BJP British Journal of Pharmacology

DOI:10.1111/bph.12132
www.brjpharmacol.org

RESEARCH PAPER

The macrocyclic tetrapeptide [D-Trp]CJ-15,208 produces short-acting κ opioid receptor antagonism in the CNS after oral administration

Correspondence
Jay P. McLaughlin, Torrey Pines Institute for Molecular Studies, 11350 SW Village Parkway, Port St. Lucie, FL 34987, USA. E-mail: jmclaughlin@tpims.org

Keywords
kappa opioid receptor; CJ-15,208; p.o. activity; cyclic tetrapeptide; antagonist; antinociception; cocaine; conditioned place preference

Received
20 September 2012
Revised
18 January 2013
Accepted
30 January 2013

Shainnel O Eans[1], Michelle L Ganno[1], Kate J Reilley[1], Kshitij A Patkar[1], Sanjeewa N Senadheera[2], Jane V Aldrich[2] and Jay P McLaughlin[1]

[1]*Torrey Pines Institute for Molecular Studies, Port St. Lucie, FL, USA, and* [2]*Department of Medicinal Chemistry, The University of Kansas, Lawrence, KS, USA*

BACKGROUND AND PURPOSE

Cyclic peptides are resistant to proteolytic cleavage, therefore potentially exhibiting activity after systemic administration. We hypothesized that the macrocyclic κ opioid receptor (KOR)-selective antagonist [D-Trp]CJ-15,208 would demonstrate antagonist activity after systemic, that is, s.c. and oral (*per os*, p. o.), administration.

EXPERIMENTAL APPROACH

C57BL/6J mice were pretreated with [D-Trp]CJ-15,208 s.c. or p.o. before administration of the KOR-selective agonist U50,488 and the determination of antinociception in the warm-water tail-withdrawal assay. The locomotor activity of mice treated with [D-Trp]CJ-15,208 was determined by rotorod testing. Additional mice demonstrating cocaine conditioned place preference and subsequent extinction were pretreated daily with vehicle or [D-Trp]CJ-15,208 and then exposed to repeated forced swim stress or a single additional session of cocaine place conditioning before redetermining place preference.

KEY RESULTS

Pretreatment with [D-Trp]CJ-15,208 administered s.c. or p.o. dose-dependently antagonized the antinociception induced by i.p. administration of U50,488 in mice tested in the warm-water tail-withdrawal assay for less than 12 and 6 h respectively. [D-Trp]CJ-15,208 also produced limited (<25%), short-duration antinociception mediated through KOR agonism. Orally administered [D-Trp]CJ-15,208 dose-dependently antagonized centrally administered U50,488-induced antinociception, and prevented stress-, but not cocaine-induced, reinstatement of extinguished cocaine-seeking behaviour, consistent with its KOR antagonist activity, without affecting locomotor activity.

CONCLUSIONS AND IMPLICATIONS

The macrocyclic tetrapeptide [D-Trp]CJ-15,208 is a short-duration KOR antagonist with weak KOR agonist activity that is active after oral administration and demonstrates blood–brain barrier permeability. These data validate the use of systemically active peptides such as [D-Trp]CJ-15,208 as potentially useful therapeutics.

Abbreviations

CJ-15,208, *cyclo*[Phe-D-Pro-Phe-Trp]; CPP, conditioned place preference; DPBS, Dulbecco's PBS; [D-Trp]CJ-15,208, *cyclo*[Phe-D-Pro-Phe-D-Trp]; GNTI, 5′-guanidinylnaltrindole; HSD, honestly significant difference; JDTic, (3*R*)-7-hydroxy-*N*-((1*S*)-1-[[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide; KOR, κ opioid receptor; KOR -/-, κ opioid receptor gene-disrupted mice; MOR, μ opioid receptor; MOR -/-, μ opioid receptor gene-disrupted mice; nor-BNI, nor-binaltorphimine; p.o., *per os*; U50,488, (±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; SNC80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]*N*,*N*-diethylbenzamide. Note that amino acids are the L-isomer unless otherwise specified; abbreviations for amino acids follow IUPAC-IUB Joint Commission of Biochemical Nomenclature (Eur J Biochem (1984) 138: 9–37)

Introduction

The κ opioid receptor (KOR) and dynorphin system play an important role in the response to stress (McLaughlin *et al.*, 2003; Shirayama *et al.*, 2004; Wee and Koob, 2010), and are thought to paradoxically potentiate the rewarding effects of cocaine (McLaughlin *et al.*, 2003; 2006) while mediating reinstatement of extinguished cocaine-seeking behaviour (Valdez *et al.*, 2007; Redila and Chavkin, 2008). Pretreatment with KOR antagonists prevents stress-induced reinstatement of extinguished cocaine-seeking behaviour (Beardsley *et al.*, 2005; Carey *et al.*, 2007; Redila and Chavkin, 2008; Aldrich *et al.*, 2009; Ross *et al.*, 2012) and decreases compulsive cocaine-seeking behaviour and intake in the absence of stress (Wee *et al.*, 2009; 2012), suggesting that KOR antagonists hold promise as medications to prevent relapse to cocaine-seeking behaviour.

Several highly selective non-peptide KOR antagonists have been identified (nor-binaltorphimine (nor-BNI), 5′-guanidinylnaltrindole and JDTic; for a review, see Metcalf and Coop, 2005). However, these prototypical KOR antagonists exhibit an unusually long duration of antagonism, lasting for weeks after a single dose (Horan *et al.*, 1991; Carroll *et al.*, 2004; Metcalf and Coop, 2005; Aldrich and McLaughlin, 2009). Unfortunately, this prolonged duration precludes certain key preclinical studies and could potentially impair clinical development. Recently, several new non-peptide KOR antagonists have been reported (Brugel *et al.*, 2010; Runyon *et al.*, 2010; Grimwood *et al.*, 2011; Mitch *et al.*, 2011; Peters *et al.*, 2011; Frankowski *et al.*, 2012), some of which demonstrated shorter durations of KOR antagonist activity (Runyon *et al.*, 2010; Peters *et al.*, 2011) or were detected for relatively short (<8 h) periods in the brain (Grimwood *et al.*, 2011; Mitch *et al.*, 2011). However, activity after p.o. administration has been reported for only four KOR selective antagonists (Beardsley *et al.*, 2005, 2010; Chang *et al.*, 2011; Mitch *et al.*, 2011), but not for any of the shorter acting antagonists, prompting a continued search for KOR antagonists.

Recent research on peptides and peptidomimetic ligands has produced KOR antagonists with selectivity, distribution and finite (≤1 day) duration of action favourable to medications development. For example, modification of dynorphin A (1-11) at the termini and incorporation of a cyclic constraint yielded zyklophin (Patkar *et al.*, 2005), a potent and highly selective KOR antagonist *in vivo* that prevents stress-induced reinstatement of previously extinguished cocaine-seeking behaviour after s.c. administration (Aldrich *et al.*, 2009). The peptide natural product CJ-15,208 was reported to antagonize a KOR agonist *in vitro* (Saito *et al.*, 2002). The L- and D-Trp stereoisomers of this macrocyclic tetrapeptide were synthesized (Dolle *et al.*, 2009; Kulkarni *et al.*, 2009; Ross *et al.*, 2010), and both found to bind to KOR with similar affinities in radioligand receptor-binding experiments (Dolle *et al.*, 2009; Kulkarni *et al.*, 2009; Ross *et al.*, 2010; 2012). [D-Trp]CJ-15,208 demonstrated potent KOR antagonism *in vitro* in a [^{35}S]GTPγS-binding assay (Dolle *et al.*, 2009; Ross *et al.*, 2012) and also exhibits KOR antagonism *in vivo*, preventing the reinstatement of extinguished cocaine conditioned place preference (CPP) after i.c.v. administration (Ross *et al.*, 2012). However, the KOR antagonist activity of the D-Trp peptide after systemic (especially oral) administration and its ability to penetrate into the CNS after systemic administration were not evaluated in these initial studies.

We hypothesized that the macrocyclic structure and relatively low molecular weight (577 g·mol^{-1}) of [D-Trp]CJ-15,208 would facilitate oral absorption while providing metabolic stability to permit CNS penetration and central KOR antagonist activity. Accordingly, we characterized [D-Trp]CJ-15,208 for KOR antagonism *in vivo* following s.c. and p.o. administration in assays of antinociception and diuresis. The CNS penetration of orally administered [D-Trp]CJ-15,208 was verified by the ability of this peptide to prevent the reinstatement of extinguished cocaine-seeking behaviour and LC-MS/MS.

