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ORIGINAL INVESTIGATION

A humanized monoclonal antibody attenuates fentanyl self-administration and reverses and prevents fentanyl-induced ventilatory depression in rhesus monkeys

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Abstract

Medications for opioid use disorder (OUD) and overdose have been available for decades, yet nearly 70% of fatal drug overdoses in the United States are attributed to the opioid receptor agonist fentanyl and its analogs. There is a pressing need for more and better medications that reduce fentanyl use and prevent overdose. A humanized (h) monoclonal antibody (mAb) targeting fentanyl, hHY6-F9, was tested for attenuating intravenous fentanyl self-administration and reversing and preventing fentanyl-induced ventilatory depression in rhesus monkeys. A single administration of hHY6-F9 significantly decreased fentanyl, but not heroin or cocaine, self-administration. In some monkeys, fentanyl self-administration remained decreased for ~2 weeks. hHY6-F9 was as effective as 32 µg/kg naloxone in reversing fentanyl-induced ventilatory depression, with a single administration protecting against fentanyl-induced ventilatory depression for 2–3 weeks. Moreover, pharmacokinetic analyses indicate that hHY6-F9 continued to sequester fentanyl in the serum for 2 weeks. This study demonstrates that hHY6-F9 selectively attenuates the positive reinforcing and ventilatory depressant effects of fentanyl, indicating its possible utility for preventing relapse and overdose.

Keywords Nonhuman primate · Opioid use disorder · Monoclonal antibody

Introduction

The Centers for Disease Control and Prevention estimates that ~70% of fatal drug overdoses in 2022 were attributed to fentanyl and its analogs (Kariisa et al. 2023). Fentanyl is a highly potent µ-opioid receptor (MOR) agonist and a common adulterant in the unregulated drug supply, thereby increasing the risk of fatal overdose due to accidental exposure (Kariisa et al. 2023; Singh et al. 2020). However, up to 45% of those testing positive for fentanyl used it intentionally (Gryczynski et al. 2019; Hochstatter et al. 2022; McKnight et al. 2023; Morales et al. 2019). Regardless of intent, the number of fentanyl-related overdose deaths remains high despite the availability of Food and Drug Administration (FDA)-approved medications for treating opioid use disorder (OUD) and overdose. OUD medications include the MOR full agonist methadone, partial agonist buprenorphine, and antagonist naltrexone. The MOR antagonists naloxone and nalmefene are available for reversing overdose.

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There are barriers to accessing and prescribing OUD medications, particularly for underprivileged communities (Mackey et al. 2020), and the high potency of fentanyl might impede the effectiveness of medications for treating OUD and reversing overdose (Chambers et al. 2023; Flynn and France 2021; Hiranita et al. 2024; Moss and Carlo 2019; Suzuki and El-Haddad 2017). Providers can be hesitant to prescribe buprenorphine or methadone due to their potential for misuse and instead prescribe naltrexone (Blanco and Volkow 2019; Mackey et al. 2020), which is a competitive MOR antagonist that attenuates reinforcing effects and prevents overdose by MOR agonists (Sullivan et al. 2006, 2013). However, antagonizing the actions of all opioid agonists would preclude using opioid agonist pain medications or methadone (Blanco and Volkow 2019). Fentanyl-targeting vaccines or monoclonal antibodies (mAbs) could be effective alternatives to currently available OUD medications. Vaccines, which stimulate the production of fentanyl-targeting polyclonal antibodies, and mAbs bind and sequester fentanyl in the serum with high selectivity to reduce drug distribution to the brain (Martinez et al. 2023; Townsend et al. 2021a), thereby attenuating the cardiorespiratory effects of fentanyl (Baehr et al. 2022a, b; Bremer et al. 2023; Chen et al. 2024). Although vaccines can be long-lasting, repeated injections are required to produce enough antibodies to block the effects of the target drug (Martinez et al. 2023; Townsend et al. 2021a), an approach that might not be effective given the high number of OUD patients who return to drug taking shortly after initiating treatment (Gossop et al. 2002; Martinez et al. 2023). In contrast, mAbs have a rapid onset of action and relatively long half-life, providing immediate and potentially long-lasting protection from relapse or overdose. If mAbs attenuate the positive reinforcing effects of fentanyl without interfering with the actions of other MOR agonists, mAbs could be effective standalone or adjuvant medications for fentanyl misuse (Baehr et al. 2020; Martinez et al. 2023).

This study characterized a humanized (h) mAb, hHY6-F9, targeting fentanyl. In vitro, hHY6-F9 has nanomolar affinity for fentanyl and lower affinity for some fentanyl analogs (e.g., acetylfentanyl; Hicks et al. 2022). Murine HY6-F9 reduces fentanyl brain concentrations and reverses the cardiorespiratory effects of fentanyl in rats (Baehr et al. 2022b) and blocks the cardiorespiratory effects of fentanyl for one week (Baehr et al. 2022b; Hicks et al. 2022). Similar effects have been reported for other fentanyl-targeting mAbs (Bremer et al. 2023; Chen et al. 2024; Triller et al. 2023). The current study extends previous research on HY6-F9 and hHY6-F9 to highly translational nonhuman primate (NHP) models of drug taking and ventilation. Given their immunological similarity to humans, NHPs are the nonhuman species most likely to accurately predict the effects of mAbs in humans (Townsend et al. 2021a). This study examined the selectivity and duration of action of hHY6-F9 by comparing its effects on intravenous (i.v.) self-administration of fentanyl, heroin, and cocaine. Opioid-targeting vaccines can decrease opioid self-administration in nonhuman species (Robinson et al. 2020; Townsend et al. 2019, 2020, 2021b); however, the effectiveness of a fentanyl-targeting, humanized mAb on fentanyl self-administration has not been well characterized. The study also explored the effectiveness and duration of action of hHY6-F9 for reversing and preventing fentanyl-induced ventilatory depression. Pharmacokinetic analyses were conducted to confirm that hHY6-F9 sequestered fentanyl in the serum and to characterize hHY6-F9 concentrations over time.

Materials and methods

Monoclonal antibody and drugs

hHY6-F9 was generated by humanizing a murine mAb isolated from mice actively immunized with a candidate fentanyl conjugate vaccine (Baehr et al. 2022b; Hicks et al. 2022). A Chinese hamster ovary (CHO)-derived stable cell line expressing hHY6-F9 was generated at Celltheon Corporation (Union City, CA; *Supplemental methods 1.a)*. Purified hHY6-F9 was tested in vitro to verify binding (*Supplemental methods 1.a*) then concentrated to 53.4 mg/mL using centrifugal protein concentrators in phosphate-buffered saline (PBS; pH 7.4; Corning™ cell culture PBS [1X]; Fisher Scientific, Hampton, NH) and stored at -80° C. hHY6-F9 was examined in mice to ensure in vivo activity (*Supplemental methods 1.b*, *results 2.a*, Figure S1). hHY6-F9 aliquots (5 mL) were received at the University of Texas Health Science Center (UTHSCSA), stored at -80° C and thawed (2–4° C) for at least 24 h before administration. Thawed hHY6-F9 not administered remained refrigerated for no longer than one month before use to avoid multiple freeze/thaw cycles. The hHY6-F9 solution was inverted several times ~ 15 min before infusing over 15–60 s, followed by a saline flush.

Fentanyl hydrochloride (HCl), heroin HCl, cocaine HCl, and naloxone HCl were provided by the National Institute on Drug Abuse Drug Supply Program. Fentanyl, heroin, and cocaine were dissolved in sterile saline; naloxone was dissolved in sterile saline (Experiment 1) or PBS (Experiments 2.1 and 2.2). All drug and vehicle solutions were drawn through a sterile polyethersulfone syringe filter (0.22 µm, 25 mm; ThermoFisher Scientific, Waltham, MA) before i.v. administration.

