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The Behavioral Effects of a Mixed Efficacy Antinociceptive Peptide, VRP26, Following Chronic Administration in Mice

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Abstract

Rationale—VRP26 displays mu opioid receptor agonist and delta opioid receptor antagonist activity *in vitro*, a pharmacological profile purported to produce reduced tolerance, dependence, and rewarding effects. We hypothesized that VRP26 would display reduced adverse effects after chronic administration as compared with the traditional opioid analgesic fentanyl.

Objective—To explore the development of tolerance, dependence and conditioned place preference of VRP26 as compared with the traditional opioid analgesic fentanyl.

Methods—The antinociceptive effects of VRP26 and fentanyl were assessed using the mouse warm water tail withdrawal (WWTW) assay. Measurement of antinociceptive tolerance and physical dependence occurred after seven days of continuous administration of either fentanyl (0.3 mg/kg/day) or VRP26 (10 mg/kg/day); tolerance was measured by a shift in the antinociceptive dose response curve in the WWTW assay. Physical dependence was determined by observation of withdrawal symptoms after precipitated withdrawal. Rewarding effects were measured by the ability of VRP26 or fentanyl to produce conditioned place preference.

Results—Fentanyl produced significant tolerance and dependence, as well as significant conditioned place preference. VRP26 produced neither tolerance nor physical dependence, nor did it produce significant conditioned place preference.

Conclusions—These results suggest that chronic treatment with VRP26 may produce less tolerance or physical dependence than chronic treatment with clinically available mu opioid analgesics such as fentanyl. Additionally, VRP26 produces less rewarding effects than fentanyl. This desirable *in vivo* profile may be due to the mixed efficacy nature of VRP26 and could provide the framework for safer opioid analgesics.

Keywords

Mixed efficacy; mu opioid receptor; delta opioid receptor; tolerance; dependence; conditioned place preference; reward

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Introduction

The growing consensus that the simultaneous modulation of multiple targets often generates a more desirable drug profile has opened new avenues of research in the development of therapeutic agents (Dietis et al. 2009; Morphy et al. 2004; Morphy and Rankovic 2009). This is of particular interest in the field of opioid analgesics, as the co-administration of a mu opioid receptor (MOR) agonist with a delta opioid receptor (DOR) antagonist has been proposed to produce MOR-mediated analgesia with reduced tolerance and dependence liabilities (Abdelhamid et al. 1991; Fundytus et al. 1995; Hepburn et al. 1997; Purington et al. 2009; Schiller 2009a). The development of antinociceptive tolerance often leads to dose escalation which may contribute to the prevalence of opioid misuse and abuse (Ballantyne and LaForge 2007). Additionally, individuals who are dependent on opioids may misuse or abuse opioids to prevent withdrawal (Bailey and Connor 2005; Ballantyne and LaForge 2007; Johnston et al. 2009; Ross and Peselow 2009). Opioid compounds that produce robust analgesia with limited development of tolerance and dependence would meet a significant unmet medical need by addressing these concerns.

For pharmacokinetic simplicity, it is advantageous to combine both MOR agonist and DOR antagonist pharmacophores into a single compound. As a result, ligands that interact simultaneously with both MOR and DOR have been the subject of much research and many peptide, peptide-like, and alkaloid ligands have been described; however, few have the desired profile both *in vitro* and *in vivo* (Anand et al. 2012; Anathan et al. 2004; Balboni et al. 2002; Bender et al. 2015; Healy et al. 2013; Purington et al. 2011; Salvadori et al. 1999; Schiller et al. 1999). The peptide DIPPψNH_2 (Schiller et al. 1999), the bivalent ligand MDAN-21 (Lenard et al. 2007), and the multifunctional opioid alkaloid UMB425 (Healy et al. 2013) all show some improvement over morphine *in vivo*, but both DIPPψNH_2 and UMB425 produce significant tolerance and dependence after chronic administration. Further, DIPPψNH_2 does not cross the blood brain barrier (Schiller 2009b) and MDAN-21 was effective in some (Aceto et al. 2012; Daniels et al. 2005), but not all (Aceto et al. 2012), measures of antinociception. While these compounds demonstrate proof of concept that MOR agonist/DOR antagonist compounds have a more desirable profile than traditional opioid analgesics, there is still room for improvement.

We have previously described a cyclic pentapeptide, VRP26 (Figure 1), which displays low nanomolar affinity for both MOR and DOR, with selectivity relative to KOR as well as agonist activity at MOR similar to morphine and antagonism at DOR *in vitro* (Mosberg et al. 2014). VRP26 produces dose-dependent antinociception in the warm water tail withdrawal (WWTW) assay in mice after peripheral administration and, more significantly, does not produce acute tolerance (Mosberg et al. 2014). VRP26 is therefore a good lead for further investigation of MOR agonist/DOR antagonist compounds as novel analgesics. In this report we explore the *in vivo* pharmacology of VRP26 to evaluate 1) its *in vivo* MOR agonist and DOR antagonist effects, 2) the development of tolerance and dependence after 7d continuous infusion of the lowest, fully effective, acute doses of fentanyl (0.3 mg/kg/day) or VRP26 (10 mg/kg/day), and 3) its rewarding effects.

MATERIALS AND METHODS

Peptide Synthesis—VRP26 (Dmt-c(SEtS)[DCys-Aci-DPen]-Ser(Glc)-NH_2) was synthesized as previously described (Mosberg et al. 2014).

