

Preclinical Efficacy and Selectivity of Vaccines Targeting Fentanyl, Alfentanil, Sufentanil, and Acetylfentanyl in Rats

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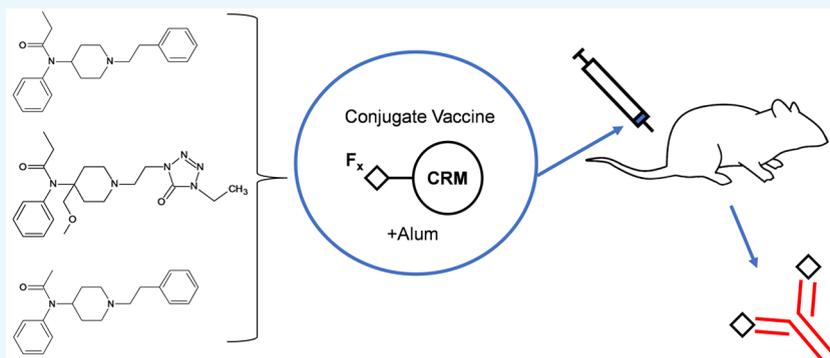
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ABSTRACT: The ongoing public health emergency of opioid use disorders (OUD) and overdose in the United States is largely driven by fentanyl and its related analogues and has resulted in over 75 673 deaths in 2021. Immunotherapeutics such as vaccines have been investigated as a potential interventional strategy complementary to current pharmacotherapies to reduce the incidence of OUD and opioid-related overdose. Given the importance of targeting structurally distinct fentanyl analogues, this study compared a previously established lead conjugate vaccine (F_1 –CRM) to a series of novel vaccines incorporating haptens derived from alfentanil and acetylfentanyl (F_8 , $9a$, $9b$, 10), and evaluated their efficacy against drug-induced pharmacological effects in rats. While no vaccine tested provided significant protection against alfentanil, lead formulations were effective in reducing antinociception, respiratory depression, and bradycardia elicited by fentanyl, sufentanil, and acetylfentanyl. Compared with control, vaccination with F_1 –CRM also reduced drug levels in the brain of rats challenged with lethal doses of fentanyl. These data further support investigation of F_1 –CRM as a candidate vaccine against fentanyl and selected analogues.

INTRODUCTION

The epidemic of opioid use disorders (OUD) and drug-related overdose fatalities has impacted the United States for decades^{1,2} and was exacerbated by the COVID-19 pandemic. Fatal drug overdoses increased by 18% to a total of 92 000 annual deaths by May 2020,^{3,4} including 62 900 opioid-related deaths. The highly potent synthetic opioid fentanyl and its analogues were implicated in a substantial portion of those fatalities, including incidents involving other opioids⁵ or nonopioids such as cocaine, methamphetamine, or other substances laced with fentanyl.^{6,7}

Currently available medications, consisting of opioid receptor agonists and antagonists, are effective; but their clinical implementation is limited by side effects, lack of access, stigma associated with opioid agonist therapy, and the requirement for detoxification prior to initiation of antagonist therapy.^{8,9} Vaccines against fentanyl or other opioids, a proposed alternative or adjunct therapy for OUD, operate through production of target-specific polyclonal antibodies, which sequester drug in the serum and reduce brain exposure

to the compound of interest. Such vaccines, which consist of an opioid-based hapten conjugated to an immunogenic carrier protein, have shown substantial preclinical efficacy as a strategy to combat OUD and drug-related overdose (reviewed in refs 10 and 11). A vaccine targeting oxycodone is currently being investigated in subjects with OUD in Phase I clinical trials.¹² Antifentanyl vaccines targeting OUD have shown efficacy in reducing antinociception, respiratory depression, and brain distribution of fentanyl in mice and rats,^{13–16} and in some studies were effective against potentially lethal fentanyl doses (i.e., 2–4 mg/kg) in mice.¹⁶ Because of their selectivity for the target drug, antifentanyl vaccines did not interfere with

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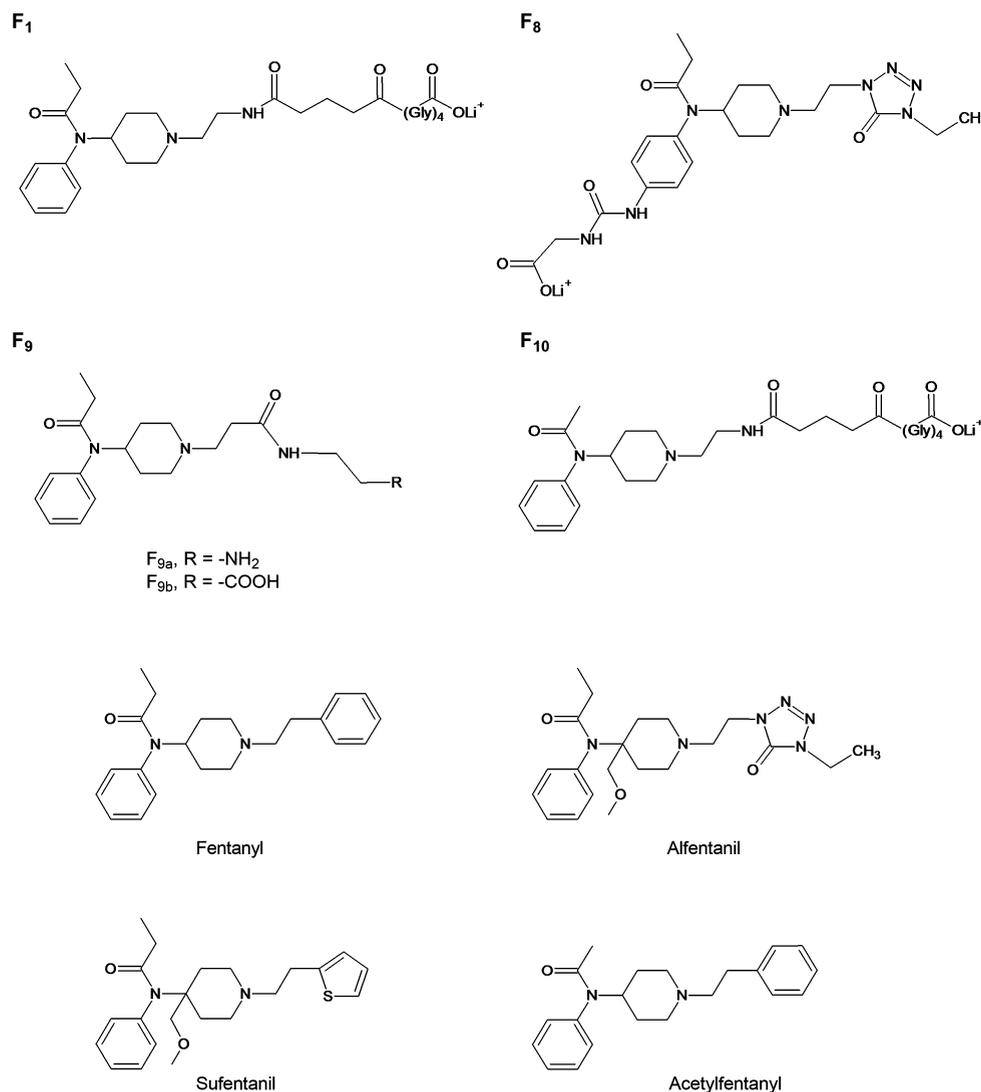


