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RESEARCH PAPER

WAY-318068: a novel, potent and selective noradrenaline re-uptake inhibitor with activity in rodent models of pain and depression

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Background and purpose: Antidepressants, which raise the CNS concentrations of 5-HT and noradrenaline, are frequently used in the treatment of chronic pain; however, it is not known if increasing CNS noradrenaline levels alone is sufficient for efficacy, in part resulting from a lack of small molecules with sufficient selectivity.
Experimental approach: In this report, we present the *in vitro* pharmacological and *in vivo* pharmacokinetic and pharmacological properties of the novel, orally available and CNS penetrant inhibitor of the noradrenaline transporter (NET), WAY-318068 (1-[(1*S*,2*R*)-1-(3,5-difluorophenyl)-2-hydroxy-3-(methylamino)propyl]-7-fluoro-3,3-dimethyl-1,3-dihydro-2*H*-indol-2-one).
Key results: WAY-318068 is a potent and effective inhibitor of the NET with a K_i of 8.7 nM in a binding assay, and an IC_{50} of 6.8 nM in an assay of transporter function, without significant binding to the dopamine transporter. Furthermore, the compound has only weak activity at the 5-HT transporter, leading to a functional selectivity of greater than 2500-fold. It is orally bioavailable with substantial quantities of the compound found in the CNS after oral dosing. As measured by microdialysis in rats, the compound causes a robust and significant increase in cortical noradrenaline levels without affecting 5-HT. WAY-318068 was effective in models of acute, visceral, inflammatory, osteoarthritic, neuropathic, diabetic and bone cancer pain, as well as in traditional models of depression at doses that do not cause motor deficits.
Conclusions and implications: Collectively, the present results support the conclusion that selectively increasing CNS levels of noradrenaline is sufficient for efficacy in models of depression and pain.
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Keywords: pain; depression; noradrenaline; 5-HT; NRI; SNRI

Abbreviations: FCA, Freund's complete adjuvant; MIA, monosodium iodoacetate; mPFC, medial prefrontal cortex; MRMT, mammary gland carcinoma; NET, noradrenaline transporter; NRI, noradrenaline re-uptake inhibitor; PPQ, para-phenylquinone; PWT, paw withdrawal threshold; RVM, rostral ventral medulla; SERT, 5-HT transporter; SNL, spinal nerve ligation; SNRI, 5-HT/noradrenaline re-uptake inhibitor; SSRI, selective 5-HT re-uptake inhibitor; STZ, streptozotocin; TST, tail suspension test; WAY-318068, (1-[(1*S*,2*R*)-1-(3,5-difluorophenyl)-2-hydroxy-3-(methylamino)propyl]-7-fluoro-3,3-dimethyl-1,3-dihydro-2*H*-indol-2-one)

Introduction

Pain is a debilitating condition that can be caused by a number of factors, including acute exposure to noxious stimuli, tissue injury and inflammation or nerve damage. Current pain treatments suffer from the presence of dose-limiting side effects, abuse potential or limited efficacy, and there is a clear medical need for improved therapies.

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Antidepressant drugs, such as those that inhibit re-uptake of noradrenaline and 5-HT (SNRIs), have been used as a first-line treatment for pain associated with diabetic neuropathy, postherpetic neuralgia, fibromyalgia, irritable bowel syndrome and interstitial cystitis (Collins *et al.*, 2000). These treatments commonly increase synaptic levels of both 5-HT and noradrenaline by inhibition of the noradrenaline (NET) and 5-HT transporters (SERT). NET and SERT are 12-transmembrane spanning proteins located in pre-synaptic and glial membranes within the CNS that act to limit the duration and magnitude of monoaminergic signalling. A role for noradrenaline in the etiology and treatment of pain has been previously described (Gebhart, 1993; Leventhal *et al.*, 2007). Noradrenaline has been shown to be an important neurotransmitter involved in the descending pain inhibitory pathway projecting from the locus coeruleus (LC) and the rostral ventral medulla (RVM) to the spinal cord (Holden *et al.*, 1999). It is generally believed that compounds that selectively affect 5-HT re-uptake (SSRIs), although effective in treating depression, have limited clinical utility as analgesics (Fishbain *et al.*, 2000). On the other hand, compounds with dual activity at both NET and SERT (SNRIs) are effective antidepressants and analgesics, leading one to postulate that elevation of both noradrenaline and 5-HT is needed for efficacy. Importantly, as pain and depression are frequently co-morbid in the clinic (Nicholson and Verma, 2004), it is possible that affecting one may indirectly affect the other. For example, SSRIs may alleviate pain only when co-morbid with depression; however, it has been postulated that increased 5-HT potentiates the activity of noradrenaline (Zhao *et al.*, 2007). Indeed, duloxetine, an SNRI with comparable potency at both transporters, was recently approved for the treatment of diabetic neuropathic pain and fibromyalgia (Bymaster *et al.*, 2005).