Drug preparation—All compounds were administered by intraperitoneal (*ip)* or subcutaneous (*sc)* injection in a volume of 10 mL/kg of body weight. Fentanyl citrate (Sigma-Aldrich, St. Louis, MO, USA), VRP26 TFA, methylnaltrexone HBr (MNTX) (Sigma-Aldrich, St. Louis, MO, USA), ketamine HCl (Henry Schein, Wixom, MI, USA) and xylazine HCl (Akorn, Decator, IL, USA) were dissolved in sterile saline (0.9% NaCl *w/v*), SNC80 was dissolved in 3% (*v/v*) 1 M HCl and brought to volume with sterile water. Naltrindole HCl (NTI) (Tocris Biosciences, Minneapolis, MN, USA) and carprofen (Zoetis Inc., Kalamazoo, MI, USA) were prepared in sterile water.

Animals—Male and female C57BL/6 wild-type mice (n= 209) or homozygous MOR knockout mice and their wild-type littermates (n = 12; Jackson Laboratory) bred in-house and weighing between 20–30g at 8–16 weeks old, were used for behavioral experiments. Mice were group-housed with free access to food and water for all experiments that did not require surgery. Mice that underwent pump implantation were single housed after surgery with free access to food and water. Experiments were conducted in the housing room, maintained on a 12h light/dark cycle with lights on at 7:00am; all experiments were conducted during the light cycle. Each mouse was used in only one experiment either for acute antinociception, antinociceptive tolerance, physical dependence, conditioned place preference, or antidepressant effects in the tail suspension test. Studies were performed in accordance with the University of Michigan Committee on the Use and Care of Animals and the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011 publication).

Antinociception—Antinociceptive effects were evaluated in the WWTW assay. Withdrawal latencies were determined by briefly placing a mouse into a cylindrical plastic restrainer and immersing 2–3 cm of the tail tip into a water bath maintained at 50°C. The latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cutoff time of 20 sec to prevent tissue damage.

Acute antinociceptive effects were determined using a cumulative dosing procedure in wild-type or transgenic mice. Each mouse received an injection of saline *ip* and then 30 min later baseline withdrawal latencies were recorded. Following baseline determinations, increasing cumulative doses of the test compound were given *ip* or *sc* at approximate 30 min intervals. Thirty min after each injection, the tail withdrawal latency was measured as described above. Injections were spaced such that there was time for each animal to be restrained, tail withdrawal latency determined, and the next injection given, before the next animal was restrained.

To determine if the antinociceptive effects were peripherally-mediated, mice were injected with either vehicle or 10 mg/kg MNTX *ip*, a peripherally-restricted opioid antagonist, after baseline determination in the WWTW assay described above. This dose of MNTX has been

shown to block peripheral, but not central, effects of opioids (Ramabadran 1982). Following vehicle or MNTX treatment cumulative dosing of test compound was completed as described above.

Effect of Chronic Drug Administration on Tolerance and Dependence

Osmotic pump calibration—Osmotic pumps that deliver 0.5 μL/hr for 7 days were purchased from Alzet (Cupertino, CA). To ensure that the osmotic pumps were delivering the correct volume and concentration of VRP26 drug solution, an osmotic pump was loaded with VRP26 and incubated in 5 mL of 50 mM Tris buffer (pH 7.4) for 1 week at 37°C in a sealed container. A standard curve for VRP26 was established using UV absorption at 230 nm. HPLC was performed on a Waters analytical HPLC; UV absorbance was monitored at 230 nm as described previously (Mosberg et al. 2014) and used to determine the amount of drug delivered under test conditions.

Osmotic pump implantation surgery—Osmotic pumps were loaded with drug or saline solution and incubated for 4 hours at 37°C in sterile saline to prime the pumps for solution delivery prior to implantation. For pump implantation surgery, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine *ip*; carprofen (5 mg/kg *sc*) was administered as an analgesic. Incisions were made between the scapulae and the subcutaneous space was cleared of connective tissue. Pumps were inserted and the incision closed with sutures. Following surgery, mice were single housed and wound healing was monitored for seven days.

For pump removal mice were anesthetized as described above, given carprofen, and a new incision was made between the scapulae. Pumps were removed and the incision closed with sutures.

Tolerance experimental design—Antinociceptive dose effect curves for VRP26 (n=12) or fentanyl (n=12) were established on the morning of day 1 using the WWTW assay. Each group was then divided such that 6 mice received pumps containing drug and 6 mice received pumps containing saline. The lowest, maximally-effective, acute dose of fentanyl (0.3 mg/kg) or VRP26 (10 mg/kg) in the WWTW assay was delivered by continuous infusion over a 24h period for 7d via osmotic pump (0.3 mg/kg/day fentanyl or 10 mg/kg/day VRP26). Immediately after determining the dose effect curve on day 1, mice were implanted with osmotic pumps (as described above) that were removed on the afternoon of day 7. Cumulative dose/effect curves were established for all mice on the morning of day 8. To determine agonist potency before and after chronic treatment with drug or vehicle, dose-response curves and ED_{50} values were calculated for each mouse. It was not expected that continuous, slow infusion of 0.3 mg/kg fentanyl over 24 h for 7 d or 10 mg/kg VRP26 over 24 h for 7 d would produce antinociceptive effects and, therefore, was not assessed.