Methods

Statement on drug and receptor nomenclature

All drug and molecular target terms conform to parameters specified in the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Chemicals

All chemicals other than [D-Trp]CJ-15,208, the D-Trp isomer of the macrocyclic tetrapeptide CJ-15,208 (Dolle *et al.*, 2009; Kulkarni *et al.*, 2009; Ross *et al.*, 2010), were obtained from Sigma-Aldrich (St. Louis, MO, USA). [D-Trp]CJ-15,208 was initially dissolved daily prior to use in ethanol and Tween-80, and sufficient warm (40°C) sterile saline added so that the final vehicle consisted of 1 part ethanol, 1 part Tween-80 and 8 parts sterile saline (0.9%). This vehicle, which has been used for the solubilization of other hydrophobic opiates for *in vivo* studies (Schmidt *et al.*, 2005; Wang *et al.*, 2005), is referred to herein as '1:1:8'.

Synthesis of cyclo[Phe-D-Pro-Phe-D-Trp] ([D-Trp]CJ-15,208)

The linear peptide D-Trp-Phe-D-Pro-Phe was synthesized by Fmoc solid phase synthesis on the 2-chlorotrityl chloride resin essentially as described previously (Ross *et al.*, 2010). The crude linear peptide was cyclized by a modification of the previously published procedure (Senadheera *et al.*, 2011). The key modifications were increasing the temperature of the cyclization reaction to 30°C for 24 h after 12 h at room temperature, and purifying the macrocyclic peptide by silica gel chromatography using a hexane/ethyl acetate step gradient (from 1:1 to 4:1).

Animals

Adult male C57BL/6J mice, weighing 20–25 g obtained from the Jackson Laboratory (Bar Harbor, ME, USA), were selected for this study because of their established responses to stress and cocaine place conditioning (McLaughlin *et al.*, 2003; Carey *et al.*, 2007; Aldrich *et al.*, 2009). Mu opioid receptor gene-disrupted (MOR -/-) and kappa opioid receptor gene-disrupted (KOR -/-) mice were obtained from colonies established at Torrey Pines Institute for Molecular Studies from homozygous breeding pairs of mice obtained from the Jackson Laboratory. All mice were kept on a 12 h light-dark cycle and were housed in accordance to the National Institute

of Health Guide for Care and Use of Laboratory Animals. All results of animal testing are reported in accordance with ARRIVE guidelines as stated by the British Journal of Pharmacology (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Antinociceptive testing

The 55°C warm-water tail-withdrawal assay was performed in mice as previously described (Ross *et al.*, 2012), with the latency of tail withdrawal from the water taken as the end point. After determining baseline tail-withdrawal latencies, mice were administered with [D-Trp]CJ-15,208 s.c. or p.o. in the 1:1:8 vehicle and tested every 10 min thereafter for up to 90 min to determine direct antinociceptive effects. To determine the antagonist effects of [D-Trp]CJ-15,208 on U50,488-induced antinociception, mice were pretreated with [D-Trp]CJ-15,208 for 2 or 3 h to preclude confounding effects of agonism produced by the macrocyclic tetrapeptide. A cut-off time of 15 s was used in this study; if the mouse failed to display a tail-withdrawal response during that time, the tail was removed from the water and the animal was assigned a maximal antinociceptive score of 100%. At each time point, antinociception was calculated according to the formula: % antinociception = 100 × (test latency – control latency)/(15 – control latency).

Diuresis testing

To measure diuresis, mice were administered either with vehicle 1:1:8 (0.3 mL per 30 g body weight, p.o.) or U50,488 (30 mg·kg^{-1}, i.p.) 3 h after p.o. administration of [D-Trp]CJ-15,208 (1–30 mg·kg^{-1}, p.o.). Mice were then administered with deionized water (0.5 mL, p.o.) and placed in the Oxymax/Comprehensive Lab Animal Monitoring System apparatus (Columbus Instruments, Columbus, OH, USA) chambers lined on the bottom with pre-weighed paper towels for 90 min. Urine output was calculated as the difference in towel weights before and after completion of the 90 min assay. Note that urine output following p.o. administration of vehicle (1:1:8; 0.46 ± 0.03 mL) did not differ significantly from that following i.p. administration of saline (0.54 ± 0.02 mL; $P = 0.07$, Student's *t*-test).

Sample preparation for LC-MS/MS analysis

Mice were orally administered with [D-Trp]CJ-15,208 (30 mg·kg^{-1}, p.o. in 1:1:8 vehicle) and euthanized at 45, 90 or 180 min post administration. Serum was obtained from blood samples (200–250 µL), and the proteins precipitated by adding 4 volumes of ice-cold acetonitrile (MeCN) followed by centrifugation at 10 000 rpm for 5 min. The supernatants were collected, dried under vacuum and reconstituted (20% MeCN, 70 µL) for analysis.

After blood collection, brains were perfused with cold Dulbecco's PBS (DPBS, 40 mL) to remove any residual blood, isolated and frozen. The brains were weighed, washed with ice-cold DPBS (4 × 1 mL) and homogenized in ice-cold DPBS (500 µL), followed by addition of ice-cold MeCN/0.1% formic acid (1 mL) and homogenized again. The homogenates were centrifuged, the supernatants collected, dried under vacuum and reconstituted (20% MeCN, 70 uL) for LC-MS/MS analysis as described below.

Instrumentation and analytical conditions

The LC-MS/MS system consisted of a 3200 Q TRAP® triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIonSpray interface (Applied Biosystems/MDS Sciex, Darmstadt, Germany) and a Shimadzu Prominence HPLC system. Separation was carried out on a C-18 reversed phase column (Luna 5 µ, 100 Å, 50 × 4.6 mm) with a C-18 reversed phase guard cartridge (Phenomenex, 4 × 3.0 mm), and the peptide eluted [retention time (t_r) = 6.4 min] using a gradient of solvents A (10 mM ammonium formate) and B (0.1% formic acid in MeCN) at 0.5 mL·min^{-1} flow rate (0–3 min: 50% B, 3–6 min: 50–95% B, 6–8 min: 95% B, 8–9 min: 95–50% B, 9–14 min: 50% B). MS instrument parameters were spray voltage 5.5 kV, curtain gas 25 psi, source temperature 700°C, ion source gas 1 70 psi, and gas 2 60 psi. The ion transitions monitored were 578.2/70.2, 578.2/217.2, 600.2/572.2 and 600.2/425.3 with 150 ms dwell time and 5 ms pause time between the transitions; the counts for the ion transitions were summed to give the peak area for [D-Trp]CJ-15,208. Blank solvent injections were run between each sample to minimize analyte carry-over from one LC-MS/MS run to the next.

Rotorod assay to determine locomotor activity

Possible sedative or hyperlocomotor effects of [D-Trp]CJ-15,208 were assessed by rotorod performance, as modified from previous protocols (Paris *et al.*, 2011). Following seven habituation trials (the last utilized as a baseline measure of rotorod performance), mice were orally administered with saline, vehicle (1:1:8) or [D-Trp]CJ-15,208 (60 mg·kg^{-1}, p.o.) and assessed after 10 min in accelerated speed trials (180 s max. latency at 0–20 rpm) over a 60 min period. Decreased latencies to fall in the rotorod test indicate impaired motor performance. Data are expressed as the percent change from baseline performance.

Cocaine CPP

Mice were conditioned with a counterbalanced cocaine CPP paradigm using similar timing as detailed previously (Carey *et al.*, 2007; Paris *et al.*, 2011; see also Figure 9A). The amount of time subjects spent in each of three compartments was measured over a 30 min testing period. Prior to place conditioning, the animals did not demonstrate significant differences in their time spent exploring the left (537 ± 12 s) versus right (568 ± 10 s) compartments, resulting in a preconditioning response of –26 ± 21 s ($P = 0.08$; Student's *t*-test). Daily for the next 2 days (for acute testing) or 4 days (for reinstatement testing; see below), mice were administered with vehicle (0.9% saline) and consistently confined in a randomly assigned outer compartment, half of each group in the right chamber, half in the left chamber. In the acute testing, mice were administered with cocaine (10 mg·kg^{-1}, s.c.), or cocaine preceded 3 h by [D-Trp]CJ-15,208 (10, 30 or 60 mg·kg^{-1}, p.o.), and confined to the opposite compartment for 30 min. Conditioned place aversion (McLaughlin *et al.*, 2006) was not detected in this study under any conditions.

Extinction

Preference tests were completed twice weekly until extinction was established over a 3 week period (see Figure 9). Extinc-

tion is defined as a statistically significant decrease in the time spent in the cocaine-paired compartment during the extinction trial as compared with the post-conditioning response after the initial 4 days of cocaine place conditioning (Carey *et al.*, 2007; Aldrich *et al.*, 2009; Ross *et al.*, 2012).

Reinstatement

Following the demonstration of extinction, reinstatement of cocaine CPP was examined after either exposure to forced swim stress (see below) or an additional cycle of cocaine place conditioning (see Figure 9A) as described previously (Carey *et al.*, 2007; Aldrich *et al.*, 2009; Ross *et al.*, 2012). Tested mice were pretreated with either vehicle (1:1:8, p.o.) or [D-Trp]CJ-15,208 (30 or 60 mg·kg^{-1}, p.o.) daily 3 h prior to forced swimming. A 2 day forced swim stress protocol was used to produce stress-induced reinstatement of cocaine CPP (Carey *et al.*, 2007; Aldrich *et al.*, 2009; Ross *et al.*, 2012). Additional mice were administered vehicle (1:1:8, p.o.) or [D-Trp]CJ-15,208 (30 or 60 mg·kg^{-1}, p.o.) on days 28 and 29, and 3 h after the final administration were given an additional session of cocaine place conditioning on day 29. On the day following the completion of stress exposure or cocaine place conditioning, mice were tested for place preference (Figure 9A).