It is less well understood if increasing noradrenaline levels without 5-HT is sufficient for activity, due, in part, to a relative lack of inhibitor compounds with sufficient selectivity for NET that would allow the conclusion that *in vivo* activity was due solely to effects of elevated noradrenaline. We previously demonstrated, using SNRI compounds with a range of potencies at SERT and NET, that efficacy in a model of visceral pain correlated with *in vitro* potency at NET, but not SERT (Leventhal *et al.*, 2007). Here, we extend these findings using a novel, orally bioavailable and highly selective noradrenaline re-uptake inhibitor (NRI), 1-[(1*S*,2*R*)-1-(3,5-difluorophenyl)-2-hydroxy-3-(methylamino)propyl]-7-fluoro-3,3-dimethyl-1,3-dihydro-2*H*-indol-2-one (WAY-318068) (McComas *et al.*, 2008; Zhang *et al.*, 2009a). WAY-318068 selectively increased CNS levels of noradrenaline, allowing us to demonstrate that this activity alone is sufficient for antidepressant and analgesic activity across a broad range of pre-clinical models.

Methods

In vitro *procedures*

Competition binding studies and functional uptake assays. Inhibition of binding of [^{3}H] nisoxetine, at a final concentration of 3 nM, to membranes prepared from Madin-Darby canine kidney (MDCK) cell line, stably transfected with hNET (MDCK-Net6) was performed as previously described (Mahaney *et al.*, 2008). Inhibition of uptake of [^{3}H]NE (16 nM) and [^{3}H]5-HT (12 nM) was performed using MDCK-Net6 and a human choriocarcinoma cell line natively expressing the human SERT (JAR cells), respectively, as previously described (Leventhal *et al.*, 2007; Mahaney *et al.*, 2008).

In vivo *procedures*

Animals. All animal care and experimental protocols were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, and were fully approved by the Wyeth Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan, IN, USA) were used, weighing 180–200 g at the start of the acute pain, inflammatory pain, microdialysis, beam walking/rotarod and pharmacokinetic experiments, or 90–110 g at the start of nerve ligation, osteoarthritis, olfactory bulbectomy and bone cancer experiments. Male CD-1 mice (Charles River, Kingston/Stoneridge, NY, USA) weighing 20–30 g were used for the *para*-phenylquinone (PPQ) and streptozotocin (STZ) models. Male Swiss Webster mice (Charles River) weighing 15–25 g were used for the tail suspension test (TST). Rodents had access to food and water *ad libitum* except when compounds were given orally and then food was removed 12 h before dosing. All experiments used group numbers of 3 (pharmacokinetics) and 10 (behaviour) per group (when overlapping dose–response experiments were performed $n = 20$) and were performed in an AAALAC-accredited facility, with randomization and assessed without knowledge of drug treatments.

Pharmacokinetic analyses. WAY-318068 (3–100 mg·kg^{-1}) was administered orally and blood collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h; from separate groups, blood and brain samples were collected 1, 3 and 5 h post-dosing to determine brain penetration. An additional group of rats was dosed intravenously with 2 mg·kg^{-1} WAY-318068 (in DMSO/80% PEG at 1 mL·kg^{-1}) to allow the determination of pharmacokinetic parameters (plasma half-life, clearance and volume of distribution). Sample analysis was performed as previously described (Sullivan *et al.*, 2007). Briefly, blood was collected in EDTA, and plasma was obtained after centrifugation at 9000× *g* for 10 min at 4°C. Whole brains were weighed and homogenized after addition of 1.2 mL of water. An aliquot of the samples (50 µL) was extracted by protein precipitation. To the aliquot was added 20 µL of a 3500 ng·mL^{-1} solution of the internal standard (a proprietary compound) and 400 µL of acetonitrile. The mixture was shaken for 2 min, centrifuged at 2500× *g* for 5 min, after which 350 µL of the supernatant was transferred using the Tomtec Quadra 96 (serial number: 196-320-585) and evaporated at 37°C under an N_2 atmosphere. The precipitated protein was then reconstituted in 400 µL of 50% acetonitrile/water and 15 µL was analysed by liquid chromatography/mass spectrometry/mass spectrometry with *m/z* transition of 379.3–348.1, and limits of quantification of 2 and 1 ng·mL^{-1} for plasma and brain respectively.