Physical dependence experimental design—On day 1 mice were implanted with pumps containing 0.3 mg/kg/day fentanyl, 10 mg/kg/day VRP26, or saline (n=6 in each group). On the morning of day 7, mice were placed individually in clear plastic observation

cages (10 in X 6 in X 8 in) without bedding and given 10 mg/kg naltrexone *ip*. Pumps were not removed prior to naltrexone administration to prevent spontaneous withdrawal from occurring. Mice were observed for signs of withdrawal for 15 min after naltrexone injection; behaviors were scored as follows and added together to generate a global withdrawal score: one point for the observation of each behavior (teeth chattering, paw tremor, piloerection, wet dog shakes or soft stool) and up to 3 points for jumps: 0 jumps (0 pts), 1–9 jumps (1 pt), 10–19 jumps (2 pts), greater than 20 jumps (3 pts).

Conditioned Place Preference and Locomotor Activity

Apparatus—A two-compartment place conditioning apparatus (MedAssociates, Inc. St. Albans, VT) was used for all conditioned place preference (CPP) studies. The compartmentalized box was divided into two equal size sections (8 in x 5 in x 5 in), accessed through a single, manual, guillotine door. The compartments differed in the wall color and floor texture (black walls with rod flooring vs. white walls with mesh flooring). Time spent in each chamber, number of beam breaks (used as a measure of locomotor activity), and number of entrances to each side were recorded using infrared photobeam detectors.

Conditioned place preference protocol—Experiments consisted of three phases: bias evaluation (2 days), conditioning (5 days), and testing (1 day).

Bias evaluation of CPP—Mice were placed randomly into one chamber on day 1 and the opposite chamber on day 2 and then allowed to freely explore the apparatus for 30 min. Mice that showed a greater than 70% pre-conditioning compartment bias were discarded from the study; two mice were discarded based on this criterion.

Conditioning phase of CPP—Mice were randomly assigned to receive test compound (fentanyl, VRP26, or saline; n=6 in each group) in the black or white chamber. During fentanyl and saline conditioning mice were given a saline injection (*ip*) and immediately placed in the saline-paired chamber for 30 min; 6 hr later mice were given an injection of fentanyl or saline (*ip*) and immediately placed in the drug-paired chamber for 30 min. As VRP26 has a slower onset than fentanyl (Mosberg et al. 2014), the conditioning sessions were modified: for VRP26 conditioning, mice were given a saline injection *ip* and returned to their home cage for 10 minutes before placement in the saline-paired chamber for 30 minutes; 6 hours later mice were given an injection of VRP26 and returned to their home cage for 10 minutes before being placed in the drug-paired chamber for 30 minutes. During all conditioning sessions, movement and activity were recorded.

Test day of CPP—Test day was always the day after the final conditioning session. Mice were randomly placed in either compartment and allowed to roam freely for 30 minutes. No injection was given on test day. Time spent in each chamber, beam breaks, and entrances to each side were recorded. CPP scores were calculated as the difference between time spent on the drug-paired side on test day and the average of time spent on the future drug-paired side on the two bias evaluations.

Tail Suspension Test (TST)—Mice were pretreated with either vehicle, 3.2 mg/kg naltrindole (NTI), 10 mg/kg VRP26, or 0.3 mg/kg fentanyl *sc* 30 min prior to injection of 3.2 mg/kg SNC80 or vehicle *sc*. Thirty minutes after SNC80 (or vehicle) injection, mice were suspended by their tail from a height of ~40 cm using tape for 6 minutes and behavior was recorded using a Sony HDR-CX220 digital camcorder. Videos were scored by observers blind to the testing conditioning and the total time mice spent immobile was summed for each animal and then averaged within each treatment group. Immobility was defined as the animal remaining motionless or making only minor, non-escape related movements.

Determination of Relative Efficacy *in vitro*—*In vitro* data for binding affinity and GTPγS binding for VRP26 and fentanyl were obtained as described in a previous report (Mosberg et al. 2014). The K_i for VRP26 and fentanyl were determined in buffer consisting of 50 mM Tris buffer, 100 mM NaCl, 5mM $MgCl_2$, 1 mM EDTA (pH 7.4) Relative efficacy was calculated using the Ehlert equation: $½(E_{maxtest}/E_{maxstandard})(1 + (K_{Dstandard}/EC_{50test}))$ = *relative efficacy* (Quock et al. 1999).

Statistics and Data Analysis—Statistical comparisons were made by either a Student's two-tailed t test, a one way, two way repeated measures, or factorial ANOVA with Tukey's post-hoc tests. Statistical analyses were performed using GraphPad Prism version 6.0 or SPSS version 22.

To calculate ED_{50} values for each mouse in the warm water tail withdrawal assay, the 50% level of maximum effect was determined from a linear regression analysis of individual latency to tail flick data, using only the linear portion of the curve and including only one dose that produced <10% of the baseline latency and one dose that produced >90% of the maximum latency. ED_{50} values from each mouse were then averaged across treatment group; group averages were compared using a Student's t test. Data are presented as mean ± standard error of the mean (SEM) for each treatment group before and after chronic treatment.

RESULTS

Comparison of acute antinociceptive effect following *ip* vs *sc* administration

The antinociceptive effects of VRP26 and fentanyl were assessed using the mouse WWTW assay after both *ip* and *sc* administration (Figure 2A). VRP26 when given *ip* or *sc* produced a maximal effect at 10 mg/kg with similar ED_{50s} (5.44 ± 0.10 mg/kg and 5.08 ± 0.23 mg/kg, respectively). Following *ip* and *sc* administration, 0.3 mg/kg fentanyl also produced similar maximal effects and had similar ED_{50s} (0.17 ± 0.013 mg/kg and 0.20 ± 0.001 mg/kg, respectively).

