

# Effects of casoxin 4 on morphine inhibition of small animal intestinal contractility and gut transit in the mouse

Glen S Patten<sup>1,2</sup>

Richard J Head<sup>1</sup>

Mahinda Y Abeywardena<sup>1,2</sup>

<sup>1</sup>CSIRO Preventative Health National Research Flagship, Adelaide, Australia;

<sup>2</sup>CSIRO Food and Nutritional Sciences, Adelaide, Australia

**Background and aims:** Chronic opioid analgesia has the debilitating side-effect of constipation in human patients. The major aims of this study were to: 1) characterize the opioid-specific antagonism of morphine-induced inhibition of electrically driven contraction of the small intestine of mice, rats, and guinea pigs; and 2) test if the oral delivery of small milk-derived opioid antagonist peptides could block morphine-induced inhibition of intestinal transit in mice.

**Methods:** Mouse, rat, and guinea pig intact ileal sections were electrically stimulated to contract and inhibited with morphine *in vitro*. Morphine inhibition was then blocked by opioid subtype antagonists in the mouse and guinea pig. Using a polymeric dye, Poly R-478, the opioid antagonists casoxin 4 and lactoferroxin A were tested orally for blocking activity of morphine inhibition of gut transit *in vivo* by single or double gavage techniques.

**Results:** The guinea pig tissue was more sensitive to morphine inhibition compared with the mouse or the rat ( $IC_{50}$  [half maximal inhibitory concentration] values as nmol/L  $\pm$  SEM were  $34 \pm 3$ ,  $230 \pm 13$ , and  $310 \pm 14$  respectively) ( $P < 0.01$ ). The inhibitory influence of opioid agonists ( $IC_{50}$ ) in electrically driven ileal mouse preparations were DADLE ([D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]-enkephalin)  $\geq$  met-enkephalin  $\geq$  dynorphin A  $\geq$  DAMGO ([D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin)  $>$  morphine  $>$  morphiceptin as nmol/L 13.9, 17.3, 19.5, 23.3, 230, and 403 respectively. The mouse demonstrated predominantly  $\kappa$ - and  $\delta$ -opioid receptor activity with a smaller  $\mu$ -opioid receptor component. Both mouse and guinea pig tissue were sensitive to casoxin 4 antagonism of morphine inhibition of contraction. In contrast to naloxone, relatively high oral doses of the  $\mu$ -opioid receptor antagonists, casoxin 4 and lactoferroxin A, applied before and after morphine injection were unable to antagonize morphine inhibition of gut transit.

**Conclusions:** Casoxin 4 reverses morphine-induced inhibition of contraction in mice and guinea pigs *in vitro* but fails to influence morphine inhibition of mouse small intestinal transit by the oral route.

**Keywords:** lactoferroxin A,  $\mu$ -opioid receptor antagonist, opioid agonists

## Introduction

Much attention has been focused on characterizing the central and systemic opioid receptor profiles of small laboratory animals used to elucidate the mechanism of morphine inhibition of gut transport.<sup>1–6</sup> A major challenge is to develop a natural or pharmaceutical agent that can relieve the constipating side effect of opioid therapy without compromising central analgesia.<sup>7</sup> However, there is no guarantee that the agent found to be efficacious in an animal model would be functional in humans. Naloxone methiodide has been developed as an antimorphine agent that could overcome constipation without antagonizing analgesia because it does not cross the blood–brain

Correspondence: Glen S Patten  
CSIRO Food and Nutritional  
Sciences, Gate 13 Kintore  
Avenue, PO Box 10041,  
Adelaide, BC 5000, Australia  
Tel +61 8 8303 8956  
Fax +61 8 8303 8899  
Email glen.patten@hsn.csiro.au

barrier,<sup>8</sup> but its therapeutic value is limited due to poor oral availability.<sup>9</sup> In this competitive field, other pharmacological agents of interest for the treatment of opioid-induced bowel dysfunction are alvimopan and methylnaltrexone,<sup>10–12</sup> but further data are required to fully assess their place in therapy.<sup>13</sup> Other compounds are now in advanced clinical trials (for example, NKTR-118, TD-1211, ALKS 37, and ADL5945) for the relief of opioid-induced constipation in chronic pain patients.<sup>14–17</sup>

Our focus has been directed towards antagonism of peripheral opioid receptors using small peptides. We have demonstrated that the methoxylated tetrapeptide sequence isolated from the  $\kappa$ -casein fraction of bovine milk, casoxin 4 (Tyr-Pro-Ser-Tyr-OCH<sub>3</sub>), antagonized morphine inhibition of electrically driven guinea pig ileum when applied specifically to the lumen.<sup>7</sup> Therefore, our hypothesis is that orally applied small milk-derived opioid antagonists can block the morphine-induced retardation of gut transit in the mouse model. Since the mouse is a well characterized model for opioid-induced analgesia and inhibition of gut transit, our first aim is to investigate the opioid agonist and antagonist profile of the electrically stimulated mouse ileum and compare this with rat and guinea pig ileum. Our second aim is to test whether two potent opioid peptide antagonist sequences found in milk peptone and whey can overcome the anti-motility properties of morphine in the mouse when administered orally.

## Materials and methods

### Animals

Male Wistar rats, male Swiss mice, and male and female guinea pigs from the Institute of Medical and Veterinary Science Animal Resource Centre (Gilles Plains, South Australia) were fed standard laboratory feed and water ad libitum. The guinea pigs were given supplements of fresh fruit and vegetables. Animals were housed and euthanized according to the guidelines of the CSIRO Food and Nutritional Sciences Animal Experimental Ethics Committee who approved the study under the guidelines of the National Health and Medical Research Council, Australian Research Council, and the Australian Vice-Chancellors' Committee – Australian code of practice for the care and use of animals for scientific purposes.<sup>18</sup> Power calculations were included in the body of the experimental design within the ethics application.

