Central Nervous System Distribution of an Opioid Agonist Combination with Synergistic Activity


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ABSTRACT

Novel combinations of specific opioid agonists like loperamide and oxymorphindole targeting the μ- and δ-opioid receptors, respectively, have shown increased potency with minimized opioid-associated risks. However, whether their interaction is pharmacokinetic or pharmacodynamic in nature has not been determined. This study quantitatively determined whether these drugs have a pharmacokinetic interaction that alters systemic disposition or central nervous system (CNS) distribution. We performed intravenous and oral in vivo pharmacokinetic assessments of both drugs after discrete dosing and administration in combination to determine whether the combination had any effect on systemic pharmacokinetic parameters or CNS exposure. Drugs were administered at 5 or 10 mg/kg i.v. or 30 mg/kg orally to institute for cancer research (ICR) mice and 5 mg/kg i.v. to Friend leukemia virus strain B mice of the following genotypes: wild-type, breast cancer resistance protein (BCRP) knockout, (triple knockout). In the combination, clearance of oxymorphindole (OMI) was reduced by approximately half, and the plasma area under the concentration-time curve (AUC) increased. Consequently, brain and spinal cord AUCs for OMI in the combination also increased proportionately. Both loperamide and OMI are P-gp substrates, but administration of the two drugs in combination does not alter efflux transport at the CNS barriers. Because OMI alone shows appreciable brain penetration but little therapeutic efficacy on its own, and because loperamide’s CNS distribution is unchanged in the combination, the mechanism of action for the increased potency of the combination is most likely pharmacodynamic and most likely occurs at receptors in the peripheral nervous system. This combination has favorable characteristics for future development.

SIGNIFICANCE STATEMENT

Opioids have yet to be replaced as the most effective treatments for moderate-to-severe pain and chronic pain, but their side effects are dangerous. Combinations of opioids with peripheral activity, such as loperamide and oxymorphindole, would be valuable in that they are effective at much lower doses and have reduced risks for dangerous side effects because the μ-opioid receptor agonist is largely excluded from the CNS.

Introduction

Opioid agonists remain the most commonly prescribed treatment of moderate-to-severe pain and have been in use for centuries (Presley and Lindsley, 2018). The efficacy of these drugs in chronic and severe pain is well characterized and has yet to be supplanted in modern clinical practice. However, along with their potent analgesic effects, opioid agonists are accompanied by well known and sometimes dangerous adverse effects like constipation, sedation, respiratory depression, a liability to dependence, and the development of tolerance (Presley and Lindsley, 2018). In recent decades, rampant overprescription of

Abbreviations: AUC, area under the concentration-time curve; AUC_{0–∞}, AUC to time infinity; AUC_{Last}, AUC to the last time point; BCRP, breast cancer resistance protein; BKO, BCRP knockout; CFA, Complete Freund’s Adjuvant; CL, systemic clearance; CL/F, apparent CL; CNS, central nervous system; DA, distributional advantage; DOR, δ-opioid receptor; F, bioavailability; f_u, undiluted free fraction; FVB, Friend leukemia virus strain B; Kp, overall tissue-to-plasma ratio or partition coefficient; K_{p,t}, tissue-to-plasma ratio at time t; K_{puu}, unbound partition coefficient; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MOR, μ-opioid receptor; MP, mobile phase; OMI, oxymorphindole; P-gp, P-glycoprotein; PKO, P-gp knockout; PWL, paw-withdrawal latency; RED, rapid equilibrium dialysis; t_{1/2}, terminal half-life; TKO, triple knockout; V, volume; WT, wild type.
opioids has led to an epidemic of opioid use disorder and overdose deaths. Although subsequent enforcement efforts and prescribing guidance from the Centers for Disease Control and Prevention hoped to stem the tide of overdose deaths (Dowell et al., 2016), there are still few alternatives to opioid agonists when it comes to pain management. The Centers for Disease Control and Prevention estimated in 2016 that approximately 20% of adults in the United States suffer from chronic pain (Dahlhamer et al., 2018). A more recent study estimated that 4.8% of adults have high-impact chronic pain, and 13.8% experience pain that limits their daily activities (Pitcher et al., 2019). This experience of pain can result in depression, anxiety, and poor overall quality of life. As a result, 3%–4% of the entire US population is prescribed opiates for long-term pain management when the benefits of opioids are thought to outweigh the inherent risks (Dowell et al., 2016). Individuals requiring long-term pain management may include patients with cancer and those with postoperative pain, or individuals experiencing neuropathic and chronic pain from a variety of causes. In light of the risks of long-term opioid use, it is imperative that new, effective treatments with minimized risks become available for these patients.

In the search for novel treatments with reduced side effects, combinations of receptor-selective opioid agonists and other compounds have shown potential for potent analgesic and antihyperalgesic effects with reduced liability to tolerance and respiratory depression. Recently published work from Bruce et al. (2019) showed that loperamide, a μ-opioid receptor (MOR) agonist, when dosed subcutaneously in a 1:1 combination with the δ-opioid receptor (DOR) agonist oxymorphindole (OMI), exhibited efficacious pain management in the face of inflammatory pain. This work is compelling in that efficacious pain management is achieved at many-fold lower doses of loperamide, and the combination is peripherally active (Bruce et al., 2019; Uhelesi et al., 2020). There is a body of evidence supporting the hypothesis that heterodimerization of MORs with DORs results in downstream signaling that is different than conventional MOR dimerization (Gomes et al., 2000, 2004; Lenard et al., 2007; Schuster et al., 2015). However, the potential for interactions in systemic disposition and central nervous system (CNS) distribution that could contribute to the synergy of these two drugs has not been determined.

The distribution of opioids to the CNS plays a crucial role in the activity and use of opioid agonists. Sedation, respiratory depression, and addiction are mediated by MOR in the CNS as shown by studies in MOR knockout mice (Matthes et al., 1996; Pattinson, 2008). Alterations in loperamide systemic pharmacokinetics or distribution to the CNS resulting from coadministration with OMI might play a role in the mechanism of action and safety of this combination. Because loperamide has long been known to be a P-glycoprotein (P-gp, ATP-binding cassette sub-family B member 1) substrate for efflux from the CNS as well as the gut (Schinkel et al., 1996), it is possible that synergistic activity between these two drugs results from alterations in systemic pharmacokinetics or CNS drug distribution. This is especially important since the efflux status of OMI has not been determined. Therefore, two possible mechanisms exist that could explain this activity: a change in systemic pharmacokinetics and distribution to the CNS or an interaction at the MOR/DOR receptor site (Fig. 1).

In this study, we sought to clarify the nature of the interaction between loperamide and OMI by determining the effect on CNS distribution and systemic pharmacokinetics of the two drugs when administered alone and in combination. The primary objective of the current study was to determine whether the synergistic effect was related to changes in pharmacokinetics or changes in pharmacodynamics.