Comparison of acute antinociceptive effects in MOR knock-out mice and wild type littermates

The antinociceptive effects of VRP26 was assessed using the mouse WWTW assay in MOR knock-out mice and their wild type littermates to determine if antinociceptive effects were mediated by MOR *in vivo* (Figure 2B). Consistent with earlier results, VRP26 produced a

maximal antinociceptive effect in wild type littermates at 10 mg/kg with and ED_{50} of 4.77 ± 0.32 mg/kg. In MOR knock-out mice, VRP26 failed to produce a significant increase in tail withdrawal latency up to 32 mg/kg. Baseline withdrawal latencies for MOR knock-out mice and their wild type counterparts were not significantly different, but 10 mg/kg VRP26 significantly increased withdrawal latency in wild type mice as compared with MOR knock-out littermates ($t(10) = 9.161$, $p < 0.0001$).

Evaluation of peripherally-mediated antinociceptive effects of VRP26

The antinociceptive effects of VRP26 were assessed using the mouse WWTW assay in animals pretreated with either vehicle or 10 mg/kg methylnaltrexone (MNTX) *ip* to determine if antinociception produced by VRP26 is peripherally-mediated *in vivo* (Figure 2C). This dose of MNTX was shown to block peripherally-mediated, but not centrally-mediated, opioid effects in mice (Ramabadran 1982). In both the vehicle and MNTX pretreated mice, VRP26 produced dose-dependent increases in tail withdrawal latency, with a maximal response at 10 mg/kg VRP26 *ip* and ED_{50} values that were not statistically different (4.73 ± 0.22 mg/kg for vehicle pretreated mice and 4.73 ± 0.13 mg/kg for MNTX pretreated mice). A two way ANOVA showed a significant effect dose of VRP26 ($F(4, 40) = 393.6$, $p < 0.0001$), no effect of pretreatment (vehicle vs MNTX) and no interaction between dose of VRP26 and pretreatment.

Chronic Treatment Studies

For both antinociceptive tolerance and physical dependence studies, mice were implanted with osmotic pumps *sc* to deliver drug continuously for 7 days. To ensure that VRP26 was stable under these conditions and eliminated from the pumps as expected, a pump loaded with VRP26 was incubated in 5 mL of 50 mM Tris buffer (pH 7.4) for seven days at 37°C. Over the course of seven days, the pump delivered >95% of the expected drug concentration as determined via analytical HPLC, indicating that the drug delivery occurred as expected and that VRP26 was stable under these conditions (data not shown).

Antinociceptive Tolerance

To explore the effects of chronic treatment with fentanyl, mice were treated for 7 days with either saline or 0.3 mg/kg/day fentanyl (Figure 3A). A factorial ANOVA comparing fentanyl dose effect curves before and after chronic treatment showed an interaction (fentanyl dose X day tested X chronic treatment; $F(4,40) = 12.17$, $p < 0.0001$), as well as a main effects of fentanyl dose ($F(4,40) = 499.91$, $p < 0.0001$), day (1 vs 8; $F(1,10) = 21.73$, $p < 0.001$), and chronic treatment (saline vs fentanyl, $F(1,10) = 7.691$, $p = 0.02$). Chronic fentanyl, but not chronic saline, treatment produced a three-fold rightward, parallel shift in the fentanyl dose response curve (chronic treatment X day interaction ($F(1,10) = 36.36$, $p = 0.01$)). After 7 days of continuous administration of 0.3 mg/kg/day fentanyl, the ED_{50} of the fentanyl dose effect curve was 3-fold larger on day 8 (0.57 ± 0.018 mg/kg) as compared with day 1 (0.18 ± 0.006 mg/kg, $t(10) = 18.71$, $p < 0.0001$). The fentanyl dose effect curves in mice treated for 7 days with saline were not different on day 1 and 8 (ED_{50} Day 1 0.18 ± 0.006 mg/kg vs ED_{50} Day 8 0.23 ± 0.04 mg/kg).

Separate groups of mice were treated for 7 days with either saline or 10 mg/kg/day VRP26 (Figure 3B). A factorial ANOVA of the VRP26 dose effect curves before and after chronic treatment showed no interaction between factors (VRP26 dose X day tested X chronic treatment). There was a main effect of VRP26 dose ($F(3,8) = 4859.98$, $p < 0.0001$), demonstrating that VRP26 produced dose-dependent increases in antinociceptive effects, but there was no effect of day tested (before and after chronic treatment) or chronic treatment (saline vs VRP26). There was no shift in the dose response curve for VRP26 before and after chronic treatment in either saline or VRP26 treated groups (Day 1 ED_{50} 5.35 ± 0.17 mg/kg saline treated group, 5.54 ± 0.12 VRP26 treated group; Day 8 ED_{50} 5.07 ± 0.23 mg/kg saline treated group, 5.16 ± 0.17 VRP26 treated group).

Physical Dependence

The effects of chronic treatment on the development of physical dependence were also explored (Figure 3C). Naltrexone (10 mg/kg, *ip*) after chronic treatment with 0.3 mg/kg/day fentanyl precipitated significantly more withdrawal signs than after chronic saline treatment ($p < 0.01$) or chronic VRP26 treatment ($p < 0.001$), (one way ANOVA, ($F(2, 15) = 10.40$; $p = 0.002$). There were no differences in the number of withdrawal signs observed following chronic treatment with 10 mg/kg/day VRP26 or saline.

