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Monoclonal Antibodies for Combating Synthetic Opioid Intoxication

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Abstract

Opioid abuse in the United States has been declared a national crisis and is exacerbated by an inexpensive, readily available, and illicit supply of synthetic opioids. Specifically, fentanyl and related analogues such as carfentanil pose a significant danger to opioid users due to their high potency and rapid acting depression of respiration. In recent years these synthetic opioids have become the number one cause of drug-related deaths. In our research efforts to combat the public health threat posed by synthetic opioids, we have developed monoclonal antibodies (mAbs) against the fentanyl class of drugs. The mAbs were generated in hybridomas derived from mice vaccinated with a fentanyl conjugate vaccine. Guided by a surface plasmon resonance (SPR) binding assay, we selected six hybridomas that produced mAbs with 10^{-11} M binding affinity for fentanyl, yet broad cross-reactivity with related fentanyl analogues. In mouse antinociception models, our lead mAb (6A4) could blunt the effects of both fentanyl and carfentanil in a dose-responsive manner. Additionally, mice pretreated with 6A4 displayed enhanced survival when subjected to fentanyl above LD₅₀ doses. Pharmacokinetic analysis revealed that the antibody sequesters large amounts of these drugs in the blood, thus reducing drug biodistribution to the brain and other tissue. Lastly, the 6A4 mAb could effectively reverse fentanyl/carfentanil-induced antinociception comparable to the opioid antagonist naloxone, the standard of care drug for treating opioid overdose. While naloxone is known for its short half-life, we found the half-life of 6A4 to be approximately 6 days in mice, thus monoclonal antibodies could theoretically be useful

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/jacs.9b04872](https://doi.org/10.1021/jacs.9b04872). MALDI-TOF spectrum, mouse titers, hybridoma characterization and additional mAb-drug SPR binding data (PDF)

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in preventing renarcotization events in which opioid intoxication recurs following quick metabolism of naloxone. Our results as a whole demonstrate that monoclonal antibodies could be a desirable treatment modality for synthetic opioid overdose and possibly opioid use disorder.

Graphical Abstract

INTRODUCTION

The ongoing rise in opioid abuse creates a dire need for fast-acting, effective therapies to combat opioid intoxication and overdose. In early August 2017, the opioid crisis was declared a national emergency in the United States due to the ever-increasing number of opioid overdose reports. The etiology of ballooning opioid-related fatalities is multifactorial and most commonly involves the abuse of powerful semisynthetic and fully synthetic opioids, i.e., heroin and fentanyl, respectively (Figure 1).^{1–4} The fentanyl class of drugs are potent mu-opioid receptor (MOR) agonists, and this pharmacological property is responsible for their pain relieving, addictive, and potentially dangerous central nervous system (CNS)-depressive effects. First discovered in 1960 by Paul Janssen,⁵ fentanyl is a Drug Enforcement Administration (DEA) schedule II drug that is routinely used in the clinic. Fentanyl can be administered intravenously for anesthesia, as well as transdermally and transmucosally via patches and lozenges, respectively, for mitigating pain in a variety of scenarios (e.g., postoperative, cancer-related and acute pain).^{6,7} However, fentanyl mirrors the abuse liability profile of heroin, and intentional abuse of prescription fentanyl patches has been reported.^{8–11}

While heroin abuse has remained a public health concern for many decades, more recently, fentanyl poses even greater danger to illicit opioid users due to its enhanced potency. Moreover, illicit supplies of fentanyl and its derivatives are frequently combined with heroin, as well as other drugs of abuse to create potent drug cocktails with a high potential to cause overdose deaths.^{4,12–14} In fact, the potency of fentanyl is approximately 100-fold greater than morphine, while one particular analogue, carfentanyl, exhibits a potency level that is a 100-fold greater than fentanyl itself (Figure 1).^{15–19} Carfentanyl, also discovered by Janssen in 1974, has long been commercially available as a tranquilizer for large animals under the name Wildnil.^{20–23} In the 2002 Moscow theater hostage crisis, it was used as a weapon in an aerosolized mixture that caused 130 casualties.²⁴ In recent years, carfentanyl in addition to fentanyl have been discovered as adulterants in commonly abused drugs, namely heroin, leading to a spike in unintentional overdoses across the country.^{25–28} As a result of carfentanyl's extreme potency and often discrete contamination with other drugs, the DEA issued an "Officer and Public Safety Alert" about the schedule II drug.²⁹

Illicit fentanyl production and distribution can be highly profitable due to the fact that fentanyl compounds are both easy and inexpensive to produce, and they exhibit potent and addictive effects. Furthermore, enforcement of US drug policy regarding synthetic opioids is difficult given the clandestine manufacturing and trafficking of fentanyl, its precursors, e.g., *N*-phenethyl-4-piperidinone (NPP), and unscheduled fentanyl analogues, which primarily originate from China.^{30,31} Alarming, overdose deaths in the US involving synthetic opioids, such as fentanyl and carfentanil have skyrocketed over the past five years, surpassing heroin as the leading cause of drug-induced fatalities.³² To give a statistic, the Centers for Disease Control and Prevention (CDC) has reported 11045 opioid overdose deaths from the period of July 2016 to June 2017, and 2275 (20.6%) tested positive for any fentanyl analogue with 1236 (11.2%) positive for carfentanil.²⁷

The rise in opioid misuse has intensified the need for new and creative ways to both treat and counter their effects. Naloxone, the mu-opioid antagonist found in the nasal spray Narcan, is effective in reversing heroin and prescription opioid-related overdose, but appears to be less effective against fentanyl and carfentanil.^{33,34} Specifically, the high potency of the fentanyl class of drugs, especially carfentanil, is reported to overwhelm the naloxone duration of action (~1 h) and therapeutic efficacy of typical naloxone doses (4 mg by nasal spray and 2 mg by autoinjector).³⁵ Fentanyl, while effective in pain medication and in anesthetic induction, is particularly dangerous when used illicitly due to its potency in respiratory depression.⁷ While the pharmacology of carfentanil is not well characterized in humans, it has similar properties to fentanyl but with greatly increased potency and a potential to cause “renarcotization” whereby a subject experiences opioid intoxication long after apparent recovery.³⁶ Although this phenomenon has not been studied in humans, it is known to occur in large animals tranquilized with carfentanil, and administration of naloxone doses of 100-fold greater than the carfentanil doses are required to prevent renarcotization.³⁷ In a clinical setting, continuous or multiple naloxone doses over time is required to sustain the reversal of fentanyl intoxication and to prevent possible renarcotization.^{38–40} Another MOR antagonist that has shown enhanced efficacy and duration of action against fentanyl and carfentanil-induced respiratory depression compared to naloxone is nalmefene.^{41,42} While antagonists are certainly useful in combatting opioid overdose, especially as a rapidly administered nasal spray, we explored an entirely new approach to treating opioid intoxication through the development of anti-fentanyl monoclonal antibodies.

Previously, we disclosed the first report of a fentanyl tetanus toxoid vaccine in 2016, which significantly shifted the fentanyl dose–effect curve in antinociceptive testing.⁴³ Moreover, this active vaccine could produce antibodies that recognized six fentanyl analogues with little cross-reactivity to oxycodone or methadone.⁴³ Despite the considerable evidence that antibodies can be useful for blocking the effects of opioids, there have been no reports of efforts to utilize a monoclonal antibody-based approach to ameliorate opioid overdose caused by fentanyl or carfentanil. However, previous studies have demonstrated the feasibility of passive immunization against fentanyl in which polyclonal antibodies with relatively weak binding affinity for fentanyl were infused into animals.^{44,45} The advantage of passive immunization against drugs is the immediate sequestration of drug molecules in peripheral blood, thus reducing drug effects on both CNS and peripherally localized organs

and tissues, most importantly the brain. A passive immunization strategy for mitigating substance use disorder has shown promise in a number of preclinical studies against nicotine,^{46–49} cocaine,^{50,51} heroin,^{52,53} and methamphetamine.^{47,54} Indeed, an antimethamphetamine vaccine has been studied in clinical trials.⁵⁵ Critical features as to why this strategy could be of enormous potential benefit include: (A) a dosing regimen that can be easily titrated and modified to match the level of drug intoxication, in contrast to vaccines; (B) the administration methods and half-life (~3 weeks) of antibodies allow for an immediate and long lasting response, which far exceeds the half-life of the synthetic opioids;^{56,57} (C) antibody affinity and specificity can be tailored to certain drug classes; (D) antibodies have inherently low toxicity because they do not modulate receptors in the brain, unlike traditional opioid pharmacotherapies, and are typically nonimmunogenic. Considering these four aspects, a monoclonal antibody (mAb) therapeutic presents a promising strategy for treating both the addictive and toxic CNS-depressive effects of synthetic opioids.