Materials and Methods

Reagents. Loperamide hydrochloride and naltrindole hydrochloride were obtained from Torcis Bioscience (via Fisher Scientific). [2H6]Loperamide was purchased from Alsachim (Illkirch-Graffensta- den, France). OMI was a gift from the laboratory of Dr. Phil Portoghese (Portoghese et al., 1988). All other chemical reagents were high-performance liquid chromatography-grade and purchased from Thermo Fisher Scientific. Rapid equilibrium dialysis plates and inserts (8 kDa molecular mass cutoff) were also purchased from Thermo Fisher Scientific.

Animals. For the behavioral experiment, adult ICR-CD1 mice (22–29 g, N = 90, male and female) were housed four (male) or five (female) to a cage and maintained on a 12-hour light/dark cycle with ad libitum access to water and food. Testing was performed during the light phase of this cycle. For the pharmacokinetic studies, male ICR mice (Charles River Laboratories) of age 8–14 weeks were used for initial studies as noted and housed in the Research Animal Resources facility in the Academic Health Center of the University of Minnesota prior to use. Subsequently, both male and female Friend leukemia virus strain B (FVB) mice of age 8–14 weeks of four different genotypes were used for transporter knockout studies. These genotypes included wild-type, Berp+/− [Berp knockout (BKO)], Mdr1a/b−/− [P-gp knockout (PKO)], and Berp−/− Mdr1a/b−/− [triple knockout (TKO)] mice (breeder pairs from Taconic Biosciences, Inc., Germantown, NY). Colonies of the FVB mice were maintained and housed in the Research Animal Resources facility at the Academic Health Center of the University of Minnesota, and animal genotypes were regularly verified by tail snip (TransnetXY, Cordova, TN). All mice for pharmacokinetic studies were maintained on a 12-hour light/dark cycle with ad libitum access to water and food. Protocols for all animal experiments received approval by the University of Minnesota Institutional Animal Care and Use Committee and were performed in accordance with the Guide for Care and Use of Laboratory Animals established by US National Institutes of Health.
Systemic Pharmacokinetics and CNS Distribution Studies. Single doses of loperamide, OMI, or a combination of the two drugs were administered to ICR mice via tail vein injection or oral gavage. Dosing formulations for both drugs were first prepared in sterile water for injection with 5% DMSO and 5% Cremophore. This solution was subsequently diluted four times in sterile water for injection to the final concentrations of 1 mg/ml for intravenous studies and 6 mg/ml for oral studies (1% DMSO, 1% Cremophore), with the exception of the first OMI intravenous study, which was diluted 2 mg/ml. The first OMI intravenous study was conducted with a dose of 10 mg/kg, which was well tolerated. However, when loperamide was initially dosed to two animals at 10 mg/kg i.v., it was found to be poorly tolerated, and the dose was lowered to 5 mg/kg. All subsequent intravenous studies for both drugs were conducted with a dose of 5 mg/kg.

After intravenous administration, blood, brain, and spinal cord samples were collected at time points from 10 minutes to 16 hours (n = 4 mice per time point). After oral administration, samples were collected from 30 minutes to 16 hours (n = 4 mice per time point). Mice were euthanized via a CO2 chamber. Blood was rapidly collected via cardiac puncture using heparinized syringes and transferred into heparinized tubes. Plasma was separated by centrifugation at 7500 rpm for 15 minutes at 4°C. Spinal cords were collected via the hydraulic extrusion method as described by Roberts et al. (2005). Briefly, after decapitation, the spinal column rostral of the pelvis was removed. Then, a saline-filled syringe fixed with a blunt-tipped needle was inserted into the caudal end of the spinal column. The plunger was depressed to extrude the spinal column fully intact. Plasma, brain, and spinal cord were stored at −80°C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Prior to analysis, brain and spinal cord were thawed and homogenized in 2x (w/v) 5% bovine serum albumin.

Translational Knockout Pharmacokinetic and CNS Distribution Studies. Systemic pharmacokinetic and CNS distribution studies to ascertain efflux transporter effects were conducted in FVB mice using intravenous administration as described above. Briefly, 5 mg/kg of loperamide, OMI, or the combination were administered via tail vein injection into wild-type, BKO, PKO, and TKO mice. Blood, brain, and spinal cord were harvested at time points ranging from 10 minutes to 16 hours (n = 4 mice per time point, 2 male, 2 female) as described above. Samples were stored at −80°C until LC-MS/MS analysis. Prior to analysis, brain and spinal cord were thawed and homogenized in 2x (w/v) 5% bovine serum albumin.

Rapid Equilibrium Dialysis for Free Fraction in Mouse Plasma and Brain Homogenate. Free fractions of loperamide, OMI, and the combination in mouse plasma and brain homogenate were determined using rapid equilibrium dialysis (RED) devices according to the manufacturer’s protocol (Thermo Fisher). For brain homogenate, brain tissue was homogenized in two volumes (w/v) of PBS (pH 7.4) using a mechanical homogenizer. Both plasma and brain homogenate were spiked with loperamide, OMI, or a 1:1 combination to a final concentration of 5 μM for each drug with 0.025% DMSO. Drug-matrix solutions (300 μl) were then added to the sample chamber, and then 500 μl of PBS (0.025% DMSO) was added to the buffer chamber. The plate was then sealed with adhesive film and incubated for 24 hours at 37°C in an orbital shaker set to 600 rpm. At 24 hours, samples were collected from both chambers and stored at −20°C until LC-MS/MS analysis. The undiluted free fraction (f_u) for both drugs was calculated with the following equation, as reported previously (Kalvass and Maurer, 2002).

\[
f_u = \frac{1}{D} \left( \frac{1}{\text{f}_{\text{r}_{\text{diluted}}} - 1} + 1/D \right)
\]

in which D is the dilution factor or 3 as noted above. LC-MS/MS Analysis. Given their widely disparate hydrophobicity, separate LC-MS/MS methods were developed for loperamide and OMI. Both methods used reverse-phase liquid chromatography via an Agilent 1200 Series HPLC connected to a TSQ Quantum Classic mass spectrometer in positive ion mode. Briefly, both drugs and their

Drug Preparation and Administration for Oral ED50 Calculation. Formulations were prepared as a solution with 5% cremophore and DMSO and subsequently diluted to administered concentrations with sterile water. Solutions were administered by oral gavage using a 20 ga × 30 mm sterile plastic feeding tube (Fine Science Tools, USA). No fluid was noted in the nose, which is an indication of aspirated solution, in any subject during or after gavage. Thermal nociceptive responses were assessed once prior to Complete Freund’s Adjuvant (CFA) administration, and a baseline was assessed after CFA administration and 1 hour after oral gavage of experimental compound.

Behavioral Measures. The Hargreaves assay was used to assess peripheral thermal nociception, as described previously (Hargreaves et al., 1988). Briefly, mice were placed in a small plastic box to restrict their movement on a heated glass floor (30°C). Animals were allowed to acclimate to the testing environment for 15 minutes prior to baseline withdrawal assessment. A radiant heat lamp was then shone on the left hind paw until the mouse withdrew the paw, and the paw latency was recorded (baseline) by a plantar stimulator antinociception meter (IITC Lifesciences, USA). A cutoff time of 20 seconds was established to prevent tissue damage. Three paw-withdrawal latencies were recorded with a minimum of 30 seconds rest time between each test.

