapon (Riches et al., 2012). Given the ever-increasing rate of overdose mortality despite the widespread distribution of naloxone/Narcan, there remains a desperate need for effective alternative treatment options. Standard therapies so far require either the presence and time-sensitive action of a caregiver to administer naloxone post-overdose, or consistent self-administrative prophylaxis in the form of buprenorphine or others, e.g.: naltrexone (Leslie et al., 2015). Currently, most standard prophylaxis programs rely on frequent dosing, given the relatively low half-lives of opioids in the body. While there are some longer-lasting buprenorphine formulations now approved for

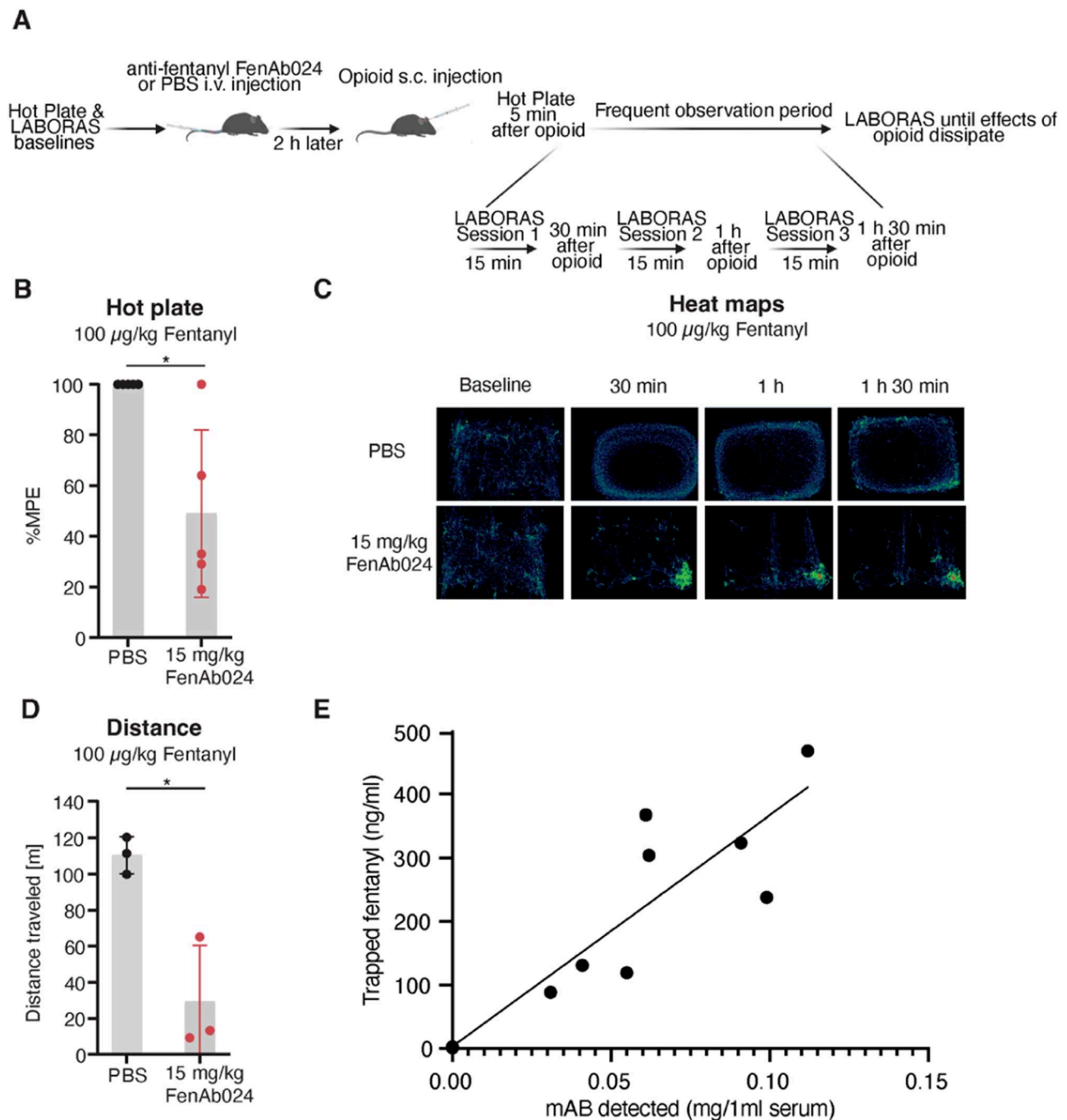


Fig. 2. The high-affinity fentanyl-specific antibody FenAb024 can shield mice from fentanyl effects. (A) Experimental setup of the behavioral assays. Mice were injected intravenously with 15 mg/kg of FenAb024 or control (PBS) 2 h before subcutaneous fentanyl injection (100 $\mu\text{g}/\text{kg}$ body weight). Hot plate was performed at baseline and 5 min after fentanyl injection. Laboratory animal behavior observation registration and analysis system (LABORAS) measurements were conducted in 3 sessions as indicated, following baseline measurements prior to injections. (B) Analgesic activity after injections was determined using the hot plate nociception assay (Cox & Weinstock, 1964). Protection from 100 $\mu\text{g}/\text{kg}$ fentanyl after passive immunization with FenAb024 is shown as percentage maximum possible effect (%MPE). %MPE was calculated as ((post-test latency – baseline)/(maximum latency (10 s) – baseline)) \times 100 (Guillemyn et al., 2012; Keyhanfar et al., 2013; Schmidt et al., 2010; Watanabe et al., 2020). Means \pm SD of 5 mice per group are depicted. * $p < 0.05$ by unpaired t test with Welch's correction. (C) Representative LABORAS heatmaps illustrating 15-minute long sessions of movement tracking of individual mice are shown from the top-down view into the cage. (D) Total distance traveled (in meters) across all LABORAS sessions is shown. Means \pm SD of 3 mice per group are depicted. * $p < 0.5$ by unpaired t test. These data are representative of several independent trials. (E) LC-MS analysis of fentanyl levels in the serum of fentanyl-challenged mice correlated to the amount of protective FenAb024 antibody detected in the serum of those mice at the time of challenge. Antibody levels were determined by sandwich ELISA using an anti-human IgG. $R^2 = 0.08209$.

clinical use (Jones et al., 2021; Lofwall et al., 2018), we and others propose immunotherapies such as vaccine and monoclonal antibody treatment, as controllable, long-lasting, and well-tolerated non-opioid

alternatives to the standard therapies (Bremer et al., 2016; Raleigh et al., 2019; Triller et al., 2023). Therapeutically, these strategies would most effectively be deployed prophylactically to prevent overdose (although