Figure 1. Structures of haptens targeting fentanyl and its analogues. A series of novel haptens based on the structures of fentanyl (F₁, F_{9a}, F_{9b}), alfentanil (F₈), and acetylfentanyl (F₁₀). The F₁ hapten has been previously described.

pharmacological activity of off-target opioids such as methadone and naloxone, or critical care medications such as anesthetics.¹⁵ Finally, such vaccines reduced the reinforcing effects of fentanyl in operant behavioral assays.^{17,18}

An important consideration for vaccines targeting the class of fentanyl-like drugs is the prevalence of fentanyl analogues.¹⁹ Because vaccine-induced antibodies are highly specific for their target opioid, it is critical for vaccine research to stay ahead of structurally diverse fentanyl analogues.²⁰ In the present study, we explore the utility of fentanyl analogue-derived haptens in conjugate vaccines targeting fentanyl, alfentanil and acetylfentanyl. Alfentanil is a fast-acting fentanyl derivative used for anesthesia,²¹ and acetylfentanyl (desmethyl fentanyl) is an impurity frequently encountered in illicit fentanyl and is less potent than fentanyl but with a narrow therapeutic window.²² Toxicology results from impaired driving cases show the frequent presence of acetylfentanyl in samples from drivers who tested negative for alcohol, but positive for fentanyl or other substances.^{23,24} Additionally, acetylfentanyl has been detected in hair samples from people who use heroin,²⁵ and in the urine of patients who tested positive for nonprescribed

opioids,²⁶ highlighting the prevalence of fentanyl analogues in illicit mixtures of fentanyl or other opioids.

Previous reports of antiopioid vaccines have shown some ability of antifentanyl vaccines to generate cross-protective antibodies against fentanyl analogues. Other groups have demonstrated *in vitro* binding of vaccine-induced polyclonal antibodies to structurally related fentanyl analogues, such as acetylfentanyl and α -methylfentanyl; however, most of these studies did not evaluate the efficacy of such antibodies against these analogues *in vivo*.^{14,16,27–29} Our previous studies of conjugate vaccines utilizing a series of fentanyl-based haptens (F₁–F₆) conjugated to either diphtheria toxoid cross reactive material-197 (CRM, or CRM₁₉₇) or keyhole limpet hemocyanin (KLH) carrier proteins showed promising efficacy in rats against fentanyl, limited protection against sufentanil, and no *in vivo* efficacy or *in vitro* cross-reactivity against alfentanil,^{13,15} supporting the exploration of alternate fentanyl-based haptens to achieve efficacy against fentanyl analogues. An additional carfentanil hapten (F₇) was evaluated but discarded from further advancement (*data not shown*). Here, we compared the efficacy of a previously reported fentanyl vaccine (F₁–CRM) to novel conjugate vaccines designed to target alfentanil (F₈–

CRM), fentanyl (F_{9a} -CRM and F_{9b} -CRM), and acetylfentanyl (F_{10} -CRM). Vaccines were evaluated for *in vivo* efficacy against drug-induced antinociception, respiratory depression, and bradycardia, while *in vitro* binding of polyclonal IgG antibodies to fentanyl and fentanyl analogues was characterized by ELISA and biolayer interferometry (BLI). Finally, the lead vaccine F_1 -CRM was evaluated for its efficacy against a lethal dose of fentanyl in rats. These results can be used to assess interaction between hapten structure, polyclonal antibody affinity, and *in vivo* efficacy of vaccines against fentanyl analogues to inform the design of vaccines targeting multiple fentanyl-class compounds.

RESULTS

Synthesis of Conjugate Vaccines against Fentanyl and Fentanyl Analogues. Haptens (Figure 1) were synthesized on the basis of the structures of alfentanil (F_8), fentanyl (F_{9a} , F_{9b}), and acetylfentanyl (F_{10}). Hapten structures were confirmed by ^1H NMR (see Supporting Information), and haptens were conjugated to CRM carrier protein. Conjugates were characterized by MALDI-TOF to determine the haptentation ratio (Table SI, Supporting Information). Because conjugate vaccines incorporating the F_1 hapten have previously shown efficacy against fentanyl,^{13,15} a lead F_1 -CRM vaccine was included to provide a basis of comparison for vaccine efficacy. Rats were immunized with CRM control; F_1 -CRM; or novel conjugates F_8 -CRM, F_{9a} -CRM, F_{9b} -CRM, or F_{10} -CRM adjuvanted with aluminum hydroxide (alum, Alhydrogel-85). Hapten-specific polyclonal serum IgG antibodies elicited by the vaccine were evaluated by ELISA on day 49 (Figure 2A), and at the termination of the experiment (Figure 2B). All vaccines elicited detectable titers against their cognate hapten.