After determining naïve paw-withdrawal latencies (PWLs), animals were briefly anesthetized using 2.5% isoflurane and 10 ml of an emulsion of 1:1 CFA in saline was injected into the left hind paw. Three to 5 days after this injection, a well characterized hyperalgesia was present in the left hind paw, and post-CFA PWLs were assessed (post-CFA) (Newbould, 1963). The experimental compounds (loperamide, OMI, or their combination) were then delivered by oral gavage. One hour after oral gavage, thermal responses were again assessed (experimental value). Each animal received only one dose of compounds or of the combination. The experimenters were not blinded to drug or concentration during compound administration or behavioral testing. One experimenter delivered compound to all subjects, and a separate experimenter performed all PWL assessment.

Data Analysis of Behavioral Measures. Thermal nociceptive responses after oral gavage of OMI, loperamide, or their combination were analyzed as a percentage of antihyperalgesia (%AH) given by the following equation:

\[
\% \text{AH} = \left( \frac{\text{post CFA value} - \text{baseline}}{\text{post CFA value} - \text{baseline}} \right) \times 100
\]

The ED50 of loperamide and loperamide in the presence of oxymorphone [Loperamide (+OMI)] were calculated using the graded dose-response curve method (Tallarida and Murray, 1987).
internal standards were extracted from plasma, brain homogenate, and spinal cord samples via liquid-liquid extraction with 5x (v/v) ethyl acetate. Samples were vortexed for 5 minutes and centrifuged. Supernatant was collected and completely dried under nitrogen, and samples were reconstituted with mobile phase (MP). For loperamide, the internal standard was [2H6]-loperamide, and for OMI, the internal standard was naltrindole. Both methods used a Phenomenex Synergi 4-μm Polar-RP column (4 μm, 75 × 2 mm) for chromatographic separation and an MP flow rate of 0.3 mL/min. For loperamide, the method was isocratic with an MP composition of aqueous phase (A) 45% distilled water with 0.1% formic acid and organic phase (B) of 55% acetonitrile with 0.1% formic acid and a total run time of 4 minutes. The OMI method used gradient elution with initial MP composition of aqueous phase (A) 75% distilled water with 0.1% formic acid and organic phase (B) 25% acetonitrile with 0.1% formic acid. The gradient was as follows: starting at 3 minutes, organic phase was increased to 90% over 0.75 minutes, held at 90% for 1.25 minutes, and decreased back to 25% over 0.5 minutes. It was then held at 25% for 4.5 minutes for a total runtime of 10 minutes. The mass-to-charge (m/z) transitions for all molecules were as follows: loperamide 478.1 → 267.3, [2H6]-loperamide 484.1 → 273.3, OMI 375.1 → 254.1, and naltrindole 415.1 → 254.1. For both methods, the standard curve was linear over the range of 0.1–1000 ng/ml (weighted 1/Y2) with coefficients of variation less than 15%. Data were acquired and analyzed using Xcalibur software. The interday variability for loperamide for all concentrations in the standard curve was less than 4%, the intraday variability was less than 7%, and the limit of quantification was 0.1 ng/ml. For OMI, the interday variability was less than 15%, the intraday variability was less than 7% and the limit of quantification was 0.1 ng/ml.

**Pharmacokinetic Data Analysis.** Plasma, brain, and spinal cord concentration-time profiles were analyzed using Phoenix WinNonlin version 8.3 (Certara USA Inc., Princeton, NJ). Brain concentrations were corrected for residual blood estimated at 1.4% of brain weight and with blood concentrations approximated by plasma concentrations (Fridén et al., 2010). Pharmacokinetic parameters and metrics were calculated by performing noncompartmental analysis. Areas under the concentration-time curves (AUCs) were determined by linear trapezoidal integration, wherein the AUC to the last time point (AUClast) was calculated directly. The AUC to infinity (AUC0→∞) was extrapolated from the last point to infinity by dividing the concentration at the last time point (Clast) by the terminal elimination rate constant (λe) as determined by the last four time points. In cases wherein the terminal slope was not sufficiently negative for the time course of these experiments to accurately extrapolate to time infinity, AUClast is reported rather than AUC0→∞. Variances for AUClast were calculated using the Bailer method as reported in Phoenix WinNonlin (Bailer, 1988). Variances for AUC0→∞ were calculated utilizing the Yuan extension of the Bailer method (Yuan, 1993).

Other pharmacokinetic parameters, including systemic clearance (CL), apparent clearance (CL/F), volume of distribution (V/F), and apparent volume of distribution (VIF) as well as the terminal half-life (t1/2) were also calculated by noncompartmental analysis in Phoenix software by the following methods:

\[
CL = \frac{Dose}{AUC_0\rightarrow\infty} \tag{3}
\]

\[
V_{ss} = MRT_{inf} \times CL \tag{4}
\]

in which MRTinf is the area under the first moment curve to infinity (AUMCinf) divided by the AUC0→∞.

\[
t_{1/2} = \frac{ln(2)}{k_e} \tag{5}
\]

in which \(k_e\) is the terminal first-order elimination rate constant associated with the log-linear portion of the concentration-time profile and is estimated by linear regression of time versus log-concentration.

The brain-to-plasma ratio, or brain tissue partition coefficient (KpBrain), for each drug was calculated as a ratio of the AUC of the brain concentration-time profile to the AUC of the plasma concentration-time profile (eq. 6). Similarly, the spinal cord-to-plasma ratio, or spinal cord tissue partition coefficient (KpSpinal Cord), was calculated as a ratio of the AUCs (eq. 7). The brain partition coefficient of free drug (KpFree) was calculated by multiplying the KpBrain by the ratio of unbound fractions in brain and plasma (eq. 8).

\[
K_{p,\text{Brain}} = \frac{AUC_{\text{Brain}}}{AUC_{\text{Plasma}}} \tag{6}
\]

\[
K_{p,\text{Spinal Cord}} = \frac{AUC_{\text{Spinal Cord}}}{AUC_{\text{Plasma}}} \tag{7}
\]

\[
K_{p,\text{Free}} = K_{p,\text{Brain}} \times \frac{f_u_{\text{Brain}}}{f_u_{\text{Plasma}}} \tag{8}
\]

The tissue-to-plasma concentration ratio at time t is used to assess the extent of drug distribution over time and will be notated as Kp, values for both brain and spinal cord. These were calculated by the following:

\[
K_p = \frac{\text{Concentration}_{\text{tissue}}}{\text{Concentration}_{\text{plasma}}} \tag{9}
\]