This study describes the development of a mAb-based therapy as an antidote for acute exposure to the potent synthetic opioid drugs like fentanyl and carfentanil. We have isolated hybridomas from mice vaccinated with a fentanylkeyhole limpet hemocyanin (Fent-KLH) conjugate, and these hybridomas produce antibodies that exhibit subnanomolar fentanyl affinity and pan-cross-reactivity with other fentanyl analogues, including carfentanil. Furthermore, we tested our lead mAb (6A4) against fentanyl and carfentanil in pharmacokinetic (PK), antinociception, and overdose assays to evaluate their therapeutic value for mitigating the effects of synthetic opioids.

RESULTS AND DISCUSSION

Monoclonal Antibody Development and SPR Affinity Profiling.

In considering vaccine design for producing antibodies with high affinity and good cross-reactivity with fentanyl analogues, we chose our previously optimized fentanyl conjugate and adjuvant system, which elicited antiserum with low nanomolar affinity for fentanyl.⁴³ Mice were immunized with the fentanyl hapten conjugated to KLH (Fent-KLH) in formulation with CpG oligodeoxynucleotide 1826 and alum adjuvants (Figure 2). Sera collected from mice throughout the vaccination schedule were analyzed by ELISA against Fent-bovine serum albumin (BSA) to determine the presence of antifentanyl antibodies (Figure 2, Figure S2, Table S1) and by an SPR competitive binding assay to determine affinity to fentanyl and two well-known methylated analogues α -methylfentanyl (aka China White), and 3-methylfentanyl (Figure S2). In this method, sera were incubated with varying concentrations of free drugs and the samples were flowed across an SPR sensor chip coated with Fent-BSA. As expected, the fentanyl vaccine exhibited robust antibody titer levels in each individual mouse and low nanomolar affinity for fentanyl. Accordingly, the mouse with the best anti-fentanyl antiserum profile was selected for mAb production: the mouse spleen was harvested to isolate B-cells, which were subsequently fused with myeloma cells. After incubation in hypoxanthine-amino-pterin-thymidine (HAT) medium, the successfully fused cells (hybridomas) were plated and screened by ELISA against Fent-BSA, revealing 41 hybridomas that tested positive for fentanyl-binding antibodies (Table S2).

Further characterization of these hybridomas was performed by testing their supernatants for binding using the SPR competitive method against a panel of 9 compounds in the fentanyl family (Table 1, Table S3). These compounds were selected based on reports of their confiscation by law enforcement according to the National Forensic Laboratory Information System.⁵⁸ On the basis of the binding profiles, six hybridomas were selected for additional rounds of subcloning and grown in larger quantities to facilitate milligram scale production and purification of their resulting mAbs. The six purified mAbs were thoroughly characterized for their fentanyl binding profile using both the competitive SPR method (Figure 3, Table S3) and a direct drug-mAb binding method for accurate K_d determination as derived from the on-and offrates (Table 2, Table S4). Using the direct method, mAbs are immobilized onto the sensor chip and varying concentrations of drugs are flowed across the chip surface. In comparing the results from these methods, the competitive IC₅₀ values were ~100-fold greater than actual K_d values. This discrepancy is caused by the requirement for increasing incubation time needed to reach equilibrium in the competition assay as mAb affinity for drug increases: below nanomolar drug affinity, the time needed to reach equilibrium between free mAb and drug-bound mAb becomes impractical for normal assay conditions. Since equilibrium is reached nearly instantaneously in the direct binding assay, it is advantageous for proper K_d characterization of purified antibodies. A further limitation to the competitive method is the skewed antibody binding to the immobilized fentanyl hapten over free drug compound due to the fentanyl hapten linker and antibody avidity. Considering the binding data, our mAbs showed unprecedented drug affinity in the picomolar range. Previous reports of antidrug mAbs have demonstrated affinity in the 10⁻⁷ to 10⁻⁹ M range.^{44,49,59-62} In comparison, our data shows mAb fentanyl affinity of at least 100-fold greater (10⁻¹¹ M), although there may be some underestimation of affinity by traditional radioimmunoassay (RIA), which was used in previous work.

The drug binding profiles of the six selected mAbs against a panel of the most potent fentanyl analogues showed the following trends: (A) affinity against fentanyl and carfentanil were excellent; (B) relatively good affinity to fentanyl analogues that varied by one methyl group at various positions on the fentanyl core; and (C) relatively weak micromolar affinity to fentanyl analogues that contained drastically different alkyl groups at the piperidine amine such as remifentanyl and alfentanil (Table 1, Table S3). Thus, there is certainly a limit to the structural range of fentanyls for which the mAbs will tolerate, and this is based on the fentanyl-like hapten structure used for immunization. The same trends were observed by the direct binding method, confirming that 6A4 and 7A8 generally showed the best drug affinity while 15F4 clearly showed the worst drug affinity (Table 2, Table S4). One mAb in particular, 6A4, showed promise in sequestering both fentanyl and its more potent analogue, carfentanil. The 7A8 mAb also appeared promising since its affinity for fentanyl was even higher than that of 6A4, albeit at the cost of carfentanil affinity. The 7A8-producing hybridoma did not produce antibody efficiently enough for us to purify it in sufficient quantities for in vivo testing; therefore, 6A4 was targeted for further in vivo evaluation for mitigating the effects of these two drugs. To ensure that 6A4 could be compatible with prescription opioid agonists and antagonists, a panel of these molecules was screened against 6A4 and virtually no cross-reactivity could be detected (Table S5).

Pharmacokinetic–Pharmacodynamic Modeling.

The key mechanism of action of antibody-based treatments for drug abuse is antibody-mediated drug sequestration in the blood, leading to reduced CNS drug concentrations and modulation of receptors in the brain.^{49,63} Specifically, depletion of fentanyl at the MOR target site via sequestration in plasma is expected to reduce the opioid pharmacodynamics in mediating antinociception and respiratory depression. Thus, we sought a pharmacokinetic strategy aiming to keep the target drug below its minimal effective concentration at its sites of action in the CNS. We have already established that polyclonal antibodies from our active vaccine increase blood concentration while decreasing brain concentration of drug compared to naive mice;⁴³ thus, we anticipated that our lead fentanyl mAb 6A4 would extend drug half-life and increase drug sequestration in the blood, thereby reducing peripheral distribution. We examined the i.v. pharmacokinetics of 50 mg/kg 6A4, 0.1 mg/kg fentanyl, and 0.025 mg/kg carfentanil alone and each opioid in combination with 6A4 with the goal of building pharmacokinetic–pharmacodynamic–efficacy (PK–PD–E) models (Figure 4A–F). A PK–PD interaction model is presented in Figure 4A where α represents the 6A4 antibody and D represents the opioid fentanyl or carfentanil. The i.v. PK profiles of the two opioids and 6A4 when singly dosed were examined and found consistent with a two compartment PK model (Figure 4A–D). On the time scale of the PK study, fentanyl and carfentanil rapidly distribute out of the plasma giving initial volumes of distribution of 33 and 24 mL respectively (Table S9). In contrast, 6A4 is initially restricted to the volume of plasma but slowly distributes to a peripheral compartment, presumably the lymph (Figure 4B). In order to mathematically model antibody–opioid interactions we must consider the plasma-based association and dissociation between the opioid and antibody. In addition, as the opioid binds to 6A4 in the plasma by the laws of mass action, the opioid redistributes back from peripheral compartments to the plasma. To mathematically capture these interactions we included a rapidly exchanging third compartment for the opioids. After obtaining the optimized microscopic PK parameters of each agent when examined alone, we then modeled the observed plasma PK of 0.1 mg/kg fentanyl or 0.025 mg/kg carfentanil when 50 mg/kg 6A4 was predosed 30 min prior to opioid injection. When obtaining fits to the combined antibody and opioid doses, the microscopic PK parameters were fixed to the values of the single agent studies while interaction parameters k_{on} and k_{off} were optimized to model the total plasma concentrations of the opioids as a function of time (Table S10).