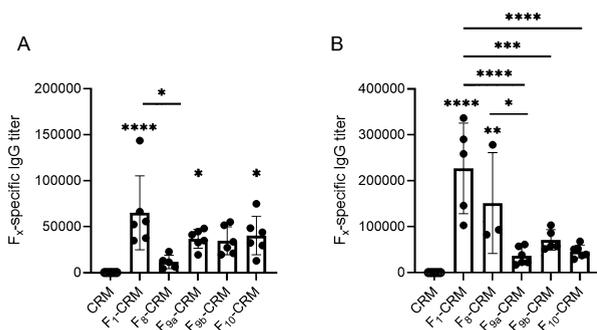


Figure 2. Vaccination with conjugates containing F_{8-10} haptens elicits hapten-specific IgG titers. Sprague–Dawley rats ($n = 6$ per group) were given an intramuscular (i.m.) immunization on days 0, 21, 42, and 63 with conjugate vaccines containing the F_1 or F_{8-10} haptens conjugated to CRM. Hapten-specific serum IgG antibody titers were evaluated by ELISA (A) 1 week after the third immunization (day 49), and (B) after completion of drug challenges (day 105). Data are expressed as mean \pm SEM. Symbols: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ vs CRM control. Brackets indicate pairwise group comparisons.

Efficacy of Conjugate Vaccines against Fentanyl and Fentanyl Analogues in Rats. Following the third vaccination, rats were challenged with fentanyl, alfentanil, and acetylfentanyl, with a minimum washout period of 1 week between challenges. In order to control for tolerance effects, rats were randomized into one of three challenge groups, in

which the order of drug challenges was rotated (such that 2 rats per group received fentanyl, alfentanil or acetylfentanyl in each challenge). The F_1 -CRM, $F_{9a/9b}$ -CRM, and F_{10} -CRM conjugate vaccines were protective against the effects of fentanyl (Figure 3), and rats vaccinated with F_8 -CRM showed some degree of protection, though only the effect on oxygen saturation was significant (Figure 3B). However, none of the vaccine groups showed any protection against alfentanil compared to CRM control (Figure S1A–C), and only $F_{9a/9b}$ -CRM and F_{10} -CRM were protective against acetylfentanyl-induced bradycardia, but not against antinociception or respiratory depression (Figure S1D–F).

Efficacy of F_8 -CRM against Alfentanil and F_{10} -CRM against Acetylfentanyl. Because the dose of these analogues in the first set of challenges (0.5 mg/kg alfentanil and 0.5 mg/kg acetylfentanyl) showed a strong effect from alfentanil but only a mild effect from acetylfentanyl by antinociception and respiratory depression in CRM control rats, we hypothesized that a lower dose of alfentanil and a higher dose of acetylfentanyl may have been required to determine whether the vaccine was able to protect from these drugs. That is, a dose of 0.5 mg/kg alfentanil may be high enough to overcome the ability of vaccine-elicited antibodies to sequester the drug, whereas 0.5 mg/kg acetylfentanyl was insufficient to produce robust antinociception, respiratory depression, or bradycardia.

To further evaluate the efficacy of vaccines against alfentanil and acetylfentanyl, rats were separated into two groups and challenged with either a lower dose of alfentanil, 0.25 mg/kg, or a higher dose of acetylfentanyl, 1.0 mg/kg (Figure 4). Rats vaccinated with F_8 -CRM were challenged with alfentanil, rats vaccinated with F_{10} -CRM were challenged with acetylfentanyl, and rats vaccinated with CRM control or F_1 -CRM were divided equally between the two challenge treatment groups. In this scenario, F_8 -CRM and F_1 -CRM did not provide significant protection against alfentanil; and the effect of F_1 -CRM and F_{10} -CRM on acetylfentanyl-induced pharmacological effects was not statistically significant compared to CRM, likely due to the small sample size of the divided CRM control and F_1 -CRM groups ($n = 3$ per group).

Efficacy of Conjugate Vaccines against Sufentanil. After completion of fentanyl, alfentanil, and acetylfentanyl challenges, rats were given one additional vaccination on day 84. Two weeks after the boost, on day 98, rats were challenged a final time with sufentanil, 0.008 mg/kg (Figure 5). None of the vaccinated rats showed significant protection from the antinociceptive effects of sufentanil; however, F_1 -CRM, F_8 -CRM, F_{9a} -CRM, and F_{10} -CRM vaccinated groups showed increased oxygen saturation at later time points (Figure 5B), indicating more rapid recovery from the effects of sufentanil.

Relative Affinity of Serum Antibodies for Fentanyl and Fentanyl Analogues. After a washout period of one week following the final challenge, blood was collected for analysis of serum antibody level and polyclonal antibody relative affinity by competitive ELISA (Table 1). Sera from all vaccine groups showed nanomolar IC_{50} values for fentanyl, though sera from the F_1 -CRM group showed the lowest IC_{50} . None of the sera showed significant binding to alfentanil, with all showing IC_{50} values above 100 μM . Similarly, affinity of all sera for sufentanil was in the micromolar range, with F_1 -CRM, F_{9a} -CRM, and F_{10} -CRM serum showing IC_{50} values between 20 and 30 μM . F_{10} -CRM, the hapten with the most structural similarity to acetylfentanyl, produced antibodies with the greatest affinity for acetylfentanyl, with IC_{50} of 6.99 nM,