In vivo *microdialysis.* Levels of WAY-318068 and monoamines (noradrenaline and 5-HT) were measured in the medial

prefrontal cortex (mPFC) of rats using *in vivo* microdialysis. Rats were anaesthetized with isoflurane (2% in O_2) and secured in a stereotaxic frame with ear and incisor bars (David Kopf, Tujunga, CA, USA). A microdialysis guide cannula (CMA/Microdialysis, Stockholm, Sweden) was directed towards the mPFC using the following coordinates: (mPFC; AP +3.2 mm; ML +0.5 mm; DV –1.8 mm relative to bregma and dura) (Paxinos and Watson, 1986). The cannula was secured to the skull using dental acrylic cement (Plastics One, Roanoke, VA, USA) and two stainless-steel screws. During the 24 h post-operative recovery period, the animals were individually housed in Plexiglas cages, and monitored. Microdialysis probes (CMA, 4 mm active membrane; OD 0.5 mm) were equilibrated according to manufacturer's specifications. Microdialysis probes were perfused with artificial CSF (aCSF; 125 mM NaCl, 3 mM KCl, 0.75 mM $MgSO_4$ and 1.2 mM $CaCl_2$, pH 7.4) at a flow rate of 1 $\mu L \cdot min^{-1}$, and implanted via the guide cannula into the mPFC. After a 3 h equilibration period, four baseline samples (30 µL) were collected into plastic tubes located in a cooled micro-fraction collector (CMA). At the end of the fourth baseline sample, the rats were given a dose of 30 $mg \cdot kg^{-1}$ WAY-318068 or vehicle (p.o.). Microdialysis samples were collected every 30 min for 4 h, and immediately placed on dry ice. At the end of the experiment, the animals were killed and probe placement was verified histologically. Animals with incorrect probe placement were excluded from the study.

For WAY-318068 analysis, microdialysis samples were desalted as described previously (Erve *et al.*, 2009). Briefly, an LTQ-Orbitrap hybrid mass spectrometer with high-resolution isolation capability (Thermo Fisher Scientific, Bremen, Germany) equipped with a TriVersa NanoMate chip-based electrospray ionization system (Advion BioSciences, Ithaca, NY, USA) was operated in the positive ionization mode with a spray voltage of 1.4–1.5 kV. ZipTips were conditioned with methanol followed by 0.1% formic acid in water. The microdialysate was passed through the ZipTip 7–10 times to trap WAY-318068 on the C18 material, and washed with 40 µL 0.1% formic acid to remove salts. Retained WAY-318068 was eluted from the ZipTip using 20 µL of 50:50 methanol : 0.1% formic acid (v/v) into a clean Eppendorf tube, and analysed immediately.

For monoamine analysis, dialysate (20 µL) from the rat mPFC was analysed by HPLC with electrochemical detection. Samples containing noradrenaline and 5-HT were separated by HPLC (C18 ODS3 column, 150 × 3.0 mm, Metachem, Torrance, CA) and detected using an ANTEC electrochemical detector (ANTEC, Rotterdam, The Netherlands) set at a potential of 0.65 V versus an Ag/AgCl reference electrode. Mobile phase (0.15 M NaH_2PO_4, 0.25 mM EDTA, 1.75 mM 1-octane sulphonic acid, 2% isopropanol and 4% methanol, pH 4.8) was delivered by a Jasco PU1580 HPLC pump (Jasco Ltd, Essex, UK) at a flow rate of 0.5 $mL \cdot min^{-1}$. Neurochemical data were compared to an external standard curve, and all data were acquired using the Atlas software package (Thermo Labsystems, Beverley, MA, USA).