Subjects and surgery

Experiment 1 included four adult rhesus monkeys (females, IR, HA; males, MA, KI; mean age ± SD, 12.4 ± 4.3 years). Experiment 2.2 included five rhesus monkeys (females, GA, KA, OR; males, MU, LA; mean age, 20.6 ± 2.9 years) and apart from monkey MU, the same monkeys were included in Experiment 2.1. All monkeys had previously self-administered or received various drugs (e.g., opioids). Monkeys in Experiments 2.1 and 2.2 were drug-free for at least 2 months before this study. Daily rations of primate chow (Harlan Teklad, Madison, WI) and fresh fruit were provided to maintain healthy weights (6.6–11.2 kg) and water was available *ad libitum* in the home cage. Monkeys were individually housed in temperature- and humidity-controlled rooms with a 14/10-hour light/dark cycle and in accordance with the UTHSCSA Institutional Animal Care and Use Committee and *Guide for the Care and Use of Laboratory Animals* (National Research Council 2010). Chronic indwelling i.v. catheters were surgically implanted (*Supplemental methods*1.d.).

Experiment 1. Self-administration general procedure

Self-administration sessions occurred twice daily (beginning at 1000 and 1400 h) in the home cage and were 90 min long (see *Supplemental methods 1.e*. for details). Briefly, 30 consecutive responses on the active lever resulted in an infusion followed by a 180-second time-out period with infusion duration varying among monkeys according to body weights. Monkeys could receive a maximum of 30 infusions each session, including an initial priming infusion.

Experiment 1: effects of hHY6-F9 on fentanyl self-administration

Fentanyl (0.032–1.0 µg/kg/infusion) and heroin (0.32–3.2 µg/kg/infusion) dose-effect curves were generated, and self-administration of 32 µg/kg/infusion cocaine, which previously resulted in a high number of infusions in all monkeys, was redetermined; fentanyl and heroin doses were tested in mixed order. Saline was intermittently substituted for drug to confirm that responding was a function of drug reinforcing effects. Each dose was available until the number of infusions obtained in 3 consecutive sessions did not vary by more than ± 20% of the mean of those sessions or was less than 10 or after a dose was available for 7 sessions. Doses of fentanyl and heroin generating the highest number of infusions were identified for each monkey. If the mean number of infusions obtained was equivalent for multiple doses, the larger dose was subsequently available for that monkey, except for monkey HA (*Supplemental results 2.b*).

Next, monkeys received an infusion of vehicle (2 mL PBS) or hHY6-F9 15 min before a morning fentanyl session; heroin was available in the afternoon session the day of treatment and, subsequently, every other day, with cocaine available in intervening afternoon sessions. All monkeys received 10 mg/kg hHY6-F9 and one monkey (IR) also received 20 and 40 mg/kg hHY6-F9. Treatment effects were examined for at least 4 consecutive sessions and until the number of fentanyl infusions obtained was within 20% of baseline for 3 consecutive sessions. Additional control experiments confirmed there was no interaction between morning and afternoon sessions (*Supplemental methods 1.e and results 2.b*).

Self-administration data analyses

The primary dependent variable was the number of infusions obtained. An unpaired, two-tailed t-test compared the number of fentanyl infusions obtained in the first 4 sessions following treatment to baseline for individual monkeys. A one-way ANOVA and Sidak multiple comparisons *post hoc* analysis compared saline infusions obtained the day before vehicle or hHY6-F9 treatment, and the effects of vehicle and hHY6-F9 or naloxone on fentanyl, heroin, or cocaine infusions obtained the first 4 days after treatment at the group level; naloxone was administered (i.v.) 15 min before a morning fentanyl session for four consecutive days. Unless otherwise indicated, data analyses were performed using GraphPad Prism (GraphPad Software, LLC, San Diego, CA).

Experiment 2: ventilation general procedure

A plexiglass plethysmography helmet (*Supplemental methods 1.f.*; Plas-Laboratories, Lansing, MI) was placed over the head with pressure changes in the helmet measured continuously and transformed by Ponemah® software (Data Sciences International, St. Paul, MN) to ventilatory rate (*f*) and tidal volume (V_T); minute volume (V_E) is $f * V_T$. The first 30 min of each session was a habituation period with the mean V_E of the last 5 min being the baseline value for that session. Sessions were 45 and 60 min long in Experiment 2.1 and 2.2, respectively.

Experiment 2.1: reversal of fentanyl-induced ventilatory depression

Saline or fentanyl was administered and the smallest dose of fentanyl that reliably decreased V_E was identified for each monkey. The effects of saline were considered stable when

V_E values 3–5 min post-infusion were within ± 1 SD from the baseline of that session, for at least 2 sessions. The effects of fentanyl were considered stable when V_E (mean, 3–5 min post-infusion) was decreased by ≥30% from baseline, for at least 2 sessions. Subsequently, fentanyl was administered followed by PBS (2 mL; control), naloxone (32 µg/kg), or hHY6-F9 (20 mg/kg); a dose of 32 µg/kg naloxone has been shown to reliably reverse ventilatory depression induced by *mu*-opioid receptor agonists (e.g., Gerak et al. 2018). PBS or naloxone was administered 5 min and hHY6-F9 8 min after fentanyl. hHY6-F9 was aseptically filtered and prepared for administration only after it was confirmed that fentanyl decreased V_E by ≥30%, thereby imposing a short delay between assessing breathing following fentanyl administration and administering hHY6-F9. Sessions occurred twice weekly with at least 3 days between tests to reduce the possibility of tolerance to fentanyl. Sessions in which only saline was given were conducted at least every other week. To determine the time-course of effects of hHY6-F9, monkeys received fentanyl, followed by PBS, 3 days and one week after hHY6-F9 treatment, then every other week, for at least 5 weeks.

Experiment 2.2: prevention of fentanyl-induced ventilatory depression

Sessions occurred twice weekly, alternating between saline and fentanyl, and included the habituation period followed by three 15-minute cycles. Saline or fentanyl was administered in the first minute of each cycle. The fentanyl dose increased by 0.25–0.5 log units each cycle to generate a dose-effect curve; dosing terminated when V_E (mean, 3–5 min post-infusion) decreased by ≥30% from baseline. For each monkey, the smallest cumulative dose of fentanyl that decreased V_E by ≥30% from baseline was identified, and dose-effect curves were redetermined. hHY6-F9 (20 or 40 mg/kg) was administered and fentanyl dose-effect curves redetermined 24 h later, then weekly; the dose range of fentanyl was adjusted downward by 0.25 log units per test as sensitivity to the ventilatory depressant effects of fentanyl recovered. Sensitivity to fentanyl was fully recovered when the fentanyl ED_{70} value of V_E (derived by calculating the slopes and y-intercepts of the fentanyl dose-effect curves) was within ± 2 SD of the fentanyl ED_{70} value determined before hHY6-F9 treatment for 2 of 3 consecutive sessions, or if a linear regression indicated no significant difference between the y-intercepts of the pre- and post-treatment fentanyl dose-effect curves.

Ventilation data analyses

The primary dependent variable was V_E, expressed as a percent change from baseline (%V_E); data analyses details for *f* and V_T are in *Supplemental methods 1.f*. Data are presented for the first 25 min of a 45-minute session (Fig. 3). Following administration of fentanyl and for each monkey, the mean %V_E 3–5 min before PBS, naloxone, or hHY6-F9 was compared to the mean %V_E 3–5 min post-treatment with a one-way ANOVA and Tukey's multiple comparisons *post hoc* analysis; the effects of PBS on V_E were determined twice and are presented as the mean and standard error, whereas naloxone and hHY6-F9 were tested once. Also for each monkey, the time course of the effects of hHY6-F9 on V_E (mean, 3–5 min post-PBS) days 0–41 after treatment was compared to control (fentanyl followed by PBS) with a one-way ANOVA and Dunnett multiple comparisons *post hoc* analysis. In Experiment 2.2, a repeated measures, mixed-effects two-way ANOVA and Dunnett multiple comparisons *post hoc* analysis compared pre- and post-treatment ED_{70} values for each hHY6-F9 dose; a Sidak multiple comparisons *post hoc* analysis compared ED_{70} values between hHY6-F9 doses at each time point. To normalize data among monkeys for statistical comparison, potency ratios for fentanyl were calculated at all time points after hHY6-F9 by dividing the post-treatment (anti-log transformed) ED_{70} value by the baseline ED_{70} value. The 95% confidence limit around the mean potency ratio for the group was considered significant if the confidence limit did not encompass 1.0. Potency ratios were calculated using Microsoft Excel® 2024 (Redmon, WA).