### Electrically driven infused guinea pig ileum

Guinea pigs of either sex weighing 400–500 g were used. After the animals were painlessly asphyxiated with CO<sub>2</sub>,

the small intestine to just above the ileocecal junction was excised and flushed with saline. The terminal 10 cm of the distal ileum was discarded. A catheter was inserted at each end of a 4–5 cm piece of ileum.<sup>7</sup> The proximal end of the ileum was plugged via the plastic cannula to a plastic sleeve over a glass inlet perfusion cannula that was integral to a glass plug that was placed into the bottom of the bath. The cannula at the distal end of the ileum was plugged into a plastic sleeve and suspended by a 10 cm rigid plastic perfusion outlet tube bent at 135° at the top with 5 cm of further outlet tubing. The outlet tube was connected at the bend to the arm of a Harvard isotonic transducer by a short length of cotton sustaining 2 g of tension. This system allows for the separation of opioid effects of the bath (or serosa) from the lumen.<sup>7</sup> Contractions were measured via a Biopac system and translated and stored using AcqKnowledge® for Windows (version 3.01; Biopac System Inc., Goleta, CA). The tissue was bathed vertically in 30 mL of a modified Krebs–Henseleit bicarbonate buffer containing in mmol/L: 118 NaCl, 25 NaHCO<sub>3</sub>, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.8 CaCl<sub>2</sub>, and 11 glucose, at pH 7.4 in a water jacketed bath at 37°C while being gently bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The contents of the bath were flushed from the bottom and withdrawn from an overflow near the top of the bath by vacuum. The ileum was initially flushed through the lumen for 30 minutes with buffer at a rate of 1.6 mL/min using a Minipuls 2 pump (Gilson Inc., Middleton, WI). The tissue was then electrically stimulated via two stainless steel electrodes, both 10 cm long, running parallel to and on opposite sides of the tissue in the bath, using square wave pulses of 60 V for 5 ms at 0.1 Hz. At least 1 hour was allowed for the tissue to stabilize. Peptide (or chemical as indicated) was added to the bath as a small bolus in saline. Dose curve effects were generated by serial addition to the bath.<sup>19,20</sup>

### Electrically driven mouse and rat ileum

Animals were killed by cervical dislocation and the small intestine removed and flushed with saline. Distal sections of ileum 4–5 cm long were dissected out and tied at each end with fine suture. The proximal end was secured to the bottom of a 30 mL organ bath and the distal end connected to the torsion arm of a Harvard isotonic transducer at minimum (0.1 g) tension to suspend the tissue. The bath contained Krebs–Henseleit bicarbonate buffer, and contractions were recorded as described above. Tissue was stimulated to contract by field stimulation created from round stainless steel electrodes at the top and bottom of the bath with the tissue situated centrally to the electrodes.

Optimal settings for ileal contraction were determined by firstly maintaining the voltage at 60 V at 0.1 Hz and incrementally increasing the length of time of the square pulse (millisecond) for the rat and mice tissue. Once the maximal time of stimulation was determined, the millisecond level was set (again with 0.1 Hz) and the voltage increased incrementally until maximal contraction had been achieved. At supramaximal settings, the tissue was then allowed 1 hour to stabilize. Opioid agonists were added to the bath as small boluses, and dose curves were generated by cumulative additions to the bath. After allowing at least 5 minutes with agonist, opioid antagonists were added stepwise to the bath.

### Small intestine transit in the mouse: single gavage experiments

Mice were injected subcutaneously (150  $\mu$ L/30 g mouse) with morphine (or morphine with naloxone) at 1.0, 1.5, or 2.0 mg/kg (of bodyweight) or saline as control as indicated and 20 minutes later given 100  $\mu$ L of a 5% solution of Poly-478 in Milli-Q Plus water (Millipore, Billerica, MA) as control or containing casoxin 4 (50 mg/kg) or lactoferroxin A (50 mg/kg) or naloxone (2 mg/kg) administered by gastric gavage.<sup>21</sup> After 45 minutes allowed for transit, the mice were euthanized by cervical dislocation, and the small intestine was cut into 8 segments of equal lengths. Segments were numbered from 1 (proximal) to 8 (distal) and all samples plus the stomach were flushed with saline up to a final volume of 6 mL at room temperature. Both the stomach flush and small intestinal flush were placed in test tubes and Poly R-478 was determined by colorimetric assay.<sup>21–23</sup> The datum for each segment was expressed as a percentage of the sum total. The dye front was measured and expressed as a percentage of the total small intestine length.

### Small intestine transit in the mouse: double gavage experiments

Mice were gavaged with 100  $\mu$ L saline containing casoxin 4 (50 mg/kg) or saline as control, and 20 minutes later injected subcutaneously with 2 mg/kg morphine or saline as control. A further 20 minutes later, the mice were gavaged with 100  $\mu$ L Poly R-478 in Milli-Q water as control or containing casoxin 4 (50 mg/kg). The total dose of casoxin 4 gavaged was therefore 100 mg/kg in 200  $\mu$ L of solution that calculates to a maximal net bolus concentration of approximately 0.7 mol/L in the stomach. Gastrointestinal transit proceeded for 45 minutes, at which time mice were euthanized and the

stomach and intestinal contents treated as described above for the single gavage experiments. After single or double gavage techniques when the contents of the stomach and small intestine were removed, no damage or bleeding was observed of the gut mucosa.

### Colorimetric assay of Poly R-478

Colorimetric assay of Poly R-478 was performed as described previously elsewhere and in our laboratory with small modifications.<sup>21–23</sup> Flush samples were homogenized for 10 seconds by Ultra-Turrax® (Janke and Kunkel, GmbH and Co., Stauffer, Germany) on setting 6. One-half mL of aliquots (in duplicates) of each homogenate were placed into plastic test tubes containing 1.5 mL 1 N KOH, vortexed, capped, and left at room temperature for 12 hours. The samples were spun for 10 minutes at 3000 rpm in a Beckman CPR centrifuge (Fullerton, CA) and the supernatants decanted into test tubes. The supernatants were read against a KOH blank at 515 nm on a Varian DMS 80 spectrophotometer (Varian Techtron Pty Ltd, Mulgrave, Australia). Several standard curves were used.