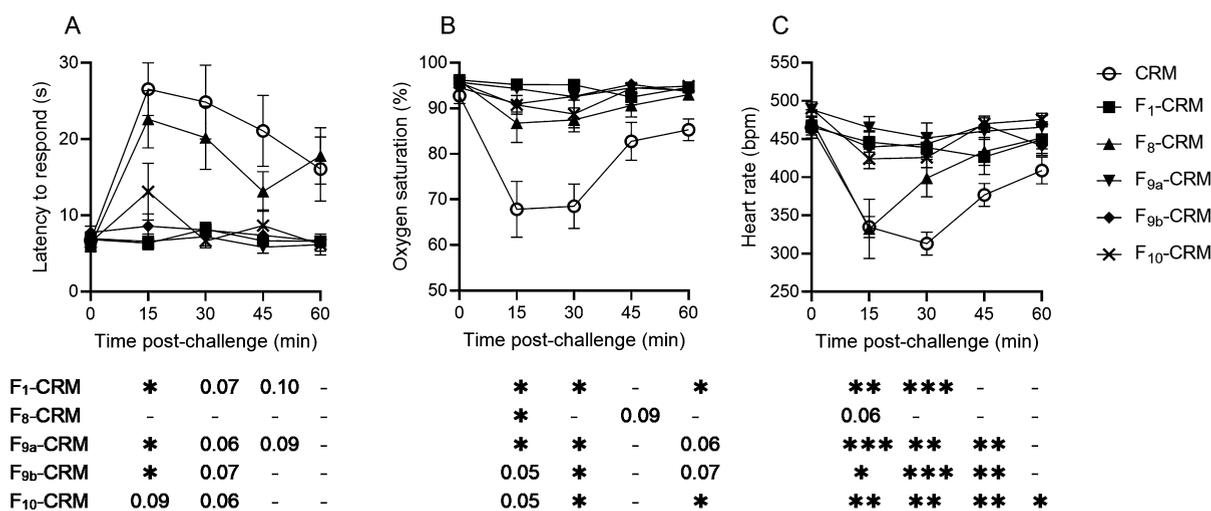


Figure 3. Efficacy of the vaccines containing the F_{8–10} haptens against fentanyl. Sprague–Dawley rats ($n = 6$, each group) were given an intramuscular (i.m.) vaccination on days 0, 21, 42, and 63 with conjugate vaccines containing the F₁ and F_{8–10} haptens or with CRM control, and were then challenged with 0.1 mg/kg fentanyl, subcutaneous (s.c.). Rats were monitored at 15 min intervals for (A) antinociception by latency to respond on a hot plate and for (B) oxygen saturation (%) and (C) heart rate measured by pulse oximetry. Data are expressed as mean \pm SEM. Below their respective graphical panels, significance of each vaccine group vs CRM control is indicated at each time point. Symbols: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to control; exact p -values are listed for $0.05 \leq p \leq 0.1$; and “–” indicates no significant difference, $0.10 \leq p$.

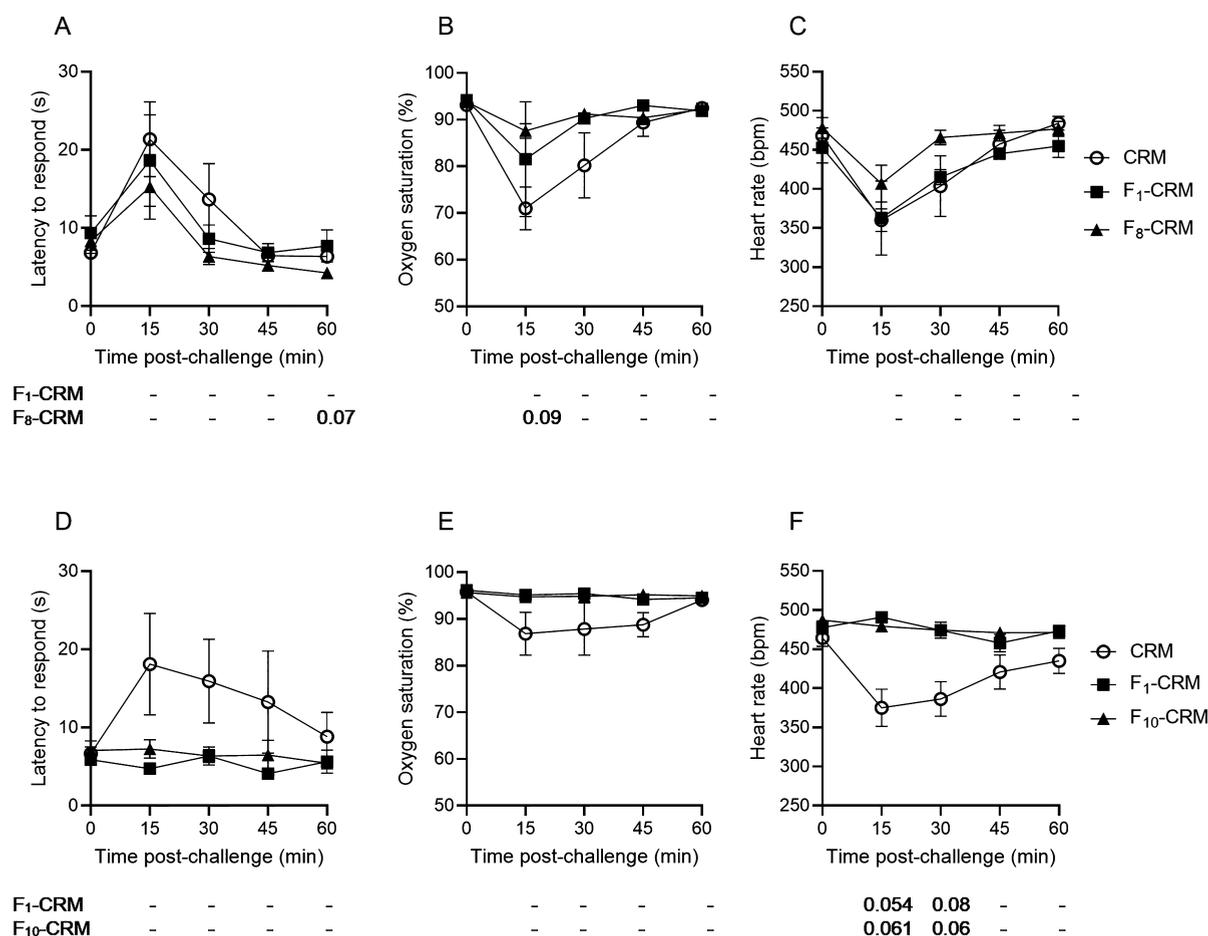


Figure 4. Efficacy of the vaccines containing the F₈ and F₁₀ haptens against alfentanil and acetylfentanyl, respectively. Rats immunized with control, F₁–CRM, or F₈–CRM were challenged with 0.25 mg/kg alfentanil, subcutaneous (s.c.) (A–C); and rats immunized with control, F₁–CRM, or F₁₀–CRM were challenged with 1.0 mg/kg acetylfentanyl s.c. (D–F). Rats were monitored at 15 min intervals for: (A,D) antinociception by latency to respond on a hot plate; (B,E) oxygen saturation (%); and (C,F) heart rate measured by pulse oximetry. Data are expressed as mean \pm SEM. Below the graphs, significance of each vaccine group vs CRM control is indicated compared to control at each time point. Symbols and statistics: exact p -values are listed for $0.05 \leq p \leq 0.1$, and “–” indicates no significant difference, $0.10 \leq p$.