As seen in Figure 4E,F, the observed plasma enrichment of fentanyl and carfentanil are well described by the PK–PD interaction model. The increases in plasma exposure to the opioids are linked to the microscopic association and dissociation rate constants of 6A4 for fentanyl and carfentanil. In the presence of 6A4, fentanyl plasma exposure increases by 700-fold while carfentanil exposure is increased by a more modest 34-fold (Figure 4, Table S9); this is reflected in the apparent in vivo binding constants for the two opioids whose best fit values are 2.6 and 500 nM respectively (Figure S10). This result is not unexpected as this pharmacokinetic phenomenon has also been observed in animals immunized with opioid conjugate vaccines targeting heroin, fentanyl, and oxycodone/hydrocodone.^{43,64,65} By modeling the PK–PD interactions we have an indirect view of the reduction in opioid exposure in the peripheral compartments, which includes the opioid site of action. From the

PK–PD model, predosed 50 mg/kg 6A4 reduced the peripheral exposure to 0.1 mg/kg fentanyl over the first hour by 50% compared to the untreated condition (Figure 4G).

The marked alteration of fentanyl pharmacokinetics relative to carfentanil in the presence of 6A4 was anticipated due to the fact that 6A4 displayed a 40-fold higher in vitro affinity to fentanyl versus carfentanil (Table 2). Moreover, the tighter binding of mAbs to fentanyl likely prevented cytochrome p450 (CYP)-mediated piperidine *N*-dealkylation to the primary metabolite norfentanyl,⁶⁶ leading to a 43-fold increase in fentanyl half-life (Table S9). The metabolism of carfentanil follows the same dealkylation pathway,⁶⁷ yet the lesser 6A4 affinity for carfentanil likely resulted in reduced shielding of carfentanil from metabolism, and only a 2-fold increase in half-life was observed (Table S9). Overall, the intravenous half-lives of fentanyl and carfentanil in mice were relatively short (~15 min) (Figure 4C,D, Table S9), but in humans the half-lives of these drugs range from 1 to 6 h or longer depending on a variety of factors such as route of administration and subject age.^{68–71} It should be noted that while 6A4 prolonged drug half-life, antidrug mAbs in general do not prolong CNS-mediated drug effects because they sequester the majority of the drug in plasma. Although a small fraction of the drug dose remains unbound in equilibrium with mAb-bound drug, this fraction is not large enough to induce observable CNS effects and is subjected to normal drug metabolism. In comparing in vitro vs in vivo outcomes, our SPR binding assay results appear to predict mAb in vivo drug binding behavior; however, we note the importance of conducting animal experiments, which can identify antibodies with unexpectedly poor in vivo stability and/or drug binding performance.⁴⁷ In evaluating the 6A4 concentration time course in mice, we observed a rapid distribution phase within the first 24 h post-i.v. infusion and a much slower elimination phase in which the antibody half-life was determined to be ~6 days (Figure 4B, Table S9). Although the 6A4 half-life is relatively short compared to some clinically used mAbs, it is similar to other antidrug mAbs tested in rodents.^{47,60,72,73}

Antinociception Testing.

Nociceptive pain is mediated by the central nervous system; therefore, hot plate and tail flick pain response tests serve as relevant behavioral models for assessing mAb-mediated antagonism of fentanyl's CNS effects. Hot plate is a measurement of supraspinally mediated nociception, while tail flick is a measurement of spinally mediated nociception. Specifically, fentanyl and related analogues activate MOR, resulting in antinociception, euphoria, and respiratory depression. For this reason, antinociception assays are often the initial screen for therapeutics that modulate psychoactivity at MOR.^{74–79} In this assay, animals are cumulatively dosed with drug and their response to thermal pain is repeatedly assessed until drug-induced antinociception reaches a predetermined cutoff point to prevent tissue damage, equivalent to 100% maximum possible effect (MPE). Thus, the assay allows for the generation of full dose-response curves in the presence and absence of treatment, revealing a rightward curve shift if a treatment is lowering drug potency. Quantitatively, this shift is represented by a ratio of treated to untreated effective dose 50 (ED₅₀) values, also known as the potency ratio. We were interested in determining the efficacy of the 6A4 mAb for attenuating fentanyl and carfentanil potency, so each drug was examined with three doses of antibody: 30, 60, and 120 mg/kg. Results are shown in Figure 5 for each test with ED₅₀ values on top (A,B) and potency ratios on bottom (C,D). All three doses of 6A4 were

effective against fentanyl in the hot plate and tail flick antinociception tests based on a one-way ANOVA ($p < 0.001$). In contrast, only the two highest doses of antibody were effective at protecting against carfentanil in hot plate antinociception ($p < 0.001$), and only the highest dose was effective in the tail flick test. These behavioral results match our binding and PK data, albeit with a lowered degree of mAb-mediated opioid antagonism against carfentanil. Despite the undeniable challenges of carfentanil's MOR affinity, the 6A4 mAb was able to blunt carfentanil's antinociception effects. Moreover, this is the first demonstration of a biologic able to alter the behavioral effects of this synthetic opioid.

Rescue Antinociception Testing.

An important application of our fentanyl mAbs is the reversal of opioid overdose. While naloxone is FDA-approved for this purpose there are certain drawbacks including a short duration of action, inability to treat prophylactically, and reduced efficacy against the most potent of opioids, especially considering the challenge of treating carfentanil "renarcotization". Although our antinociception experiments in Figure 5 established mAb efficacy, they do not represent a particularly realistic treatment scenario, that is, mAb is administered prior to drug. In an attempt to parallel a more clinical-relevant scenario, we administered mAb after a fully antinociceptive opioid dose (approximately 2 times the ED₅₀ dose) to assess the mAb's ability to rescue mice from fentanyl/carfentanil effects. To perform this procedure, baseline nociception responses were measured, carfentanil or fentanyl was administered, and nociception responses were measured again at the approximate $t_{\max}$ (i.e., 15 min post i.p. drug administration). As shown in Figure 6, the administered opioid doses induced 100% MPE in the mice. At 30 min, rescue treatment was intravenously administered consisting of either 6A4 mAb or naloxone, and nociception responses were monitored for two additional hours.

Notably, rescue of both fentanyl and carfentanil effects was achieved not only with naloxone, but also with the 6A4 mAb as early as 30 min post-treatment and is evidenced by the marked decrease in the animals' latency to nociception toward baseline levels (Figure 6). While naloxone produced better efficacy compared to the mAb at the earliest time point (60 min) due to its relatively rapid biodistribution as a lipophilic small-molecule, still 6A4 was able to achieve significance versus control in 3 out of the 4 tests (Figure 6). More importantly, 6A4 appeared to outperform naloxone at the later time points, most likely because of its much longer half-life. This was particularly pronounced at the latest time point in the carfentanil tail flick test when naloxone failed to achieve significance vs control while the mAb remained effective. The loss in naloxone efficacy at the later time point could be indicative of renarcotization in which naloxone is metabolized beyond what is required for effective antagonism of opioid effects; however, the drawback of this experiment is that renarcotization cannot actually be observed in mice due to rapid rodent metabolism of fentanyl/carfentanil compared to humans and large animals. It should be noted that hot-plate antinociception in control animals returned to baseline more rapidly than tail-flick likely because of differences in drug pharmacokinetics in the spinal cord vs the brain, which primarily mediate the tail flick and hot plate nociceptive responses, respectively.