### Drugs

Drugs used were: morphine hydrochloride (Fauldings, Adelaide, South Australia);  $\beta$ -casomorphin[1–4] amide (morphiceptin), [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]-enkephalin (DADLE), [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin (DAMGO), dynorphin<sub>[1–13]</sub> A, ibuprofen, naloxone hydrochloride, Poly R-478, and other chemicals from Sigma-Aldrich Chemical Company (Sydney, Australia); met-enkephalin and custom synthesis of casoxin 4 and lactoferroxin A from Auspep (Melbourne, Victoria, Australia).

### Statistical analyses

Data were usually shown as mean  $\pm$  SEM (standard error of the mean). The effects of opioid agonists and antagonists on small intestinal contractility or transit time were determined using two-tailed Student's *t*-tests when comparing between two sets of data or by 2-way ANOVA for three or more sets of data (Sigma-Stat 3.1; Jandel Scientific, Corte Madera, CA). When significance was obtained by ANOVA ( $P < 0.05$ ), post tests by Bonferroni were performed or by using Dunnett's multiple comparison post tests using suitable controls. The EC<sub>50</sub> (half maximal effective concentration) and IC<sub>50</sub> (half maximal inhibitory concentration) values and maximal contraction values were determined from concentration dose curves using Graph fits in Prism (version 4.0; GraphPad Software, San Diego, CA) with  $R^2$  values  $> 0.99$ .

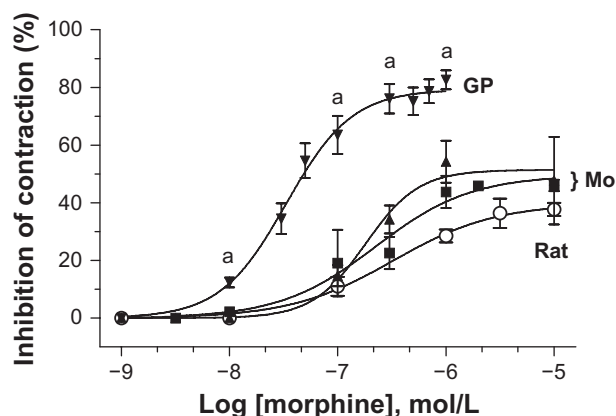
## Results

### Electrically driven ileum of small animal models

For the mouse, electrical square pulses of 3–5 milliseconds were required to stimulate maximal movement (results not shown) of mouse ileum at 60 V and 0.1 Hz. This resulted in a large relaxation phase that was followed by a smaller contraction phase. The  $IC_{50}$  for morphine inhibition for the electrically driven contractions in the mouse ileum was  $180 \pm 14$  nmol/L (Figure 1). It was found that  $0.1 \mu\text{mol/L}$  ibuprofen reduced the basal tone and routinely abolished the majority of the relaxation phase revealing predominantly the contraction phase of smooth muscle action. However, in this system, ibuprofen did not significantly change the sensitivity to morphine ( $IC_{50}$   $230 \pm 15$  nmol/L), compared with morphine alone,  $180 \pm 14$  nmol/L (Figure 1). In all subsequent experiments, ibuprofen was included 5–10 minutes before the addition of opioid agonist or antagonist. The relative sensitivities to morphine inhibition using the electrical stimulation ( $IC_{50}$  as nmol/L  $\pm$  SEM) regimens were for the guinea pig ( $34 \pm 3$ ), compared with the mouse ( $230 \pm 15$ ) and rat ( $310 \pm 17$ ), which was significantly lower in the guinea pig compared with both mouse and rat ( $P < 0.001$ ) and between the mouse and the rat ( $P < 0.05$ ) as shown in Figure 1 ( $P < 0.01$ ).

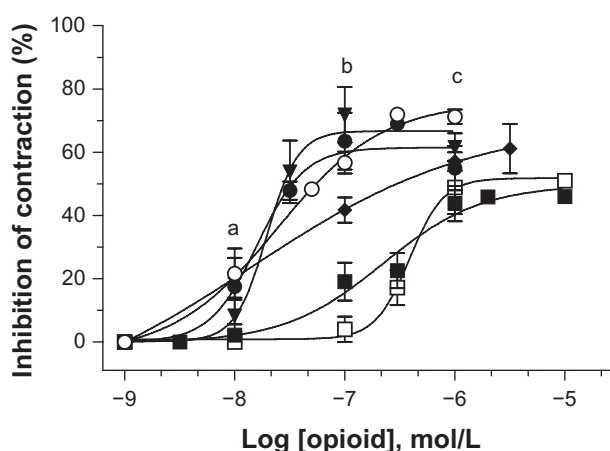
### Effect of opioid agonists on electrically driven contraction in mouse ileum

The effect of a range of opioid receptor-specific agonists on the inhibition of electrically driven contractions in the mouse ileum is given in Figure 2. The relative order of



**Figure 1** Morphine inhibition of electrically driven contractions in guinea pig (▼), rat (○), and mouse (▲) ileum. Mouse was also in the presence (■) of  $0.1 \mu\text{M}$  ibuprofen. Each point is the mean  $\pm$  SEM for ileal tissue from 15–19 guinea pigs and 3–4 rats or mice performed in duplicate. Significant differences between species is indicated at  $10^{-8}$ ,  $10^{-7}$ ,  $3 \times 10^{-7}$ , and  $10^{-6}$  mol/L as determined by 2-way ANOVA and Dunnett post tests as  $P < 0.01$  (letter a) for guinea pig compared with rat or mouse.

**Abbreviations:** ANOVA, analysis of variance; GP, guinea pig; Mo, mouse; SEM, standard error of the mean.



**Figure 2** Effect of opioid agonists on inhibition of the electrically driven mouse ileum with  $0.1 \mu\text{mol/L}$  ibuprofen. Opioids: (■) morphine, (○) DAMGO, (▼) dynorphin $_{[1-13]}$ -A, (●) met-enkephalin, (◆) DADLE, (□) morphiceptin. Each point represents the mean  $\pm$  SEM at each dose tested on  $n = 3-4$  mice in duplicate. On the graph, the letter "a" as determined by ANOVA and Bonferroni post tests, at  $10^{-8}$  mol/L, the % inhibition of electrically driven contraction by DAMGO, DADLE, and met-enkephalin are significantly higher than morphine, dynorphin $_{[1-13]}$ -A, and morphiceptin; for "b" at  $10^{-7}$  mol/L, DAMGO, dynorphin $_{[1-13]}$ -A, DADLE, and met-enkephalin are higher than morphine and morphiceptin; and "c" at  $10^{-6}$  mol/L, DAMGO is higher than morphine ( $P < 0.05$ ).