Acute analgesia. The effect of WAY-318068 and reboxetine on acute analgesia was investigated using the tail flick and hot plate assays as previously described (Sullivan *et al.*, 2007). For the tail flick, rats were placed on the apparatus (Ugo Basile, Varese, Italy), and an infrared beam was focused onto the tail, 5 cm from the tip. The latency to tail flick was assessed with cut-off set at 30 s and the intensity set at 35%. For the hot plate, rats were placed on a metal plate maintained at 52°C (Columbus Instruments, Columbus, OH, USA). The latency to nocifensive response, defined as hind paw lift, flutter, licking or escape behaviour, was measured with cut-off set at 30 s. For both assays, baseline latency was determined, and 1 h later rats received a single dose of 1, 3, 10, 30 or 100 $mg \cdot kg^{-1}$ WAY-318068 p.o., 10 or 30 $mg \cdot kg^{-1}$ reboxetine, p.o., 10 $mg \cdot kg^{-1}$ morphine (s.c. positive control) or vehicle. Latencies were determined at 1, 3 and 5 h following drug administration.

PPQ model of visceral pain. The ability of WAY-318068 to attenuate acute visceral (abdominal) pain was assessed following an i.p. injection of 2 $mg \cdot kg^{-1}$ of *PPQ* in 4% ethanol as previously described (Siegmund *et al.*, 1957; Leventhal *et al.*, 2007). The mice received 0.3, 1, 3, 10 or 30 $mg \cdot kg^{-1}$ WAY-318068 p.o. and were individually placed in a Plexiglas cage. Sixty minutes after WAY-318068 administration, PPQ was injected, and the total number of abdominal constrictions was recorded for 1 min periods starting 5 and 10 min following PPQ injection. *S*-duloxetine (30 $mg \cdot kg^{-1}$, p.o.), administered 60 min prior to testing, was used as a positive control.

Inflammatory hyperalgesia. The efficacy of WAY-318068 against hyperalgesia associated with inflammation was investigated using the Freund's complete adjuvant (FCA) and carrageenan models, as previously described (Bingham *et al.*, 2007; Sullivan *et al.*, 2007). For the FCA model, paw withdrawal threshold (PWT) to a noxious mechanical stimulus was determined using an analgesymeter (model 7200, Ugo Basile) with cut-off set at 250 g, and the end point was taken as complete paw withdrawal. PWT was determined once for each rat at each time-point. Baseline PWT was determined, after which rats were anaesthetized with isoflurane (2% in O_2) and received an intraplantar injection of 50% FCA (50 µL, diluted in saline) to the left hind paw. Twenty-four hours after FCA injection, pre-drug PWTs were measured, after which rats received 1, 3, 10, 30 or 100 $mg \cdot kg^{-1}$ WAY-318068 p.o., 30 $mg \cdot kg^{-1}$ celecoxib (positive control) or vehicle, and were tested 1, 3, 5 and 24 h later. For the carrageenan model, paw withdrawal latency to a radiant heat stimulus was determined by placing rats in a Plexiglas box on a glass surface maintained at 32°C (IITC, Woodland Hills, CA, USA). The plantar surface of the paw was then exposed to a beam of radiant heat. Latency to paw withdrawal was measured to the nearest 0.1 s with a maximal cut-off of 20 s. Baseline latencies were determined, after which rats received an intraplantar injection of 2% carrageenan (50 µL, dissolved in water). Three hours after carrageenan injection, rats received 10, 30 or 100 $mg \cdot kg^{-1}$ WAY-318068 p.o., 10 $mg \cdot kg^{-1}$ indomethacin (positive control) or vehicle and were tested 1 h later.

Monosodium iodoacetate (MIA) model of osteoarthritis pain. The MIA model of osteoarthritis in rats was used as previously described (Pomonis *et al.*, 2005). Osteoarthritis was induced using intra-articular injection of MIA. Rats were anaesthetized with isoflurane (2% in O_2) and received an injection of 1 mg

MIA (20 μL in saline) into the left knee joint using a 27-gauge needle inserted through the patellar tendon. Behavioural assessment of osteoarthritis-related pain behaviour was determined using weight bearing (Stoelting, CA, USA). Baseline behaviour was assessed prior to induction of osteoarthritis. Three weeks following injection of MIA, baseline weight bearing was determined, after which rats received 10 or 30 mg·kg^{-1} WAY-318068 p.o. or vehicle, and were tested 1, 3 and 5 h later. Three separate readings were taken for each time-point.