To our knowledge, this experiment is the first demonstration that an antidrug antibody can actually reverse opioid effects, in contrast to previous studies on opioid vaccines, which require prophylactic immunization in order to mitigate opioid effects. Interestingly, since the mAbs cannot cross the blood brain barrier, they must act by shifting the biodistribution of fentanyl/carfentanil away from the CNS and into peripheral blood where the opioids can no longer activate MOR. This unique mechanism of action differs from traditional antagonists such as naloxone, which rapidly enter the brain and directly compete with opioids for MOR binding. One drawback of this experiment is that antinociception is not directly representative of the intoxicating effects of fentanyl and carfentanil. A better model for carfentanil intoxication is observing respiratory depression via plethysmography and has been used to study fentanyl vaccines and nalmefene.^{42,80} While future studies investigating anti-synthetic opioid mAbs should employ plethysmography, the antinociception model is at least adequate to establish proof-of-principle that mAbs can reverse opioid intoxication. This is because the anesthetic and analgesic effects of synthetic opioids occur simultaneously and at similar doses, as is the case for most MOR agonists.^{81,82}

Mouse Lethality Challenge.

Given that the 6A4 mAb mitigated opioid effects at sublethal doses in PK and behavioral assays, we advanced 6A4 to testing against >10-fold greater fentanyl doses to observe the potential of the mAb to protect mice from fentanyl-induced lethality. Mice were dosed with either 3 mg/kg or 6 mg/kg of fentanyl that had been prophylactically given 0, 30, 60, or 120 mg/kg 6A4 prior to the fentanyl dose. At 3 mg/kg, on a per mole basis, fentanyl is in 44, 22 and 11-fold excess of the 6A4 doses, respectively. Respiratory depression is the driver of lethality and occurs nearly instantaneously upon fentanyl injection. In our experiment, most animals lost consciousness and died within 5 min as depicted by the survival curves (Figure 7, Table S12). At 3 mg/kg fentanyl, 66% of the untreated mice died in under 3 min indicating that mice have an LD₅₀ for fentanyl close to 3 mg/kg. Notably, 6A4 produced a dose-dependent enhancement of survival, at 30 mg/kg 6A4 prolonged the time until death of the mice while 60 mg/kg 6A4 increased overall survival to 66%. At 120 mg/kg 6A4, all mice survived the 3 mg/kg fentanyl dose; thus, even when a dose of fentanyl exceeds the total binding capacity of the mAb present, there is sufficient plasma sequestration at the 120 mg/kg 6A4 dose to prevent lethal respiratory failure (Figure 7A). The 6 mg/kg dose of fentanyl was lethal to all untreated mice, which died within 30 s. The 30, 60, and 120 mg/kg doses of 6A4 prolonged survival times dose-dependently while one-fourth of the 120 mg/kg dose cohort survived the 6 mg/kg fentanyl dose (Figure 7B). By the laws of mass action, antibody-opioid sequestration is most effective at lower opioid concentrations since at higher opioid doses the antibody becomes over-whelmed, however, our results indicate that an antibody with a K_d similar to 6A4 can be administered at lower doses and still blunt the opioid effects. At higher opioid doses, the principles are the same but the antibody must scavenge more opioid, i.e., a higher dose of antibody is required to have the same efficacy against the opioid.

Modeled Translation to Human Opioid-Induced Respiratory Depression.

Rodents are considerably less sensitive to fentanyl induced respiratory depression than primates as reflected in the fentanyl LD₅₀ differences reported for rats and monkeys which

are 3 mg/kg and 0.03 mg/kg respectively.^{83,84} From the respiratory depression studies in healthy volunteers, humans are likely to have a fentanyl LD₅₀ comparable to monkeys.⁸⁵ By pooling the study data in reports of human fentanyl PK and fentanyl respiratory depression studies we built a human fentanyl PK–PD model of respiratory depression. One study (Harper et al.)⁸⁵ examined fentanyl doses of 0.09 mg to 0.54 mg and reported respiratory activity from 2 min to 4 h. By combining the 0.17 mg human fentanyl PK study of Bower and Hull⁸⁶ with the respiratory suppression study of Harper et al., we calculated the plasma concentrations at each dose and respiratory time point (Figure 8A). We plotted respiratory rate as a function of plasma concentration and determined that respiratory depression is a direct function of fentanyl plasma concentration having an EC₅₀ of 7.8 ± 0.8 nM (Figure 8B). To model the pharmacokinetics of a human anti-fentanyl antibody, we used a general IgG antibody PK profile.⁸⁷ Next, we constructed the human version of the antibody opioid PK–PD model and then conducted a sweep of perspective k_{on} and k_{off} rate constants to identify a minimum criterion that would allow a candidate antibody to rescue respiratory depression at a reasonable dose (Table S11). Our analysis indicates that to be effective at a 500 mg dose or lower, the antibody should have an association rate constant (k_{on}) of at least $1 \text{ nM}^{-1} \text{ h}^{-1}$ and a dissociation rate constant (k_{off}) of no more than 0.7 h^{-1} . Since, on the time scale of PK, respiratory depression is in rapid equilibrium with plasma fentanyl concentration, it is essential that the antibody rapidly sequesters fentanyl. Our model predicts that candidate antibodies with tighter overall binding, which have maximized k_{off} but possess slower k_{on} , will be less effective at retarding the rapid onset of respiratory depression. To illustrate, a simulation in Figure 8C presents the predicted degree of respiratory suppression that will occur following a 3 mg i.v. fentanyl dose in humans with or without a 500 mg dose of an anti-fentanyl antibody with a k_{on} of $1.1 \text{ nM}^{-1} \text{ s}^{-1}$ and a k_{off} of 0.73 h^{-1} given 24 h prior to the fentanyl challenge. For a 60 kg human, a 3 mg fentanyl dose is at 0.05 mg/kg which exceeds the monkey LD₅₀ of 0.03 mg/kg. An antibody possessing these binding kinetics blocks most of the respiratory depression that a potentially fatal dose of fentanyl would produce (Figure 8C).

Our modeling efforts predict a distinct link between antifentanyl mAb binding kinetics and mAb efficacy in mitigating opioid-induced respiratory depression in humans; thus, development of future human anti-opioid antibodies should include an ex vivo assessment of opioid binding kinetics (preferably in plasma at 37 °C), similar to our current work in developing a murine anti-fentanyl mAb. The candidate antibodies could then be ranked in mice to validate the link between inhibition of respiratory depression and the optimized association and dissociation rate constants with the selected candidate antibody. Further examination in monkeys would enhance translatability to humans given similar fentanyl interspecies sensitivity and much greater opioid sensitivity compared to rodents.

Cost-effectiveness of using mAbs in the clinic for opioid overdose reversal and prevention could be a problem given that small-molecule antagonists are cheaper to manufacture. In terms of the required antibody dose, our modeling predictions suggest that 500 mg antibody could theoretically be effective against a typical LD₅₀ fentanyl dose. Comparing this dose to typical mAb doses used for cancer immunotherapy shows that 500 mg falls within the typical range for oncology mAbs (200–1000 mg),⁸⁸ yet anticancer antibodies require multiple weekly doses to achieve efficacy, while just one anti-opioid mAb infusion would be

immediately effective. Taken together, we expect the cost of anti-opioid immunotherapy to be more expensive than antagonists but less expensive than cancer immunotherapy.