Statistical analysis

Student's *t*-tests and ANOVA with Bonferroni or Tukey's honestly significant difference (HSD) *post hoc* tests were used as appropriate to analyse tail-withdrawal data as described previously (Ross *et al.*, 2012). Rotorod data were analysed via repeated measures ANOVA, with drug treatment condition as a between-groups factor. For all repeated measures ANOVA, simple main effects and simple main effect contrasts are presented following significant interactions. CPP data are presented as the difference in time spent in drug- and vehicle-associated chambers, and were analysed via one-way ANOVA with the difference in time spent on the treatment- versus vehicle-associated side as the dependent measure and conditioning status as the between-groups factor. Where appropriate, Tukey's HSD *post hoc* tests were used to assess group differences. Effects were considered significant when $P < 0.05$. All effects are expressed as mean ± SEM.

Results

Agonist and antagonist testing of systemically administered [D-Trp]CJ-15,208 in the mouse 55°C warm-water tail-withdrawal assay

[D-Trp]CJ-15,208 produced only limited (<25%) but significant antinociception (Figure 1A) in C57BL/6J wild-type mice as compared with vehicle ($F_{(4,264)} = 24.5$, $P < 0.0001$; two-way ANOVA) when administered through either the s.c. or p.o. routes. Significant antinociception lasted no more than 70 min after the largest dose tested (60 mg·kg^{-1}, p.o.; $F_{(7,264)} = 19.3$, $P < 0.0001$; two-way ANOVA with Bonferroni *post hoc* testing). Vehicle administration (1:1:8, s.c. or p.o.) did not significantly increase tail-withdrawal latencies over the baseline response over time ($F_{(1,126)} = 1.25$, $P = 0.27$; two-way repeated measures ANOVA).

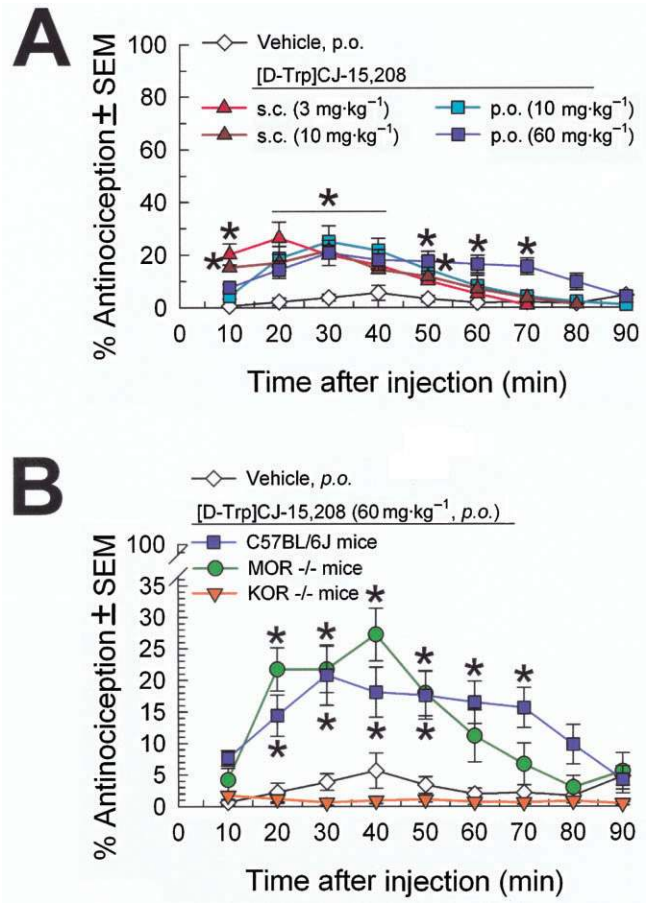

Figure 1

Assessment of [D-Trp]CJ-15,208-mediated antinociception using the 55°C warm-water tail-withdrawal assay with repeated measurement over time. (A) The antinociceptive activity of [D-Trp]CJ-15,208 was assessed *in vivo* following s.c. (3 and 10 mg·kg^{-1}) or p.o. (10 and 60 mg·kg^{-1}) administration in C57BL/6J wild-type mice. Administration of vehicle (1:1:8) alone had no significant effect. (B) Characterization of [D-Trp]CJ-15,208-induced (60 mg·kg^{-1}, p.o.) antinociception in MOR -/- mice (circles) and KOR -/- mice (triangles). Note that the *Y*-axis scale is changed from that used in (A); data collected from C57BL/6J wild-type mice (squares) are included for comparison. Mean % antinociception ± SEM from six to eight mice for each group is presented. *Significantly different from vehicle response, $P < 0.05$; two-way repeated measures ANOVA with Bonferroni *post hoc* test.

Additional characterization of [D-Trp]CJ-15,208-induced (60 mg·kg^{-1}, p.o.) antinociception was performed with MOR -/- and KOR -/- mice (Figure 1B). [D-Trp]CJ-15,208 produced significant antinociception in MOR -/- mice ($F_{(4,208)} = 35.7$, $P < 0.0001$; two-way repeated measures ANOVA) that was not significantly different from the response of wild-type mice ($P > 0.05$, Bonferroni *post hoc* testing). In contrast, [D-Trp]CJ-15,208 did not exhibit significant antinociception in KOR -/- mice when compared with vehicle ($P > 0.05$, Bonferroni *post hoc* testing).

Consistent with results following i.c.v. administration (Ross *et al.*, 2012), a 2 h pretreatment with [D-Trp]CJ-15,208 resulted in significant dose-dependent KOR antagonism, pre-

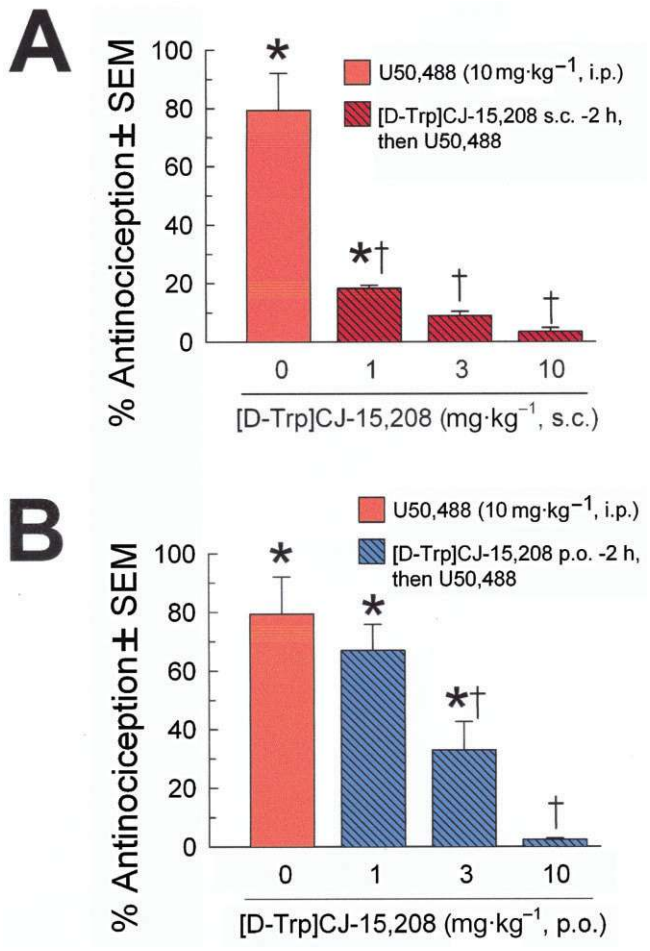

Figure 2

Dose-dependent antagonism of U50,488-induced antinociception in the mouse 55°C warm-water tail-withdrawal assay by [D-Trp]CJ-15,208 pretreatment. The antinociceptive effects of U50,488 (10 mg·kg^{-1}, i.p.) were determined 40 min after administration in mice pretreated 2 h with vehicle or [D-Trp]CJ-15,208 (1–10 mg·kg^{-1}) administered by either the (A) s.c. or (B) p.o. routes. Mean % antinociception ± SEM from eight mice for each group is presented. *Significantly different from the baseline tail-withdrawal latency. †Significantly different from U50,488 alone-induced antinociception; one-way ANOVA with Tukey's HSD *post hoc* test.