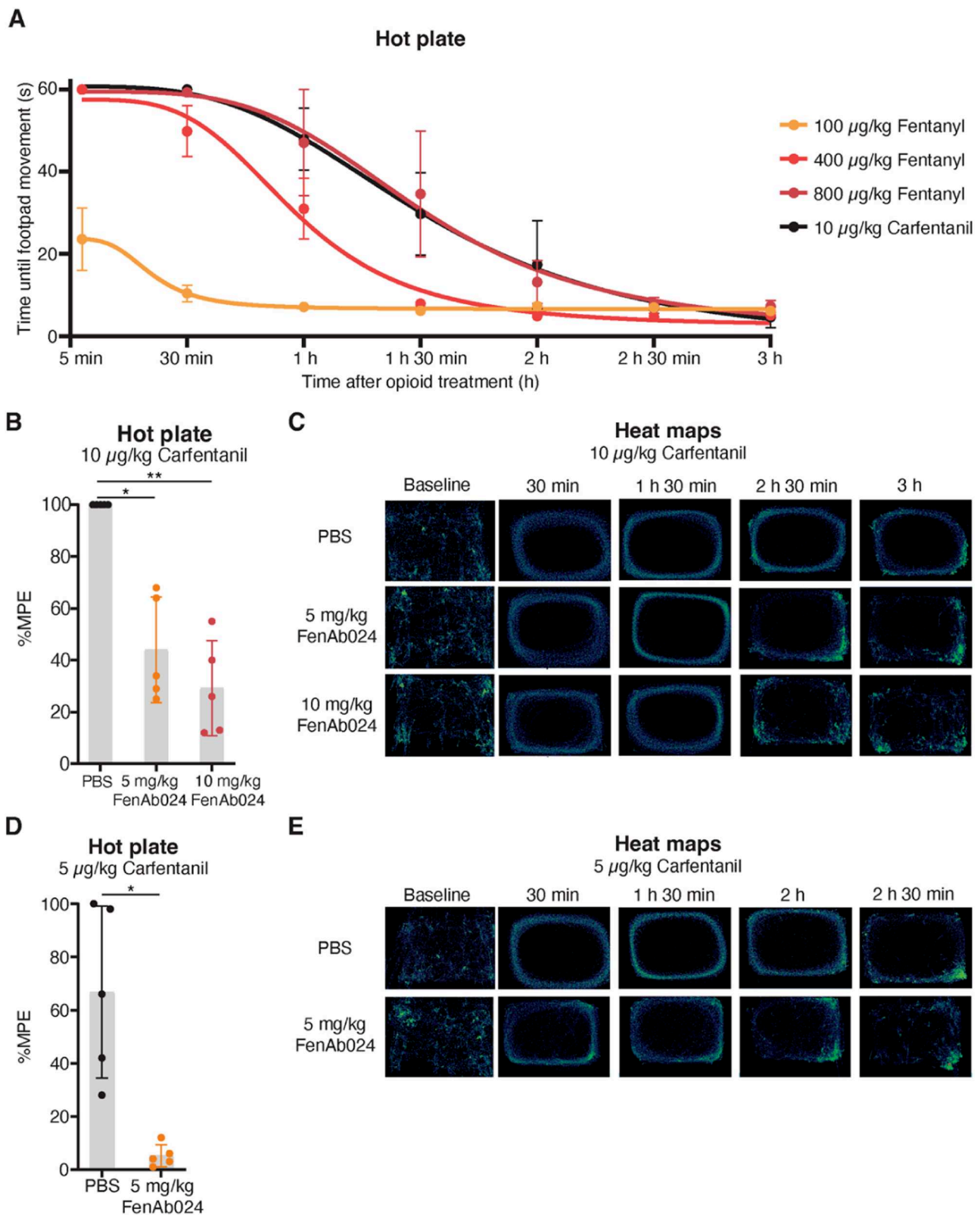


Fig. 3. FenAb024 can protect mice from carfentanil pharmacological effects. (A) Analgesic activity was measured via the hot plate assay after injection of 100 µg/kg, 400 µg/kg and 800 µg/kg body weight fentanyl and 10 µg/kg body weight carfentanil, shown in time until footpad movement at 5 min and then every 30 min up to 3 h after opioid injection. Means ± SEM for each group are depicted. (B) Hot plate measurements were performed at baseline and 5 min after carfentanil injection. Protection from 10 µg/kg carfentanil after passive immunization with FenAb024 is shown as percentage maximum possible effect (%MPE). %MPE was calculated as (post-test latency – baseline)/(maximum latency (60 s) – baseline) × 100 (Raleigh et al., 2019). Means ± SD of 5 mice per group are depicted. *p < 0.05, **p < 0.01 by Dunn’s multiple comparisons test. (C) Representative LABORAS heatmaps illustrating 15-minute long sessions of movement tracking of individual mice. (D) Behavioral protection from 5 µg/kg carfentanil is shown as described in (B). *p < 0.05 by unpaired t test with Welch’s correction. (E) LABORAS heatmaps for mice injected with 5 µg/kg carfentanil, similar to (C).

there is some evidence that vaccine approaches also have the potential to reduce opioid addiction (Pravetoni and Comer, 2019)). For example, an antibody could be administered to individuals in rehabilitation centers at the time of departure, with that individual also receiving additional subcutaneous antibody doses on a monthly basis. These doses could even be self-administered at home to increase compliance, as has become standard for several clinically approved mAb therapies (Deleuran et al., 2020; Dupixent, n.d.; Malani et al., 2021). The antibody would then consistently remain above the threshold required to