CONCLUSIONS

In this work we disclose the first report of a monoclonal antibody that can mitigate the effects of fentanyl and carfentanil in mouse models. The six mAbs described were generated from rodent immunization with a fentanyl conjugate vaccine and selected using an SPR-based competitive binding assay. Characterization of the six mAbs using a direct SPR binding method revealed very high affinity of the antibodies for fentanyl and carfentanil in the 10^{-11} M range, and this data led us to choose 6A4 as our lead mAb. In basic behavioral tests for opioid psychoactivity, the 6A4 mAb attenuated the anti-nociceptive effects of both fentanyl and carfentanil in a doseresponsive manner. Furthermore, 6A4 reversed preexisting fentanyl/carfentanil antinociception and was comparable in efficacy to naloxone. Our results suggest that monoclonal antibodies could be useful in humans for combatting opioid overdose. Since there is no expected interaction between naloxone and 6A4, there is potential for the two strategies to be combined with naloxone as a fast-acting component and a monoclonal antibody as a long acting component. In fact, polyclonal antibodies elicited by a fentanyl vaccine did not interfere with naloxone-mediated antagonism of fentanyl effects.⁸⁰ While it remains unclear how clinically effective mAbs can be compared to antagonists for overdose reversal, our lethality experiment and modeling predictions strongly support the efficacy of mAbs for providing prophylactic anti-opioid protection. Once dosed, a typical antibody remains present at efficacious concentrations several weeks later opening the potential for its use as an out-patient therapy against overdose or to protect first responders reporting to an illicit fentanyl drug site. In the case of opioid overdose reversal, one might envision immediate intranasal administration of naloxone by paramedics followed by intravenous antibody administration in the emergency room. Although cost-effectiveness is a potential problem for using mAbs against opioid intoxication, our modeling predicts that a one-time 500 mg dose would be effective against an LD₅₀ fentanyl dose; thus, anti-opioid immunotherapy should be no more expensive than cancer immunotherapy.

An important feature of antidrug antibodies is their unique mechanism of action in redistributing drug away from the CNS without direct modulation of MOR, allowing for the administration of adjunct pharmacotherapy such as buprenorphine to relieve opioid cravings during drug detoxification. Because they are cross-reactive only to the fentanyl class of compounds without antagonism of the MOR, our antibodies would also be useful in combating designer fentanyl analogues, such as acetyl fentanyl, while still enabling the use of prescription opioids like oxycodone and hydrocodone. Using the antibody development platform established in this work, we envision the implementation of this platform to generate antibodies to other dangerous designer drugs or existing drugs of abuse. The application of our methodology to humanized rodent models combined with fluorescence activated cell sorting (FACS) could enable an efficient pipeline for generating fully human antibodies that could be rapidly advanced to the clinic. Furthermore, our modeling work suggests that in vitro screening for optimal antibody-drug binding kinetics and performing in vivo PK experiments would be highly translational in producing an effective anti-opioid mAb therapeutic.

Following our demonstration of the 6A4 mAb in preventing and reversing the acute antinociceptive and lethal effects of fentanyl/carfentanil, future preclinical studies should also investigate the predicted ability of the mAb for reducing drug-seeking behavior in drug self-administration models: we anticipate the mAb could be useful not only for overdose reversal but also for treating substance use disorder. While extended mAb administration is fairly impractical, short-term mAb treatment during the initial immunization period of an opioid vaccine (designed for long-term efficacy) would be useful in helping to achieve or maintain drug abstinence during the initial period when vaccine-mediated immunity has not fully matured. This treatment strategy of combined mAb + active vaccine has already been explored in preclinical models for nicotine and methamphetamine dependence.^{89,90} Overall, immunotherapeutic options are only now being realized for their potential in treating opioid abuse and overdose, and our latest advancement in developing an anti-fentanyl mAb further supports the utility of an antibody-based approach in combatting the current opioid crisis.

MATERIALS AND METHODS

Animals.

All studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee and all protocols adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Mice were group-housed in an AAALAC-accredited vivarium containing temperature and humidity-controlled rooms and kept on a reverse light cycle (lights on: 9PM to 9AM). All behavioral procedures were performed during the dark phase.

Vaccines, Immunizations, and Hybridoma Generation.

The fentanyl-BSA and KLH conjugates were synthesized according to our literature procedure,⁴³ and afforded hapten to protein ratios of 15:1 according to MALDI-TOF mass analysis of the fentanyl-BSA conjugate (Figure S1). Following hapten-protein coupling, conjugates were diluted to 25% (v/v) with glycerol to give a final protein concentration of 1.3 mg/mL and stored at -80 °C. On the day of vaccination, conjugates (166 µg/dose) were thawed and formulated with CpG ODN 1826 (62 µg/dose) and 1:1 v/v Inject alum. Female A/J mice were immunized i.p. on weeks 0, 3, and 8 and bled on weeks 4 and 9. A final i.v. infusion of conjugate was performed on week 13. Three days later, one animal was sacrificed, and its spleen was extracted, homogenized and splenocytes were washed with RPMI medium. The resulting B-cells were fused with X63Ag8.653 nonproducing myeloma cells using PEG 1500 and selected by culturing in HAT medium. Of the plated hybridomas, 41 were positive for hapten binding by ELISA, 6 were subcloned once (2F12, 3H2, 6A4, 7A8, 12A1, 15F4) and 2 were subcloned twice (6A4, 7A8). All monoclonal antibodies were isotyped (Table S2).

Antibody Production and Purification.

Murine hybridoma cells were cultured at 37 °C in a humidified incubator with 5% CO₂ saturation. For antibody production, media consisted of RPMI 1640 supplemented with Gentamicin, HEPES, nonessential amino acids, sodium pyruvate and 8% ultralow IgG FBS (all from Gibco.) Cell culture supernatant was purified using GammaBind G (GE

Healthcare) as per manufacturer instructions. Briefly, samples were loaded onto a GammaBind G column then washed using 0.15 M NaCl in 10 mM sodium phosphate, pH 7.0. Antibodies were eluted using 0.5 M acetic acid, pH 3.0, and the acid neutralized with 1 M Tris base, pH 9.0, followed by dialysis of the antibody solution against PBS, pH 7.4. As needed, antibodies were concentrated using Amicon Ultra-15 centrifugal filters (Millipore), typically to about 30 mg/mL.

ELISA.

Corning 3690 half area, 96-well microtiter plates were coated with 33 ng fentanyl-like-BSA antigen in PBS per well then dried overnight at 37 °C. After blocking in 5% skim milk, six serial dilutions of each serum sample were run alongside 12 serial dilutions of 6A4 antibody (100–0.05 ng/mL) as a standard curve. Serum samples and 6A4 antibody were both diluted in 2% BSA in PBS as was the secondary antibody. After a 2 h incubation at 37 °C in a moist chamber, plates were washed 10 times with water, and donkey antimouse HRP secondary antibody (Jackson ImmunoResearch) was added and incubated for 1 h as described above. After washing 10 times with water, TMB (Thermo Fisher) was added and plates were stopped using 2 M H₂SO₄ and then read at 450 nm. Data were analyzed using GraphPad PRISM 6 using a nonlinear Michaelis–Menten fit of the 6A4 standards to interpret unknown serum concentrations of 6A4.

For initial screening of mouse serum and hybridoma supernatants, a similar procedure was used for end point titer determinations. For isotyping, goat antimouse antibodies recognizing kappa/lambda LC or gamma 1/2a/2b HC were used for plate coating.

SPR.