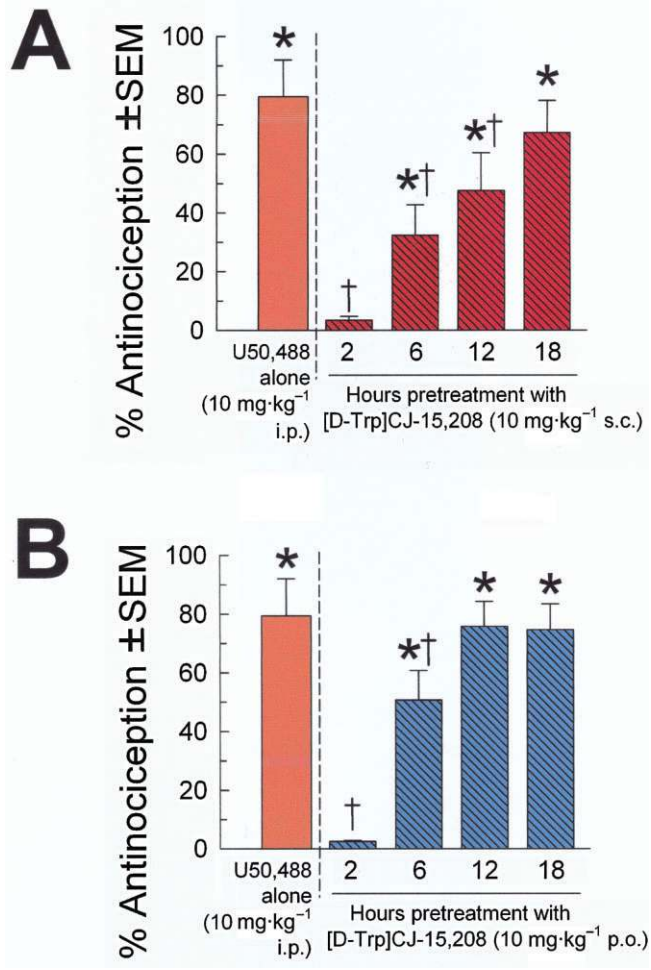

Figure 3

Duration of [D-Trp]CJ-15,208-mediated antagonism of U50,488-induced antinociception in the mouse 55°C warm-water tail-withdrawal test. Tail-withdrawal latencies were determined 40 min after administration of U50,488 (10 mg·kg^{-1}, i.p.) following pretreatment with [D-Trp]CJ-15,208 (10 mg·kg^{-1}) at the times indicated on the horizontal axis either (A) s.c. or (B) p.o. Mean % antinociception ± SEM from eight mice for each group is presented. *Significantly different from the baseline tail-withdrawal latency. †Significantly different from U50,488-induced antinociception alone; one-way ANOVA with Tukey's HSD *post hoc* test.

venting antinociception induced by the KOR agonist U50,488 (10 mg·kg^{-1}, i.p.), after either s.c. ($F_{(4,63)}$ = 59.8, P < 0.0001; one-way ANOVA; Figure 2A) or p.o. administration ($F_{(4,63)}$ = 46.8, P < 0.0001; Figure 2B). Significant KOR antagonism produced by a single dose (10 mg·kg^{-1}) of [D-Trp]CJ-15,208 lasted at least 12 h after s.c. ($F_{(5,79)}$ = 32.5, P < 0.0001; one-way ANOVA; Figure 3A) and at least 6 h after p.o. administration ($F_{(5,79)}$ = 60.7, P < 0.0001; one-way ANOVA; Figure 3B).

Orally administered [D-Trp]CJ-15,208 crosses the blood–brain barrier to antagonize centrally-located KOR

Oral administration of [D-Trp]CJ-15,208 3 h prior to testing antagonized the antinociceptive effect of U50,488 administered centrally (100 nmol, i.c.v., Figure 4). [D-Trp]CJ-15,208-mediated antagonism of CNS KOR was dose-dependent, with significant effects after pretreatment with doses of 30 and 60 mg·kg^{-1}, p.o. ($F_{(4,59)}$ = 189.8, P < 0.0001; one-way ANOVA with Tukey's HSD *post hoc* test; Figure 4).

To further confirm that [D-Trp]CJ-15,208 was both absorbed after oral administration and crossed the blood–brain barrier, blood and perfused brains were collected from mice 45, 90 and 180 min after p.o. administration of the macrocyclic peptide (30 mg·kg^{-1}, p.o.). The presence of [D-Trp]CJ-15,208 in both serum and brain homogenate samples was detected at all three time points after p.o. administration by LC-MS/MS analysis (Figure 5), indicating oral absorption and penetration into the CNS.

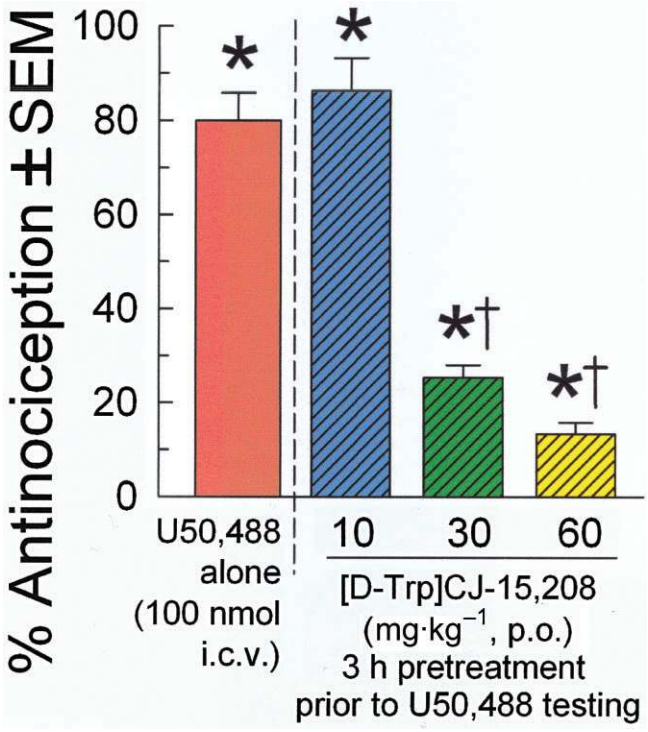

Figure 4

Orally administered [D-Trp]CJ-15,208 crosses the blood–brain barrier to antagonize U50,488-induced antinociception in the mouse 55°C warm-water tail-withdrawal test. Antinociception induced by centrally administered U50,488 (100 nmol, i.c.v.) was antagonized in mice peripherally pretreated 3 h with [D-Trp]CJ-15,208 (10, 30 or 60 mg·kg^{-1}, p.o.). Tail-withdrawal latencies were measured 20 min after injection of U50,488. Mean % antinociception ± SEM from six to eight mice for each group is presented. *Significantly different from the baseline tail-withdrawal latency. †Significantly different from U50,488-induced antinociception alone; one-way ANOVA with Tukey's HSD *post hoc* test.

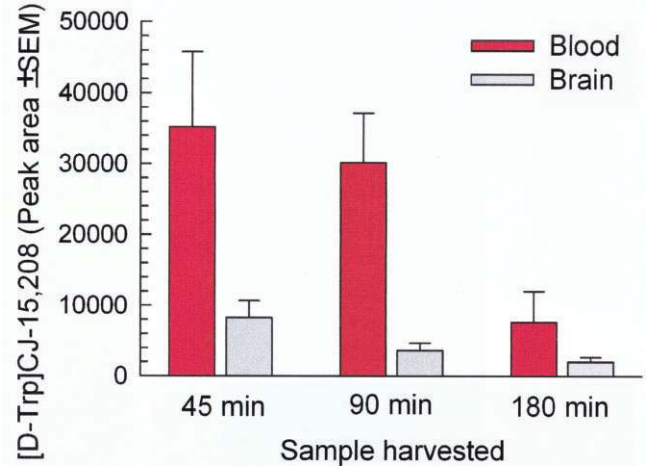

Figure 5

LC-MS/MS detection of [D-Trp]CJ-15,208 in blood and brain after p.o. administration. Samples were collected 45, 90 and 180 min after administration of [D-Trp]CJ-15,208 (30 mg·kg^{-1}, p.o.). Mean peak area ± SEM from four separate mice for each time point is presented.

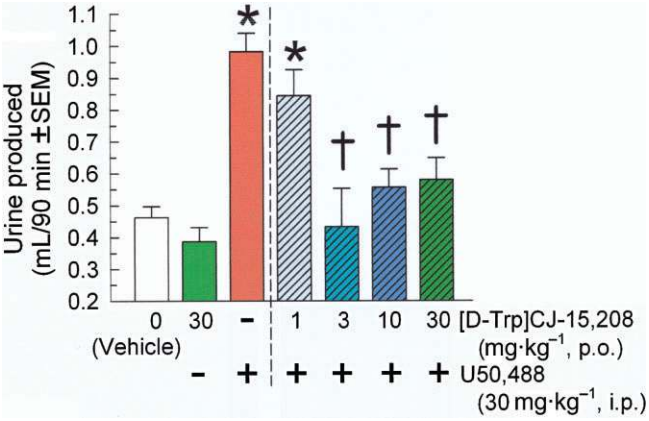

Figure 6

Effects of [D-Trp]CJ-15,208 and vehicle on U50,488-induced diuresis. Diuretic effects of vehicle alone (i.p., white bar) or U50,488 (30 mg·kg^{-1}, i.p., orange bar) were measured during a 90 min time period in mice. Additional mice were pretreated with [D-Trp]CJ-15,208 (1–30 mg·kg^{-1}, p.o.) 3 h prior to U50,488 (striped bars) or vehicle (open green bar) as denoted by plus and minus signs under bars. Mean urine produced ± SEM from 7 to 12 mice for each group is presented. *Significantly different from the vehicle response. †Significantly different from U50,488-induced diuresis; one-way ANOVA with Tukey's HSD *post hoc* test.