protect an individual from a certain lethal dose of fentanyl while that individual remains at risk of relapsing, a period that in some cases can last several years. Additionally, these mAb therapies have potential uses in chemical defense settings, wherein first responders and soldiers at risk of fentanyl/carfentanil exposure could protect themselves from toxicity.

With our trypanosome-based VAST platform technology (Triller et al., 2023), we generated several antibodies specific for both fentanyl and carfentanil. Our lead candidate FenAb024 shows robust *in vitro* binding to fentanyl and its derivatives (Fig. 1), displays strong *in vitro* stability properties (Fig. 1), and demonstrates *in vivo* efficacy against behavioral opioid effects (Figs. 2 and 3). We could observe protective effects of 15 mg/kg FenAb024 against 100 µg/kg fentanyl in the hot plate nociception assay and faster normalization of behavioral opioid-related phenotypes such as the circular running pattern and distance traveled. At baseline, mice were introduced to the LABORAS home cage and they were more motile as they explored their surroundings, which is evident by the heat maps (Figs. 2 and 3). FenAb024-injected mice followed a trajectory more closely aligned with baseline, yet displayed reduced mobility, resting in a corner of the cage after exploration. This phenotype could be explained by mice acclimating to the cage and thus reflective of the true normal behavior of mice during the day, or it could be explained by mice experiencing some form of weariness in this assay system. Others have shown that this physiology, described as “freezing” or “crouching”, can potentially be related to anxiety (Blanchard et al., 1986). Future work could utilize different behavioral tests to determine whether indeed this phenotype is anxiety-related.