Conditioned Place Preference

The rewarding effects of both fentanyl and VRP26 were explored using the conditioned place preference (CPP) assay (Figure 4). Fentanyl produced robust dose-dependent increases in time spent on the fentanyl-paired side of the apparatus (one way ANOVA: ($F(4,36) = 5.31$, $p = 0.0018$, Figure 4A). Mice conditioned with 0.3 mg/kg or 1.0 mg/kg fentanyl, spent more time on the drug-paired side of the apparatus than animals conditioned with saline ($p < 0.01$). While conditioning with VRP26 slightly increased time spent on the VRP26-paired side of the apparatus, the difference between saline conditioning and VRP26 conditioning was not statistically different at any of the doses tested (Figure 4B).

Locomotor activity was recorded during all conditioning sessions. Fentanyl produced dose-dependent increases in locomotor activity (main effect of dose: $F(4,37) = 11.90$, $p < 0.0001$), with increases in activity on both day 1 and day 5 at 0.3 mg/kg *ip* ($p < 0.05$) and 1.0 mg/kg *ip* ($p < 0.0001$) compared with saline (Figure 4C). However, there were no interactions between day and dose demonstrating that fentanyl-induced locomotor activity did not change with daily administration for 5 d. VRP26 also produced dose-dependent increases in locomotor activity on both day 1 and day 5 (Figure 4D); a factorial ANOVA of these data showed a main effect of dose ($F(3, 62) = 3.71$, $p = 0.02$), as well as an interaction of day X dose ($F(3, 31) = 3.65$, $p = 0.02$). 32 mg/kg VRP26 *ip* increased locomotor activity on day 1 only ($p < 0.05$) and 10 mg/kg VRP26 *ip* increased locomotor activity ($p < 0.05$) on day 5 only.

Antagonism of DOR

We sought to confirm that VRP26 was a centrally-active DOR antagonist by examining whether it was capable of blocking the antidepressant-like effects of a DOR agonist, SNC80, in the tail suspension test (TST; Figure 5). Animals were pretreated with vehicle, the

prototypic DOR antagonist naltrindole (NTI, 3.2 mg/kg), VR26 (10 mg/kg), or fentanyl (0.3 mg/kg), then administered either 3.2 mg/kg SNC80 or vehicle. A two way ANOVA showed a significant main effect of SNC80 dose ($F(1, 51) = 123.8$, $p < 0.0001$) and pretreatment (NTI, VRP26, fentanyl, or vehicle, $F(3, 51) = 8.518$, $p < 0.0001$)), as well as an interaction of pretreatment X dose ($F(3, 51) = 12.50$, $p < 0.0001$). Mice treated with 3.2 mg/kg SNC80 (*sc*) alone displayed a decrease in immobility in the mouse TST as compared with vehicle treated mice ($p < 0.0001$). This SNC80-induced decrease in immobility was blocked by pretreatment with 3.2 mg/kg NTI *sc* ($p < 0.0001$); NTI pretreated mice had immobility scores that were not statistically different from immobility in vehicle treated mice. Pretreatment with 10 mg/kg (*sc)* VRP26 partially blocked SNC80-induced decreases in immobility, such that VRP26 pretreatment was significantly different from both SNC80 alone ($p < 0.001$) and vehicle pretreatment ($p < 0.01$). VRP26 alone has no effect on immobility as compared with control. Pretreatment with fentanyl had no effect on immobility scores in vehicle- or SNC80-treated mice.

Determination of Relative Efficacy *in vitro*

The relative efficacy of VRP26 and fentanyl at MOR were determined *in vitro* relative to the standard agonist DAMGO to examine if the lack of tolerance to chronic treatment with VRP26 is due to differences in relative efficacy. It was found that fentanyl is more efficacious (relative efficacy 0.5) than VRP26 (relative efficacy 0.28).

DISCUSSION and CONCLUSIONS

We have previously described the novel peptide MOR agonist/DOR antagonist compound, VRP26, which produced opioid receptor-mediated, dose-dependent antinociception *in vivo* after peripheral administration, without the development of acute tolerance (Mosberg et al. 2014). Consistent with its *in vitro* profile, VRP26 also demonstrates MOR agonist and DOR antagonist activity *in vivo*. VRP26 produces maximal antinociceptive effects in WWTW by MOR activation; these antinociceptive effects are attenuated by administration of a MOR antagonist and genetic deletion. Additionally, by blocking DORs, VRP26 attenuates the antidepressant-like effects of the DOR agonist SNC80 in the tail suspension test. Although VRP26 levels in brain tissue were not measured directly, VRP26 likely enters the central nervous system, as it attenuates the centrally-mediated antidepressant-like effects and its antinociceptive effects are not blocked by the peripherally-restricted opioid antagonist methylnaltrexone (MNTX). Together these results suggest that the antinociceptive effects of VRP26 are not mediated by peripheral mechanisms alone; however, future PK studies will examine CNS penetration of VRP26 *in vivo.*

In this report we evaluated the development of tolerance and physical dependence following chronic administration of VRP26. In contrast to their acute antinociceptive effects, fentanyl and VRP26 have significantly different effects following chronic administration. Continuous administration of 10 mg/kg/day VRP26 does not shift its antinociceptive dose effect curve (Figure 3B) in this warm water tail withdrawal procedure, demonstrating that, unlike fentanyl (Figure 3A), antinociceptive tolerance does not develop to VRP26 under these conditions.