Serum hHY6-F9 concentrations

Serum hHY6-F9 concentrations from whole blood collected in Experiments 2.1 and 2.2 (see *Supplemental methods 1.g* for sampling details) were assessed using an ELISA assay (Pravetoni et al. 2012). The 96-well ELISA plates (Costar®, Corning, Corning, NY) were coated with 5 ng/well Bovine Serum Albumin (BSA) conjugate to F_1 hapten or unconjugated BSA control in carbonate buffer (pH 9.6) overnight at 4° C. Plates were washed between steps with PBS with 0.05% Tween 20 (Thermo Fisher Scientific, Waltham, MA), then blocked with 0.5% fish gelatin (Sigma Aldrich, St. Louis, MO) for 1 h at room temperature.

To quantify serum hHY6-F9 concentrations, known hHY6-F9 concentrations (0.02–1000 ng/mL) were added alongside serum samples, which were serially diluted starting at 1:100, and incubated for 1 h at room temperature. Concentrations of hHY6-F9 were detected with a secondary antibody (goat anti-human IgG (H+L)-horseradish peroxidase; Alpha Diagnostics, San Antonio, TX). Reactivity levels were visualized with 3,3,5,5 tetramethylbenzidine substrate (Life Technologies, Carlsbad, CA) for 10 min, followed by adding 1 N hydrochloric acid. Absorbances were read at OD_{450}. ED_{50} values of hHY6-F9 concentrations

were calculated using a four-parameter logistic regression of absorbance curves, and the concentration of hHY6-F9 in individual samples was calculated using the ED_{50} value from the standard curve.

Serum fentanyl concentrations

Serum fentanyl and norfentanyl concentrations from whole blood collected in Experiments 2.1 and 2.2 were quantified with LC-MS/MS; serum naloxone concentrations were quantified for Experiment 2.1. Liquid chromatography was conducted using an Agilent 1290 Infinity II system (Agilent, Santa Clara, CA) coupled with a Poroshell 120 EC-C18 column. An Agilent 6470 ESI triple quadrupole mass spectrometer was used for detection, with analyte concentrations determined through mass hunter quantitation software (Agilent). Calibration curves ranged from 5 to 1000 ng/mL, with internal standard solutions prepared at 100 ng/mL. Sample preparation involved protein precipitation from plasma using acetonitrile, followed by centrifugation and evaporation of solvents. The resulting solution was diluted and transferred to extraction cartridges for purification (sampling and LC-MS/MS details are in *Supplemental methods 1.h.*).

ELISA and LC-MS/MS data analyses

The primary dependent variables were serum hHY6-F9 concentrations in ELISA analyses and serum fentanyl and norfentanyl concentrations in LC-MS/MS analyses; serum naloxone concentration analyses are in *Supplemental methods 1.f.* In Experiment 2.1 and for each variable, the effects of hHY6-F9 at each time point were compared to control (fentanyl followed by PBS) with a one-way ANOVA and Dunnett multiple comparisons *post hoc* analysis. In Experiment 2.2 and for each variable, pre- and post-treatment effects with each hHY6-F9 dose, and between hHY6-F9 doses, were compared with a repeated measures, mixed-effects two-way ANOVA and Tukey's multiple comparisons *post hoc* analysis. Using a linear trapezoidal method, a non-compartmental analysis was performed with the PKSolver 2.0 plugin for Microsoft Excel (Zhang et al. 2010) to calculate $t_{1/2}$ and C_0 from serum hHY6-F9 concentrations analyzed for Experiment 2.2.

Results

Experiment 1

Baseline self-administration of fentanyl, heroin, and cocaine is shown in *Supplemental results 2.b* and Figure S3. The unit dose of fentanyl yielding the highest number of infusions varied among monkeys (IR, 1.0 µg/kg/infusion; KI and MA, 0.32 µg/kg/infusion; HA, 0.1 µg/kg/infusion), whereas all monkeys obtained a high number of infusions with 3.2 and 32 µg/kg/infusion of heroin and cocaine, respectively. All monkeys received 10 mg/kg hHY6-F9; because 10 mg/kg hHY6-F9 did not affect fentanyl self-administration in monkey IR, that monkey was also tested with 20 and 40 mg/kg hHY6-F9 with 3.5–5.5 weeks between treatments. Fentanyl self-administration was modestly decreased in IR after treatment with 40 mg/kg hHY6-F9 ($p = 0.059$; Figs. 1 and 2d). For the group, hHY6-F9 significantly decreased fentanyl infusions obtained ($F_{(8, 24)} = 21.06$, $p < 0.0001$; mean ± 1 SEM; 11.6 ± 3.9) in the 4 sessions after treatment, compared with vehicle (23.4 ± 2.5; $p < 0.005$; Fig. 1). There was no significant effect of vehicle or hHY6-F9 on the number of heroin (after vehicle: 22.0 ± 2.8; after hHY6-F9: 24.5 ± 2.1) or cocaine (after vehicle: 24.8 ± 2.1; after hHY6-F9: 24.6 ± 1.6) infusions obtained (Figs. 1 and 2). Because the duration of effect of hHY6-F9 on fentanyl self-administration varied among monkeys, from 8 to 36 days, the number of fentanyl infusions obtained varied from 230 to 644 and total fentanyl intake varied from 190 to 2350 µg (Fig. 2). Neither heroin nor cocaine self-administration was significantly altered in any monkey after hHY6-F9 treatment. A dose of 0.1 mg/kg naloxone administered prior to a morning session when fentanyl was available for self-administration significantly decreased the number of fentanyl infusions received ($p < 0.0001$; Fig. 1 and Table S1) without affecting self-administration of heroin in the afternoon session that day (Table S1).

Experiment 2.1

The dose of fentanyl that significantly decreased V_E varied among monkeys, (5.6–23.7 µg/kg; Fig. 3) producing an average (± SEM) reduction to 54.3 (7.0)%. Figure 3 shows the effects of PBS, naloxone, and hHY6-F9 on fentanyl-suppressed V_E. For each monkey, there was a significant main effect of treatment (OR: $F_{(5, 12)} = 64.02$; GA: $F_{(5, 12)} = 59.78$; KA: $F_{(5, 12)} = 104.90$; LA: $F_{(5, 12)} = 34.27$; $p < 0.0001$ for all monkeys). PBS had no effect on fentanyl, whereas naloxone significantly increased (reversed) fentanyl-suppressed V_E ($p < 0.01$). hHY6-F9 significantly increased fentanyl-suppressed V_E in three monkeys (OR, GA, KA: $p < 0.0001$). In the fourth monkey (LA), V_E gradually increased to ~10% below baseline 18 min after treatment. When monkeys were subsequently tested with fentanyl (Fig. 3.e), there was a significant main effect of hHY6-F9 in 3 monkeys (OR: $F_{(6, 14)} = 7.77$; GA: $F_{(6, 14)} = 43.23$; KA: $F_{(6, 14)} = 96.71$; $p < 0.001$). hHY6-F9 significantly attenuated the effects of fentanyl for 13–41