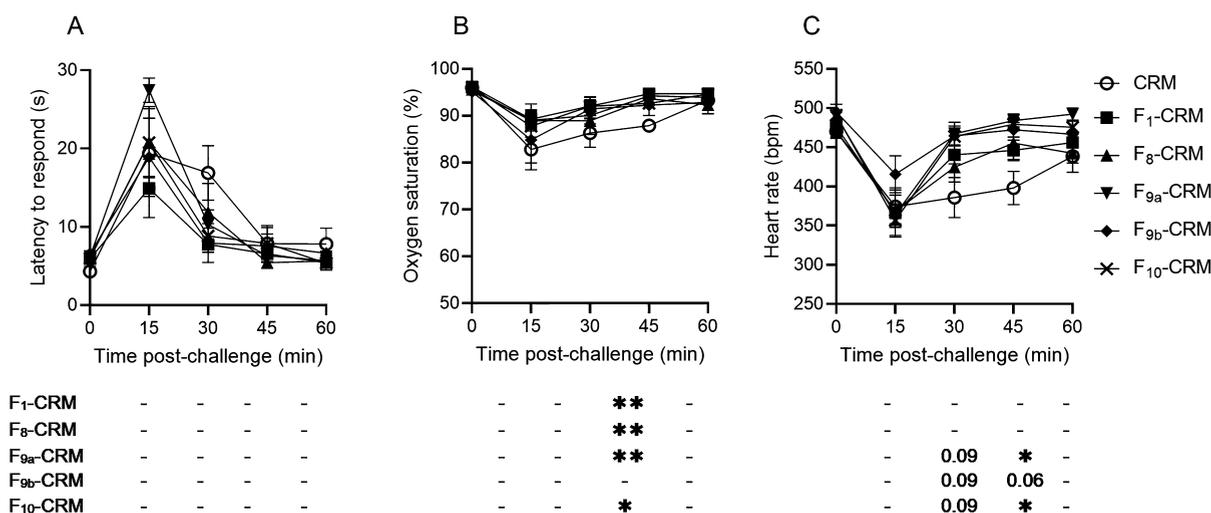


Figure 5. Efficacy of vaccines containing F₈₋₁₀ haptens against sufentanil. Rats vaccinated with F₁ and F₈₋₁₀ conjugate vaccines or CRM control were challenged with 0.008 mg/kg sufentanil, subcutaneous (s.c.). Rats were monitored at 15 min intervals for (A) antinociception by latency to respond on a hot plate, (B) oxygen saturation (%), and (C) heart rate. Data are expressed as mean ± SEM. Below their respective graphs, significance of each vaccine group vs CRM control is indicated at each time point. Symbols and statistics: **p* ≤ 0.05, ***p* ≤ 0.01 compared to control; *p*-values are listed for 0.05 ≤ *p* ≤ 0.1; and “-” indicates no significant difference, 0.10 ≤ *p*.

Table 1. F₈₋₁₀ Relative Affinity^a

	F ₁ -CRM	F ₈ -CRM	F _{9a} -CRM	F _{9b} -CRM	F ₁₀ -CRM
fentanyl (nM)	17.2 ± 14.8	141.3 ± 63.5	25.9 ± 17.7	69.7 ± 69.2	50.1 ± 26.1
alfentanil (μM)	243	348	203.9	181.4	180.2
acetylfentanyl (nM)	28.59	2090	584	763	6.99
sufentanil (μM)	25.82	>100	20.81	>100	29.04

^aFentanyl IC₅₀ expressed as mean ± SEM from all sera in group, *n* = 6; IC₅₀ for analogues obtained using serum pooled from all samples in each group. Maximum detection limit for alfentanil was 1 mM; detection limit for fentanyl, sufentanil and acetylfentanyl was 100 μM.

though F₁-CRM serum also showed a relatively high affinity with an IC₅₀ of 28.6 nM.

In order to estimate cross-reactivity between polyclonal antibodies against fentanyl-, alfentanil-, and acetylfentanyl-targeting haptens, *in vitro* binding of pooled serum from each vaccine group was evaluated against biotinylated F₁, F₈, and F₁₀ haptens by biolayer interferometry (BLI). Serum from all vaccine groups showed binding to both F₁-biotin and F₁₀-biotin, and only serum from F₈-CRM vaccinated rats showed significant interaction with F₈-biotin (Figure S2). Serum from rats vaccinated with F₁-CRM showed the highest response values to both F₁-biotin and F₁₀-biotin, consistent with the F₁-specific titer obtained by ELISA (Figure 2).

Efficacy of Lead Conjugate Vaccine F₁-CRM against High Fentanyl Doses. To evaluate efficacy of the lead F₁-CRM vaccine against high doses of fentanyl, a separate cohort of rats was vaccinated with F₁-CRM or with CRM control. After the last vaccination, rats were given doses of 0.25 mg/kg of fentanyl every 15 min to a total cumulative dose of 2.25 mg/kg (Figure 6). The F₁-CRM vaccine shifted the ED₅₀ for respiratory depression approximately 3.5-fold from 0.139 to 0.523 mg/kg (Figure 6A), and reduced fentanyl-induced bradycardia (Figure 6B). Individual rats were euthanized if oxygen saturation dropped below 50% (Figure 6C), or after the final fentanyl dose was administered, and brain and serum were collected for analysis of fentanyl distribution. The overall effect of F₁-CRM on preventing respiratory arrest was not significant (*p* = 0.35), though F₁-CRM-immunized rats showed increased concentration of fentanyl in serum and

reduced distribution to brain (Figure 6D,E). To determine the effect of F₁-CRM vaccine on mortality from acute fentanyl exposure, a separate cohort of rats was vaccinated with F₁-CRM or with CRM control. After the last vaccination, rats were given a bolus dose of 2.25 mg/kg, and survival was recorded at 1 and 4 h postfentanyl challenge (Figure 6F). At 4 h postadministration more rats survived in the vaccinated group compared to control, though the difference was not significant.