**Abbreviations:** DADLE, [D-Ala $^2$ , D-Leu $^5$ ]-enkephalin; DAMGO, [D-Ala $^2$ , N-Me-Phe $^4$ , Gly-ol $^5$ ]-enkephalin; SEM, standard error of the mean.

potency (opioid receptor subtype,  $IC_{50}$  in nmol/L  $\pm$  SEM) was DADLE ( $\delta$ ,  $13.9 \pm 2.1$ )  $\geq$  met-enkephalin ( $\delta$ ,  $17.9 \pm 3.3$ )  $\geq$  dynorphin $_{[1-13]}$  A ( $\kappa$ ,  $19.5 \pm 4.0$ )  $\geq$  DAMGO ( $\mu$ ,  $23.3 \pm 4.1$ )  $>$  morphine ( $\mu$ ,  $230 \pm 13$ ) and morphiceptin ( $\mu$ ,  $403 \pm 19$ ). The  $IC_{50}$  values for DADLE, met-enkephalin, and dynorphin $_{[1-13]}$  A were significantly lower than for morphine, and the morphine value was lower than for morphiceptin ( $P < 0.05$ ). The differences between the various opioid agonists at  $10^{-8}$ ,  $10^{-7}$ , and  $10^{-6}$  mol/L are given in the legend to Figure 2. The  $\mu$ -opioid receptor agonists (morphine and morphiceptin) had maximal inhibition of 48% and 50%, whereas the more active agents ranged between 64% and 76% maximal inhibition. It is of note that the dose response curve shapes of the various opioid agonists are different in Figure 2. However, similar variability in curve shapes has been observed for the inhibition of electrically driven contractions by enkephalins in vitro for the guinea pig ileum.<sup>7</sup>

### Antagonist effect of casoxin 4 against morphine in the mouse and guinea pig

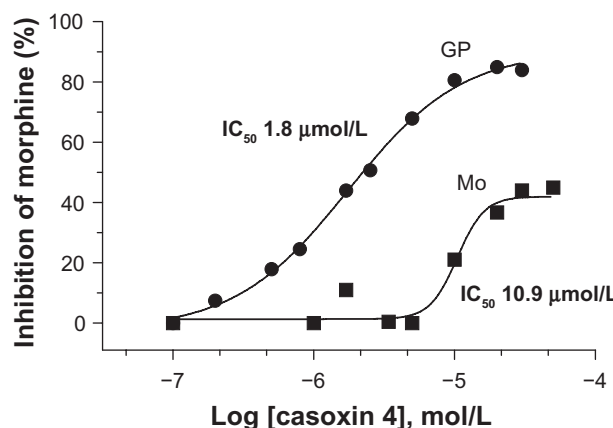
Concentrations of morphine required to inhibit electrically driven contractions by approximately 50% in the guinea pig and mouse were  $0.1$  and  $1.0 \mu\text{mol/L}$  respectively. The  $\mu$ -specific opioid antagonist, casoxin 4, one of the smallest and most potent opioid antagonist peptides yet described, is a synthetic tetrapeptide fragment of casoxin 6.<sup>24,25</sup>



The potency of casoxin 4 to antagonize morphine inhibition of electrically driven contractions is shown in Figure 3. The  $IC_{50}$  for casoxin 4 reversal of morphine inhibition is  $2.1 \pm 0.2 \mu\text{mol/L}$  for the guinea pig and  $11.3 \pm 0.5 \mu\text{mol/L}$  for the mouse, respectively, which were significantly different by Student's *t*-test ( $P < 0.001$ ). Using the electrical regimes employed here, the guinea pig was far more sensitive and had a higher maximal inhibition to morphine compared with the mouse. Furthermore, casoxin 4 overcame only 50% of the morphine inhibition of electrically driven contraction in the mouse. It was found that all inhibition of contractility could be antagonized by 0.1–1.0  $\mu\text{mol/L}$  naloxone (results not shown).

### Effects of casoxin 4, lactoferroxin A, and naloxone against morphine inhibition of gut transit in the mouse

We have previously demonstrated that casoxin 4 antagonizes the inhibitory effect of morphine on electrically driven contraction when specifically applied to the lumen of the isolated intact guinea pig ileum.<sup>7</sup> In this report we have shown that casoxin 4 also inhibits morphine when added nonspecifically to the serosal side of the mouse ileum. We have further investigated whether casoxin 4 and another milk-derived oligopeptide, lactoferroxin A, a  $\mu$ -opioid receptor antagonist isolated from the enzymatic digestion of human lactoferrin (H-Tyr-Leu-Gly-Ser-Gly-Tyr-OCH<sub>3</sub>), can antagonize the morphine inhibition

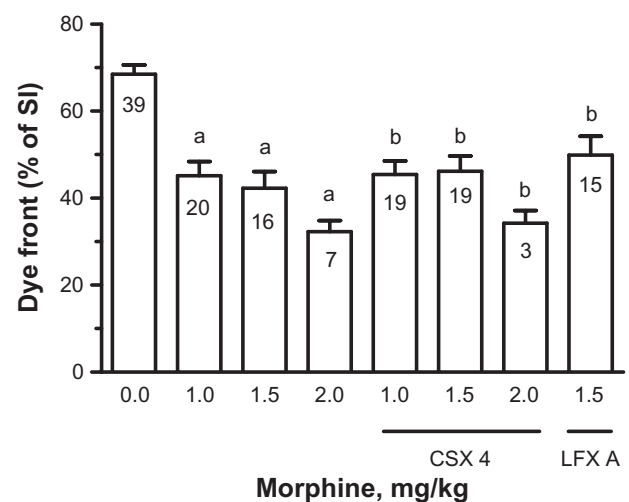


**Figure 3** Casoxin 4 antagonism of morphine inhibition of electrically driven contraction in the mouse pre-incubated with 0.1  $\mu\text{mol/L}$  ibuprofen (■) and guinea pig ileum (●). The concentrations of morphine used to induce approximately 50% of contraction were 1  $\mu\text{mol/L}$  for the mouse and 0.1  $\mu\text{mol/L}$  for the guinea pig. This is a representative graph of three determinations on different animals in duplicate. The  $IC_{50}$  values for casoxin 4 for  $n = 3$  determinations in duplicate for the guinea pig and  $n = 3$  determinations in duplicate for the mouse were  $2.1 \pm 0.2 \mu\text{mol/L}$  and  $11.3 \pm 0.5 \mu\text{mol/L}$ , respectively, which were significantly different by two-tailed Student's *t*-test ( $P < 0.001$ ).