While we attribute the lack of tolerance development under these conditions to be due to DOR antagonism, it is possible that differences in tolerance development between fentanyl and VRP26 could be due to differences in relative efficacy. High efficacy agonists have large receptor reserves and, therefore, are less affected by the loss of receptors on the cell surface as the result of desensitization and down-regulation (i.e., greater receptor reserve). Therefore, we evaluated the relative efficacy of VRP26 and fentanyl *in vitro* as compared to the standard MOR agonist DAMGO. Fentanyl is more efficacious (relative efficacy 0.5) than VRP26 (relative efficacy 0.28) *in vitro,* suggesting that the difference in antinociceptive tolerance is not due to greater relative efficacy and receptor reserve. The pharmacokinetic properties of fentanyl and VRP26 should also be considered when discussing the development of antinociceptive tolerance. VRP26 has a slightly shorter duration of action than fentanyl (Mosberg et al. 2014), and as a result, continuous infusion of fentanyl may achieve a higher steady-state concentration than VRP26, which could contribute to the difference in tolerance development between fentanyl and VRP26. It is also possible that a more aggressive dosing regimen or longer treatment with VRP26 might produce antinociceptive tolerance. These points warrant further investigation with repeated and prolonged treatment regimens using larger doses of VRP26 that produce acute antinociceptive effects. However, based on the current data, we would expect less tolerance to develop to VRP26 than to opioid analgesics such as morphine or fentanyl. Additionally, tolerance to the antinociceptive effects of VRP26 may develop differentially across various pain assays, especially procedures more relevant to clinical settings such as chronic pain or pain-suppressed behavior. Future studies with VRP26 will evaluate longer drug exposures, different types of pain (e.g., chronic pain), and development of cross-tolerance with traditional opioid agonists.

We also examined the development of physical dependence following seven day continuous treatment of fentanyl or VRP26. Fentanyl produced significantly more physical dependence than VRP26 (Figure 3C), as naltrexone failed to precipitate withdrawal signs in VRP26-treated mice. Opioid analgesics with reduced development of physical dependence fulfill an unmet medical need as opioid dependence may lead to misuse and abuse of opioids to avoid withdrawal symptoms (Bailey and Connor 2005; Ballantyne and LaForge 2007; Johnston et al. 2009; Ross and Peselow 2009). In this report, we also explored the rewarding properties of VRP26 by examining its ability to induce conditioned place preference. In contrast to fentanyl, VRP26 produced only modest conditioned place preference and locomotor stimulation, suggesting that VRP26 may be less rewarding than fentanyl and may differentially affect dopaminergic reward pathways.

In conclusion, mixed efficacy compounds, such as VRP26, may be safer alternatives to traditional opioid analgesics as they could provide continued pain relief without tolerance, dependence, and abuse liability. Taken together, these data support the idea that mixed efficacy MOR agonist/DOR antagonist compounds have distinctly different pharmacological actions *in vivo* as compared with traditional opioid analgesics. VRP26 represents an advancement in the field as it has balanced affinity between MOR and DOR, as well as significantly better bioavailability after peripheral administration when compared to previously published compounds (Dietis et al. 2009; Healy et al. 2013). While the molecular mechanism by which antagonism of DOR regulates MOR-mediated effects is unclear,

studies have suggested that these effects may be due to receptor oligomerization, alterations in receptor surface expression or recycling, or changes in intracellular signaling cascades (Breitwieser 2004; Cahill et al. 2007; Gomes et al. 2011; Hanyaloglu et al. 2002; Law et al. 2005; Milligan 2010; Wisler et al. 2014). Regardless of the mechanism, mixed efficacy opioid ligands, such as VRP26, may present an effective analgesic alternative to traditional opioids, thereby meeting a significant unmet medical need for analgesics with limited tolerance, dependence, and abuse liability.

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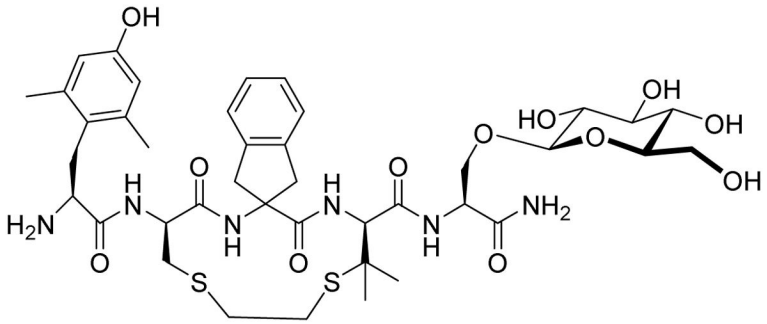

Figure 1.

Structure of MOR agonist/DOR antagonist peptide VRP26 (Dmt-c(SEtS)[DCys-Aic-DPen]-Ser(β-glucose)-NH_2