DISCUSSION

This study sought to evaluate the efficacy of novel antifentanyl and antiacetylfentanyl conjugate vaccines against fentanyl and selected fentanyl analogues in rats. The efficacy of these and of the antifentanyl vaccines F₁-CRM and F_{9a/9b}-CRM against fentanyl analogues including alfentanil, sufentanil, and acetylfentanyl, were fully characterized by evaluating the relative affinity of antibodies against these analogues *in vitro*, and *in vivo* efficacy was tested against opioid-induced antinociception, respiratory depression, and bradycardia. This approach builds upon reports of other antifentanyl and antifentanyl analogue vaccines currently under investigation. Specifically, other groups have demonstrated *in vitro* affinity of fentanyl vaccine-induced polyclonal antibodies for some structurally related fentanyl analogues, such as acetylfentanyl and α-methylfentanyl, but the efficacy of such antibodies against these compounds was not evaluated *in vivo*.^{14,16} Evaluations of multitarget vaccines such as heroin/fentanyl vaccines have shown specificity of serum antibodies for

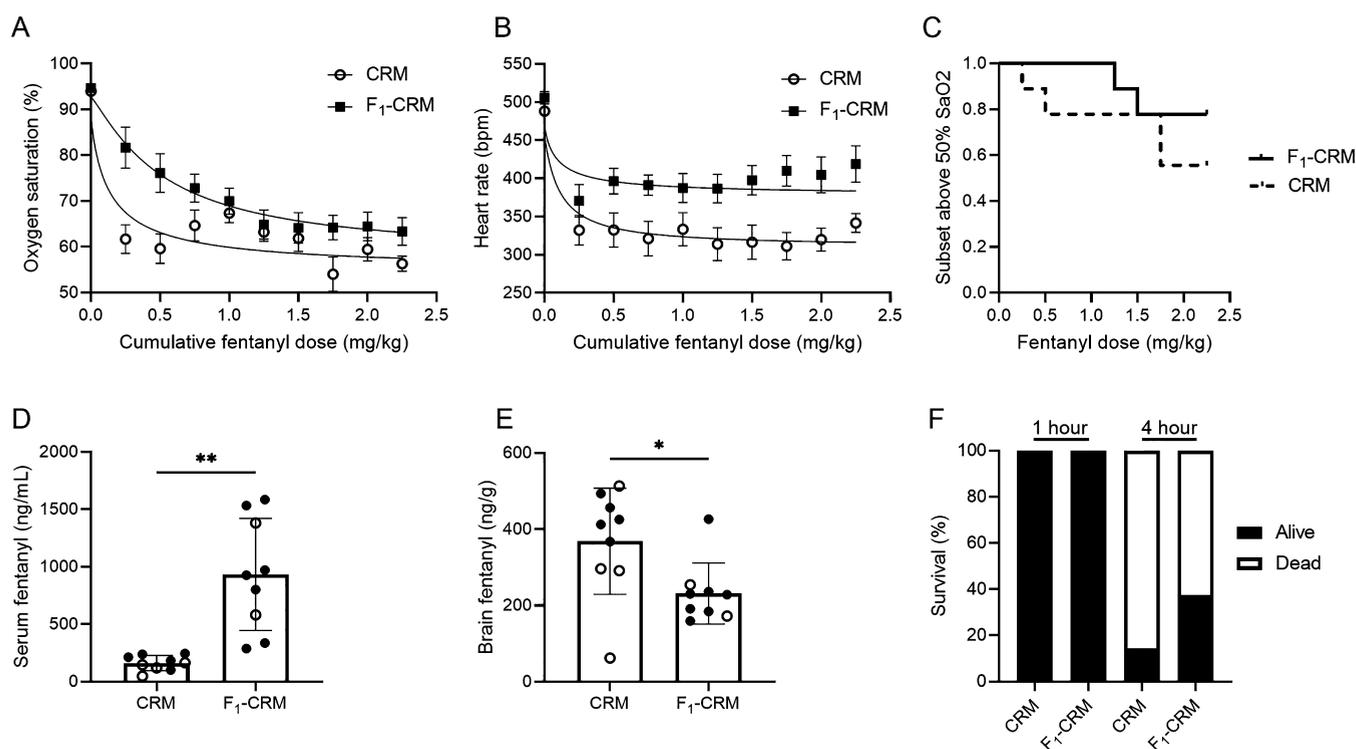


Figure 6. Efficacy of a fentanyl vaccine against higher fentanyl doses in rats. Rats ($n = 9$ per group) were given an intramuscular (i.m.) immunization on days 0, 21, 42, and 63 with either CRM control or the F_1 -CRM conjugate adsorbed on alum. A week after the fourth vaccination, rats were challenged with doses of 0.25 mg/kg fentanyl subcutaneous (s.c.) every 15 min, to a final cumulative dose of 2.25 mg/kg or until oxygen saturation was measured at <50%. (A) Respiratory depression measured by oxygen saturation (%) and (B) bradycardia measured as heart rate (bpm) over the course of the experiment. (C) Survival curve indicating subset of rats above 50% oxygen saturation; once <50% oxygen saturation was reached or at a cumulative dose of 2.25 mg/kg fentanyl, rats were euthanized and fentanyl concentration was quantified in serum and brain tissue. Fentanyl concentration calculated in (D) serum and (E) brain; open circles indicate rats that did not receive the full cumulative dose. Data are expressed as mean \pm SEM. Symbols: * $p \leq 0.05$; ** $p \leq 0.01$. (F) A separate cohort of rats was immunized as above and challenged with a bolus dose of 2.25 mg/kg fentanyl. Survival was assessed at 1 and 4 h postfentanyl.

multiple opioid targets but did not assess efficacy against fentanyl analogues other than those targeted by the vaccine.^{27,28,30–32} Recent vaccines targeting carfentanyl have demonstrated *in vitro* cross-reactivity against multiple fentanyl analogues and *in vivo* efficacy against both fentanyl and carfentanyl,²⁹ and against fentanyl-carfentanyl admixtures,³³ but were not evaluated *in vivo* against other fentanyl analogues.