**Abbreviations:** GP, guinea pig;  $IC_{50}$ , half maximal inhibitory concentration; Mo, mouse.

of gut transit in the mouse model.<sup>26,27</sup> Morphine doses of 1.0, 1.5, and 2.0 mg/kg (subcutaneous) significantly inhibited the transit of the dye Poly R-478 in the small intestine of the mouse (Figure 4). After 45 minutes transit time, the percentage of Poly R-478 remaining in the stomach for saline control, 1.5 mg/kg, and 2.0 mg/kg morphine, subcutaneous, (number of mice) were:  $14.2 \pm 2.8$  (24),  $22.5 \pm 2.1$  (16) and  $31.2 \pm 7.8$  (6), respectively. There was significant difference between the groups ( $P = 0.02$ ), with 2.0 mg/kg morphine being significantly higher than saline control ( $P < 0.05$ ). Casoxin 4 gavaged at 50 mg/kg did not significantly lower the Poly R-478 content of the stomach at the two doses of morphine. Casoxin 4 and lactoferroxin A gavaged at 50 mg/kg with the dye 20 minutes after injection of morphine also failed to significantly overcome the morphine inhibition of small intestinal transit (Figure 4).

Since morphine is also known to increase sphincter tone and inhibit gastric emptying and hence may prevent the gavaged opioid antagonist reaching the small intestine at amounts that could antagonize morphine, a double gavage experiment was designed to potentially overcome this problem.<sup>28</sup> However, gavaging the mouse with 50 mg/kg casoxin 4, 20 minutes before injection of 2 mg/kg morphine and 20 minutes after with 50 mg/kg casoxin 4, also failed to



**Figure 4** Effect of opioid antagonists on morphine inhibition of small intestinal transit in the mouse. The dye front represents the percentage of the total SI that the dye Poly R-478 has traveled. Morphine was injected subcutaneously at the concentrations indicated 20 minutes before the gavage of dye-containing saline as control or CSX 4 (50 mg/kg of bodyweight) or LFX A (50 mg/kg). Total gut transit time was 45 minutes. Results are mean  $\pm$  SEM, with the number of mice indicated inside the bar. Morphine at 1.0, 1.5, and 2.0 mg/kg significantly inhibited transit (a, ANOVA,  $P < 0.01$ ) compared with control (0 mg/kg). The corresponding CSX 4 or LFX A-treated mice had SI transit dye fronts that were also significantly different compared with control (b, ANOVA,  $P < 0.001$ ) but not significantly different from the corresponding morphine dosage treatment alone.

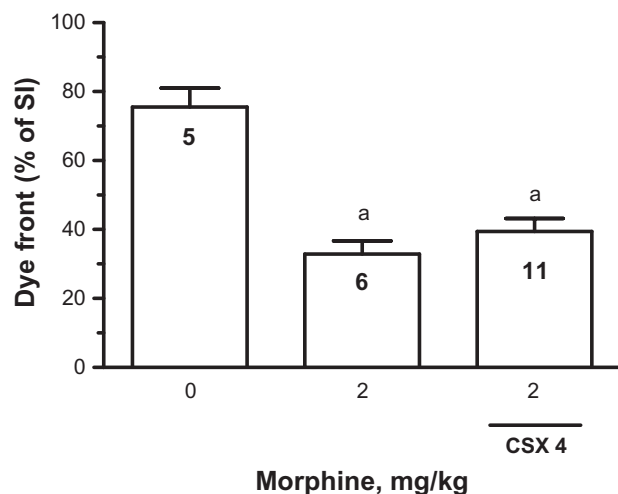
**Abbreviations:** ANOVA, analysis of variance; CSX 4, casoxin 4; LFX A, lactoferroxin A; SEM, standard error of the mean; SI, small intestine.

significantly reverse the morphine inhibition of gut transit (Figure 5).

The alkaloid opioid antagonist naloxone, however, significantly reversed the morphine inhibition of mouse gut transit when added either subcutaneously with morphine or orally 20 minutes after subcutaneous injection of morphine gavaged with the Poly R-478 (Figure 6).

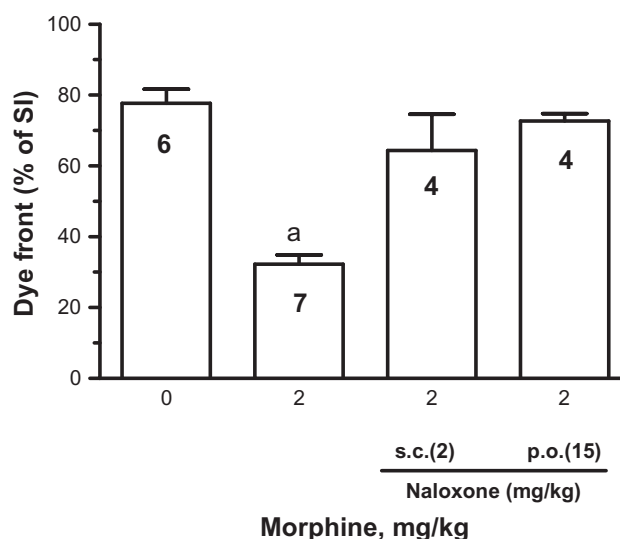
## Discussion

The mouse and rat ileum were electrically stimulated to contract by having the tissue under minimal tension and inducing current for a similar time required for the guinea pig.<sup>7,25,26</sup> In the mouse, this electrically induced activity was predominantly a relaxation followed by a smaller contractile phase. It was found that the nonsteroidal anti-inflammatory drug, ibuprofen, which is commonly used to indirectly deduce the possible role of prostaglandins,<sup>29</sup> reduced the basal tone and unmasked the contractile component that was inhibited by morphine. Previous investigations with rodents, using various electrical geometries, have shown the mouse to be insensitive to morphine inhibition<sup>3</sup> and that the rat had fast