The oral bioavailability (F) of both drugs was calculated by eq. 9:

\[
F = \left( \frac{[AUC_{(0\rightarrow\infty),\text{plasma}}]}{[AUC_{(0\rightarrow\infty),\text{plasma}}]} \right) \cdot \left( \frac{\text{Dose}_{\text{IV}}}{\text{Dose}_{\text{oral}}} \right) \tag{10}
\]

The distributional advantage (DA) achieved in mice lacking efflux transporters at the CNS barriers was calculated by the following equation:

\[
DA(\text{brain or spinal cord}) = \frac{K_{p,\text{Brain}} \text{ or spinal cord transporter knockout mice}}{K_{p,\text{Brain or spinal cord wild - type mice}}} \tag{11}
\]

**Statistical Analysis.** Data are represented as mean ± S.D. when applicable. For the behavioral study, the data were analyzed by nonlinear regression, fitting an [agonist] versus response curve to compare ED50 values by GraphPad Prism (version 8.4; Graphpad Software, La Jolla, CA), with a null hypothesis that the ED50s for both datasets were equal. To compare AUCs among studies and between different tissues, a two-tailed unpaired t test was performed in Graphpad Prism with a null hypothesis that AUCs were equal. One-way ANOVA with Tukey's multiple comparisons test was performed to compare AUCs among wild-type (WT) and transporter knockout mice in Graphpad. A significance level of \(P < 0.05\) was considered significant in all tests.

**Results**

**Oral ED50 of Loperamide with and without OMI.** The oral ED50 for loperamide was 51.8 mg/kg, and the oral ED50 for loperamide with OMI was 0.68 mg/kg. The best-fit models for the dose-response curves resulted in a rejection of the null hypothesis (\(P < 0.01\)), indicating that the potency of loperamide is increased when administered in combination with OMI. OMI individual ED50 could not be determined from these data.

**Loperamide Disposition in ICR mice.** To determine whether the coadministration of OMI and loperamide changes their CNS distribution or systemic pharmacokinetics, the two drugs were dosed alone and in combination. Brain, plasma, and spinal cord were collected, and the concentrations of drug in each tissue were determined by LC-MS/MS. The total
Loperamide (5 mg/kg) IV

Plasma Brain Spinal Cord

OMI + Lop (1:1 5 mg/kg) IV Loperamide

Plasma Brain Spinal Cord

Comparison of Kp\(_{\text{Brain}}\) Lop IV

Comparison of Kp\(_{\text{Spinal Cord}}\) Lop IV

![Graphs and figures](image)

**Fig. 3.** Loperamide (Lop) intravenous pharmacokinetics and CNS distribution in ICR mice. (A) Plasma, brain, and spinal cord concentration-time profiles after a single intravenous dose (5 mg/kg) of loperamide. (B) Plasma, brain, and spinal cord concentration-time profiles after a single intravenous dose of loperamide (5 mg/kg) coadministered with OMI (5 mg/kg). (C) Kp\(_{\text{Brain}}\) of loperamide from the pharmacokinetic studies described by (A and B). (D) Kp\(_{\text{Spinal Cord}}\) from the pharmacokinetic studies described by (A and B).

Accordingly, the overall Kp\(_{\text{Brain}}\) and Kp\(_{\text{Spinal Cord}}\) as calculated by AUC ratios were also less than 1 for both discrete dosing and combination studies, which was expected, as loperamide is a P-gp substrate (Table 1). Loperamide appears to reach a rapid distributional equilibrium in the CNS, as Kp\(_{\text{Brain}}\) did not change over the time course in either brain or spinal cord.

The pharmacokinetic parameters for loperamide alone and in combination with OMI were also listed in Table 1. There was no apparent difference among the parameters of t\(_{1/2}\), CL, or V in the two studies. The difference among AUCs in plasma, brain, and spinal cord for loperamide alone and loperamide in combination with OMI was nonsignificant (P = 0.966, P = 0.312, and P = 0.779, respectively).

**OMI Disposition in ICR Mice.** The total (bound + unbound drug) plasma, brain, and spinal cord concentration-time profiles for single intravenous dose of OMI (10 mg/kg) were shown in Fig. 3, A and B. The plasma, brain, and spinal cord concentrations were below the limit of quantification for these studies at 12- and 16-hour time points, and therefore these were not included. Concentration-time profiles in all tissues exhibited biexponential decline over time. For loperamide alone, brain concentrations were significantly lower than that of plasma (P < 0.001), and spinal cord concentrations were lower than that of brain and significantly lower than plasma (P < 0.001) for the duration of the time course. For loperamide in combination with OMI, the same trend was observed. The tissue-to-plasma concentration ratios over time (Kp\(_{\text{Brain}}\) and Kp\(_{\text{Spinal Cord}}\), Fig. 3, C and D) remain less than 1 for the duration of the time course.

**TABLE 1**

Summary pharmacokinetic parameters for loperamide and OMI in ICR mice after intravenous administration alone and in combination OMI exposures are dose-normalized. A two-tailed unpaired t test was performed to compare AUCs among tissues (see results) and between the same tissues in discrete vs. combination studies (P < 0.001). Results are presented as mean or mean ± S.D.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Loperamide Alone (5 mg/kg)</th>
<th>Loperamide in Combination (5 mg/kg)</th>
<th>OMI Alone (dose-normalized)</th>
<th>OMI in Combination (Dose-Normalized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(_{1/2}) (h)</td>
<td>1.16</td>
<td>1.23</td>
<td>3.35</td>
<td>3.9</td>
</tr>
<tr>
<td>CL (l/h)/kg</td>
<td>3.6</td>
<td>3.6</td>
<td>12</td>
<td>6.6</td>
</tr>
<tr>
<td>V (l/kg)</td>
<td>4.7</td>
<td>4.9</td>
<td>17.6</td>
<td>11.6</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) Plasma (h*ng)/ml</td>
<td>1389 ± 140</td>
<td>1374 ± 299</td>
<td>84 ± 6*</td>
<td>149 ± 16*</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) Brain(h*ng)/g</td>
<td>257 ± 55</td>
<td>347 ± 65</td>
<td>175 ± 15</td>
<td>219 ± 20</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) Spinal Cord (h*ng)/g</td>
<td>163 ± 32</td>
<td>151 ± 28</td>
<td>43 ± 3</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>Kp(_{\text{Brain}})</td>
<td>0.19</td>
<td>0.25</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Kp(_{\text{Spinal Cord}})</td>
<td>0.12</td>
<td>0.11</td>
<td>0.51</td>
<td>0.42</td>
</tr>
</tbody>
</table>
and OMI in combination with loperamide (5 mg/kg) in ICR mice are shown in Fig. 4, A and B. The plasma, brain, and spinal cord concentrations show a pronounced distributional phase in both the discrete and combination studies. Brain and spinal cord concentrations are greater than plasma in the terminal phase for both studies, and this is apparent in Fig. 4C, wherein KptBrain (calculated as concentration ratios over time) is greater than unity at 2 hours for both OMI alone and OMI in combination. Similarly, in both the discrete dosing and combination studies, the KptSpinal Cord is greater than unity at 4 hours after administration (Fig. 3D). OMI appears to take longer than loperamide to reach dynamic equilibrium between plasma and the CNS, as the Kp for both brain and spinal cord reaches a maximum around 8–12 hours after administration.