Orally administered [D-Trp]CJ-15,208 dose-dependently antagonizes KOR agonist-mediated diuresis

Mice were pretreated 3 h with [D-Trp]CJ-15,208 (1–30 mg·kg^{-1}, p.o.) before administration of vehicle (i.p.) or U50,488 (30 mg·kg^{-1}, i.p.). Treatment with the macrocyclic peptide had no effect on diuresis as compared with vehicle administration (0.39 ± 0.04 vs. 0.46 ± 0.03 mL urine, Figure 6). As expected, [D-Trp]CJ-15,208 treatment produced significant dose-dependent antagonism of U50,488-induced diuresis ($F_{(6,57)}$ = 10.3, P < 0.0001; one-way ANOVA), returning urine output to baseline values at a dose of 3 mg·kg^{-1}, p.o.

Oral administration of [D-Trp]CJ-15,208 does not alter locomotor activity

Mice were pretreated orally with saline, vehicle (1:1:8) or [D-Trp]CJ-15,208 (60 mg·kg^{-1}, p.o.) and tested on the rotorod apparatus for 60 min. Although all animals showed significant improvement in the latency to fall over time compared with initial baseline ($F_{(2,126)}$ = 7.67, P < 0.0001; two-way ANOVA, P > 0.05, Bonferroni *post hoc* test; Figure 7), [D-Trp]CJ-15,208 treatment did not produce significant differences as compared with saline or vehicle-treated mice at any time point ($F_{(2,126)}$ = 0.06, P = 0.991; two-way ANOVA). There was no significant difference in effect between the two vehicle (saline or 1:1:8) treatments (data not shown).

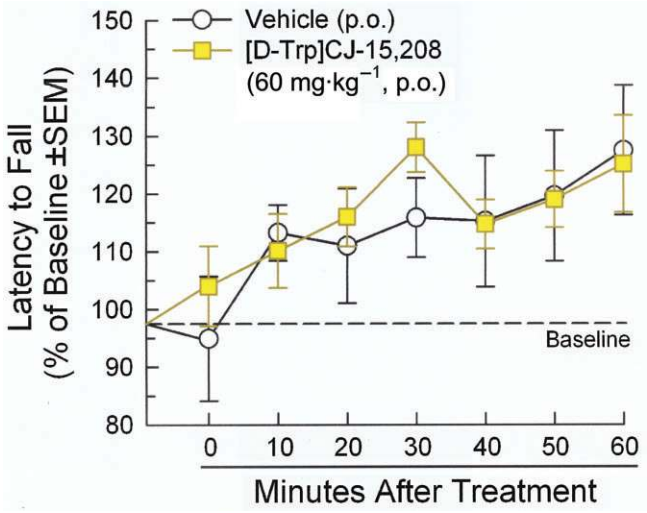

Figure 7

Locomotor activity of [D-Trp]CJ-15,208 in the mouse rotorod assay. Mice were administered with vehicle (1:1:8) or [D-Trp]CJ-15,208 orally (60 mg·kg^{-1}, p.o.). The graph depicts the latency to fall from the rotorod as the mean percent change from baseline performance ± SEM from eight mice for each treatment group.

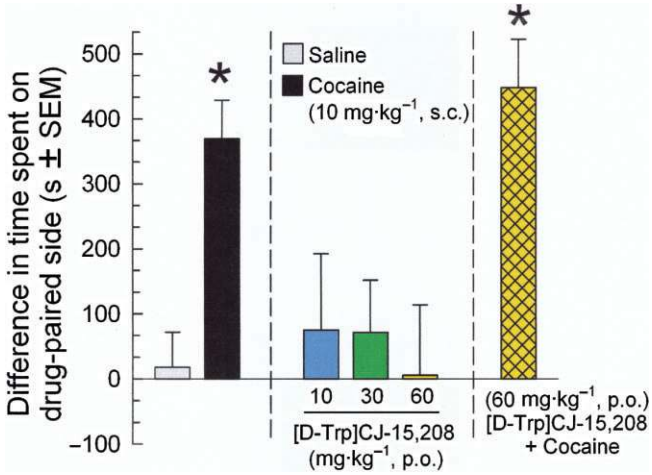

Figure 8

[D-Trp]CJ-15,208 alone did not produce conditioned place preference or alter cocaine-conditioned place preference. Mice received saline (grey bar), cocaine (10 mg·kg^{-1}, s.c.; black bar) or [D-Trp]CJ-15,208 (10, 30 or 60 mg·kg^{-1}, p.o., central bars) daily for 2 days. Additional mice received [D-Trp]CJ-15,208 (60 mg·kg^{-1}, p.o.) 3 h prior to administration of cocaine and place conditioning for each of 2 days. Mean difference in time spent on the drug-paired side ± SEM is presented from 14 to 24 mice per treatment condition. *Significantly different from saline CPP, $P < 0.05$; one-way ANOVA with Tukey's HSD *post hoc* test.

Oral administration of [D-Trp]CJ-15,208 does not produce CPP or aversion, and has no effect on cocaine CPP

Mice demonstrated significant place preference following 2 days of place conditioning with cocaine ($F_{(6,230)} = 10.7$, $P < 0.0001$; one-way ANOVA followed by Tukey's HSD *post hoc* test; Figure 8), which was significantly greater than the response demonstrated by saline place conditioned mice ($P < 0.01$, Tukey's HSD *post hoc* test). Mice place conditioned with saline (p.o.) or [D-Trp]CJ-15,208 alone (10, 30 or 60 mg·kg^{-1}, p.o. daily) demonstrated neither significant preference nor aversion for the macrocyclic peptide-paired side ($P > 0.05$, Tukey's HSD *post hoc* test; Figure 8, middle bars). Moreover, when administered daily 3 h before cocaine, [D-Trp]CJ-15,208 (60 mg·kg^{-1}, p.o.) did not alter subsequent cocaine CPP ($P > 0.05$, Tukey's HSD *post hoc* test; Figure 8, rightmost bar).

Oral administration of [D-Trp]CJ-15,208 prevents stress-induced reinstatement of extinguished cocaine CPP

Following 4 days of cocaine place conditioning, mice showed a significant preference for the drug-paired side ($F_{(3,408)} = 31.44$, $P < 0.0001$; one-way ANOVA with Tukey's HSD *post hoc* test; Figure 9B, black bar). Extinction was observed within 3 weeks of place conditioning, at which point mice were pretreated orally once daily for 2 days with vehicle (1:1:8, p.o.) or [D-Trp]CJ-15,208 (30 or 60 mg·kg^{-1}, p.o.), and exposed to forced swim stress or an additional cycle of cocaine place conditioning (see schematic, Figure 9A). Mice pretreated with vehicle (1:1:8, p.o.) demonstrated significant reinstatement of cocaine CPP after exposure to forced swimming ($F_{(5,356)} = 21.8$, $P < 0.0001$; one-way ANOVA followed by Tukey's HSD *post hoc* test) or cocaine ($F_{(5,352)} = 24.1$, $P < 0.0001$; one-way ANOVA followed by Tukey's HSD *post hoc* test; Figure 9B). Oral [D-Trp]CJ-15,208 pretreatment dose-dependently prevented stress-induced reinstatement ($P < 0.01$ after pretreatment with 60 mg·kg^{-1}, p.o., Tukey's HSD *post hoc* test; Figure 9B, central bars), but was without effect on cocaine-induced reinstatement ($P > 0.05$, Tukey's HSD *post hoc* test; Figure 9B, rightmost bars).

Discussion and conclusions

Pretreatment with KOR-selective antagonists can block stress-induced reinstatement of cocaine-seeking behaviour (Beardsley *et al.*, 2005; 2010; Carey *et al.*, 2007; Aldrich *et al.*, 2009), a property now also demonstrated by [D-Trp]CJ-15,208 after p.o. administration. A growing body of evidence suggests that the prolonged stress-induced release of dynorphins (Shirayama *et al.*, 2004), the endogenous agonists for KOR, results in both the potentiation of the rewarding effects of cocaine (McLaughlin *et al.*, 2003) and reinstatement of extinguished cocaine-seeking behaviour (Valdez *et al.*, 2007; Redila and Chavkin, 2008). While acute administration of KOR agonists blocks cocaine self-administration (Glick *et al.*, 1995; Shippenberg *et al.*, 2007) and is not reinforcing (Koob *et al.*, 1986; McLaughlin *et al.*, 2006), repeated exposure to KOR agonists has been shown to paradoxically increase cocaine-seeking behaviour (Kuzmin *et al.*, 1997; Negus, 2004; McLaughlin *et al.*, 2006). Thus, the prophylactic use of KOR antagonists to prevent stress-induced relapse to cocaine-seeking behaviour in abstinence subjects may be of significant therapeutic benefit.