Neuropathic hyperalgesia. The L5 spinal nerve ligation (SNL) model was used as a model of nerve injury-related pain in rats as previously described (LaBuda and Little, 2005). PWTs to a noxious mechanical stimulus were determined using an analgesymeter (model 7200, Ugo Basile) with the cut-off set at 250 g, and the end point was taken as complete paw withdrawal. Baseline PWT was determined prior to SNL surgeries. Nerve injury was produced by tight ligation of the L5 spinal nerve (LaBuda and Little, 2005). Twenty-one days following nerve ligation, pre-drug PWTs were measured, after which rats received a single dose of 1, 3, 10, 30 or 100 mg·kg^{-1} WAY-318068 p.o., 10 or 30 mg·kg^{-1} reboxetine p.o., 100 mg·kg^{-1} gabapentin (i.p. positive control) or vehicle. PWTs were again determined 1, 3, 5 and 24 h post-drug administration.

Bone cancer-induced allodynia. To examine the potential effects of WAY-318068 on pain behaviour induced by bone cancer, a group of rats was injected with active or heat-killed mammary gland carcinoma cells (MRMT-1). Tactile thresholds were assessed using a series of calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA). Assessment of tactile allodynia was measured as the hind PWT that produced a 50% likelihood of a withdrawal using the up–down method, as described previously (Sullivan *et al.*, 2007). Baseline thresholds were determined before surgery, after which rats were anaesthetized with isoflurane (2% in O_2). A 1 cm rostral–caudal incision was made to expose the tibia, and using a 25-gauge needle, the bone was pierced 5 mm below the knee joint distal to the epiphyseal growth plate. The needle was inserted at an angle allowing access to the intramedullary bone canal. Using a micro syringe (Hamilton, Reno, NV, USA), 3 μL of live or heat-killed MRMT-1 cells in suspension at a density of 0.3×10^6 cells·mL^{-1} was injected into the tibia. Bone wax (Ethicon, Somerville, NJ, USA) was used to seal the injection site and the incision closed with surgical clips. Three weeks post-injection, baseline behaviour was determined again, after which rats received 30 or 100 mg·kg^{-1} WAY-318068 p.o. or vehicle, and were tested 1, 3 and 5 h later.

STZ model of diabetic neuropathy. To examine the potential effects of WAY-318068 and reboxetine on diabetic neuropathy, the STZ model was used as previously described (Gabra *et al.*, 2005). Six weeks after STZ administration (60 mg·kg^{-1}, i.p., 2 mL·kg^{-1} in 0.02 M citrate buffer at pH 4.5), hyperalgesia was assessed using the hot plate assay. The mice received 3, 10 or 30 mg·kg^{-1} WAY-318068 p.o., 10 mg·kg^{-1} reboxetine p.o., 300 mg·kg^{-1} gabapentin (positive control) or vehicle, and were tested 1 and 3 h later.

TST. To examine the antidepressant effects of WAY-318068, the mice were tested in the TST. The mice were treated with 10, 30 or 100 mg·kg^{-1} WAY-318068 p.o. or vehicle 60 min prior to the 6 min test session. After this pretreatment period, the mice were suspended upside down by taping their tails to a flat metal bar connected to a force transducer within a sound attenuation chamber (Medical Associates, St Albans, VT, USA). The bar is positioned such that the mouse is unable to reach the top or sides of the chamber. Data are represented as total immobility time (s).

Olfactory bulbectomy. The rats were anaesthetized with isoflurane (2% in O_2), two burr holes (2 mm diameter) drilled 8.0 mm from bregma, and ±2 mm from the midline and the olfactory bulbs removed by aspiration. Haemostatic sponge was inserted to prevent blood loss, and the wounds were closed using wound clips. The animals were weighed, handled and received either 10 mg·kg^{-1} WAY-318068 or vehicle p.o. daily for 14 days. At day 14, 4 h post-dosing, the rats were placed in an open field apparatus for a 5 min locomotor activity test using Ethovision (Noldus, Wageningen, The Netherlands) consisting of a 90 × 90 cm opaque plexiglass box with 30 cm high walls. Locomotor activity data were acquired by Ethovision video tracking system (Noldus). Data are shown as total distance moved (cm).

Ataxia/motor coordination. To examine the potential effects of WAY-318068 and reboxetine on motor performance, the rats were tested using the accelerating rotarod (Columbus Instruments) and beam walking assays. For the rotarod, speed was set to accelerate from 4 to 40 rpm over 300 s, with the maximum time spent on the rotarod set at 300 s. The rats received two training trials on the first day; were fasted overnight; and then received a single dose of 10, 30 or 100 mg·kg^{-1} WAY-318068 p.o.; 10, 30 or 100 mg·kg^{-1} reboxetine p.o.; or 10 mg·kg^{-1} morphine (s.c.) as a positive control. The rats were tested on the rotarod 1, 3 and 5 h following drug administration. For the beam walking assay, the rats were given an exposure trial to cross an elevated wooden beam (constructed in-house with dimensions of 2.5 cm wide by 114 cm long elevated by 34 cm) and enter a darkened chamber, after which baseline latency to cross the beam was measured for each rat. Cut-off latency was set at 60 s. Twenty-four hours after baseline testing, the rats received 10, 30 or 100 mg·kg^{-1} WAY-318068 p.o.; 10 mg·kg^{-1} morphine (s.c. positive control); or vehicle, and were tested 1, 3 and 5 h later.