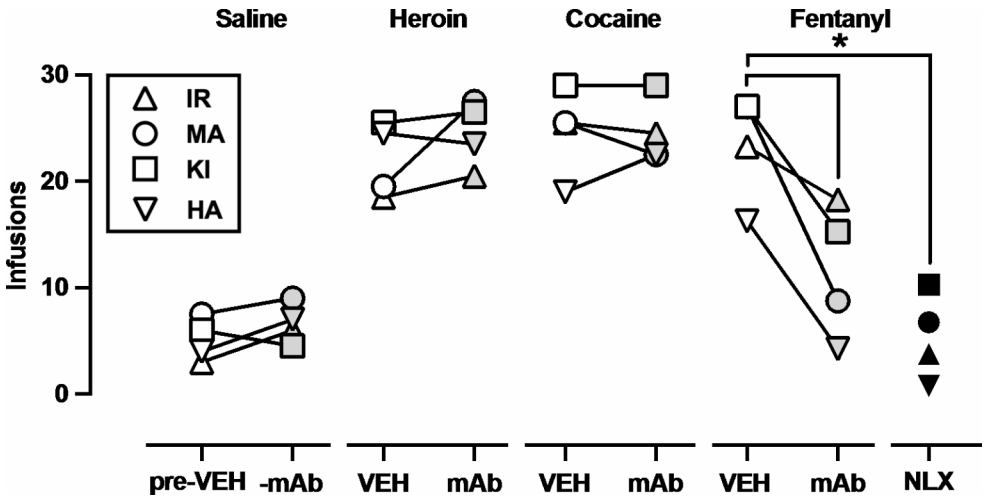

Fig. 1 The mean number of infusions obtained prior to and following treatment with vehicle (VEH, open symbols), hHY6-F9 (mAb, shaded symbols; IR, 40 mg/kg; MA, KI, HA, 10 mg/kg), or naloxone (NLX, black symbols; 0.1 mg/kg) for individual monkeys. Saline data represent the mean number of saline infusions obtained in the morning and afternoon sessions the day prior to vehicle or hHY6-F9 treatment (pre-VEH or -mAb). Heroin (3.2 µg/kg/infusion) and cocaine (32 µg/kg/infusion) data represent the mean of the first two afternoon sessions when each drug was available, and fentanyl (IR, 1.0 µg/kg/infusion; KI and MA, 0.32 µg/kg/infusion; HA, 0.1 µg/kg/infusion) data represent the mean of the first four fentanyl sessions. *$p<0.05$

days in three monkeys (OR, GA, KA; $p<0.005$). There was no effect of hHY6-F9 on fentanyl in monkey LA at any time after treatment. The effects of fentanyl, PBS, naloxone, and hHY6-F9 on *f* and V_T are in *Supplemental methods 1.f*, *results 2.c*, and Figures S4-S5.

Serum concentrations of hHY6-F9, fentanyl, and norfentanyl are in Fig. 4.a-c and of naloxone are in *Supplemental results 2.c* and Figure S6. After treatment, hHY6-F9 concentrations increased significantly ($F_{(6,\ 21)}=81.33$, $p<0.005$) from (mean [± SD]) 0.2 (0.5) to 717 (141.7) µg/mL and remained increased for 7 days. Similarly, the day of treatment with hHY6-F9 and fentanyl, serum concentrations of fentanyl (35.8 ± 20.6 to 3111.6 ± 1725.7 ng/mL; $F_{(6,\ 21)}=8.61$; $p<0.0001$) and norfentanyl (3.5 ± 1.7 to 32.7 ± 18.0 ng/mL; $F_{(6,\ 21)}=4.57$; $p<0.05$) were significantly increased with norfentanyl concentrations being significantly increased for 3 days ($p<0.05$).

Experiment 2.2

Individual ED_{70} values before and after treatment with 20 and 40 mg/kg hHY6-F9 are in Supplemental Table S2, and the initial effects of hHY6-F9 on V_E and potency ratios are in Fig. 5. The potency of fentanyl to decrease V_E varied among monkeys, with ED_{70} values (mean [± SD)] ranging from 0.9 (1.6) to 23.6 (1.6) µg/kg before treatment with 20 mg/kg hHY6-F9; when fentanyl dose-effect curves were redetermined before treatment with 40 mg/kg hHY6-F9, ED_{70} values ranged from 1.4 (1.2) to 27.0 (1.0) µg/kg. Twenty-four hours after receiving 20 mg/kg hHY6-F9, the fentanyl dose-effect curves were shifted (mean [± SD]) 3.46 (0.5)-fold rightward in three monkeys (GA, KA, LA), reflecting a decrease in the potency of fentanyl to suppress V_E. In monkey OR, who was the most sensitive to fentanyl before treatment (ED_{70}: 0.9 ± 1.6 µg/kg), hHY6-F9 shifted the fentanyl dose-effect curve 13.6-fold rightward. In monkey MU, the largest cumulative dose of fentanyl given 24 h after hHY6-F6 did not decrease V_E by ≥30%, precluding the determination of an ED_{70} value. Excluding monkey MU, the ED_{70} values 24 h following hHY6-F9 ranged from 12.3 to 92.5 µg/kg; sensitivity to the effects of fentanyl on V_E returned to control in 3–4 weeks. Similarly, 40 mg/kg hHY6-F9 shifted the fentanyl dose-effect curves for GA, KA, LA and MU an average of 4.43 (2.7)-fold rightward 24 h following treatment; monkey OR remained the most sensitive to fentanyl with 40 mg/kg hHY6-F9 shifting her dose-effect curve 18.8-fold rightward. Analysis of ED_{70} values indicated a significant main effect of time ($F_{(1.26,\ 5.02)}=7.35$, $p=0.039$). There were no significant differences between ED_{70} values after either hHY6-F9 dose, nor any difference between doses, at any time point (that might be impacted by an inability to derive ED_{70} values for some hHY6-F9 tests when fentanyl did not decrease V_E by ≥30%). The 95% confidence limits calculated for the mean potency ratios at each pre- and post-hHY6-F9 time point, and for each hHY6-F9 dose, were not significant, apart from days 8 and 15 after treatment with 20 mg/kg hHY6-F9, likely due to the potency differences among monkeys.

Figure 4.d-f shows the effects of 20 and 40 mg/kg hHY6-F9 on serum hHY6-F9, fentanyl, and norfentanyl