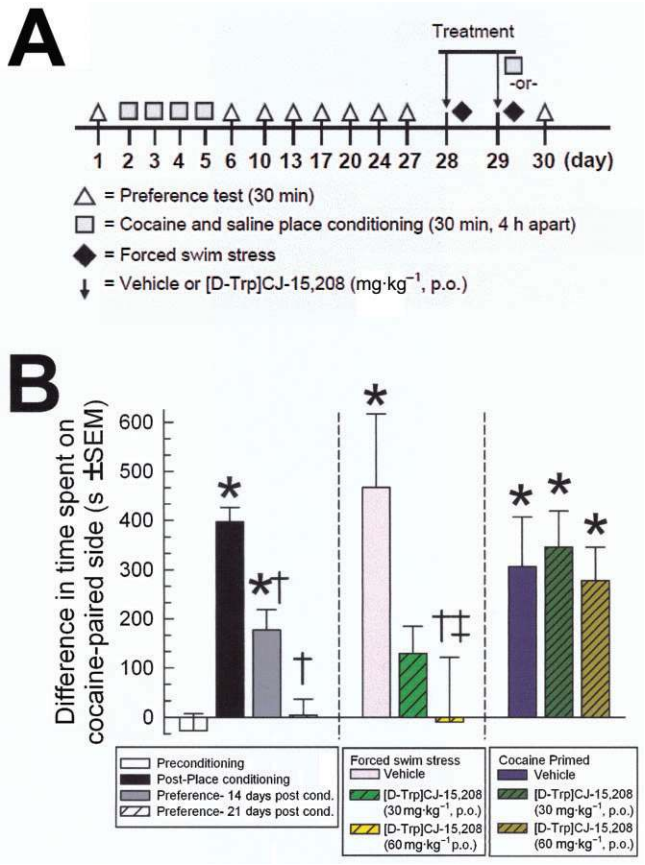

Figure 9

Stress-induced reinstatement of cocaine CPP prevented by pretreatment with [D-Trp]CJ-15,208. (A) Schematic of reinstatement and testing protocol. Vehicle (1:1:8, p.o.) or [D-Trp]CJ-15,208 (30 or 60 mg·kg^{-1}, p.o.) was administered on days 28 and 29, 3 h prior to initial exposure to forced swim stress (diamonds); for cocaine place conditioning the mice were treated on days 28 and 29 with vehicle or peptide, followed by cocaine place conditioning 3 h after the injection (square) on day 29. (B) Mice exhibited significant preference for the cocaine-paired (10 mg·kg^{-1}, s.c. daily for 4 days) environment, with extinction occurring by 3 weeks later (left bars). Mice were then exposed to forced swim stress (centre bars) or an additional round of cocaine place conditioning (right bars), reinstating preference in vehicle-treated mice (open bars). Pretreatment with [D-Trp]CJ-15,208 (30 or 60 mg·kg^{-1}, p.o., striped bars) prevented stress-induced reinstatement of place preference (centre bars), but was ineffective at blocking cocaine-induced reinstatement (rightmost bars). Mean difference in time spent on the drug-paired side ± SEM is presented from 14 to 19 mice per treatment condition; cocaine place conditioning data on left represent combined responses of 103 mice used in this experiment. *Significantly different from preconditioning place preference response (leftmost bar), $P < 0.01$. †Significantly different from post-CPP response (black solid bar, left), $P < 0.01$. ‡Significantly different from stress-induced reinstatement of place preference response (centre), $P < 0.005$; ANOVA followed by Tukey's HSD *post hoc* test.

Recent results also suggest a broader therapeutic potential for KOR-selective antagonists in treating the abuse and relapse to abuse of a number of reinforcing drugs. Of particular interest, KOR antagonists have recently been shown over time to decrease both compulsive cocaine-seeking behaviour and intake in the absence of stress (Wee *et al.*, 2009; 2012). They may also have potential in the treatment of opiate abuse, based on promising results with a 'functional κ opioid receptor antagonist' (buprenorphine plus naltrexone) in heroin addicts (Rothman *et al.*, 2000; Gerra *et al.*, 2006) and in rodent studies of morphine CPP and the reinstatement of extinguished morphine CPP (Cordery *et al.*, 2012). In addition, KOR antagonists attenuated symptoms of nicotine withdrawal (Jackson *et al.*, 2010) and stress-induced reinstatement of nicotine-seeking behaviour (Jackson *et al.*, 2012).

The KOR antagonism produced by [D-Trp]CJ-15,208 was of relatively short duration compared with the activity of prototypical small molecule KOR antagonists such as nor-BNI (Metcalf and Coop, 2005). The prolonged KOR antagonism of these prototypical KOR antagonists, which is the subject of ongoing study (Melief *et al.*, 2011; Munro *et al.*, 2012), may complicate preclinical studies, and potentially impair clinical development. The recent termination of the phase 1 clinical trials of JDTic due to undisclosed adverse effects (ClinicalTrials.gov, NCT01431586) has further spurred the search for new, safe and shorter acting KOR-selective antagonists. As discussed above, while several new non-peptide KOR antagonists have recently been reported, including some with finite durations of KOR antagonist activity (Runyon *et al.*, 2010; Peters *et al.*, 2011) or with short residence time in the brain (Grimwood *et al.*, 2011; Mitch *et al.*, 2011) after peripheral administration, reports of orally active KOR antagonists have been limited to only four KOR-selective antagonists: JDTic (Beardsley *et al.*, 2005), an analogue of JDTic (Beardsley *et al.*, 2010), an aminobenzyloxyarylamide (Mitch *et al.*, 2011), and a biphenylsulfonamide (Chang *et al.*, 2011). To date, p.o. activity has not been reported for any of the shorter acting small molecule KOR antagonists. Thus, to the best of our knowledge, the present demonstration of the activity of [D-Trp]CJ-15,208 after p.o. administration represents the first report of an orally active KOR-selective antagonist with finite duration of KOR antagonist activity, which is very promising and clearly a desirable trait for potential therapeutic development.

[D-Trp]CJ-15,208 does demonstrate limited (<25%), brief antinociception after peripheral administration in wild-type mice. The lack of antinociception in KOR -/- (but not MOR -/-) mice suggests that [D-Trp]CJ-15,208 possesses weak KOR agonist activity. The natural product, CJ-15,208 with L-Trp, also exhibits both KOR agonist and antagonist activities, although the agonism of that compound is much more robust and is partially mediated through MOR as well as KOR (Ross *et al.*, 2012; Aldrich *et al.*, 2013). Notably, the agonist activity of [D-Trp]CJ-15,208 did not confound the antagonist testing performed here, as animals were pretreated 2–3 h with the macrocyclic tetrapeptide to avoid any agonist effects. It is conceivable that the weak agonist activity of [D-Trp]CJ-15,208 could produce antagonism by inducing desensitization of KOR (McLaughlin *et al.*, 2004) at the higher doses tested orally here. However, this seems unlikely, given that i.c.v. administration of [D-Trp]CJ-15,208 at doses that produced robust KOR antagonism (1 and 3 nmol, i.c.v.) did not exhibit antinociception (<5%) (Ross *et al.*, 2012), providing strong evidence that KOR antagonism is not simply due to receptor desensitization.

The development of peptidic ligands selective for KOR potentially offers several additional advantages (Aldrich and McLaughlin, 2009), including the high specificity of peptides for their targets, low toxicity, minimal drug–drug interactions and low accumulation in tissues (Marx, 2005). Notably, a number of peptides with favourable pharmacokinetic properties have been developed as therapeutics (Vlieghe *et al.*, 2010).

The key finding in the present study is that the macrocyclic peptide [D-Trp]CJ-15,208 demonstrated dose-dependent KOR antagonism in the CNS after oral administration. The demonstration of biological activity by peptides after p.o. administration is relatively rare (Aldrich and McLaughlin, 2012). Linear peptides such as dynorphin A typically undergo rapid metabolism by proteases (Reed *et al.*, 2003; Klintenberg and Andren, 2005) that limit their therapeutic use. Incorporation of structural modifications (e.g. unnatural and D-amino acids) into linear peptides can impart sufficient metabolic stability to facilitate activity after systemic administration (Aldrich and McLaughlin, 2012), as shown by E-2078, a metabolically stable analogue of dynorphin A-(1-8) which exhibits analgesic activity in humans after intramuscular injection (Fujimoto and Momose, 1995). In contrast, macrocyclic peptides are typically stable to proteases and are instead metabolized by cytochrome P_{450} enzymes involved in drug metabolism (Christians and Sewing, 1993; Delaforge *et al.*, 1997). Moreover, their macrocyclic structure often results in intramolecular hydrogen bonding which can facilitate membrane permeability (Rezai *et al.*, 2006), thereby enhancing oral absorption and CNS penetration. While novel, the oral activity of [D-Trp]CJ-15,208 is not without precedence, given that p.o. formulations of the immunosuppressant macrocyclic peptide drug cyclosporine are used clinically (Diasio and LoBuglio, 1996).