The overall KpBrain was 2.0 for OMI dosed alone and 1.4 for OMI dosed in combination (Table 1). The overall KpSpinal Cord was 0.51 in the discrete dosing study and 0.42 in the combination study.

There was no difference in the half-life of OMI between the discrete and combination studies. To compare the AUCs between discrete dosing and the combination, dose-normalized AUCs were used, as the two studies were performed at different doses. When dose-normalized AUCs were compared, the plasma AUC0–∞ in the combination study (149 ± 16 hour*ng/ml) was significantly higher than the plasma AUC0–∞ for OMI alone (84 ± 6 hour*ng/ml, P < 0.001, Table 1). There also appeared to be evidence of an increase in the dose-normalized AUC for both brain and spinal cord, although the difference was not statistically significant. For an additional comparison, the WT FVB studies for OMI alone and in combination with loperamide were used, as these studies were performed at the same dose. In WT FVB mice, the brain exposure of OMI was higher in combination with loperamide (P = 0.0012); however, the overall brain-to-plasma ratio was unchanged, indicating that the brain exposure increased in proportion to the plasma (Table 4).

Regarding OMI systemic exposure, because the AUC depends on dose and clearance, assuming linear pharmacokinetics, the most likely explanation for an increase in the AUC is a reduction in the systemic clearance of OMI. This is evident in a decreased clearance from 12 (l/h)/kg for OMI alone to 6.6 (l/h)/kg for OMI in combination (Table 1). Further evidence for linear pharmacokinetics and a reduction in systemic clearance can also be taken from the WT FVB studies, wherein the CL was nearly 10 (l/h)/kg for OMI alone and 5 (l/h)/kg for OMI in combination (Table 4). Additionally, the systemic clearance is similar between the ICR mice (dosed at 10 mg/kg) and the WT FVB mice (dosed at 5 mg/kg). Between those two studies, the AUCs were proportional, and the dose-normalized plasma AUCs for OMI intravenous in ICR and wild-type FVB are not significantly different (P = 0.19), indicating no significant strain differences.

**Loperamide PO Systemic Pharmacokinetics and CNS Distribution in ICR Mice.** Loperamide and OMI were also administered orally to assess systemic pharmacokinetics, CNS distribution, and bioavailability of the drugs when dosed in combination. The total plasma, brain, and spinal cord concentration-time profiles for loperamide in mice when dosed at 30 mg/kg alone and in combination with OMI (30 mg/kg) are shown in Fig. 5, A and B. Similar to the intravenous studies, the brain and spinal cord concentrations in the PO study are less than the plasma for the
duration of the time course, and therefore the $K_{pBrain}$ and $K_{pSpinal Cord}$ were also less than unity (Fig. 5, C and D). However, these concentration-time profiles in both studies show some evidence of multiple peaks, possibly because loperamide undergoes enterohepatic recycling (Miyazaki et al., 1979). The $t_{max}$ (time of maximum concentration in plasma) occurred at 1 hour for loperamide alone and at 4 hours for loperamide with OMI. The half-life for loperamide alone and in combination was 7 and 3.1 hours, respectively (Table 2). The CL/F for loperamide when dosed alone was similar to CL/F in the combination study, and the differences in the AUCs between the two studies for plasma, brain, and spinal cord were all nonsignificant ($P<0.49$, $P<0.150$, and $P<0.720$, respectively), and in accord with the intravenous studies, the bioavailability ($F$) was also not different ($F=0.19$ and $F=0.25$, Table 2).

**OMI Oral Systemic Pharmacokinetics and CNS Distribution in ICR Mice.** The total concentration-time profiles for OMI administered at 30 mg/kg alone and in combination are shown in Fig. 6, A and B. The $t_{max}$ occurred at 1 hour for OMI alone and at 30 minutes for OMI in combination with loperamide. The $K_{pBrain}$ for OMI alone and in combination showed a similar trend as in the intravenous studies; however, the concentration in brain did not surpass the concentration in plasma until after 4 hours in the PO studies. The overall brain-to-plasma ratio for OMI was greater than 1 for both the discrete and combination studies and was similar ($K_{pBrain}=1.27$ and 1.5, respectively; Table 2). In accordance with the intravenous

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Loperamide Alone (30 mg/kg)</th>
<th>Loperamide in Combination (30 mg/kg)</th>
<th>OMI Alone (30 mg/kg)</th>
<th>OMI in Combination (30 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>7</td>
<td>3.1</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>CL/F (l/h)/kg</td>
<td>19</td>
<td>15.5</td>
<td>22.3</td>
<td>51.2</td>
</tr>
<tr>
<td>V/F (l/kg)</td>
<td>192</td>
<td>70.3</td>
<td>80</td>
<td>162</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>235</td>
<td>370</td>
<td>345</td>
<td>186</td>
</tr>
<tr>
<td>$t_{max}$ (h)</td>
<td>0.5</td>
<td>4</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ Plasma (h*ng)/ml</td>
<td>1577 ± 427</td>
<td>1936 ± 290</td>
<td>1386 ± 191*</td>
<td>745 ± 166*</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ Brain (h*ng/g)</td>
<td>94 ± 13</td>
<td>162 ± 40</td>
<td>1762 ± 296</td>
<td>1121 ± 282</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ Spinal Cord (h*ng/g)</td>
<td>304 ± 220</td>
<td>223 ± 44</td>
<td>744 ± 273</td>
<td>615 ± 262</td>
</tr>
<tr>
<td>$K_{pBrain}$</td>
<td>0.06</td>
<td>0.08</td>
<td>1.27</td>
<td>1.5</td>
</tr>
<tr>
<td>$K_{pSpinal Cord}$</td>
<td>0.19</td>
<td>0.12</td>
<td>0.54</td>
<td>0.82</td>
</tr>
<tr>
<td>$F$</td>
<td>0.19</td>
<td>0.25</td>
<td>0.55</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**TABLE 2**
Summary pharmacokinetic parameters for loperamide and OMI in ICR mice after oral administration alone and in combination
A two-tailed unpaired $t$ test was performed to compare AUCs among tissues (see results) and between the same tissues in discrete vs. combination studies (*$P<0.014$). Results are presented as mean or mean ± S.D.
studies, the concentrations of OMI in spinal cord were less than brain and only surpassed plasma concentrations at later time points. The overall spinal cord-to-plasma ratios were 0.54 for OMI alone and 0.82 in combination with loperamide (Table 2). The plasma AUC for OMI alone was significantly greater than AUC plasma for OMI in the combination (P = 0.014). In accord with the intravenous studies, the oral bioavailability of OMI when dosed orally with loperamide was reduced from 0.55 to 0.17 (Table 2). However, when comparing the AUCs for brain and spinal cord for OMI alone and OMI in combination, the AUCs were found to be not significantly different (P = 0.111 and P = 0.735, Table 2).