IC₅₀ Ranking for Hybridoma Culture Supernatant.—The binding IC₅₀ for 41 mouse hybridoma culture supernatants and free fentanyl or fentanyl derivatives was determined by competitive binding assay by surface plasmon resonance (SPR) using a Biacore 3000 instrument (GE Healthcare Life Sciences) equipped with a research-grade CM5 sensor chip. The ligand, fentanyl-BSA conjugate, was immobilized onto the chip surface using Amine Coupling Kit (BR-1000–50, GE Healthcare Life Sciences) as follows: (1) All four flow cells were activated for 7 min with a 1:1 mixture of 0.1 M NHS and 0.4 M EDC at a flow rate of 10 µL/min. (2) The fentanyl-BSA conjugate resuspended in 10 mM sodium acetate (pH 4.0) was immobilized at a density of 2500 RU on flow cell 2 (Fc2), whereas flow cell 1 (Fc1) was immobilized with BSA (resuspended in 10 mM sodium acetate, pH 4.0) at a similar density to serve as reference surface. (3) All surfaces were blocked with 7 min injection of 1.0 M ethanolamine-HCl (pH 8.5). All assays were conducted at a flow rate of 30 µL/min at 25 °C, using 1×HBS-EP+buffer (BR-1006–69, GE Healthcare Life Sciences) as running buffer. For initial IC₅₀ ranking, each hybridoma culture supernatant was 1:100 diluted in running buffer and the diluted sample was incubated with corresponding free compound at various concentrations (see Table S6) at room temperature for 30 min before injection. For each analysis cycle, one preincubated sample was injected for 300 s over all flow cells, followed by 150 s of dissociation in running buffer. The relative response at the end of dissociation (reference flow cell subtracted, i.e., Fc2 – Fc1 for fentanyl-BSA) was recorded in the sensogram and was used for initial binding evaluation and IC₅₀ ranking. After each

sample analysis, all flow cells were regenerated with 30 s injection of 10 mM Gly-HCl, pH 1.5, before the next cycle of analysis. The IC₅₀ value for each hybridoma-compound pair was estimated and scored from the relative responses with various concentration of compound against response from no compound control (see spreadsheet in Supporting Information). On the basis of these ranked IC₅₀ values, six hybridomas, including 2F12, 3H2, 6A4, 7A8, 12A1, and 15F4, were selected for subcloning, expanding, mAb production, and further binding characterizations.

Fine IC₅₀ Determination.—To determine more accurate IC₅₀s for selected mAbs, a competitive SPR assay with 12-point fentanyl or carfentanil dilutions were conducted. The experimental setup was similar as described in IC₅₀ ranking section, except the concentration series used was listed in Table S7. The data were analyzed with PRISM 6, and the IC₅₀ value for each mAb-compound pair was derived from a nonlinear fit of the competitive binding curve in PRISM 6.

Determination of Binding Kinetics for Selected mAbs by Biacore 8K (6 mAbs and 9 Compounds).—The binding kinetics for selected mAbs and fentanyl and its derivatives were determined by SPR using a Biacore 8K instrument (GE Healthcare Life Sciences) equipped with a Series S CM5 sensor chip. Six selected mAbs were immobilized into individual channels on a sensor chip surface using Amine Coupling Kit as follows: (1) The flow cell 2 surface of each channel was activated for 7 min with a 1:1 mixture of 0.1 M NHS and 0.4 M EDC at a flow rate of 10 μ L/min, whereas flow cell 1 of each channel was not activated. (2) Selected mAbs resuspended in 10 mM sodium acetate (pH 5.5), except 12A1 in 10 mM sodium acetate (pH 4.5), were injected over activated Fc2 for 120 s at 10 μ L/min in each channel separately. (3) All flow cell surfaces were blocked with a 7 min injection of 1.0 M ethanolamine-HCl (pH 8.5) at a flow rate of 10 μ L/min. All assays were conducted at a flow rate of 30 μ L/min at 25 °C, using 1 $\times$ PBS-P+ buffer (28–9950-84, GE Healthcare Life Sciences) as running buffer. To determine the binding kinetics, we used a predefined single-cycle kinetics method (SCK, Biacore 8K control software ver. 1.1.1.7442) with four analyte concentrations as follows: (1) 4 $\times$ startup cycles (each cycle includes 300 s of running buffer injection and 600 s of dissociation, all at a flow rate of 30 μ L/min, the chip surface was regenerated with Gly-HCl (pH 1.5) for 30 s) were conducted before SCK analysis. (2) For SCK analysis, each compound was prepared in running buffer as shown in Table S8. The compound with various concentrations was injected for 120 s consecutively followed by 10 800 s of dissociation in running buffer. (3) The sensor chip surface was regenerated with 30 s of injection of Gly-HCl (pH 1.5) solution before next cycle of SCK analysis. A blank running buffer injection was also conducted before each compound run using exactly the same conditions for SCK analysis. All data were collected by Biacore control software in a result file. The run data sets stored in the result file were then analyzed by Biacore 8K evaluation software (ver. 1.1.1.7442) using a predefined fragment/LWM single-cycle kinetics method. Each SCK analysis data set was double referenced with signal from reference Fc1 and blank running buffer injection and was fitted using a 1:1 binding model.

Drugs.

Fentanyl and methylated analogues were provided by NIDA Drug Supply as solids. Opioids for cross-reactivity screening and deuterated drug standards were obtained from Cerilliant as 0.5 or 1 mg/mL solutions in organic solvent. Carfentanil was obtained from Cayman Chemical as a 0.1 or 1 mg/mL solution in methanol. For animal experiments, freebase fentanyl was dissolved in saline containing 10% v/v dimethylsulfoxide and 10% v/v Tween 80. Freebase carfentanil was directly diluted into saline for injection into animals, and 6A4 mAb was administered in a pH 7.4 PBS vehicle. The volume per injection of drugs and mAb was typically 5 mL/kg.

In Vivo Pharmacokinetics.

Male Swiss Webster mice ($n = 6$ per group) were intravenously administered via retroorbital injection fentanyl, carfentanil, 6A4 antibody, or a combination of either drug + 6A4 mAb. For the combination, mAb was administered 30 min prior to drug. Retroorbital bleeds were taken from three animals at each time point, alternating the other three animals every other time point.

PK–PD Modeling.

Noncompartmental analysis was conducted in Microsoft Excel 16.24 using standard equations.⁹¹ PK-PD modeling was conducted in Berkeley Madonna 9.1.0 using the Rosenbrock Stiff numerical integrator. Opioid PK was initially evaluated as a two compartment model which produced initial volumes of distribution in excess of plasma. Since the anti-opioid antibody is restricted to plasma and in order to capture the binding and plasma sequestration of the opioid, a third compartment in rapid equilibrium with the central compartment and described by k_{13} and k_{31} was added where the ratio k_{13}/k_{31} is equal to $V_{(\text{opioid})}/V_{(\text{antibody})}$, i.e., the initial volume of distribution of the opioid divided by the initial volume of distribution of the antibody. To achieve rapid equilibrium the value of k_{13} was assigned an arbitrarily large value relative to the values of k_{12} and k_{10} . Next, the best fit values of k_{10} and k_{12} from the original opioid 2 compartment model were increased by multiplying them by $V_{(\text{opioid})}/V_{(\text{antibody})}$. The new 3 compartment opioid model reproduced the originally modeled plasma PK curve reported previously in humans.⁸⁶ Mouse 6A4 pharmacokinetics were evaluated using a two compartment model. Human “generic” antibody pharmacokinetics were taken from Dirks and Meibohm 2010.⁸⁷ When evaluating combined opioid antibody studies, the microscopic PK parameters were locked to the single agent values, but two new parameters, k_{on} and were introduced and adjusted to find best fit values that described the observed total plasma opioid concentration. The model did not account for opioid plasma protein binding, and therefore k_{on} is an apparent value. The following equation could be used to correct for opioid plasma protein binding: $k_{\text{on}(\text{corrected})} = k_{\text{on}}/\text{fraction unbound opioid}$. The peripheral compartment of antibody PK is presumably the lymph. Binding and release of opioid can also occur in this compartment but the opioid concentration in this compartment is unknown; thus, the model was simplified to assume opioid binding and release only occur in the central compartment (plasma). As well, opioid bound antibody is assumed to have identical PK to free antibody.