In the present study, all vaccines generated fentanyl-specific antibody titers and were protective against 0.1 mg/kg fentanyl to some degree, though the alfentanil F_8 -CRM was least efficacious. This result matched with the *in vitro* affinity of antibodies for fentanyl measured by competitive ELISA, in which F_8 -CRM serum showed the lowest relative affinity for fentanyl *in vitro* (Table 1). In initial challenges of 0.5 mg/kg alfentanil and acetylfentanyl, none of the vaccines were protective against alfentanil, and only F_{9a} -CRM, F_{9b} -CRM, and F_{10} -CRM were effective at reducing acetylfentanyl-induced bradycardia. In follow-up experiments using 0.25 mg/kg alfentanil, F_8 -CRM did not have significant impact on alfentanil-induced effects; and with 1.0 mg/kg acetylfentanyl, the effects of F_1 -CRM and F_{10} -CRM were not statistically significant ($p = 0.08$ and $p = 0.06$, respectively); however, it is possible that F_{10} -CRM would be protective in a challenge with higher statistical power ($n > 6$). Finally, some degree of efficacy was seen against 0.008 mg/kg sufentanil, which is consistent with previous reports of F_1 -CRM vaccine efficacy against this fentanyl analogue in rats.¹⁵

Differences in efficacy can be attributed in part to conjugate properties including hapten chemistry, linker length, and haptenation ratio (Table SI, Supporting Information). The F_{10} hapten differs from F_1 only in the presence of the methyl group that distinguishes fentanyl from acetylfentanyl, and both conjugates displayed similar haptenation ratios. The F_1 -CRM and F_{10} -CRM vaccines produced serum antibody response with nanomolar affinity for both fentanyl and acetylfentanyl (Table 1), with F_1 -CRM producing higher relative affinity for fentanyl (17.2 nM) and F_{10} -CRM producing higher relative affinity for acetylfentanyl (6.99 nM). In comparison to F_1 -CRM, F_{9a} -CRM and F_{9b} -CRM had shorter linker length and lower haptenation ratios (17.0 for F_1 -CRM vs 4.2 for F_{9a} -CRM and 8.5 for F_{9b} -CRM), and though all three conjugates produced antibodies with nanomolar affinity for fentanyl, F_1 -CRM generated antibodies showing the lowest IC_{50} (Table 1).

Interestingly, F_8 -CRM was effective against fentanyl *in vivo* but not against alfentanil. The relative affinity of serum from rats vaccinated with F_8 -CRM indicated substantially higher affinity of polyclonal antibodies for fentanyl than for alfentanil (141 nM vs 348 μ M). Conversely, BLI analysis of F_8 -CRM binding to the biotinylated haptens indicated relatively similar levels of binding of serum antibodies to F_1 , F_8 , and F_{10} . Importantly, the F_8 hapten differs from the alfentanil molecule in that it lacks the methoxymethyl moiety on the 4-position of the central piperidine, possibly accounting for the low relative

affinity of F₈-immunized serum for free alfentanil observed in the competitive ELISA assay.

Differences in efficacy may also arise from the relative potencies of fentanyl versus its analogues; as previously reported,¹⁵ testing of vaccine efficacy across multiple analogues *in vivo* is complicated by different pharmacokinetic and pharmacodynamic profiles of the individual target drugs. For example, in this study alfentanil and acetylfentanyl required higher doses to achieve a clinically relevant effect compared with fentanyl (Figure 4, Figure S2). Whereas a dose of 0.1 mg/kg fentanyl induced an approximately 30% reduction in oxygen saturation in CRM control-vaccinated rats (Figure 3), a 2.5- to 5-fold higher dose of alfentanil was required to produce the same effect. Conversely, sufentanil required a lower dose (0.008 mg/kg) to produce a reduction in oxygen saturation (Figure 5). Therefore, it is possible that even a small amount of cross-reactive antibodies may be sufficient to reduce brain concentration of sufentanil to a measurable effect from vaccine. Hence, it is important to evaluate vaccine efficacy against a battery of opioid-induced pharmacological effects.

Because of its demonstrated efficacy in previous work and here, F₁-CRM was concluded to be the best candidate for further preclinical development out of the conjugates evaluated. The lead vaccine F₁-CRM produced antibodies with the highest *in vitro* affinity for fentanyl and was effective at preventing fentanyl-induced respiratory depression and bradycardia. Therefore, it was selected for additional efficacy testing against higher fentanyl doses. This is one of few reports specifically testing antifentanyl vaccines against a lethal fentanyl challenge in rodents, though the overall effect of F₁-CRM on survival was not significant.¹⁶ Given that control and actively vaccinated rats were resilient to fentanyl-induced fatal overdose particularly in the first 1–2 h after exposure, it is possible that rodents are not suitable species to test for the efficacy of medications against opioid-induced overdose because very high doses of opioids are required for lethality, which may translate into higher drug plasma concentrations than those found in humans. It is indeed possible that other species, for example, ferrets³⁴ or large animals (e.g., pigs^{35,36}) may be required to study clinically relevant overdose scenarios. Overall, these results highlight the importance of evaluating the applicability of antifentanyl vaccines *in vivo* both for broad efficacy against a variety of fentanyl analogues of clinical interest, and for efficacy of fentanyl vaccines against high doses of fentanyl relevant for use as a strategy for overdose prevention.

EXPERIMENTAL PROCEDURES

Synthesis and Conjugation of F_{8–10}-Based Conjugate Vaccines. See the Supporting Information for details of hapten synthesis and conjugation. Haptens were synthesized and then conjugated by carbodiimide coupling chemistry to CRM from either Fina Biosolutions (F_{9a}, F_{9b}, F₁₀) or Pfenex (F₁, F₈) for vaccines, or to bovine serum albumin (BSA) for ELISA. The F₁-CRM vaccine was prepared as previously described.¹⁵ Conjugates were purified by ultrafiltration (Amicon) and stored at 2.5 mg/mL in sterile PBS pH 7.2.

Animals. All studies were approved by the University of Minnesota Institutional Animal Care and Use Committee, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed. Male Sprague–Dawley rats (Envigo), 8 weeks on arrival, were housed with a 14/10 h

light/dark cycle and fed *ad libitum*. Rats were allowed 1 week habituation period prior to initiation of experiments.

Immunization. Vaccines consisted of 60 μg CRM control or F₁-CRM or F_{8–10}-CRM conjugate adsorbed on 90 μg alum adjuvant (Alhydrogel-85, Invivogen) in sterile saline to a final volume of 150 μL. Rats (*n* = 6 per group) were given an intramuscular (i.m.) immunization in both rear thigh muscles (75 μL each side) on days 0, 21, 42, 63, and 84. Serum samples were collected for analysis of serum antibody level by tail vein sampling on day 49, 1 week after the third vaccination, and on day 105, 1 week after completion of all drug challenges.