**Loperamide Disposition and CNS Distribution in FVB Knockout Mice with and without OMI.** To determine the contribution of P-gp and BCRP to the pharmacokinetics and CNS distribution of OMI and loperamide alone and in combination, 5 mg/kg of both drugs and the combination were administered intravenous to WT, BKO, PKO, and TKO FVB mice. Concentration-time profiles for loperamide alone are shown in Supplemental Fig. 1. Loperamide disposition in the wild-type mice when administered alone is similar to its disposition in the ICR mice, and the plasma AUCs for loperamide intravenous in ICR and wild-type FVB studies are not significantly different (P = 0.56), indicating no significant strain differences. The concentration-time profiles in the BKO mice also have similar kinetics and distribution to WT mice (Supplemental Fig. 1, A and B), with brain and spinal cord concentrations lower than that of plasma. However, in the PKO and TKO mice, brain and spinal cord concentrations are higher than plasma for the duration of the time course. The terminal slopes for brain and spinal cord in these genotypes were not sufficiently negative to accurately extrapolate to time infinity, and therefore AUC Last is reported for these tissues rather than AUC0→∞. Upon comparison, the brain AUCs in the PKO and TKO mice were significantly higher than that of the WT mice (padj < 0.001, and padj = 0.009, respectively) as well as BKO mice (padj < 0.001 and padj = 0.015, respectively), but the brain AUCs in the PKO and TKO mice were not significantly different. The spinal cord AUCs in the PKO and TKO mice were also significantly higher than in the WT (padj = 0.001 and padj = 0.003, respectively) and the BKO mice (padj = 0.001 and padj = 0.004, respectively). This agrees with the prior characterization of loperamide as a P-gp substrate (Schinkel et al., 1996). These data, for the first time, characterize the contribution of P-gp to efflux of loperamide from mouse spinal cord.

Additionally, the plasma terminal phase in both the PKO and TKO mice shows a reduced slope (Supplemental Fig. 1, C and D). When the plasma AUC0→∞ was compared among the four genotypes, it was found that the WT AUC was not significantly different from the BKO mice (P adj = 0.988) or the TKO mice (p adj = 0.3151). However, the plasma AUC in PKO mice was significantly greater than in the WT mice (p adj = 0.001), and the PKO and TKO mice were not significantly different (p adj = 0.152). The systemic clearance appears to be reduced in mice lacking P-gp.

The concentration-time profiles and overall PK parameters for loperamide when administered with OMI show the same trends as the discrete dosing studies in all four genotypes (Supplemental Fig. 3, A–D; Table 3). Again, the distribution of loperamide into the CNS is significantly
increased in mice lacking P-gp. The half-life for loperamide also appears to be increased in mice lacking P-gp, and the clearance appears to be reduced. Similar to the ICR mouse studies, the addition of OMI did not significantly alter the plasma, brain, or spinal cord AUCs in the WT FVB mice (Table 3, $P = 0.914$, $P = 0.139$, $P = 0.617$, respectively).

When the $K_{p_{\text{Brain}}}$ and $K_{p_{\text{Spinal Cord}}}$ for loperamide alone are plotted over time, it is apparent that the mice with functional P-gp have similar tissue-to-plasma ratios (Fig. 7, A and B). This is also reflected in the DA for loperamide in both brain ($DA_{\text{Brain}}$) and spinal cord ($DA_{\text{Spinal Cord}}$), which is around 2 in the BKO mice (Table 5). Alternatively, the mice lacking P-gp have much higher tissue-to-plasma ratios over time for both brain and spinal cord (Fig. 7, A and B), with distributional advantages around and above 40 (Table 5). These same patterns are mirrored in the

### TABLE 3

Summary pharmacokinetic parameters of loperamide in WT, BKO, PKO, and TKO FVB mice after a single intravenous dose (5 mg/kg) and after coadministration with OMI (5 mg/kg)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>Wild-Type</th>
<th>BKO</th>
<th>PKO</th>
<th>TKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loperamide alone</td>
<td>$t_{1/2}$ (h)</td>
<td>1.85</td>
<td>1.7</td>
<td>7.8</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>CL (l/h/kg)</td>
<td>2.9</td>
<td>2.6</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>V (l/kg)</td>
<td>6.6</td>
<td>7.0</td>
<td>10.6</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0-\infty}$ or (last) Plasma (h*ng)/ml</td>
<td>1686 ± 413</td>
<td>1909 ± 312</td>
<td>4302 ± 781</td>
<td>2838 ± 273</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0-\infty}$ or (last) Brain (h*ng)/g</td>
<td>173 ± 78</td>
<td>397 ± 105</td>
<td>17,503 ± 5100</td>
<td>13,832 ± 3503</td>
</tr>
<tr>
<td></td>
<td>$K_{p_{\text{Brain}}}$</td>
<td>0.10</td>
<td>0.21</td>
<td>4.1</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>$K_{p_{\text{Spinal Cord}}}$</td>
<td>0.05</td>
<td>0.11</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Loperamide in combination</td>
<td>$t_{1/2}$ (h)</td>
<td>2.05</td>
<td>2.4</td>
<td>4.17</td>
<td>9.54</td>
</tr>
<tr>
<td></td>
<td>CL (l/h/kg)</td>
<td>2.9</td>
<td>2.4</td>
<td>0.98</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>V (l/kg)</td>
<td>6.9</td>
<td>5.67</td>
<td>4.46</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0-\infty}$ or (last) Plasma (h*ng)/ml</td>
<td>1744 ± 333</td>
<td>2080 ± 207</td>
<td>5266 ± 923</td>
<td>4860 ± 1377</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0-\infty}$ or (last) Brain (h*ng)/g</td>
<td>460 ± 188</td>
<td>216 ± 27</td>
<td>13,350 ± 2012</td>
<td>14,566 ± 2713</td>
</tr>
<tr>
<td></td>
<td>$K_{p_{\text{Brain}}}$</td>
<td>0.06</td>
<td>0.10</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>$K_{p_{\text{Spinal Cord}}}$</td>
<td>0.05</td>
<td>0.07</td>
<td>2.0</td>
<td>1.89</td>
</tr>
</tbody>
</table>

**Fig. 7.** Loperamide CNS distribution in FVB transporter knockout mice. (A) $K_{p_{\text{Brain}}}$ of loperamide in WT, BKO, PKO, and TKO FVB mice. (B) $K_{p_{\text{Spinal Cord}}}$ of loperamide in WT, BKO, PKO, and TKO FVB mice. (C) $K_{p_{\text{Brain}}}$ of loperamide when coadministered with OMI in WT, BKO, PKO, and TKO FVB mice. (D) $K_{p_{\text{Spinal Cord}}}$ of loperamide when coadministered with OMI in WT, BKO, PKO, and TKO FVB mice.
tissue-to-plasma ratios for loperamide in the combination study, wherein mice with functional P-gp have lower tissue-to-plasma ratios than the mice lacking P-gp (Fig. 7, C and D). Interestingly, the DA for brain and spinal cord may be reduced when loperamide is dosed in combination with OMI (Table 5).