The dose-dependent antagonism by orally administered [D-Trp]CJ-15,208 of centrally (i.c.v.) administered U50,488-induced antinociception and the prevention of stress-induced reinstatement of cocaine-seeking behaviour strongly indicate that the macrocyclic peptide crosses the blood–brain barrier to reach KOR in the CNS. LC-MS/MS analysis verified the presence of [D-Trp]CJ-15,208 in both serum and perfused brain following its p.o. administration. The systemic administration of this very hydrophobic macrocyclic peptide necessitated the use of solubilizers [ethanol and Tween-80 (polysorbate 80)], potentially raising questions concerning the effect these agents could have on the CNS activity and/or absorption of the macrocyclic peptide. Tween-80 is commonly used to solubilize and administer compounds *in vivo*, including other opioids (Schmidt *et al.*, 2005; Wang *et al.*, 2005), and is present in a number of clinical formulations such as p.o. formulations of cyclosporine (Strickley, 2004). While intravenous administration of both ethanol and Tween-80 has been reported to enhance blood–brain barrier penetration of drugs (Hanig *et al.*, 1972; Azmin *et al.*, 1985; Sakane *et al.*, 1989), the small amounts administered orally in the present study (0.025 mL each) are unlikely to affect the CNS penetration of the macrocyclic peptide, and much higher oral doses of Tween-80 ($6.4\ g{\cdot}kg^{-1}{\cdot}day^{-1}$ for 28 days) were tolerated in C57BL/6J mice without evidence of cytotoxicity (Li *et al.*, 2011). It should be noted, however, that Tween-80 may inhibit efflux proteins such as P-glycoprotein, although the evidence is mixed (Goole *et al.*, 2010).

In conclusion, oral administration of the macrocyclic tetrapeptide [D-Trp]CJ-15,208 produced a dose-dependent, KOR-selective antagonism that lasted less than 12 h. To the best of our knowledge, this represents the first report of an orally active KOR-selective antagonist with a finite (≤1 day) duration of activity. Furthermore, mice pretreated orally with [D-Trp]CJ-15,208 demonstrated dose-dependent antagonism of U50,488 administered centrally, and [D-Trp]CJ-15,208 could be detected by LC-MS/MS in both serum and perfused brain samples, indicating orally administered [D-Trp]CJ-15,208 is both absorbed from the gastrointestinal tract and crosses the blood–brain barrier to antagonize KOR in the CNS. Finally, p.o. administration of [D-Trp]CJ-15,208 prevented the stress-, but not cocaine-, induced reinstatement of cocaine CPP, consistent with previous demonstrations with KOR antagonists. Together, these data validate the use of systemically active peptidic ligands such as [D-Trp]CJ-15,208 as potentially useful therapeutics for treating relapse to drug abuse.

Acknowledgements

This work was supported by NIDA grants DA023924 and DA032928 (to J. V. A.) and the State of Florida, Executive Office of the Governor's Office of Tourism, Trade, and Economic Development (to J. P. M.).

Conflict of interest

The authors state no conflict of interest.

References

Aldrich JV, McLaughlin JP (2009). Peptide kappa opioid receptor ligands: potential for drug development. AAPS J 11: 312–322.

Aldrich JV, McLaughlin JP (2012). Opioid peptides: potential for drug development. Drug Disc Today Technol 9: e23–e31.

Aldrich JV, Patkar KA, McLaughlin JP (2009). Zyklophin, a systemically active selective kappa opioid receptor peptide antagonist with short duration of action. Proc Natl Acad Sci USA 106: 18396–18401.

Aldrich JV, Senadheera SN, Ross NC, Ganno ML, Eans SO, McLaughlin JP (2013). The macrocyclic peptide natural product CJ-15,208 is orally active and prevents reinstatement of extinguished cocaine seeking behavior. J Natural Products 76: 433–438.

Alexander SP, Mathie A, Peters JA (2011). Guide to receptors and channels (GRAC), 5th edn. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Azmin MN, Stuart JF, Florence AT (1985). The distribution and elimination of methotrexate in mouse blood and brain after concurrent administration of polysorbate 80. Cancer Chemotherapy Pharmacol 14: 238–242.

Beardsley P, Gerald P, James H, Carroll F (2010). Effectiveness of analogs of the kappa opioid receptor antagonist (3R)-7-Hydroxy-N-((1S)-1-{[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl}-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide (JDTic) to reduce U50,488-induced diuresis and stress-induced cocaine reinstatement in rats. Psychopharmacology (Berl) 210: 189–198.

Beardsley PM, Howard JL, Shelton KL, Carroll FI (2005). Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. Psychopharmacology (Berl) 183: 1–9.

Brugel TA, Smith RW, Balestra M, Becker C, Daniels T, Hoerter TN *et al*. (2010). Discovery of 8-azabicyclo[3.2.1]octan-3-yloxy-benzamides as selective antagonists of the kappa opioid receptor. Part 1. Bioorg Med Chem Lett 20: 5847–5852.

Carey AN, Borozny K, Aldrich JV, McLaughlin JP (2007). Reinstatement of cocaine place-conditioning prevented by the peptide kappa-opioid receptor antagonist arodyn. Eur J Pharmacol 569: 84–89.

Carroll I, Thomas JB, Dykstra LA, Granger AL, Allen RM, Howard JL *et al*. (2004). Pharmacological properties of JDTic: a novel kappa-opioid receptor antagonist. Eur J Pharmacol 501: 111–119.

Chang C, Byon W, Lu Y, Jacobsen LK, Badura LL, Sawant-Basak A *et al*. (2011). Quantitative PK-PD model-based translational pharmacology of a novel kappa opioid receptor antagonist between rats and humans. AAPS J 13: 565–575.

Christians U, Sewing KF (1993). Cyclosporin metabolism in transplant patients. Pharmacol Ther 57: 291–345.

Cordery SF, Taverner A, Ridzwan IE, Guy RH, Delgado-Charro MB, Husbands SM *et al*. (2012). A non-rewarding, non-aversive buprenorphine/naltrexone comination attenuates drug-primed reinstatement to cocaine and morphine in rats in a conditioned place preference paradigm. Addict Biol DOI: 10.1111/adb.12020

Delaforge M, Andre F, Jaouen M, Dolgos H, Benech H, Gomis JM *et al*. (1997). Metabolism of tentoxin by hepatic cytochrome P-450 3A isozymes. Eur J Biochem 250: 150–157.

Diasio RB, LoBuglio AF (1996). Immunomodulators: immunosuppressive agents and immunostimulants. In: Hardman JG, Limbird LE (eds). Goodman and Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition. McGraw-Hill: New York, pp. 1291–1308.

Dolle RE, Michaut M, Martinez-Teipel B, Seida PR, Ajello CW, Muller AL *et al*. (2009). Nascent structure-activity relationship study of a diastereomeric series of kappa opioid receptor antagonists derived from CJ-15,208. Bioorg Med Chem Lett 19: 3647–3650.

Frankowski KJ, Hedrick MP, Gosalia P, Li K, Shi S, Whipple D *et al*. (2012). Discovery of small molecule kappa opioid receptor agonist and antagonist chemotypes through a HTS and hit refinement strategy. ACS Chem Neurosci 3: 221–236.

Fujimoto K, Momose T (1995). Analgesic efficacy of E-2078 (dynorphin analog) in patients following abdominal surgery. Jpn J Anesth 44: 1233–1237.

Gerra G, Fantoma A, Zaimovic A (2006). Naltrexone and buprenorphine combination in the treatment of opioid dependence. J Psychopharmacol 20: 806–814.

Glick SD, Maisonneuve IM, Raucci J, Archer S (1995). Kappa-opioid inhibition of morphine and cocaine self-administration. Brain Res 681: 147–152.

Goole J, Lindley DJ, Roth W, Carl SM, Amighi K, Kauffmann JM *et al*. (2010). The effects of excipients on transporter mediated absorption. Int J Pharm 393: 17–31.

Grimwood S, Lu Y, Schmidt AW, Vanase-Frawley MA, Sawant-Basak A, Miller E *et al*. (2011). Pharmacological characterization of 2-methyl-N-((2′-(pyrrolidin-1-ylsulfonyl) biphenyl-4-yl)methyl)propan-1-amine (PF-04455242), a high-affinity antagonist selective for kappa opioid receptors. J Pharmacol Exp Ther 339: 555–566.

Hanig JP, Morrison JM, Jr, Krop S (1972). Ethanol enhancement of blood-brain barrier permeability to catecholamines in chicks. Eur J Pharmacol 18: 79–82.