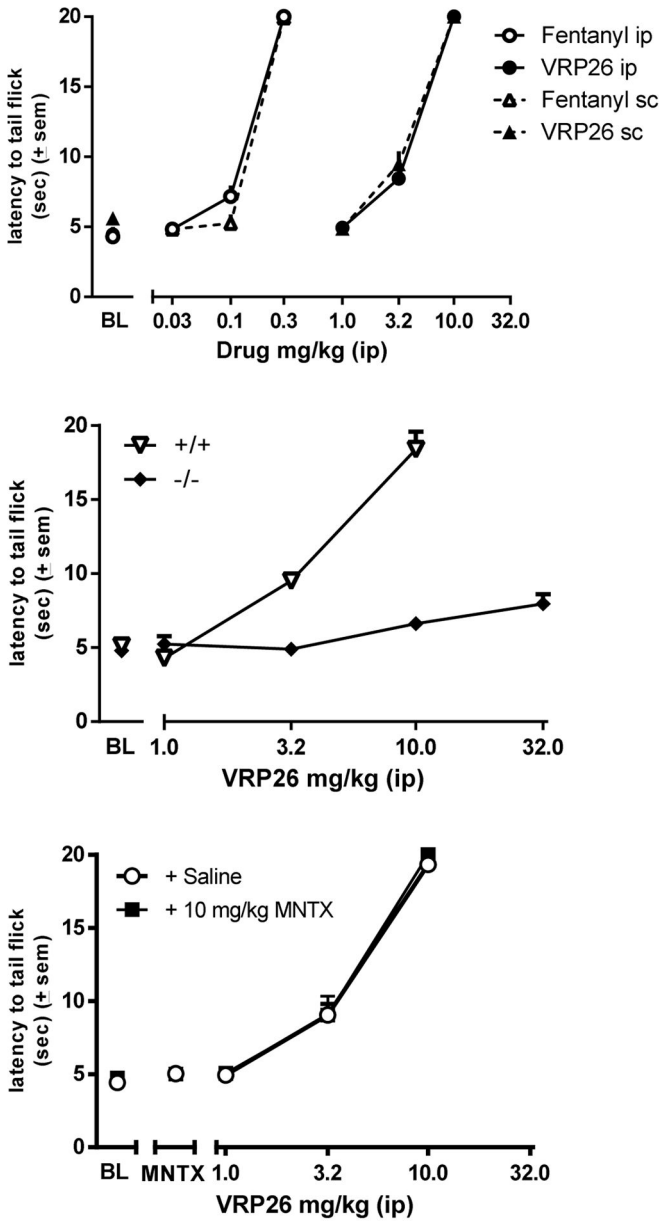

Figure 2.

(A) Cumulative antinociceptive dose response curves for fentanyl *ip* (n=6), fentanyl *sc* (n=4), VRP26 *ip* (n=6), and VRP26 *sc* (n=4) in the mouse warm water tail withdrawal assay. (B) Cumulative antinociceptive dose response curves for VRP26 *ip* in mu knock-out mice (n=6) and their wild type littermates (n=6) in the mouse warm water tail withdrawal assay (C) Cumulative antinociceptive dose response curves for VRP26 in animals pretreated with vehicle (n=6) or 10 mg/kg methylnaltrexone (MNTX, n=6) a peripherally-restricted opioid antagonist. Data are plotted as mean +/− standard error of the mean (SEM) for all groups.

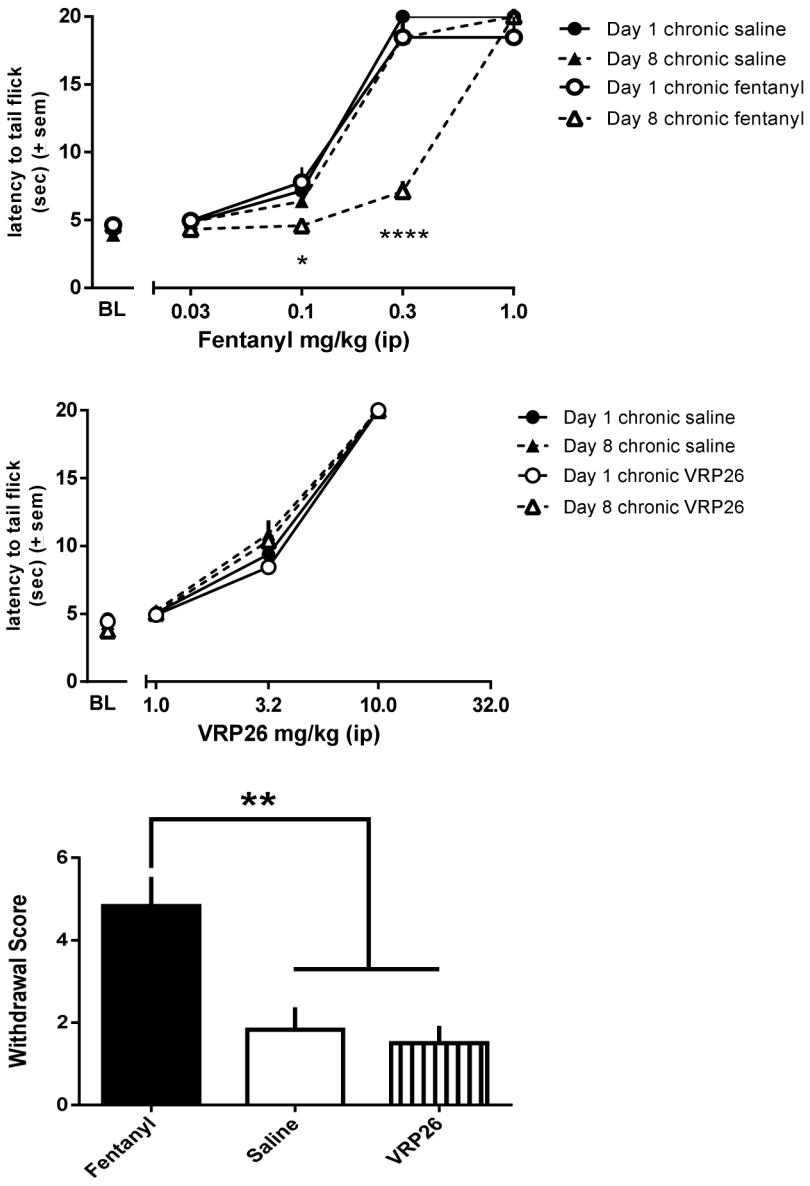

Figure 3.