Analysis of results. Data are shown as mean ± SD (*in vitro*) or SEM (*in vivo*). K_i and IC_{50} values were determined using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Pharmacokinetic parameters were calculated by non-compartmental approaches using WinNonlin Professional 4.1 (Pharsight, Mountain View, CA, USA). Statistical significance was determined on untransformed data using a one-way (PPQ, TST, olfactory bulbectomy) or a two-way ANOVA (acute pain, FCA, carrageenan, MIA, PSN, SNL, bone cancer, rotarod, beam walking, microdialysis) using a customized SAS-Excel application (SAS Institute, Cary, NC, USA) with across-group comparisons (all treatments to vehicle) being reported. Significant effects were analysed further by subsequent least

significant difference analysis. The level of significance was set at $P < 0.05$.

Materials. Celecoxib was purchased from Toronto Research Chemicals (Toronto, Canada), and gabapentin, reboxetine and duloxetine from Organix (Woburn, MA, USA). WAY-318068 was synthesized by Wyeth Chemical Sciences (Collegeville, PA, USA) as described by Mahaney *et al.* (2007). All other compounds and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All drugs were administered in 2% Tween 80/0.5% methylcellulose as free base equivalent p.o. in a volume of 10 mL·kg^{-1} unless otherwise stated. All drug/molecular target nomenclature follows Alexander *et al.* (2009).

Results

WAY-318068 is a selective inhibitor of the NET

In a radioligand binding assay using membrane preparations generated from cells expressing the hNET, WAY-318068 competed with [^{3}H]nisoxetine with a K_i value of 8.65 ± 0.35 nM. Desipramine was used as a standard and generated a K_i value of 1.95 ± 0.59 nM in agreement with literature values. In a functional noradrenaline uptake assay, using whole cells expressing the hNET, WAY-318068 blocked the uptake of [^{3}H] noradrenaline, and a calculated mean IC_{50} value of 6.83 ± 3.55 nM was generated in this assay (Table 1), supporting the classification of WAY-318068 as a potent NRI. In addition, WAY-318068 did not displace mazindol, up to a concentration of 10 μM, in a binding assay using the human dopamine transporter. To determine the selectivity of WAY-318068 versus a closely related monoamine transporter, SERT, the compound was tested in a functional 5-HT uptake bioassay. Evaluation of WAY-318068 revealed weak activity at the SERT (IC_{50} = 19.55 ± 0.13 μM), demonstrating a functional selectivity between the two monamine trnasporters of more than 2500-fold (Table 1). In addition, WAY-318068 was also evaluated for activity at a panel of 63 other receptors, ion channels, enzymes and transporters (see Table 2 for list; NovaScreen, Hanover, MD, USA). At a concentration of 10 μM, WAY-318068 was inactive (<35% inhibition of binding) at 60 of these targets with significant activity only at the NET (101%), the L-type calcium channel (65%) and site 2 of the sodium channel (85%), allowing us to estimate IC_{50} values of 8 μM for the calcium, and 6 μM for the sodium channels. Further in-house assays with the $Na_v1.2$ sodium channel provided an IC_{50} of 27 μM, confirming WAY-318068 is a highly selective compound. Importantly, there was no binding to the SERT (–0.55% inhibition at 10 μM, Table 2) confirming our assessment of low functional activity at the human SERT. Data from assays of NET and SERT function were also generated for desipramine, reboxetine, duloxetine, paroxetine and fluoxetine, providing selectivity ratios of 115, 105, 0.74, 0.05 and 0.02 respectively. WAY-318068, therefore, displays potent activity at the hNET with a very high degree of selectivity over the closely related SERT.