Predicting effective mAb doses against various opioids in humans is challenging for several reasons; antibodies primarily interact with circulating drugs in the serum, while unbound drugs in circulation are influenced by tissue distribution effects. Therefore, it is critical to understand both the effective amount of opioid trapped by a given dose of antibody as well as the hypothetical maximum amount of opioid that could be trapped by a given dose of antibody. Given that the maximum fentanyl:mAb binding ratio is 2 fentanyl molecules per 1 IgG antibody, we calculate that the doses mentioned above lead to a 1.5-fold molar excess of fentanyl molecules to available antibody binding sites. Therefore, the hypothetical maximum amount of fentanyl that 15 mg/kg of antibody could trap in this assay system is only 2/3rds of the total amount of injected drug in this system (where the drug dosage is experimentally controlled). This proved sufficient to note a decrease in opioid-related responses. The increased serum concentration of fentanyl in mice administered with the antibody supports the hypothesis that FenAb024 traps fentanyl within the bloodstream and limits its redistribution to the opioid receptors in the CNS and elsewhere. The efficacy of opioid binding antibodies is thus dependent on this serum-trapping mechanism, but also the confounding element of the general unpredictability of the quantity of fentanyl administered in clinical settings.

In more conventional antibody therapeutics, the Fc-region drives critical effector functions required for, for example, pathogen clearance and destruction. This is highlighted in the cases of malaria and COVID vaccines. Specifically, IgG and IgM antibodies produced as a result of immunization with the MSP1 SumayaVac-1 malaria vaccine, can stimulate numerous Fc-mediated effector mechanisms essential to malaria protection, such as phagocytosis, production of INF- γ and complement activation (Rosenkranz et al., 2023). A similar observation can be made for most COVID vaccines as well; Fc engagement is necessary for

vaccine-induced antibody-mediated protection against the SARS-CoV-2 virus, resulting again in activation of phagocytosis and complement pathways (Mackin et al., 2023). An open question in opioid-related immuno-therapeutics is whether any specific effector functions are actually necessary. Here, the antibody FenAb024 has a murine variable region and a human IgG1 Fc. Further, others have used antibody scFv fragments to achieve similar protective effects in rodent models (Eubanks et al., 2023). Together, these data suggest that at least the initial protective capacity of these mAbs is independent of any particular effector functions, an observation further supported by Huseby Kelcher et al. (2021). However, antibody-opioid complexes are then likely to remain in circulation for some amount of time, and understanding the physiological impact that these complexes may or may not have over that time remains an important future direction.

Here, and previously using alternative strategies (Triller et al., 2023), we have correlated the amount of fentanyl trapped in the serum of the mice with the concentration of antibody present in the serum at the time of challenge. The positive correlations that we observe allow for a uniquely strong predictability of therapeutic efficacy compared to antibodies for non-drug targets, given the mechanism of protection. By contrast, it remains challenging to predict what minimal dose of antibody will protect a human from, for example, any given infectious agent. Instead, here the amount of fentanyl that can be trapped in the serum is directly dependent on the amount of antibody present in the serum at the time - a “numbers game”. Antibodies that have affinities as high as FenAb024 are thus likely to protect from easily predictable levels of opioid in humans, which presents a unique advantage in the context of clinical trial design.

Since carfentanil is approximately 80–100-fold more potent than fentanyl, a substantially lower dose of carfentanil (compared to fentanyl) is required for the observance of any effects on mouse behavior (Fig. 3A). In these experiments, the number of circulating bioavailable molecules of carfentanil at a dose of 5 µg/kg is substantially lower than the number of circulating molecules of fentanyl at a dose of 100 µg/kg. Therefore, the hypothetical amount of drug that one would need to trap with an antibody in order to protect from toxicity should be concomitantly lower in carfentanil’s case in the context of these doses. This is indeed what we have observed where only 5 mg/kg of FenAb024 was needed to protect from this dose of carfentanil, while we needed more mAb to protect from 100 µg/kg fentanyl. This observation is also partially dependent on the antibody affinity being high-enough to capture the drug *in vivo* at the molar concentrations that are being deployed here. However, since the effects of carfentanil on mouse behavior are similar at these dosed concentrations, one could hypothesize that similar observations could be made in humans. That is to say that, for example, a lower dose of FenAb024 may be needed to protect humans from a medically relevant dose of carfentanil compared to a medically relevant dose of fentanyl, even though its observed affinity for carfentanil is slightly lower.