**Figure 5** Effect of double gavage of CSX 4 on morphine inhibition of transit in the SI of the mouse. The dye front represents the percentage of the total length that the dye Poly R-478 has traveled along the length of the SI. The control group (no morphine) was gavaged with saline 20 minutes before the injection of saline subcutaneously, followed 20 minutes later by gavage of dye-containing saline. The morphine group was gavaged with saline 20 minutes before being injected subcutaneously with morphine (2 mg/kg), followed 20 minutes later by gavage of dye. The third group was gavaged with CSX 4 (50 mg/kg) 20 minutes before subcutaneous injection of morphine (2 mg/kg), followed 20 minutes later by gavage of dye containing CSX 4 (50 mg/kg). Total gut transit time was 45 minutes. Results are mean  $\pm$  SEM, with the number of mice indicated within the bar. The morphine group and morphine group with CSX 4 were significantly different from the control group (a, ANOVA,  $P < 0.01$ ). There was no significant difference between the morphine group and the morphine group with CSX 4.

**Abbreviations:** ANOVA, analysis of variance; CSX 4, casoxin 4; SEM, standard error of the mean; SI, small intestine.



**Figure 6** Effect of naloxone subcutaneously or orally on morphine inhibition of transit in SI of the mouse. The dye front represents the percentage of the total SI that the dye Poly R-478 has traveled along the length of the SI. Saline alone, or morphine (2 mg/kg), or morphine plus naloxone (2 mg/kg each) were injected subcutaneously 20 minutes before the gavage of dye  $\pm$  naloxone (15 mg/kg). Total gut transit time was 45 minutes. Results are mean  $\pm$  SEM with the number of mice indicated inside the bar. Morphine at 2 mg/kg significantly inhibited transit (a, ANOVA,  $P < 0.01$ ) compared with control as 0 mg/kg morphine. Treatment with morphine with naloxone added subcutaneously or with morphine at the same time as gavage of dye (orally) were significantly different from morphine treatment alone (2 mg/kg) ( $P < 0.01$ ) but not significantly different from control as 0 mg/kg morphine.

**Abbreviations:** ANOVA, analysis of variance; p.o., orally; s.c., subcutaneously; SEM, standard error of the mean; SI, small intestine.

atropine-sensitive contraction that produced noncholinergic after-contractions.<sup>5</sup> However, we determined that the length of duration of single square pulses of the small intestine of the rat and mouse was slightly longer but similar to that described in the guinea pig.<sup>30,31</sup>

In the systems described, morphine significantly inhibits electrically induced contractions in the mouse and rat intestine at  $IC_{50}$  values higher than for the guinea pig which is in concordance with other opioid studies.<sup>3,5,32</sup> This follows from the profound effect that morphine and other opiates have on the opioid receptor system by delaying gastric emptying and small intestinal transit in these rodents.<sup>2,4,9,33,34</sup> The rat is a common laboratory animal used in gut physiology studies and was included with the comparison of mouse and guinea pig in this study to determine cross-species sensitivities that may give an insight into the level of peptide antagonists that might be required in subsequent anti-morphine or other bioactive gut trials.<sup>32,35,36</sup> In the electrically driven mouse model, for the opioid agonists tested, we have determined that the  $\Delta$ -selective agonists DADLE and metenkephalin, and the  $\kappa$ -selective agonist dynorphin A were only slightly more

potent than the  $\mu$ -selective agonist DAMGO. However, all these agonists were much more potent than the nonselective  $\mu$ -receptor agonists morphine and morphiceptin. The results are consistent with the findings of others who concluded that the mouse ileum is predominantly a  $\kappa$ - and  $\Delta$ -opioid receptor system.<sup>3,4,6</sup>

In this study, the tetrapeptide casoxin 4, one of the smallest and most potent peptide opioid antagonists described,<sup>7,24,25</sup> blocked morphine inhibition of electrically induced contraction in the mouse and guinea pig (see Figure 3). This result is in accordance with our previous study where casoxin 4 and its potentially peptidase-resistant analog [D-Ala<sup>2</sup>]-casoxin 4,<sup>7,37</sup> blocked the morphine inhibition of electrically induced contraction of guinea pig ileum when applied to the luminal side in an attempt to mimic the situation in vivo.<sup>7</sup> Naloxone also antagonizes morphine inhibition in both species but at a lower concentration, probably due to its higher bioavailability and efficacy. Experiments were therefore conducted to determine if naturally occurring peptide sequences had biological potency in the mouse gut transit assay that has been shown to be sensitive to opioid inhibition.<sup>4,6</sup> Both the  $\mu$ -specific opioid receptor antagonists, casoxin 4 and lactoferroxin A, when added orally in high dose did not antagonize morphine (subcutaneously) induced inhibition of mouse gut transit time. The influence of a two-fold higher dose of casoxin 4 gavaged 20 minutes before and 20 minutes after morphine injection was also without any effect. However, naloxone had a profound effect on reversing morphine inhibition of intestinal transit time. We had already shown that casoxin 4 and naloxone reverses the inhibition of contraction mediated by morphine in vitro. The failure of casoxin 4 but not naloxone to influence gastrointestinal transit modified by morphine is probably due to a number of factors. These could include degradation of the peptide by peptidases, low tissue penetration to the myenteric plexus, and an IC<sub>50</sub> difference in the mouse compared with the guinea pig. The potential to use D-amino acid substitutions in the small peptide opioid antagonists as previously employed by us to limit peptidase activity,<sup>7,38</sup> and increase bioavailability is also a possibility. At the current time it is not known which of these influences predominates. The major aim of this study was to present the small peptide opioid antagonists orally and assess antagonism of morphine. Therefore, a limitation of this study is not knowing the potential opioid antagonistic effects of casoxin 4 and lactoferroxin A when administered subcutaneously or intravenously. Further, since casoxin 4

and [D-Ala<sup>2</sup>]-casoxin 4 can antagonize morphine inhibition of electrically driven contractions on both serosal and luminal side of the guinea pig ileum in vitro,<sup>7</sup> it would be instructive to test the casoxins in vivo orally in the guinea pig and assess gut transit after subcutaneous morphine administration.