Retroanalysis of Human Respiratory Depression.

Human respiratory depression study data as a function of fentanyl i.v. dose and time were taken from a previously reported study.⁸⁵ The fentanyl plasma concentrations corresponding to each time and dose respiratory depression datum was computed using data from a human fentanyl PK study⁸⁶ by assuming PK dose linearity over the dose range examined. Next, study results of respiratory depression were sorted and then plotted by plasma fentanyl concentration. The following response equation was fit to the study data:

$$\text{respiratory rate} = \frac{\text{undosed rate}}{1 + \frac{[\text{fentanyl}]_{\text{plasma}}}{\text{EC}_{50}}}$$

Simulations of Human Respiratory Depression.

Plasma fentanyl concentration is no longer predictive of respiratory depression upon antibody sequestration of fentanyl. Instead, moles of fentanyl in the rapid exchange compartment were used to calculate respiratory rate with an apparent respiratory depression EC_{50} of 108 nmol.

Fentanyl/Carfentanil MS Sample Preparation.

Frozen mouse blood samples were thawed on ice, and 60 μL blood was pipetted into a new tube along with 8 μL of 50 ng/mL fentanyl or carfentanil-d5 in MeOH. After vortexing, 120 μL of 50 mM K_2CO_3 solution and 420 μL of 7:3 hexane/ethyl acetate were added. The samples were vortexed for 15 s and centrifuged at 3000 rpm for 5 min. The top solvent layers were pipetted into new tubes and evaporated by Genevac for 1 h. The resulting residues were dissolved in 68 μL MeOH and analyzed on an LC-MS/MS instrument (see below). Standards were prepared in the same manner by extracting blood spiked with fentanyl or carfentanil ranging in concentration from 5000 ng/mL to 1.6 ng/mL. The limit of detection was determined to be around 1.6 ng/mL.

LC-MS/MS Instrumentation.

All samples were run on a Waters Xevo TQ-XS triple quad instrument.

For carfentanil, m/z 395.1 $\rightarrow$ 335.12 was the quantitative transition state and m/z 395.1 $\rightarrow$ 112.9 the qualitative transition. For the d5 internal standard, m/z 400.15 $\rightarrow$ 340 was used.

Carfentanil MS parameters:

- 395.1 $\rightarrow$ 335.12, cone voltage (CV) = 62 V, collision energy (CE) = 16 V
- 395.1 $\rightarrow$ 112.9, CV = 62 V, CE = 26 V
- 400.15 $\rightarrow$ 340, CV = 40 V, CE = 16 V

For fentanyl, 337.1 $\rightarrow$ 187.99 was the quantitative transition state, and m/z 337.1 $\rightarrow$ 104.91 was the qualitative transition. For the d5 internal standard, m/z 342.1 $\rightarrow$ 188 was used.

Fentanyl MS parameters:

- 337.1 → 187.99, CV = 78 V, CE = 18 V
- 337.1 → 104.91, CV = 78 V, CE = 28 V
- 342.1 → 188, CV = 16 V, CE = 24 V

LC conditions were the same for all compounds. The column was a Waters 21 × 50 mm BEH C18, UPLC column with a 1.7 μm particle size. For the mobile phase, water/0.1% formic (A) and acetonitrile/0.1% formic (B) were used. Flow rate was 0.3 mL/min and 5 μL sample was injected. The LC method was as follows:

- $T = 0$ min, 90:10 (A:B)
- $T = 5$ min, 5:96 (A:B)
- $T = 8$ min, 5:95 (A:B)
- $T = 8.5$ min, 90:10 (A:B)
- $T = 12$ min, stop.

Antinociception.

Male Swiss Webster mice ($n = 8$ per group) were tested for cumulative drug response in primarily supraplinal (hot plate) and spinal (tail flick) behavioral tests as previously described.⁹² In the hot plate test, the mouse was observed in an acrylic cylinder (14 cm diameter × 22 cm) on a 55 °C surface. The latency to perform one of the following nociceptive responses: licking of hind paw, shaking/withdrawal of hind paw or jumping, was timed with a 35 s cutoff to prevent tissue damage. The tail immersion test was performed using an IITC Life Science Tail Flick Analgesia Meter to measure time of tail withdrawal from a heated light beam (45% active intensity) with a cutoff of 10 s to prevent tissue damage. Since tail flick is a reflexive behavior, hot plate was always performed first. Immediately following both antinociceptive assays, fentanyl or carfentanil was injected intraperitoneally. The fentanyl doses tested were 0.2, 0.4, 0.6, and 0.8 mg/kg and the carfentanil doses tested were 0.01, 0.02, 0.04, 0.06, and 0.08 mg/kg to generate a full dose-response curve. For the control, an extra set of ($n = 4$) mice received smaller doses (0.05, 0.1, 0.15, 0.2 mg/kg fentanyl and 0.005, 0.01, 0.015, and 0.02 mg/kg for carfentanil) to more accurately assess the ED₅₀. Testing for all animals was repeated in 15 min intervals, following each injection with increase cumulative dosing until full antinociception (cut off times surpassed) was observed in both assays. Percent maximum possible effect (%MPE) was calculated from time by the equation:

$$\%MPE = \frac{(\text{test} - \text{baseline})}{(\text{cutoff} - \text{baseline})} \times 100$$

The resulting % MPE versus log(dose) curve was fit using a log(agonist) vs normalized response nonlinear regression in Graph-Pad PRISM 6. The ED₅₀ bars in Figure 5 are represented as the mean ± SEM and were determined for each antinociception test and individual treatment groups.

Rescue Antinociception.

Male Swiss Webster mice ($n = 6$ per group) were measured for baseline response in two nociception tests, hot plate and tail flick, as described above. After 15 min, mice were administered either carfentanil (0.01 mg/kg) or fentanyl (0.2 mg/kg) intraperitoneally to induce full antinociception, and the hot plate and tail flick responses were measured 15 min post drug administration. At 30 min post drug administration mice were administered either 6A4 mAb (120 mg/kg) or naloxone (10 mg/kg) intravenously via retroorbital injection. In the fentanyl group, nociception was measured again 60, 90, and 120 min post drug administration. In the carfentanil group, nociception was measured again 60, 90, and 150 min post drug administration. In Figure 6, points represent the mean $\pm$ SEM.

Lethal Fentanyl Challenge.

Male Swiss Webster mice ($n = 4$ per group) were administered a retroorbital i.v. injection of 0, 30, 60, and 120 mg/kg 6A4 mAb. After 3.5 and 4.5 h, 6 mg/kg and 3 mg/kg fentanyl, respectively, were given to the mice via retroorbital i.v. injection, and the mice were continuously monitored for 1 h. The time to death was recorded when both loss of breathing and reflex was observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structures and approximate relative potencies of morphine, heroin, fentanyl, and carfentanil.

Figure 2.

Illustration of the research strategy and timeline. Fentanyl hapten was conjugated to carrier protein keyhole limpet hemocyanin (KLH), then formulated with CpG oligodeoxynucleotide (ODN) 1826 and Alhydrogel (alum) to form the fentanyl vaccine. Immunization of mice proceeded according to the timeline shown. Serum IC₅₀ values for fentanyl are shown at weeks 4 and 9. Following spleen harvest, isolated splenocytes were fused with myeloma cells, and resulting hybridomas were screened for binding against a panel of fentanyl analogues. Final purified mAbs were evaluated in mice for fentanyl and carfentanil pharmacokinetics, antinociception, and overdose lethality.

Figure 3.

Competitive SPR binding curves for 6 selected mAbs against (A) fentanyl and (B) carfentanil. Resulting IC₅₀ values are shown in panel (C). IC₅₀ values were determined using purified antibodies and are expressed as mean ± SEM. Fields marked with n.a. indicate an IC₅₀ > 1000 nM.