Table 1 Functional properties of WAY-318068 at NET and SERT

Compound	*NET IC_{50} (nM)*	*SERT IC_{50} (nM)*	*SERT/NET selectivity*
WAY-318068	6.83 ± 3.55	19 550 ± 127.28	2862
Desipramine	3.41 ± 1.58	393.00 ± 8.91	115
Reboxetine	3.24 ± 1.03	241.93 ± 39.05	75
Duloxetine	3.80 ± 0.00	2.80 ± 0.00	0.74
Paroxetine	57.20 ± 60.53	3.07 ± 3.67	0.05
Fluoxetine	381.10 ± 235.02	9.35 ± 2.71	0.02

Inhibition of noradrenaline uptake in MDCK-Net6 cells stably transfected with hNET, and inhibition of 5-HT uptake in JAR cells (the human choriocarcinoma cell line) which natively express hSERT. IC_{50} values represent the means ± SD of at least three independent determinations.

WAY-318068 is orally bioavailable and CNS penetrant

The pharmacokinetic profile of WAY-318068 (chemical structure shown in Figure 1A) was examined in rats (Figure 1). Mean WAY-318068 plasma concentrations were determined following a single 2 mg·kg^{-1} i.v. dose. Among the pharmacokinetic parameters calculated after i.v. administration using a non-compartmental model were the following: plasma half-life, 1.5 ± 0.3 h; clearance rate, 39 ± 6.3 mL·min^{-1}·kg^{-1}; volume of distribution, 4.2 ± 1.4 L·kg^{-1} (n = 3 rats). Plasma concentrations of WAY-318068 were also measured following a single p.o. dose of 3, 10 and 100 mg·kg^{-1} (Figure 1B,C). Working with a detection limit of 2 ng·mL^{-1}, plasma concentrations were measurable at all doses. Higher plasma levels and exposures occurred in a non-linear fashion with increasing dose. In addition, all doses resulted in sustained plasma levels up to 5 h with the highest dose resulting in substantial levels (>1000 ng·mL^{-1}) present 24 h post-dosing. Penetration into the CNS was determined 1, 3 and 5 h after a single 30 mg·kg^{-1} p.o. dose of WAY-318068. The resulting plasma-versus-brain exposures were as follows: plasma, 3522 h*ng·mL^{-1}; brain, 3809 h*ng·g^{-1} tissue. The mean brain-to-plasma ratio for WAY-318068 at this dose was calculated at 1.08. The percent unbound fraction in the brain (brain %Fu) is 0.95. In addition, the compound was detected in microdialysis samples collected from the mPFC between 90 and 180 min post-dosing (Figure 1D). These data indicate that substantial quantities of WAY-318068 are found in the CNS, and in particular in discrete areas such as the mPFC after oral dosing.

WAY-318068 selectively increases brain levels of noradrenaline in the CNS

WAY-318068 was administered to rats in order to examine its neurochemical effects (Figure 2) in the mPFC. Levels of noradrenaline and 5-HT were measured by microdialysis prior to and following a single administration of 30 mg·kg^{-1} WAY-318068. A stable baseline level for noradrenaline and 5-HT was determined for 90 min prior to dosing; WAY-318068 significantly elevated NE, but not 5-HT, from 90 to 240 min post-dosing (the latest time-point studied). The maximal increase in cortical noradrenaline was 533% above baseline.

WAY-318068 reduces acute pain and pain associated with inflammation

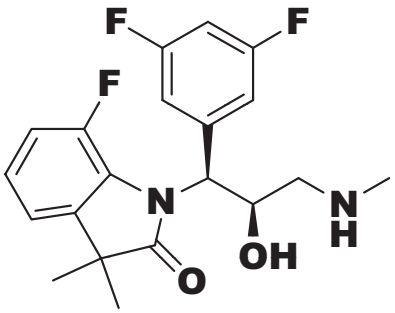
We tested the effects of WAY-318068 on acute nociception using the tail flick and hot plate assays. Oral administration