To further develop FenAb024 for most potential clinical applications, the antibody needs to be fully humanized and optimized for *in vivo* stability. An antibody with a murine variable region will not be suitable for long-term therapeutic administration, as antibodies targeting FenAb024 will be elicited soon after the first administration. However, the chemical defense-related use-case may only require a one-time administration of the antibody, and thus the existing chimeric FenAb024 already has human-applicable potential.

A comprehensive characterization of FenAb024’s safety profile through further preclinical studies is essential to evaluate potential off-target effects of the antibody. However, at least so far, the literature suggests that anti-opioid specific antibodies do not have any impact on the behavior of mice, at least in the context of these particular model systems and assays (Triller et al., 2023, Baehr et al., 2020). Additionally, fentanyl and its derivatives are known to cause respiratory depression. This is not something we have explored in the current manuscript, however, a similar study with rodents illustrated that fentanyl indeed

causes respiratory depression in the animals (Smith et al., 2019) that can be reversed with naloxone. Furthermore, an engineered human antibody fragment can reverse carfentanil-induced respiratory depression (Eubanks et al., 2023). Whether FenAb024 can also be used for such a purpose remains to be investigated, although we hypothesize that to be the case based on its other protective properties.

In conclusion, FenAb024 shows promise as an immunotherapeutic solution against the opioid crisis. It effectively protects against fentanyl and carfentanil in murine pre-clinical studies, via a serum-trapping mechanism. Future optimization for clinical use is needed, along with further investigation to understand the long-term impacts of antibody-based opioid prophylaxis on drug metabolism.

CRediT authorship contribution statement

Anastasia Gkeka: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Katharina Urban:** Investigation, Formal analysis, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Dennis Greiner:** Writing – review & editing, Methodology, Investigation. **Monica Chandra:** Data curation, Investigation, Methodology, Writing – review & editing. **Joseph Verdi:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Carly Baehr:** Investigation, Methodology, Writing – review & editing. **Marco Pravetoni:** Conceptualization, Writing – review & editing. **F. Nina Papavasiliou:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. **C. Erec Stebbins:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. **Sandra Ruf:** Writing – review & editing, Methodology, Investigation. **Selina Pollich:** Investigation, Methodology, Writing – review & editing. **Tom Sundermann:** Investigation, Methodology, Writing – review & editing. **Yosip Kelemen:** Investigation, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joseph Verdi reports financial support was provided by National Institute on Drug Abuse. Nina Papavasiliou reports financial support was provided by Helmholtz Foundation. Erec Stebbins reports financial support was provided by Helmholtz Foundation. Joseph Verdi reports a relationship with Hepione Therapeutics Inc. that includes: employment and equity or stocks. Nina Papavasiliou reports a relationship with Hepione Therapeutics Inc. that includes: equity or stocks. Erec Stebbins reports a relationship with Hepione Therapeutics Inc. that includes: board membership and equity or stocks. Yosip Kelemen reports a relationship with Hepione Therapeutics Inc. that includes: employment. Joseph Verdi reports a relationship with Panosome GmbH that includes: employment and equity or stocks. Nina Papavasiliou reports a relationship with Panosome GmbH that includes: board membership and equity or stocks. Erec Stebbins reports a relationship with Panosome GmbH that includes: board membership and equity or stocks. Anastasia Gkeka reports a relationship with Panosome GmbH that includes: employment and equity or stocks. Katharina Urban reports a relationship with Panosome GmbH that includes: employment and equity or stocks. Monica Chandra reports a relationship with Panosome GmbH that includes: employment and equity or stocks. Dennis Greiner reports a relationship with Panosome GmbH that includes: employment. Sandra Ruf reports a relationship with Panosome GmbH that includes: equity or stocks. Selina Pollich reports a relationship with Panosome GmbH that includes: employment. Joseph Verdi has patent pending to Hepione Therapeutics Inc. Nina Papavasiliou has patent pending to Hepione Therapeutics Inc. Erec Stebbins has patent pending to Hepione Therapeutics Inc. Yosip Kelemen has patent pending to Hepione Therapeutics

Inc. Marco Pravetoni has patent pending to University of Minnesota. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.toxlet.2024.03.008](https://doi.org/10.1016/j.toxlet.2024.03.008).

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