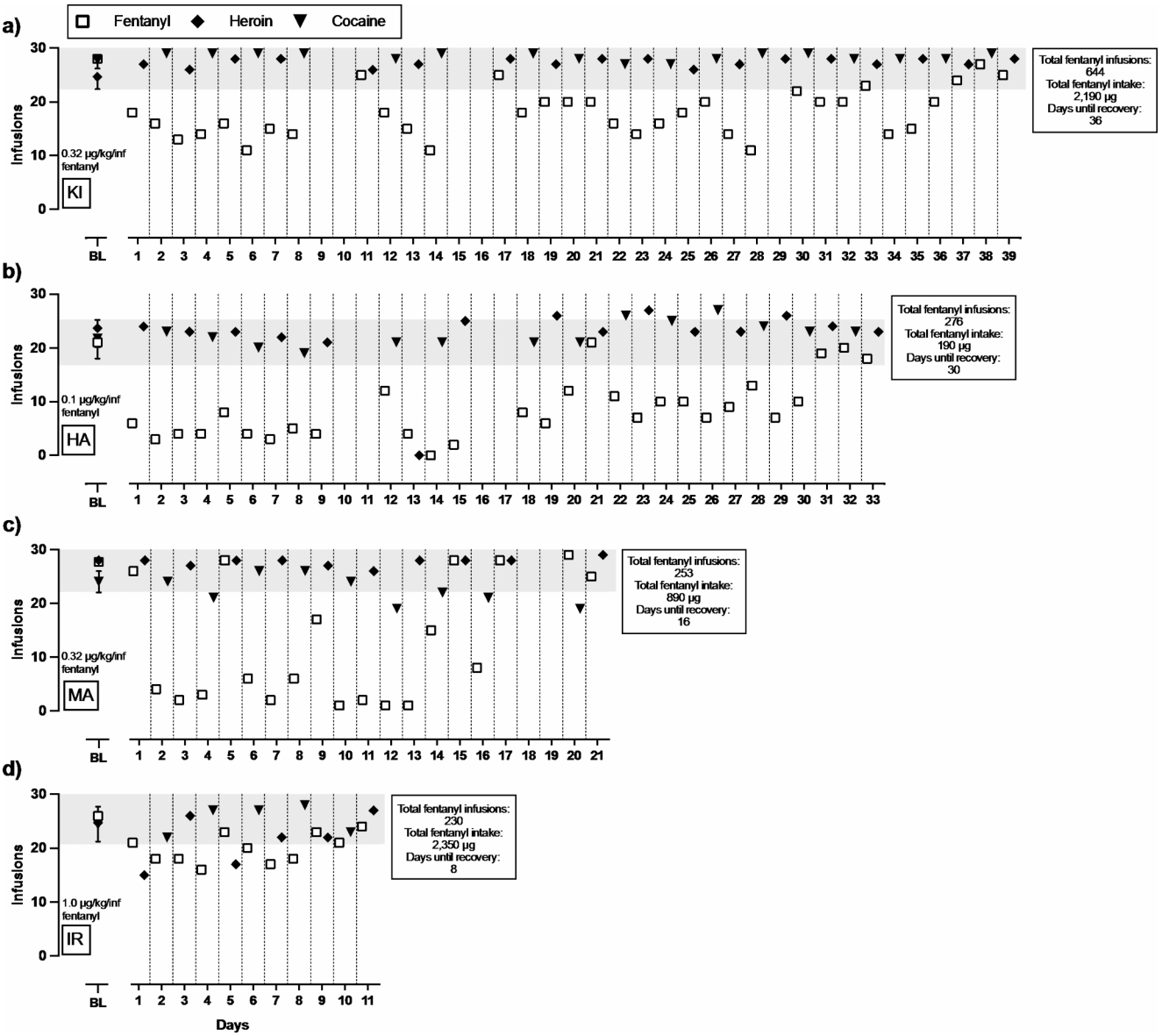

Fig. 2 Effects of hHY6-F9 on fentanyl, heroin, and cocaine self-administration compared to baseline (BL; error bars represent ± 1 SD from the mean). hHY6-F9 (IR, 40 mg/kg; MA, KI, HA, 10 mg/kg) was administered (i.v.) 15 min prior to the morning fentanyl session on day 1. The unit dose of fentanyl self-administered is indicated in each panel (IR, 1.0 µg/kg/infusion; KI and MA, 0.32 µg/kg/infusion; HA, 0.1 µg/kg/infusion); all monkeys self-administered 3.2 µg/kg/infusion heroin and 32 µg/kg/infusion cocaine. Fentanyl self-administration was considered having recovered when the number of infusions obtained was within ± 20% from baseline (shaded area) for three consecutive sessions; days until fentanyl recovered and total fentanyl intake and fentanyl infusions obtained over that time are indicated for each monkey. Missing data points are attributed to laboratory closures

concentrations. There was a significant interaction ($F_{(4, 15)} = 24.59$, $p < 0.0001$) between dose ($F_{(1, 4)} = 81.47$, $p = 0.0008$) and time ($F_{(4, 16)} = 82.86$, $p < 0.0001$) on hHY6-F9 concentrations. There was no difference in hHY6-F9 concentrations before administering 20 or 40 mg/kg hHY6-F9 (mean ± 1 SD; 1.1 ± 0.7 or 10.2 ± 9.6 µg/mL, respectively). Twenty-four hours after treatment, both doses of hHY6-F9 significantly increased hHY6-F9 concentrations ($p < 0.05$); however, 40 mg/kg hHY6-F9 produced a ~ 2-fold greater increase (1476.5 ± 367.3 µg/mL; $p < 0.05$) compared with 20 mg/kg hHY6-F9 (661.8 ± 92.5 µg/mL). One week later, concentrations remained significantly increased and differed between doses (20 mg/kg: 230.3 ± 43.8 µg/mL; 40 mg/kg: 546.2 ± 234.5 µg/mL; $p < 0.05$). Similarly, there was a significant interaction ($F_{(4, 15)} = 5.28$, $p = 0.0074$) between hHY6-F9 doses ($F_{(1, 4)} = 7.77$, $p = 0.0494$) and time ($F_{(4, 16)} = 24.50$, $p < 0.0001$) on serum fentanyl concentrations. Fentanyl concentrations were significantly increased one day after treatment with 20 (2365.1 ± 1457.9 ng/mL) or 40 mg/