Analysis of Serum Antibody Level. Antibody analysis was performed via indirect ELISA; briefly, 96-well plates were coated with 5 ng/well of the corresponding BSA conjugate or unconjugated BSA as control in 50 mM Na₂CO₃ buffer, pH 9.6 (Sigma-Aldrich, St. Louis, MO), and blocked with 1% porcine gelatin (Sigma-Aldrich). Plates were incubated with serum samples diluted in 1× PBS + 0.05% Tween-20 (PBS-T, Thermo Fisher), then washed and incubated with a horse-radish peroxidase (HRP)-conjugated goat antirat IgG (Jackson ImmunoResearch) to assess hapten-specific serum IgG. HRP activity was quantitated with *o*-phenylenediamine substrate (SigmaFast OPD, Sigma-Aldrich) by absorbance at 492 nm on a 96-well plate reader (Tecan Infinite).

Competitive Binding ELISA. Determination of relative affinity by competitive binding ELISA was performed essentially as described.¹⁵ Briefly, 96-well plates were coated with 0.5 ng/well F₃-BSA³⁷ and blocked with 1% gelatin, and fentanyl or fentanyl analogues were added to the wells with concentrations ranging from 1 × 10⁻⁴ M to 1 × 10⁻¹⁰ M. Plates were incubated with diluted serum in the presence of a competitor, washed with PBS-T, incubated with HRP-conjugated goat antirat IgG, and quantitated with OPD substrate as above. The relative affinity of serum antibodies was calculated as IC₅₀, or the concentration of competitor that resulted in 50% decrease in antibody binding.

Drug Challenges. Fentanyl citrate, alfentanil HCl, and sufentanil citrate were obtained from Boynton Pharmacy (University of Minnesota). Acetylfentanyl HCl was obtained through the NIDA drug supply. For acute drug challenges, each drug was diluted in sterile saline and given as a single bolus dose administered subcutaneous (s.c.). Challenges occurred across 3 weeks starting on day 56, with each subject receiving one drug challenge per week. Rats were randomized to receive fentanyl (0.1 mg/kg), alfentanil (0.5 mg/kg), or acetylfentanyl (0.5 mg/kg) in the first challenge, followed by the other analogues in subsequent challenges, in order to control for tolerance effects due to repeated exposure to fentanyl and fentanyl analogues. On day 77, after the initial three challenges were completed, alfentanil (0.25 mg/kg) and acetylfentanyl (1.0 mg/kg) were given to the corresponding vaccine group (F₈-CRM for alfentanil or F₁₀-CRM for acetylfentanyl), with CRM control and F₁-CRM groups randomized to receive either alfentanil or acetylfentanyl. A final challenge of sufentanil (0.008 mg/kg) was given to all groups on day 98.

Opioid-Induced Antinociception, Respiratory Depression, and Bradycardia. Rats were allowed to acclimate to the testing environment for 1 h prior to experiments, and baseline measurements were taken 15 min prior to drug challenge. Opioid-induced antinociception was measured by latency to respond on a hot plate (Columbus Instruments, Columbus, OH) set to 54 °C, and opioid-induced respiratory

depression (percent oxygen saturation, SaO₂) and bradycardia (heart rate in beats per minute, BPM) were measured with a MouseOx Plus pulse oximeter (Starr Life Sciences, Oakmont, PA). Antinociception and oximetry measurements were taken at 15 min intervals postdrug administration for a total of 60 min.

F₁–CRM Vaccine Efficacy in Lethal Fentanyl Challenge. Rats ($n = 9$ per group) were given an intramuscular (i.m.) vaccination on days 0, 21, 42, and 70 with either F₁–CRM or CRM control. Serum was collected via tail vein sampling on day 50 for analysis of fentanyl-specific antibody levels. On day 84, rats were allowed to acclimate to the testing environment for 1 h, followed by a baseline measurement of oxygen saturation and heart rate. Rats were then given 0.25 mg/kg fentanyl s.c. every 15 min, to a maximum cumulative dose of 2.25 mg/kg. Prior to each successive dose, rats were monitored by oximetry for respiratory depression and bradycardia. Following the final oximetry measurement, or after occurrence of respiratory arrest, rats were euthanized via CO₂ inhalation, and blood and brain were collected for analysis of fentanyl concentration by liquid chromatography coupled with mass spectrometry (LC–MS).

LC–MS Analysis of Fentanyl Concentration. Determination of fentanyl concentration in brain and serum was performed as described.^{13,15} Briefly, serum was prepared from whole blood by centrifugation, and brain tissue was homogenized; serum and brain homogenate were processed with acetonitrile, supernatant was extracted with Bond Elut extraction cartridges (Agilent), and the serum and brain were reconstituted in ammonium formate mobile phase buffer. Samples were analyzed on reverse-phase C18 column coupled with G6470 triple quadrupole mass spectrometry system (Agilent), and peak integration was performed with Mass Hunter software.

Statistical Analysis. Fentanyl-specific serum IgG antibody titers were compared by one-way ANOVA followed by Sidak's multiple comparisons test. Latency to respond on hot plate, oxygen saturation (SaO₂), and heart rate (beats per minute, BPM) over time were compared using two-way ANOVA or mixed-effects analysis with Dunnett's multiple comparisons test to evaluate significance versus the CRM control group at each time point. Fentanyl-induced mortality during lethal fentanyl overdose was analyzed with Fisher's exact test. Fentanyl serum and brain concentrations were compared with Welch's t test. All analyses were conducted in Prism v9.1 (GraphPad, San Diego, CA).

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c00820>.

Details on hapten synthesis and conjugation, conjugate characterization, assessment of serum by BLI, *in vivo* data from additional alfentanil and acetylfentanyl challenges, and tables comparing haptentation ratio of conjugates and overall efficacy of conjugates against described drug challenges (PDF)

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Author Contributions

S.E.A., S.P.R., and M.P. conceived of the project and designed the experiments. C.B., C.R., A.K., R.J., and V.G. performed the experiments. C.B. and M.P. wrote and edited the manuscript.

Notes

The authors declare the following competing financial interest(s): Pravetoni, Averick, and Runyon are inventors of provisional application No. 62/989,41, "Fentanyl haptens, fentanyl hapten conjugates, and methods for making and using." The other authors declare no competing financial interest.

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■ ABBREVIATIONS

OD, opioid use disorder; CRM, diphtheria toxoid cross-reactive molecule; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; BLI, biolayer interferometry

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