Figure 4. PK-PD modeling of fentanyl and carfentanil in the presence and absence of 6A4 mAb. (A) Diagram of PK-PD model. Plasma pharmacokinetics of a (B) 50 mg/kg 6A4 dose, (C) 0.1 mg/kg fentanyl dose, (D) 0.025 mg/kg carfentanil dose, (E) 0.1 mg/kg fentanyl dose 30 min after 50 mg/kg 6A4, (F) 0.025 mg/kg carfentanil dose 30 min after 50 mg/kg 6A4. (G) Best fit moles of fentanyl in the rapidly exchanging peripheral compartment following a 0.1 mg/kg i.v. dose of fentanyl with or without treatment of 50 mg/kg 6A4 30 min prior to fentanyl. For all panels, mice ($n = 6$) were administered drugs and mAb intravenously and bled per time point, alternating three mice every other time point. Blood samples were analyzed by LC-MS/MS to measure drug concentrations and by ELISA to measure mAb concentrations.

Figure 5.

Dose-ranging efficacy of 6A4 against fentanyl and carfentanil antinociception in mice. (A) Results from antinociceptive tests in i.v. 6A4-treated mice ($n = 6$) using i.p. fentanyl. (B) Results from antinociceptive tests in i.v. 6A4-treated mice ($n = 6$) using i.p. carfentanil. (C) Potency ratios for antibody treatments against fentanyl. (D) Potency ratios for antibody treatments against carfentanil. Significance is denoted by an asterisk from a one-way ANOVA and a Dunnett post hoc test when comparing antibody treated groups to controls. ** $P < 0.01$, *** $P < 0.001$ versus control. The dashed line in potency panels denotes control levels.

Figure 6.

6A4 and naloxone rescue mice from fentanyl/carfentanil-induced antinociception. Baseline nociception was measured at -15 min, then carfentanil (0.01 mg/kg) or fentanyl (0.2 mg/kg) was administered intraperitoneally at $t = 0$. Nociception was measured again at 15 min post drug administration, then rescue treatment of either 120 mg/kg 6A4 mAb (90 mg/kg for fentanyl) or 10 mg/kg naloxone was administered intravenously at 30 min. Nociception was measured again at times 60 , 90 , and 120 or 150 min. (A) Results of hot plate evaluation of carfentanil, carfentanil + naloxone, and carfentanil + 6A4 mAb. (B) Results of hot plate evaluation of fentanyl, fentanyl + naloxone, and fentanyl + 6A4 mAb. (C) Results of tail flick evaluation of carfentanil, carfentanil + naloxone, and carfentanil + 6A4 mAb. (D) Results of tail flick evaluation of fentanyl, fentanyl + naloxone, and fentanyl + 6A4 mAb. Significance is denoted by an asterisk from a two-way ANOVA and a Dunnett post hoc test when comparing treatment groups to control. $**P < 0.01$, $***P < 0.001$ versus control. Dashed lines denote the antinociception cutoff times (100% maximum possible effect).

Figure 7. Prophylactic treatment with 6A4 mAb prevents fentanyl-induced lethality. Mice ($n = 4$ per group) were administered i.v. 6A4 at the indicated doses. Subsequently, mice were given an i.v. fentanyl challenge at doses of (A) 3 mg/kg or (B) 6 mg/kg and time to death was recorded.

Figure 8.

Modeling mAb-mediated antagonism of fentanyl-induced respiratory depression in humans.

(A) Modeled human PK for 0.09, 0.18, 0.36, and 0.54 mg fentanyl doses based on a previous report.⁸⁶ (B) The apparent response curve of respiratory suppression in healthy volunteers as a function of fentanyl plasma concentration.⁸⁵ (C) Simulations of respiratory suppression following a 3 mg i.v. dose of fentanyl in humans treated or untreated with a 500 mg injection of an anti-fentanyl antibody with a k_{on} of $1.1 \text{ nM}^{-1} \text{ h}^{-1}$ and k_{off} of 0.73 h^{-1} , 24 h prior to fentanyl administration.

Table 1.

Opioid Affinity Profiling of Six Selected Antibodies against a Panel of Nine Fentanyl Analogues^a

Drug	Drug IC ₅₀ of mAbs against Fent-BSA					
	2F12	3H2	6A4	7A8	12A1	15F4
Fentanyl	1-10nM	1-10nM	~1nM	1-10nM	1-10nM	>10nM
Carfentanil	1-10nM	1-10nM	1-10nM	1-10nM	>>10nM	>100nM
3-Methylfentanyl	<10nM	~10nM	>10nM	<10nM	~10nM	>10nM
α-Methylfentanyl	<10nM	<10nM	<10nM	~10nM	<10nM	>10nM
Acetyl fentanyl	<10nM	<10nM	<10nM	<10nM	<10nM	>10nM
Butyrfentanyl	<10nM	<10nM	<10nM	<10nM	<10nM	>10nM
p-Tolyfentanyl	<10nM	<10nM	>10nM	>10nM	<10nM	>10nM
Alfentanil	>>1μM	>>1μM	>>1μM	>>1μM	>>1μM	>>1μM
Remifentanil	~1μM	>1μM	~1μM	<1μM	>>1μM	>>1μM
Key	>100nM	>>1μM	>1μM	>1μM	~1μM	<1μM
>>10nM	>10nM	~10nM	<10nM	<10nM	1-10nM	~1nM

A. Fentanyl hapten: R₁=(CH₂)₂COOH, R₆=Bn
B. Fentanyl: R₁=Me, R₆=Bn
C. Acetyl fentanyl: R₁=H, R₆=Bn
D. Butyrfentanyl: R₁=Pr, R₆=Bn
E. Tolyfentanyl: R₁=Me, R₂=Me, R₆=Bn
F. 3-Methylfentanyl: R₁=Me, R₂=Me, R₃=Me, R₆=Bn
G. α-Methylfentanyl: R₁=Me, R₄=Me, R₆=Bn
H. Alfentanil: R₅=CH₂OMe, R₆=-CH₂NHCOCH₃
I. Remifentanyl: R₅=CH₂COOMe, R₆=CH₂COOMe
J. Carfentanil: R₁=Me, R₅=COOMe, R₆=Bn
Unspecified R Groups = H

^a Approximate competitive IC₅₀ values are shown in the table with color coding and were determined by screening hybridoma supernatants against four dilutions of the listed drug compounds by the SPR method.

Table 2.Fentanyl and Carfentanil Binding Kinetics of Six Monoclonal Antibodies^a

mAb	Fentanyl			Carfentanil		
	k_a (M ⁻¹ s ⁻¹)	k_d (S ⁻¹)	K_D (M)	k_a (M ⁻¹ s ⁻¹)	k_d (S ⁻¹)	K_D (M)
2F12	1.21×10^6	5.42×10^{-5}	4.47×10^{-11}	1.68×10^6	1.57×10^{-3}	9.33×10^{-10}
3H2	1.02×10^6	2.32×10^{-5}	2.27×10^{-11}	6.89×10^5	1.27×10^{-3}	1.85×10^{-9}
6A4	4.71×10^7	6.50×10^{-4}	1.38×10^{-11}	1.16×10^7	6.14×10^{-3}	5.29×10^{-10}
7A8	2.96×10^7	1.67×10^{-4}	5.64×10^{-12}	4.28×10^6	1.62×10^{-4}	3.78×10^{-11}
12A1	1.18×10^6	1.41×10^{-5}	1.19×10^{-11}	3.50×10^5	1.20×10^{-4}	3.43×10^{-10}
15F4	1.92×10^6	8.64×10^{-3}	4.49×10^{-9}	1.67×10^6	1.25×10^{-2}	7.49×10^{-8}

^a On-rates (k_a) and off-rates (k_d) of drug binding were determined by immobilizing the six mAbs on an SPR sensor chip and flowing varying concentrations of fentanyl and carfentanil across the chip while measuring time-dependent changes in SPR signal.