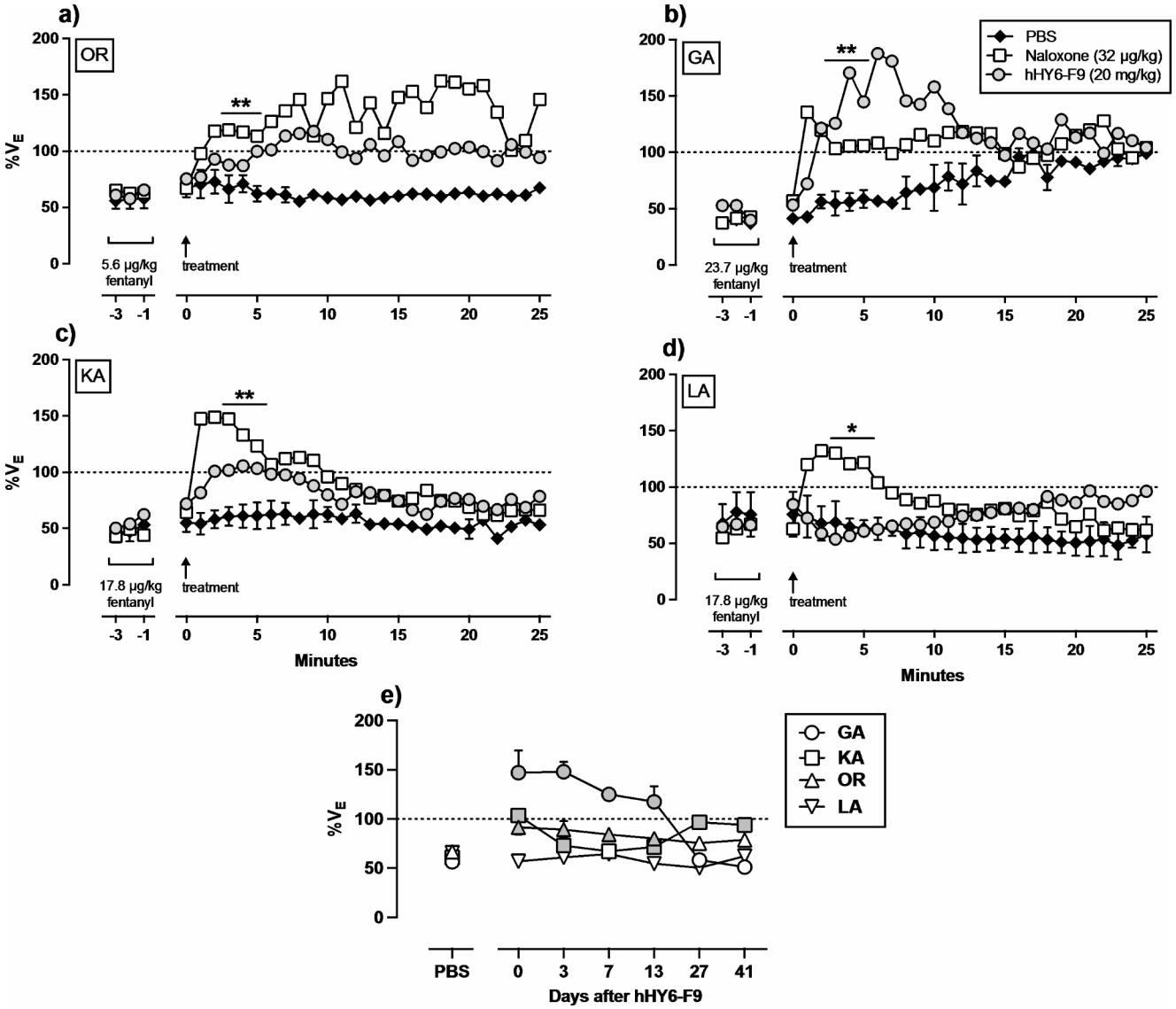

Fig. 3 Experiment 2.1 (reversal): (Panel a-d) The effects of on minute volume (V_E), expressed as a percentage of baseline (%V_E; dashed line) representing V_E in the 3 min immediately before treatment with vehicle (phosphate buffered saline, PBS), naloxone (32 µg/kg), or hHY6-F9 (20 mg/kg). The dose of fentanyl administered is indicated in each panel (OR, 5.6 µg/kg; GA, 23.7 µg/kg; KA and LA, 17.8 µg/kg). Asterisks indicate that the effects of **naloxone and hHY6-F9 on %V_E (mean, 3–5 min post-infusion) were both significantly ($p<0.05$) different from PBS (twice determined; error bars represent ± SEM), or the effects of *only naloxone were significantly ($p<0.05$) different from PBS. (Panel e) The time-course of effects of hHY6-F9 on the effects of fentanyl on V_E. hHY6-F9 was administered after fentanyl (day 0); in subsequent sessions (days 3–41), PBS vehicle was administered after fentanyl. Shaded symbols indicate %V_E (mean ± SD, 3–5 min post-infusion) was significantly ($p<0.05$) greater than control

kg hHY6-F9 (4289.4 ± 1168.6 ng/mL), compared to baseline (55.9 ± 95.6 ng/mL); fentanyl concentrations remained significantly increased one week after 40 mg/kg hHY6-F9 (1934.6 ± 1500.2 ng/mL; $p<0.005$). There was a dose-dependent difference 1 and 8 days after treatment ($p<0.005$). Moreover, there was significant interaction ($F_{(4, 15)}=3.18$, $p=0.044$) and main effect of time ($F_{(4, 16)}=12.69$, $p<0.0001$) with serum norfentanyl concentrations increasing one day after receiving 20 mg/kg hHY6-F9 (21.7 ± 12.1 ng/mL), compared to baseline (1.8 ± 1.7 ng/mL), and one day (34.5 ± 17.1 ng/mL) and 8 days (26.0 ± 22.6 ng/mL) after treatment with 40 mg/kg hHY6-F9 ($p<0.0003$). There was a significant dose-dependent difference in norfentanyl concentrations 1 and 8 days after treatment ($p<0.05$). A non-compartmental analysis of serum hHY6-F9 concentrations showed that the mean (± SD) $t_{1/2}$ was 7.5 (± 2.7) or 7.9 (± 1.4) days following treatment with 20 or 40 mg/kg hHY6-F9, respectively; C_0 was 662 (± 92) or 1436 (± 331) µg/mL, and thereby proportional to hHY6-F9 dose.

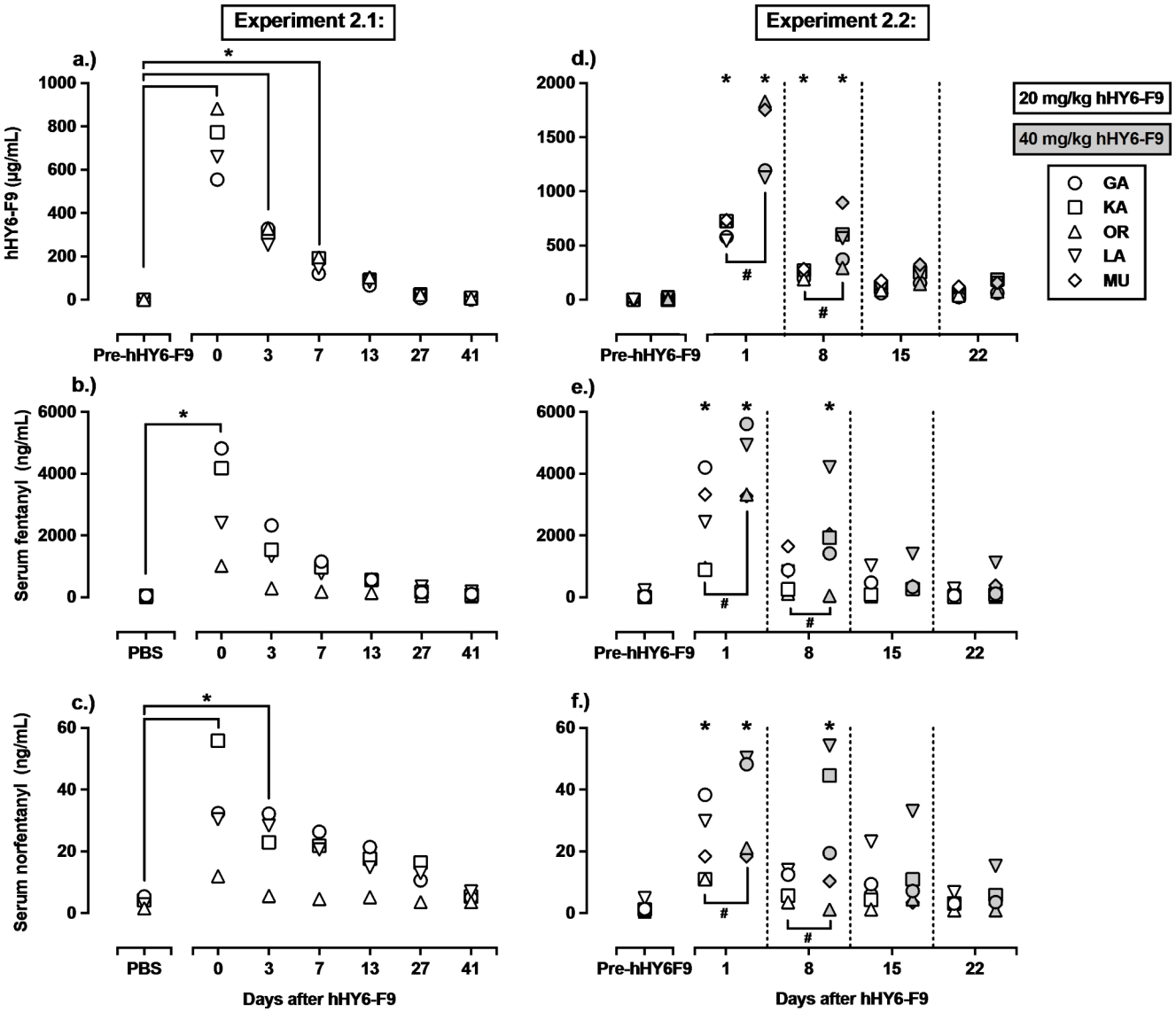

Fig. 4 Serum hHY6-F9, fentanyl, and norfentanyl concentrations for experiments 2.1 (reversal) and 2.2 (prevention). Symbols represent individual monkeys; monkey MU did not participate in Experiment 2.1. For Experiment 2.1: Serum hHY6-F9 concentrations (**a**) prior to (pre-hHY6-F9) and following hHY6-F9 treatment (20 mg/kg; administered on day 0) are shown. Fentanyl (**b**) and norfentanyl (**c**) concentrations are shown following a control session (fentanyl followed by PBS), the day of hHY6-F9 treatment (day 0), and on days 3–41. Blood specimens were collected 60 min after fentanyl was administered; the fentanyl dose varied among monkeys (5.6–23.7 µg/kg, *Methods*). *$p<0.05$. For experiment 2.2: Serum hHY6-F9 (**a**), fentanyl (**b**), and norfentanyl (**c**) concentrations before and after administration of 20 (open symbols) and 40 mg/kg (shaded symbols) hHY6-F9. Blood specimens were collected 60 min after the first dose of fentanyl was administered; the fentanyl dose varied among monkeys and time points (*Methods*). Blood was inadvertently not collected for monkey KA (squares) 24 h after receiving 40 mg/kg hHY6-F9. *Indicates a significant ($p<0.05$) difference between the pre- and post-hHY6-F9 treatment values. $^{\#}$indicates a significant ($p<0.05$) difference between hHY6-F9 doses

Discussion

This study examined the pharmacodynamic and pharmacokinetic properties of the fentanyl-targeting mAb hHY6-F9. Due to their rapid onset of action and potentially long half-life, fentanyl-targeting mAbs could be an alternative or adjuvant treatment for OUD, specifically for preventing relapse and overdose. The present study reports the selectivity, magnitude of antagonism, and time-course of effects of hHY6-F9 using translational i.v. self-administration and ventilation procedures in rhesus macaques.