Pilot trials aimed at overcoming the constipating side effects of morphine analgesia or methadone therapy have successfully used intravenous methylnaltrexone, which is a peripherally restricted,  $\mu$ -opioid receptor antagonist.<sup>39</sup> It is of note that oral bioavailability of methylnaltrexone is low, with plasma levels not correlating with its actions in the gut, suggesting a predominantly local luminal action in the gut.<sup>31</sup> However, oral application of enteric-coated methylnaltrexone has also prevented opioid-induced delay in oral-cecal transit in normal volunteers<sup>40,41</sup> and has recently been approved in several countries for subcutaneous injections for the treatment of opioid bowel dysfunction in advanced illness for which the patient is receiving palliative care and when laxative therapy is insufficient.<sup>42</sup> Further, in a recent randomized controlled trial, coadministration of prolonged-release oral naloxone and prolonged release oral oxycodone to patients with chronic pain receiving stable oxycodone therapy is associated with a significant improvement in bowel function compared with oxycodone alone, with no reduction in analgesia.<sup>43</sup> Phenyl piperidine derivatives have been characterized that can reverse the constipating effect of morphine on gut transit of mice at doses that are below that required to significantly antagonize the central analgesic effects of morphine.<sup>9</sup> Selective small molecule opioid receptor-like (ORL1) antagonists have also been produced.<sup>44,45</sup>

The results demonstrate that casoxin 4, by an action on  $\mu$ -opioid receptors, reverses the morphine inhibition of contraction in the isolated mouse and guinea pig intestinal tissue. The  $\mu$ -opioid receptor component of inhibition is greater in the guinea pig than in the mouse. Finally, it was found that the results did not confirm our hypothesis. Casoxin 4, and lactoferroxin A, failed to achieve a significant inhibition unlike the morphine inhibition of gastrointestinal transit in the mouse, which was reversed by naloxone. Further investigation is needed to determine the usefulness of these peptides in reversing opioid-induced inhibition of gastrointestinal motility.

## Disclosure

The authors report no conflicts of interest in this work.