Cumulative antinociceptive dose response curve for fentanyl *ip* in mouse warm water tail withdrawal assay before and after (A) 7 days of chronic 0.3 mg/kg/day fentanyl treatment *sc* (n=6) or 7 days chronic saline treatment *sc* (n=6) (B) 7 days of chronic 10 mg/kg/day VRP26 treatment *sc* (n=6) or 7 days chronic saline treatment *sc* (n=6). (C) Global withdrawal scores for animals treated for 7 days with either 0.3 mg/kg/day fentanyl (n=6), 10 mg/kg/day VRP26 (n=6), or saline (n=6) *sc*. Withdrawal was precipitated with 10 mg/kg naltrexone *ip* and jumps, wet dog shakes, paw tremor, soft stool, teeth chattering, and piloerection were all counted as physical symptoms of withdrawal. Data are plotted as mean +/− standard error of the mean (SEM) for all groups. * p< 0.05; **p< 0.01; **** p< 0.0001

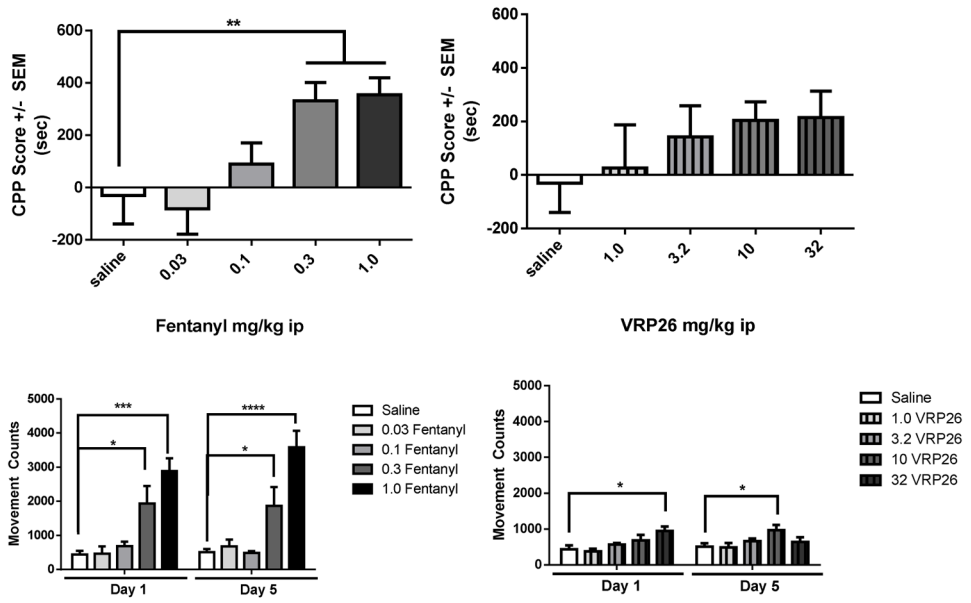

Figure 4.

Conditioned place preference (CPP) scores for animals trained for 5 days on various doses of fentanyl or VRP26 (*ip*). CPP scores are defined as the difference between time spent on drug paired side pre- and post- conditioning measured in seconds. (A) Animals conditioned with fentanyl showed significant place preference at doses of 0.3 and 1.0 mg/kg (*ip*) ($p < 0.01$). (B) Animals conditioned with VRP26 did not show significant place preference at any of the tested doses. Dose response curves for locomotor activity over 30 mins for (C) fentanyl and (D) VRP26 on the afternoon of Day 1 and Day 5. Data are plotted as mean +/− standard error of the mean (SEM) for all groups (n=7–11 for each group) * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$

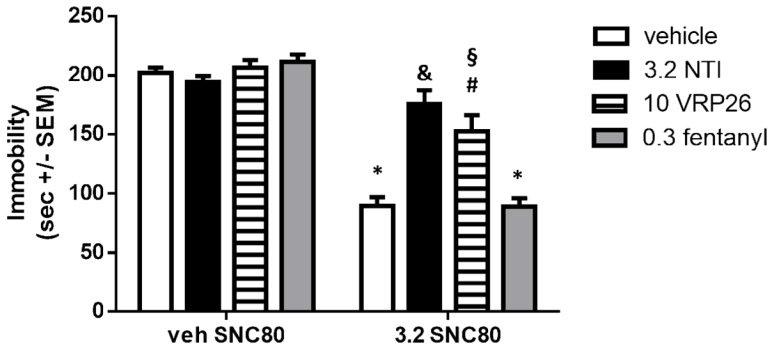

Figure 5.

Animals were pretreated with either vehicle, 3.2 mg/kg naltrindole (NTI), 10 mg/kg VRP26, or 0.3 mg/kg fentanyl *sc.* Thirty minutes later animals were given 3.2 mg/kg SNC80 or vehicle *sc.* After 30 minutes, animals were suspended by their tail and their behavior was recorded for 6 minutes. Immobility was defined as the animal remaining motionless or making only minor, non-escape related movements. Data are plotted as mean +/− standard error of the mean (SEM) for all groups (n=6–10 for each group) * denotes $p < 0.0001$ relative to veh/veh, § denotes $p < 0.01$ relative to veh/veh # denotes $p < 0.001$ relative to veh/SNC80, & denotes $p < 0.0001$ relative to veh/SNC80.