Murine and chimeric HY6-F9 were shown to have nanomolar affinity for fentanyl and norfentanyl (Hicks et al. 2022). Because murine and chimeric mAbs can evoke immunogenicity in NHPs and humans, HY6-F9 was humanized (i.e., hHY6-F9) and maintained nanomolar affinity for

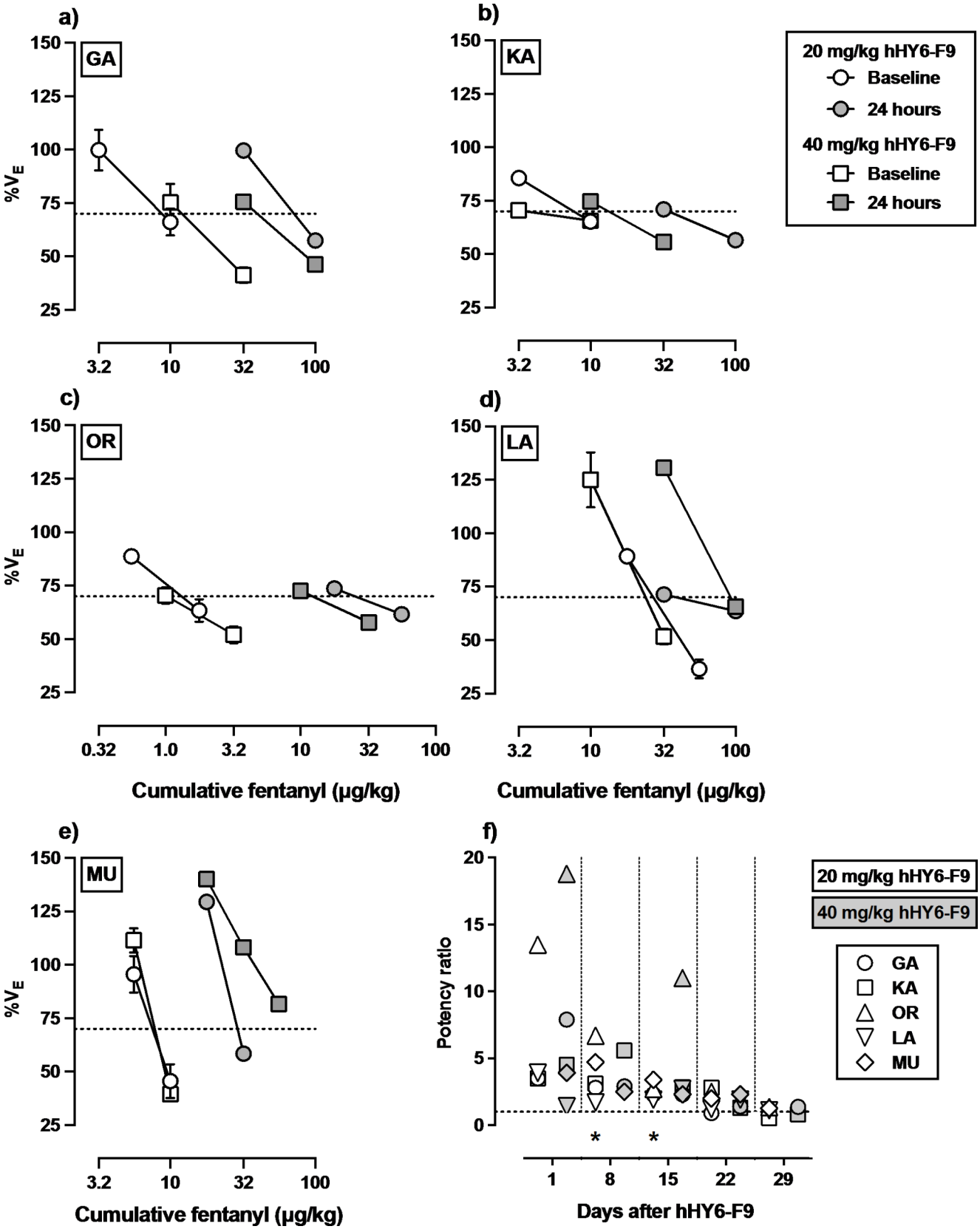
a)
GA
b)
KA
20 mg/kg hHY6-F9
Baseline
24 hours
40 mg/kg hHY6-F9
Baseline
24 hours
c)
OR
d)
LA
%V_E
150
125
100
75
50
25
3.2
10
32
100
0.32
1.0
Cumulative fentanyl (µg/kg)
e)
MU
f)
Potency ratio
20
15
10
5
0
*
1
8
15
22
29
Days after hHY6-F9
20 mg/kg hHY6-F9
40 mg/kg hHY6-F9
GA
KA
OR
LA
MU

Fig. 5 Experiment 2.2 (prevention): (Panels a-e) The potency of fentanyl to decrease minute volume, expressed as a percentage of baseline ($\%V_E$), by ≥30% (dashed line) in individual monkeys prior to (baseline, open symbols) and 24 h (shaded symbols) following administration of 20 (circles) or 40 mg/kg (squares) hHY6-F9. Baseline fentanyl dose-effect curves were determined at least twice prior to hHY6-F9; therefore, error bars represent ±SEM of the mean. (Panel f) Potency ratios, calculated by dividing the ED_{70} for fentanyl following treatment with 20 (open symbols) or 40 mg/kg (shaded symbols) hHY6-F9 in individual monkeys and at each time point after hHY6-F9 treatment. *Confidence limits not encompassing 1.0 indicate a significant difference compared to baseline in the potency of fentanyl at the group level (horizontal dashed line); potency ratios could not be calculated for some monkeys at some time points (see *Results*)

fentanyl (7 nM; *Supplemental results*; Hicks et al. 2022). However, neither the selectivity of hHY6-F9 in vivo nor its effects on fentanyl self-administration had been examined. This study demonstrates that a single administration of hHY6-F9 attenuates fentanyl but not heroin of cocaine self-administration. The magnitude and duration of effect varied among individual monkeys, and those differences are likely relative to the mAb: fentanyl ratio. Studies showing dose-dependent effects of mAbs support the notion that their effectiveness can be contingent on the mAb: fentanyl ratio (Baehr 2022b, Khaimraj et al. 2024). For example, in Khaimraj et al. (2024), a 2-fold molar excess of mAb (40 mg/kg HY6-F9) to an 11-fold molar excess of fentanyl (2 mg/kg HY6-F9) ratio relative to 0.1 mg/kg fentanyl, dose-dependently reduced fentanyl distribution to the brain in mice. Similarly, 10 and 40 mg/kg of the mAb CSX-1004 dose-dependently blocked the effects of fentanyl on food-maintained responding in squirrel monkeys, shifting the dose-effect curve 4.8- and 15.8-fold rightward, respectively (Bremer et al. 2023). In the present study, 40 mg/kg of hHY6-F9 modestly decreased fentanyl self-administration in the monkey (IR) self-administering the largest unit dose of fentanyl (1.0 µg/kg/infusion) for less than one week. In contrast, 10 mg/kg hHY6-F9 decreased self-administration of smaller unit doses of fentanyl (0.1–0.32 µg/kg/infusion) for ~2 weeks. The number of days until fentanyl self-administration was considered recovered was determined using a relatively conservative criterion that does not reflect the gradual return to baseline, as shown in Fig. 3. Nevertheless, these results are consistent with the notion that the effects of a mAb are related to the mAb: fentanyl ratio.