**OMI Disposition and CNS Distribution in FVB Transporter Knockout Mice with and without Loperamide.** When OMI was administered alone in WT and BKO FVB mice, it showed similar distribution kinetics, with the brain and spinal cord concentrations surpassing that of plasma at the later time points (Supplemental Fig. 2, A and B). However, the PKO and TKO mice showed higher concentrations of OMI in brain and spinal cord throughout the time course. The terminal slopes for brain and spinal cord in these genotypes were not sufficiently negative to accurately extrapolate to time infinity, and therefore AUCLast is reported for these tissues rather than AUC0–∞. When the AUCs were compared, differences among brain and spinal cord AUCs in WT and BKO mice were not distinguishable (padj = 0.551 and padj = 0.999, respectively). The PKO and TKO mice, however, did have significantly higher AUCs than the WT mice and the BKO mice for both brain and spinal cord (padj < 0.001 for all cases). When the KPBrain and KPspinal Cord were compared over time, it was apparent that the mice with functional P-gp trend closely together at ratios near 1, lower than that of the PKO and TKO mice (Fig. 8, A–D). The DAbrain for OMI in the knockouts was around 1 for the BKOs and 8 in the two genotypes lacking P-gp. The DASpinal Cord was less than 1 in the BKOs and greater than 8 in the P-gp knockouts and triple knockouts (Table 5). All of these data indicate that OMI is a P-gp substrate but not a BCRP substrate.

For OMI discrete dosing, there was no difference in the plasma AUC or the half-life in any of the genotypes, implying that the systemic clearance and volume of distribution of OMI were not altered significantly by a lack of P-gp or BCRP (Table 4). The concentration-time profiles for OMI in combination with loperamide were similar to OMI alone for all four genotypes (Supplemental Fig. 4). As observed in the ICR OMI intravenous studies, the clearance of OMI is reduced by approximately half when administered in combination with loperamide (Table 4), and as previously stated, the AUCBrain for OMI in WT FVB mice is significantly higher when dosed in combination with loperamide, but the overall KPBrain did not change. Also, as previously stated, the dose-normalized plasma AUCs for OMI intravenous in ICR and wild-type FVB are not significantly different (P = 0.19), indicating no significant strain differences. Interestingly, the reduction in clearance in the presence of loperamide that is observed in the ICR mice as well as the WT FVB mice is also consistent across all FVB genotypes when comparing OMI discrete dosing and the combination. This indicates that neither P-gp nor BCRP are likely to be involved in the mechanism of the interaction resulting in reduced systemic clearance of OMI.

**TABLE 5**

Distributional advantage for brain and spinal cord in the three genotypes of transporter knockout FVB mice (BKO, PKO, and TKO mice) Determined by a ratio of Kps in each tissue to the corresponding Kp in wild-type FVB mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue</th>
<th>Loperamide Alone</th>
<th>Loperamide in Combination</th>
<th>OMI Alone</th>
<th>OMI in Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKO</td>
<td>DAbrain</td>
<td>2.1</td>
<td>0.38</td>
<td>1.2</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>DAspinal Cord</td>
<td>2.2</td>
<td>1.4</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>PKO</td>
<td>DAbrain</td>
<td>4.1</td>
<td>9.6</td>
<td>8.1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>DAspinal Cord</td>
<td>40</td>
<td>40</td>
<td>25</td>
<td>3.9</td>
</tr>
<tr>
<td>TKO</td>
<td>DAbrain</td>
<td>49</td>
<td>11</td>
<td>8.4</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>DAspinal Cord</td>
<td>54</td>
<td>38</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
</table>
RED for Free Fraction in Mouse Plasma and Brain Homogenate. The free fraction in plasma and brain was determined by rapid equilibrium dialysis. The brain unbound partition coefficient (Kpuu) was determined using the brain partition coefficients from wild-type FVB mouse studies, as all of these studies were carried out at the same dose. The unbound fractions and Kpuu are reported in Table 6. There was a significant increase in the unbound fraction of loperamide in plasma in the presence of OMI ($P < 0.001$). However, there was no detectable difference in the unbound fraction of loperamide in brain homogenate with the presence of OMI. The fraction unbound of OMI was much higher than that of loperamide in both plasma and brain ($P < 0.001$ in both cases), but there was no change in the unbound fraction of OMI in the presence of loperamide in either plasma ($P = 0.165$) or in brain homogenate ($P = 0.222$). The Kpuu for both drugs was unchanged by the presence of the other. The Kpuu for loperamide alone was 0.1 versus 0.11 in the presence of OMI, and the Kpuu for OMI alone was 0.44 versus 0.42 in the presence of loperamide. This indicates that the brain penetration of both drugs is not significantly altered by a change in protein binding when they are administered in combination.

**Table 6**

Unbound fractions of loperamide and OMI in brain and plasma determined from in vitro RED experiment after 24-hour incubation in five replicates.

Unbound partition coefficients determined using data from WT FVB mice. Results are presented as mean ± S.D.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Plasma $f_u$ (mean ± S.D.)</th>
<th>Brain $f_u$ (mean ± S.D.)</th>
<th>Kpuu (wild-type FVB mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loperamide</td>
<td>0.0183 ± 0.0011*</td>
<td>0.0154 ± 0.0022</td>
<td>0.10</td>
</tr>
<tr>
<td>Loperamide (with OMI)</td>
<td>0.0357 ± 0.0053*</td>
<td>0.0140 ± 0.0016</td>
<td>0.11</td>
</tr>
<tr>
<td>OMI</td>
<td>0.192 ± 0.0754</td>
<td>0.0841 ± 0.0142</td>
<td>0.44</td>
</tr>
<tr>
<td>OMI (with loperamide)</td>
<td>0.253 ± 0.0482</td>
<td>0.0951 ± 0.0187</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Discussion**

Opioid agonists have a long history of effective use in the treatment of pain. However, along with their benefits come a number of caveats and risks like tolerance, dependence, and death (Presley and Lindsley, 2018). This has led to demand for more prudent prescription practices and alternatives to the conventional use of opioids, and one promising avenue is through combinations of biased opioid agonists targeting the μ- and δ-opioid receptors. Loperamide is an MOR-agonist that is already Food and Drug Administration–approved as an anti-diarrheal medication, and oxymorphindole is a novel DOR-agonist with high specificity (Takemori et al., 1992). In combination, these drugs have shown to be peripherally active with synergistic efficacy (Bruce et al., 2019; Uhelski et al., 2020) or at least significantly increased potency of loperamide, depending on the route of administration (Fig. 2). Although there are proposed mechanisms of action for this significant increase in potency, it could be due to a change in pharmacokinetics or CNS penetration (Fig. 1). Our pharmacokinetic assessment sought to clarify whether these drugs have a pharmacokinetic interaction. In the present study, we administered loperamide and OMI alone and in
combination to both wild-type ICR mice and four genotypes of FVB mice. The ICR mouse studies provide continuity with previously published pharmacodynamic studies and give information about the systemic disposition of both drugs, whereas the FVB mouse studies provide further insight into potential interactions of the two drugs at efflux transporters in the barriers of the CNS.