## References

- Schulz R, Wuster M, Herz A. Centrally and peripherally mediated inhibition of intestinal motility by opioids. *Naunyn-Schmiedeberg's Arch Pharmacol*. 1979;308:255–260.
- Galligan JJ, Burks TF. Centrally mediated inhibition of small intestinal transit and motility by morphine in the rat. *J Pharmacol Exp Ther*. 1983; 226:57–61.
- Smith CFC, Waldron C, Brook NA. Opioid receptors in the mouse ileum. *Arch Int Pharmacodyn*. 1988;291:122–131.
- Porreca F, Mosberg HI, Hurst R, et al. Roles for mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *J Pharmacol Exp Ther*. 1984;230:341–348.
- Coupar IM, de Luca A. Opiate and opiate antidiarrhoeal drug action on isolated rat intestine. *J Auton Pharmacol*. 1994;14:69–78.
- Broccardo M, Improta G, Tabacco A. Central effects of SNC 80, a selective and systemically active  $\delta$ -opioid receptor agonists, on gastrointestinal propulsion in the mouse. *Eur J Pharmacol*. 1998;342:247–251.
- Patten GS, Head RJ, Abeywardena MY, et al. An apparatus to assay opioid activity in the infused lumen of the intact isolated guinea pig ileum. *J Pharmacol Toxicol Methods*. 2001;45:39–46.
- Milne RJ, Coddington JM, Gamble GD. Quaternary naloxone blocks morphine analgesia in spinal but not intact rats. *Neurosci Lett*. 1990; 114:259–264.
- Zimmerman DM, Gidda JS, Cantrell BE, et al. Discovery of a potent peripherally selective trans-3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid antagonist for the treatment of gastrointestinal motility disorders. *J Med Chem*. 1994;37:2262–2265.
- Neary P, Delaney CP. Alvimopan. *Expert Opin Investig Drugs*. 2005; 14:479–488.
- Becker G, Blum HE. Novel opioid antagonists for opioid-induced bowel dysfunction and postoperative ileus. *Lancet*. 2009;373:1198–1206.
- The role of opioid receptor antagonists in the treatment of opioid-induced constipation: a review. *Adv Ther*. 2010;27:714–730.
- McNicol ED, Boyce D, Schumann, et al. Mu-opioid antagonists for opioid-induced bowel dysfunction. *Cochrane Database Syst Rev*. 2008;2: CD006332.
- Medical News Today. Phase 2 data from oral NKTR-118 presented at American College of Gastroenterology in San Diego. 2009. Available from: <http://www.medicalnewstoday.com/articles/168923.php>. Accessed Dec 10 2010.
- Clinical Trials. Theravance begins proof of concept TD-1211 phase 2 trial. 2010. Available from: [http://clinicaltrials.pharmaceutical-business-review.com/news/theravance\\_begins\\_proof\\_of\\_concept\\_td1211\\_phase\\_2\\_trial\\_100415](http://clinicaltrials.pharmaceutical-business-review.com/news/theravance_begins_proof_of_concept_td1211_phase_2_trial_100415). Accessed Dec 10 2010.
- Clinical Trials. A study of RDC-1036 (ALKS 37) in adults with opioid-induced constipation (OIC). 2010. Available from: <http://clinicaltrials.gov/ct2/show/NCT01100151>. Accessed Dec 10 2010.
- The Medical News. Adolor initiates ADL5945 phase 2 proof-of-concept study for patients with OIC. 2010. Available from: <http://www.news-medical.net/news/20101019/Adolor-initiates-ADL5945-Phase-2-proof-of-concept-study-for-patients-with-OIC.aspx>. Accessed Dec 10 2010.
- Bajka BH, Clarke JM, Topping DL, et al. Butyrylated starch increase large bowel butyrate levels and lowers colonic smooth muscle contractility in rats. *Nutr Res*. 2010;30:427–434.
- Patten GS, Bird AR, Topping DL, et al. Dietary fish oil alters the sensitivity of guinea pig ileum to electrically driven contractions and 8-iso-PGE<sub>2</sub>. *Nutr Res*. 2002;22:1413–1426.
- Patten GS, Bird AR, Topping DL, et al. Effects of convenience rice congee supplemented diets on guinea pig whole animal and gut growth, caecal digesta SCFA and in vitro ileal contractility. *Asia Pacific J Clin Nutr*. 2004;13:92–100.
- Shinohara H, Williams C, Koldovsky O. The use of Poly R-478 as a marker to determine gastric emptying and intestinal propulsive motility in suckling rats. *Physiol Res*. 1995;44:281–286.
- Stahl GE, Fayer JC, Ling SC, et al. Comparison of nonabsorbable markers Poly R-478 and [14C]PEG-4,000 for use in developmental absorption studies. *J Pediatr Gastroenterol Nutr*. 1991;12: 485–493.
- Patten GS, Augustin MA, Sanguansri L, et al. Site specific delivery of microencapsulated fish oil to the gastrointestinal tract of the rat. *Dig Dis Sci*. 2009;54:511–521.
- Chiba H, Yoshikawa M. Biological functional peptides from food proteins: new opioid peptides from milk proteins. In: Feeney RE, Whitaker JR, editors. *Protein Tailoring for Food and Medical Uses*. New York, NY: Marcel Dekker; 1986:123–152.
- Yoshikawa M, Tani F, Ashikaga T, et al. Purification and characterization of an opioid antagonist from a peptic digest of bovine  $\kappa$ -casein. *Agric Biol Chem*. 1986;50:2951–2953.
- Yoshikawa M, Tani F, Chiba H. Structure-activity relationship of opioid antagonist peptides derived from milk proteins. In: Shiba T, Sakakibara S, editors. *Peptide Chemistry*. Osaka, Japan: Protein Research Foundation; 1987:473–476.
- Tani K, Kunihiro I, Chiba H, et al. Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agric Biol Chem*. 1990;54:1803–1810.
- Buente L, Fioramonti J. Action of opiates on gastrointestinal function. In: Grundy D, Read NW, editors. *Bailliere's Clin Gastroenterol International Practice and Research*. Volume 2. London, UK: Bailliere Tindall; 1988:123–139.
- Kokoska ER, Smith GS, Deshpande Y, et al. Indomethacin increases susceptibility to injury in human gastric cells independent of PG synthesis inhibition. *Am J Physiol Gastrointest Liver Physiol*. 1998;38: G620–G628.
- Schaumann W. The paralyzing action of morphine on the guinea-pig ileum. *Br J Pharmacol Chemother*. 1955;10:456–461.
- Paton WDM, Visi ES. The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *Br J Pharmacol*. 1969;35:10–28.
- Cucumel K, Bagnol D, Moinier D, et al. The rat dermorphin-like immunoreactivity is supported by an aminopeptidase resistant peptide. *J Neuroimmunol*. 1998;81:211–224.
- Galligan JJ, Mosberg HI, Hurst R, et al. Cerebral delta opioid receptors mediate analgesia but not the intestinal motility effects of intracerebroventricularly administered opioids. *J Pharmacol Exp Ther*. 1984;229:641–648.
- Coupar IM. The peristaltic reflex in the rat ileum: evidence for functional  $\mu$ - and  $\delta$ -opiate receptors. *J Pharm Pharmacol*. 1995;47: 643–646.
- Davis TP, Gillespie TJ, Shook J, et al. Changes in opioid receptor selectivity following processing of peptide E: effect on gut motility. *Gastroenterology*. 1991;100:1603–1615.
- Ghayur MN, Gilani AH, Houghton PJ. Species differences in the gut stimulatory effects of radish seeds. *J Pharm Pharmacol*. 2005;57: 1493–1501.
- Matthies H, Stark H, Hartrodt B. Derivatives of  $\beta$ -casomorphins with high analgesic potency. *Peptides*. 1984;5:463–470.
- Yuan CS, Foss JF, O'Connor M, et al. Effects of intravenous methylnaltrexone on opioid-induced gut motility and transit time changes in subjects receiving chronic methadone therapy: a pilot study. *Pain*. 1999;83:631–635.
- Foss JF. A review of the potential role of methylnaltrexone in opioid bowel dysfunction. *Am J Surg*. 2001;182(Suppl):19S–26S.
- Yuan CS, Foss JF, O'Connor M, et al. Effects of enteric-coated methylnaltrexone in preventing opioid-induced delay in oral-cecal transit time. *Clin Pharmacol Ther*. 2000;67:398–404.
- Yuan CS, Foss JF. Methylnaltrexone: investigation of clinical applications. *Drug Dev Res*. 2000;50:133–141.
- Greenwood-van Meerveld B, Standifer KM. Methylnaltrexone in the treatment of opioid-induced constipation. *Clin Expt Gastroenterol*. 2008;1:49–58.



43. Meissner W, Leyendecker P, Mueller-Lissner S, et al. A randomised controlled trial with prolonged-release oral oxycodone and naloxone to prevent and reverse opioid-induced constipation. *Eur J Pain*. 2009; 13:56–64.
44. Thomas JB, Mascarella SW, Burgess JP, et al. N-substituted octahydro-4a-(3-hydroxyphenyl)-10a-methylbenzo[g]isoquinolines are opioid receptor pure antagonists. *Bioorg Med Chem Lett*. 1998;8: 3149–3152.
45. Thomas JB, Zheng X, Mascarella SW, et al. N-substituted 9 $\beta$ -methyl-5-(3-hydroxyphenyl)morphans are opioid receptor pure antagonists. *J Med Chem*. 1998;41:4143–4149.

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