The pharmacokinetics of fentanyl might also contribute to the variability observed in this study. Fentanyl and its metabolite, norfentanyl, can accumulate peripherally and be redistributed back into the serum (Palmer 2010; Schneider and Brune 1985, 1986). Fentanyl was self-administered nearly daily for several months before hHY6-F9 treatment, albeit in small unit doses. Therefore, serum fentanyl concentrations might not be due exclusively to fentanyl intake during a single session. While having some off-target binding to norfentanyl, hHY6-F9 has > 10-fold greater affinity for fentanyl compared with norfentanyl (Supplemental Figure S1b). Moreover, the concentration of norfentanyl cannot exceed that of fentanyl, and the pharmacokinetic data reported herein show substantially lower serum concentrations of norfentanyl compared with fentanyl. It is also possible there is a gradual dissociation of the mAb-fentanyl complex that impacts the effects of hHY6-F9. This type of "regeneration" of unbound mAbs is hypothesized to extend the binding capacity of a methamphetamine-targeting mAb, ch-mAb7F9 (Stevens et al. 2014). Finally, the pharmacokinetics of hHY6-F9, bound or unbound, are not well understood; however, it is hypothesized to be excreted or catabolized similarly to many therapeutic mAbs (Ryman and Meibohm 2017).

The present study extends findings in rats (Baehr 2022b; Hicks et al. 2022) and demonstrates that, like naloxone, hHY6-F9 rapidly reverses the ventilatory depressant effects of fentanyl (in three monkeys). hHY6-F9 gradually increased V_E towards baseline in monkey LA; although not tested, a larger hHY6-F9 dose might be more effective in LA. Variability in the effects of the mAb among monkeys is unsurprising and might predict similar variability in humans, as is the case with some medications. While naloxone increased ventilation to ~20–50% above baseline in all monkeys, a similar increase was observed with hHY6-F9 only in monkey GA. Naloxone can stimulate the sympathetic nervous system, cause cardiovascular instability, and increase respiration (Feria et al. 1990; Kanof et al. 1991; Mills et al. 1988; Rzasa Lynn and Galinkin 2018). That hHY6-F9 might not cause sympathetic arousal could enhance its potential utility as a reversal agent, although further studies are needed and there are challenges and likely limitations to using mAbs as reversal agents (see below). Studies on storage conditions (e.g., room temperature) are needed to further assess the feasibility of using mAbs in various real-world settings. mAbs are typically stored frozen or refrigerated, which might not be practical for some indications (e.g., rescue from overdose). Alternative routes of administration must also be investigated. Some therapeutic mAbs for other indications are given subcutaneously or intramuscularly (Kosten and Owens 2005), and intranasal administration of a mAb therapy for COVID-19 patients was recently approved (Moreira et al. 2021), suggesting these routes could be considered for hHY6-F9. In general, intravenous administration is not practical for rescuing overdose victims.

A single administration of hHY6-F9 protected against fentanyl-induced ventilatory depression for ≥2 weeks in some monkeys. Analyses of serum hHY6-F9, fentanyl, and norfentanyl concentrations are consistent with the extended duration of hHY6-F9 in sequestering fentanyl. The magnitude of antagonism of two hHY6-F9 doses was also

characterized. hHY6-F9 (20 and 40 mg/kg) decreased the potency of fentanyl by ~3.5- and 4.5-fold, respectively, with some monkeys receiving cumulative fentanyl doses as large as 100 µg/kg without a significant decrease in ventilation. A non-compartmental analysis indicated that both hHY6-F9 doses had a mean half-life of ~7.6 days and protected from the effects of fentanyl for 2–3 weeks. Statistical analyses did not identify a dose-dependent effect for magnitude of antagonism despite significant differences in serum hHY6-F9 concentrations. This lack of dose relatedness might result from variability in the sensitivity of monkeys to fentanyl. Doses of 10 and 40 mg/kg of the mAb CSX-1004 shifted the fentanyl dose-effect curve dose-dependently for ventilation (5- and 14-fold rightward, respectively); however, the 4-fold dose range in that study was larger than the dose range in the current study (Bremer et al. 2023).

There are potential challenges regarding the utility of mAbs for treating OUD (i.e., preventing relapse and overdose) or reversing overdose, including the following: (1) patients might consume greater amounts of fentanyl in an attempt to overcome the effects of the mAb; (2) because many individuals consume drug mixtures, the effectiveness of a mAb could be limited due to its selectivity (e.g., for fentanyl); (3) selectively blocking the reinforcing effects of fentanyl could result in drug switching, whereby a person takes an opioid that the mAb cannot sequester (Martinez et al. 2023); (4) the dose of mAb would have to be sufficiently large to have a high probably of being effective in all or nearly all patients (e.g., it is unclear why hHY6-F9 was not effective in one monkey in this study); and (5) the need to administer the mAb intravenously would preclude its use as a overdose rescue agent and possibly limit its utility for other indications. Further studies on the magnitude of antagonism by hHY6-F9 could inform whether overcoming the effects of fentanyl is a legitimate concern. Similar concerns of overcoming MOR blockade have been raised for naltrexone, although it is unclear whether this is a significant impediment to treatment (Sullivan et al. 2013). The ability of hHY6-F9 to attenuate the effects of drug mixtures would likely depend on the ratio of fentanyl in the mixture – the greater the fentanyl ratio, the more effective hHY6-F9 will be. In addition to fentanyl, potent fentanyl analogs (e.g., carfentanil) are commonly found in drug mixtures. Although hHY6-F9 has limited affinity for some fentanyl analogs in vitro, it is possible that off-target binding of hHY6-F9 with fentanyl analogs could block or diminish their effects (Suzuki and El-Haddad 2017). Finally, although drug switching might be a legitimate concern in patients treated with hHY6-F9, its selectivity for fentanyl would preserve the therapeutic actions of buprenorphine and methadone, which can attenuate drug craving, drug taking, and relapse (Blanco and Volkow 2019). hHY6-F9 might enhance the therapeutic effects of buprenorphine or methadone by sequestering fentanyl in the serum, thereby increasing MOR occupancy by buprenorphine or methadone. hHY6-F9 could protect from overdose if a patient treated with buprenorphine or methadone used fentanyl. Preventing overdose for several weeks would be especially advantageous for this approach since resumption of drug taking increases risk of overdose (Sordo et al. 2017; Williams et al. 2020). Given its selectivity, hHY6-F9 might also be effective in patients treated with other MOR agonists for pain.

This study demonstrates that the fentanyl-targeting, humanized mAb hHY6-F9 selectively decreases fentanyl self-administration for ≥2 weeks, supporting the view that hHY6-F9, and possibly other fentanyl-targeting mAbs, might be effective for treating OUD. This study also shows that hHY6-F9 rapidly reverses the ventilatory depressant effects of fentanyl, with a single treatment protecting from potentially lethal doses of fentanyl for up to 3 weeks. Taken together, hHY6-F9 could be an effective standalone or adjuvant treatment for OUD and specifically for preventing relapse and overdose.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00213-025-06751-9.

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Author contributions Designed experiments: Galbo-Thomma, Hiranita, Taylor, Maguire, Baehr, Pravetoni, France. Performed experiments: Galbo-Thomma, Marecki, Kim, Hiranita, Taylor, Hicks, Gebo, Khaimraj. Performed statistical analyses: Galbo-Thomma, Marecki, Kim, Hiranita, Hicks. Wrote or contributed to the writing of the manuscript: Galbo-Thomma, Marecki, Kim, Hiranita, Taylor, Maguire, Hicks, Baehr, Pravetoni, France.

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Data availability Source data for all figures and tables in the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The mAb described herein has been disclosed through patent applications (inventors: Pravetoni, Baehr, Hicks). M. Pravetoni is the founder of CounterX Therapeutics, Inc. Other authors have no financial conflicts or competing interests to declare.

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