Horan P, Taylor J, Yamamura HI, Porreca F (1991). Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. J Pharmacol Exp Ther 260: 1237–1243.

Jackson KJ, Carroll FI, Negus SS, Damaj MI (2010). Effect of the selective kappa-opioid receptor antagonist JDTic on nicotine antinociception, reward, and withdrawal in the mouse. Psychopharmacology (Berl) 210: 285–294.

Jackson KJ, McLaughlin JP, Carroll FI, Damaj MI (2012). Effects of the kappa opioid receptor antagonist, norbinaltorphimine, on stress and drug-induced reinstatement of nicotine reward. Psychopharmacology (Berl) (April 20 E-pub ahead of print).

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Klintenberg R, Andren PE (2005). Altered extracellular striatal in vivo biotransformation of the opioid neuropeptide dynorphin A(1-17) in the unilateral 6-OHDA rat model of Parkinson's disease. J Mass Spectrom 40: 261–270.

Koob GF, Vaccarino FJ, Amalric M, Bloom FE (1986). Neurochemical substrates for opiate reinforcement. NIDA Res Monogr 71: 146–164.

Kulkarni SS, Ross NC, McLaughlin JP, Aldrich JV (2009). Synthesis of cyclic tetrapeptide CJ 15,208: a novel kappa opioid receptor antagonist. Adv Exp Med Biol 611: 269–270.

Kuzmin AV, Semenova S, Gerrits MA, Zvartau EE, Van Ree JM (1997). Kappa-opioid receptor agonist U50,488H modulates cocaine and morphine self-administration in drug-naive rats and mice. Eur J Pharmacol 321: 265–271.

Li X, Wang L, Li Y, Ho Y, Yang D, Chen Y *et al*. (2011). Polysorbates as novel lipid-modulating candidates for reducing serum total cholesterol and low-density lipoprotein levels in hyperlipidemic C57BL/6J mice and rats. Eur J Pharmacol 660: 468–475.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McLaughlin JP, Marton M, Chavkin C (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J Neurosci 23: 5674–5683.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA *et al*. (2004). Prolonged kappa-opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. J Biol Chem 279: 1810–1818.

McLaughlin JP, Land BB, Li S, Pintar JE, Chavkin C (2006). Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. Neuropsychopharmacology 31: 787–794.

Marx M (2005). Watching peptide drugs grow up. Chem Eng News 83: 17–24.

Melief EJ, Miyatake M, Carroll FI, Beguin C, Carlezon WA Jr CBM *et al*. (2011). Duration of action of a broad range of selective κ-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. Mol Pharmacol 80: 920–929.

Metcalf MD, Coop A (2005). Kappa opioid antagonists: past successes and future prospects. AAPS J 7: E704–E722.

Mitch CH, Quimby SJ, Diaz N, Pedregal C, de la Torre MG, Jimenez A *et al*. (2011). Discovery of aminobenzyloxyarylamides as kappa opioid receptor selective antagonists: application to preclinical development of a kappa opioid receptor antagonist receptor occupancy tracer. J Med Chem 54: 8000–8012.

Munro TA, Berry LM, Van't Veer A, Beguin C, Carroll FI, Zhao Z *et al*. (2012). Long-acting kappa opioid antagonists nor-BNI, GNTI and JDTic: pharmacokinetics in mice and lipophilicity. BMC Pharmacol 12: 5.

Negus SS (2004). Effects of the kappa opioid agonist U50,488 and the kappa opioid antagonist norbinaltorphimine on choice between cocaine and food in rhesus monkeys. Psychopharmacology (Berl) 176: 204–213.

Paris JJ, Reilley KJ, McLaughlin JP (2011). Kappa opioid receptor-mediated disruption in novel object recognition: relevance for psychostimulant treatment. J Addiction Res Ther S4: pii:007.

Patkar KA, Yan X, Murray TF, Aldrich JV (2005). N^{α}-BenzylTyr^{1},cyclo(D-Asp^{5},Dap^{8})]-dynorphin A-(1-11)NH_2 cyclized in the 'address' domain is a novel kappa-opioid receptor antagonist. J Med Chem 48: 4500–4503.

Peters MF, Zacco A, Gordon J, Maciag CM, Litwin LC, Thompson C *et al*. (2011). Identification of short-acting kappa-opioid receptor antagonists with anxiolytic-like activity. Eur J Pharmacol 661: 27–34.

Redila VA, Chavkin C (2008). Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. Psychopharmacology (Berl) 200: 59–70.

Reed B, Zhang Y, Chait BT, Kreek MJ (2003). Dynorphin A(1-17) biotransformation in striatum of freely moving rats using microdialysis and matrix-assisted laser desorption/ionization mass spectrometry. J Neurochem 86: 815–823.

Rezai T, Bock JE, Zhou MV, Kalyanaraman C, Lokey RS, Jacobson MP (2006). Conformational flexibility, internal hydrogen bonding, and passive membrane permeability: successful in silico prediction of the relative permeabilities of cyclic peptides. J Am Chem Soc 128: 14073–14080.

Ross N, Reilley K, Murray T, Aldrich J, McLaughlin J (2012). Novel opioid cyclic tetrapeptides: Trp isomers of CJ-15,208 exhibit distinct opioid receptor agonism and short-acting kappa opioid receptor antagonism. Brit J Pharmacol. 165: 1097–1108.

Ross NC, Kulkarni SS, McLaughlin JP, Aldrich JV (2010). Synthesis of CJ-15,208, a novel κ-opioid receptor antagonist. Tetrahedron Lett 51: 5020–5023.

Rothman RB, Gorelick DA, Heishman SJ, Eichmiller PR, Hill BH, Norbeck J *et al*. (2000). An open-label study of a functional opioid κ antagonist in the treatment of opioid dependence. J Substance Abuse Treat 18: 277–281.

Runyon SP, Brieaddy LE, Mascarella SW, Thomas JB, Navarro HA, Howard JL *et al*. (2010). Analogues of (3R)-7-hydroxy-N-[(1S)-1-{[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-pipe ridinyl]methyl}-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxami de (JDTic). Synthesis and in vitro and in vivo opioid receptor antagonist activity. J Med Chem 53: 5290–5301.

Saito T, Hirai H, Kim YJ, Kojima Y, Matsunaga Y, Nishida H *et al*. (2002). CJ-15,208, a novel kappa opioid receptor antagonist from a fungus, Ctenomyces serratus ATCC15502. J Antibiot (Tokyo) 55: 847–854.

Sakane T, Tanaka C, Yamamoto A, Hashida M, Sezaki H, Ueda H *et al*. (1989). The effect of polysorbate 80 on brain uptake and analgesic effect of D-kyotorphin. Int J Pharm 57: 77–83.

Schmidt MD, Schmidt MS, Butelman ER, Harding WW, Tidgewell K, Murry DJ *et al*. (2005). Pharmacokinetics of the plant-derived kappa-opioid hallucinogen salvinorin A in nonhuman primates. Synapse 58: 208–210.

Senadheera SN, Kulkarni SS, McLaughlin JP, Aldrich JV (2011). Improved synthesis of CJ-15,208 isomers and their pharmacological activity at opioid receptors. In: Lebl M (ed.). Building Bridges: The Proceedings of the 22nd American Peptide Symposium. American Peptide Society: San Diego, CA, pp. 346–347.

Shippenberg TS, Zapata A, Chefer VI (2007). Dynorphin and the pathophysiology of drug addiction. Pharmacol Ther 116: 306–321.

Shirayama Y, Ishida H, Iwata M, Hazama GI, Kawahara R, Duman RS (2004). Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. J Neurochem 90: 1258–1268.

Strickley RG (2004). Solubilizing excipients in oral and injectable formulations. Pharm Res 21: 201–230.

Valdez GR, Platt DM, Rowlett JK, Ruedi-Bettschen D, Spealman RD (2007). Kappa agonist-induced reinstatement of cocaine seeking in squirrel monkeys: a role for opioid and stress-related mechanisms. J Pharmacol Exp Ther 323: 525–533.

Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M (2010). Synthetic therapeutic peptides: science and market. Drug Disc Today 15: 40–56.

Wang Y, Tang K, Inan S, Siebert D, Holzgrabe U, Lee DY *et al*. (2005). Comparison of pharmacological activities of three distinct kappa ligands (Salvinorin A, TRK-820 and 3FLB) on kappa opioid receptors in vitro and their antipruritic and antinociceptive activities in vivo. J Pharmacol Exp Ther 312: 220–230.

Wee S, Koob GF (2010). The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. Psychopharmacology (Berl) 210: 121–135.

Wee S, Orio L, Ghirmai S, Cashman JR, Koob GF (2009). Inhibition of kappa opioid receptors attenuated increased cocaine intake in rats with extended access to cocaine. Psychopharmacology (Berl) 205: 565–575.

Wee S, Vendruscolo LF, Misra KK, Schlosburg JE, Koob GF (2012). A combination of buprenorphine and naltrexone blocks compulsive cocaine intake in rodents without producing dependence. Sci Transl Med 4: 146ra110.