The results from the loperamide intravenous administration studies in ICR mice as well as WT FVB mice indicate that OMI has no significant effect on loperamide systemic pharmacokinetics or CNS distribution that might alter the activity or safety of this drug. With reference to safety, MOR agonists are of particular concern, as the reward signaling and adverse effects of dependence and respiratory depression are mediated by MORs in the CNS (Matthes et al., 1996; Pattinson, 2008). The present studies show no change in the $K_{p}$Brain, $K_{p}$Spinal Cord, or the $K_{p}$unbound, indicating that OMI has no effect on the CNS partitioning or active concentration of loperamide in the brain. This supports previous studies that show this combination has reduced liability for respiratory depression (a centrally mediated adverse effect) compared with that of other, more brain-penetrant MOR-agonists (Bruce et al., 2019). After oral administration of the combination, there is no significant change in loperamide's systemic disposition, CNS distribution, or oral bioavailability. Given that the combination was administered at doses nearly 10-fold higher than the oral ED$_{50}$ for this pharmacokinetic assessment, the likelihood of a pharmacokinetic interaction that changes the efficacy or safety of loperamide is even lower at the therapeutic oral doses and would be of little concern for future development of this combination therapy.

In assessing the disposition and CNS distribution of OMI for the first time, it was found that OMI has appreciable CNS penetration and that the plasma and CNS exposure of OMI is increased in the presence of loperamide. According to the intravenous administration studies in ICR and WT FVB mice, the increase in CNS exposure of OMI is proportional to the increased plasma exposure. Additionally, the unbound fraction of OMI does not change in the presence of loperamide in either brain or plasma, and therefore the unbound partitioning of OMI into the brain is also unchanged. Furthermore, given OMI's tolerability in ICR mice at a higher dose of 10 mg/kg i.v. and the fact that DORs do not promote the undesirable effect respiratory depression, the distribution of OMI to the CNS is not a present concern with regard to safety. In fact, certain DORs have been shown to modulate some opioid effects, such as tolerance; therefore, OMI CNS penetration could be an advantage of the combination (Zhu et al., 1999; Pradhan et al., 2009).

Regarding the systemic disposition of OMI, the increase in the plasma AUC of OMI when coadministered with loperamide in both the ICR intravenous studies as well as the WT intravenous studies is due to the reduction in clearance observed in the presence of loperamide. The metabolism and elimination of OMI have not been previously characterized, and therefore, we cannot speculate on a potential mechanism. However, given the fact that the reduction in clearance was observed in the combination studies in all four FVB genotypes, it is unlikely that either P-gp or BCRP play a role in this particular interaction. In the case of oral administration, the systemic exposure of OMI is significantly reduced. This is likely due to the decreased bioavailability of OMI when administered in the combination. According to the pharmacodynamic data, significantly lower oral doses of the combination show increased potency, and therefore a potential reduction in the bioavailability of OMI is not likely to be a limitation of the combination.

With regard to an interaction at the CNS barriers, a significant factor in many drugs’ CNS distribution to the brain and spinal cord is efflux by ATP-binding cassette transporters. Loperamide has been previously characterized as a P-gp substrate (Schinkel et al., 1996) but not a BCRP substrate, and how these transporters factor into loperamide's spinal cord distribution was unknown. Our studies show that loperamide is not a strong BCRP substrate and that P-gp plays a significant role in excluding loperamide from spinal cord. Furthermore, the substrate status of OMI has never been determined. The current study indicates that OMI is a substrate of P-gp but not a substrate of BCRP. Additionally, because neither drug shows increased CNS tissue partitioning after coadministration in the studies described herein, there is no evidence that P-gp is saturated when the drugs are coadministered. A large body of research shows that efflux transport systems at the blood-brain barrier are robust even in cases when the blood-brain barrier is disrupted either by the presence of a tumor or by artificial means (Goutal et al., 2018; de Gooijer et al., 2021; Griffith et al., 2021). Although some studies have shown that the administration of loperamide with P-gp modulators and inhibitors like quinidine could pose the risk of classic opioid effects (Sadeque et al., 2000), postmarketing assessments of loperamide when administered in combination with a variety of other P-gp substrates show that MOR-associated adverse effects are unlikely to occur, implying that loperamide’s access to the CNS is not enhanced to a therapeutically significant extent (Vandenbossche et al., 2010).

Although the CNS exposure of OMI may be increased after intravenous administration of the combination due to a reduction in its systemic clearance, the unbound CNS partitioning of both drugs is unchanged in the combination. Given the fact that the drugs appear to have no significant interaction at the CNS barriers and the fact that OMI is not an efficacious analgesic agent on its own, the most likely mechanism for the interaction between loperamide and OMI is an alteration in pharmacodynamics at receptors in the peripheral nervous system. Earlier research has shown colocalization of MOR and DOR receptors and evidence of heterodimerization, especially in inflammatory pain states (Gomes et al., 2004; Bruce et al., 2019). Other previous studies have indicated that the synergy between specific MOR and DOR agonists requires protein kinase C ε, and that DOR agonism is retained only in the case of biased signaling wherein specific agonists promote DOR and MOR phosphorylation but not DOR and MOR internalization (Pradhan et al., 2009; Schuster et al., 2015; Derouiche et al., 2020). The mechanism of synergy for OMI and loperamide is therefore most likely that MORs and DORs form heteromers that remain localized at the cell membrane of primary afferents and retain protein kinase C ε-dependent signaling.

This conclusion regarding the peripherally mediated activity of OMI and loperamide is another promising step in the development of peripherally restricted opioids for the management of chronic and severe pain that significantly reduce the potential for tolerance, dependence, and overdose deaths. No peripherally restricted opioids have been approved for the treatment of chronic pain, but their development is of increasing interest. A number of bispecific agonists have been proposed, and previous work shows that bispecific agonists with a specific linker length have pronounced synergy and modulation of undesirable side effects (Daniels et al., 2005; Lenard et al., 2007; Ding et al.,
2018, Lei et al., 2020). This strategy is attractive for future drug development, and accounting for biased signaling of peripherally restricted combinations of MOR and DOR agonists will likely lead to the development of safer and more effective analgesics.

**Authorship Contributions**

*Participated in research design:* Kim, Fairbanks, Wilcox, Elmqquist, 
*Conducted experiments:* Griffith, Kim, Bruce, Peterson, Kitto, 
*Mohammad, Rathii, 
*Performed data analysis:* Griffith, Kim, Bruce.

Wrote or contributed to the writing of the manuscript: Griffith, 
*Bruce, Peterson, Kitto.*

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**References**