Table 2 List of pharmacological targets evaluated by NovaScreen

Target	*Ligand*	*Target*	*Ligand*
Adenosine receptor, non-selective	[^{3}H]5′-*N*-ethylcarboxamidoadenosine	Nicotinic, neuronal	[^{3}H]Epibatidine
Adrenoceptor, α_1	[^{3}H]7-Meoxy-prazosin	NET	[^{3}H]Nisoxetine
Adrenoceptor, α_2	[^{3}H]RX 821002	Opioid, non-selective	[^{3}H]Naloxone
Adrenoceptor, β non-selective	[^{3}H]DHA	Orphanin, ORL1	[^{3}H]Nociceptin
Dopamine transporter	[^{3}H]WIN 35,428	5-HT transporter	[^{3}H]Citalopram
Dopamine receptor, non-selective	[^{3}H]Spiperone	5-HT receptor non-selective	[^{3}H]*d*-Lysergic acid diethylamide
$GABA_A$, receptor agonist site	[^{3}H]GABA	σ, Non-selective	[^{3}H]Ditolylguanidine
$GABA_A$, BDZ, α_1	[^{3}H]CGP 54626A	Oestrogen	[^{125}I]3,17B-oestradiol, 16a
$GABA_B$ receptor	[^{3}H]Flunitrazepam	Testosterone	[^{3}H]Methyltrienolone
Glutamate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	[^{3}H]α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	Potassium channel, ATP-sensitive	[^{3}H]Gilbenclamide
		Potassium channel, Ca^{2+} activated	[^{125}I]Apamin
Glutamate, *N*-methyl-D-aspartate agonist site	[^{3}H]CGP 39653	Potassium channel, $[Kr\}^+$ (hERG)	[^{3}H]Astemizole
Glutamate, AMPA site	[^{3}H]AMPA	Sodium site 2	[^{3}H]Batrachotoxin A 20-α-benzo
Glutamate, kainate site	[^{3}H]Kainic acid	Calcium channel, type L	[^{3}H]Nitrendipine
Histamine receptor, H_1	[^{3}H]Pyrilamine	Calcium channel, type N	[^{125}I]Conotoxin GVIA
Histamine, H_2	[^{125}I]Aminopotentidine	Nitric oxide synthase	[^{3}H]*N*ω-nitro-L-arginine
Histamine, H_3	[^{3}H]*N*-a-MeHistamine	Leukotriene receptor, LTB_4 (BLT)	[^{3}H]LTB4
Melatonin receptor, non-selective	[^{125}I]2-Iodomelatonin	Leukotriene, LTD_4 (CysLT1)	[^{3}H]LTB4
Muscarinic receptor, non-selective, central	[^{3}H]Quinuclidinyl benzilate (QNB)	Thromboxane A_2 receptor	[^{3}H]SQ 29,548
Muscarinic, non-selective, peripheral	[^{3}H]QNB	Corticotropin-releasing factor, non-selective	[^{125}I]-Tyr0-CRF
Muscarinic receptor, M_1	[^{3}H]Scopolamine	Angiotensin II receptor, AT_1	[^{125}I]Sar1-Ile8) Angiotensin
Muscarinic receptor, M_2	[^{3}H]-*N*-methyl scopolamine	Angiotensin II, receptor AT_2	[^{125}I]Tyr4-Angiotensin II
Oxytocin receptor	[^{3}H]Oxytocin	Cholecystokinin, receptor CCK_1 (CCK_A)	[^{125}I]-CCK8
Platelet-activating factor (PAF)	Hexadecyl^{3}H]acetyl-PAF	Cholecystokinin, receptor CCK_2 (CCK_B)	[^{125}I]-CCK8
Thyrotropin-releasing hormone (TRH)	[^{3}H](3MeHis2)TRH	Endothelin, ET-B	[^{125}I]-Endothelin-1
Bradykinin receptor, BK_2	[3H]Bradykinin	Glutamic acid decarboxylase	[^{14}C]Glutamic Acid
Endothelin receptor, ET_A	[^{125}I]Endothelin-1	Ach esterase	Acetylthiocholine
Endothelin receptor, ET_B	[^{125}I]Endothelin-1	Monoamine oxidase-A	[^{14}C]5-Hydroxytryptamine
Galanin receptor, non-selective	[^{125}I]Galanin	Monoamine oxidase-B sd	[^{14}C]Phenylethylamine
Neurokinin receptor, NK1	[^{3}H](Substance P)		
Neurokinin receptor, NK2	[^{125}I]NKA		
Vasoactive intestinal peptide, receptor non-selective	[^{125}I]VIP		
Vasopressin receptor, V_1	[^{3}H]Phenyl 3,4,5-8-Arg-Vasopr		
Choline acetyl transferase	[^{14}C]Acetyl Coenzyme		

CGP 39653, (*RS*)-(*E*)-2-amino-4-phosphonomethyl-3-heptenoic acid; MDL-105,519, 3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1-*H*-indole-2-carboxylic acid; RX 821002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxan-2-yl)-4,| 5-dihydro-1*H*-imidazoline.

A



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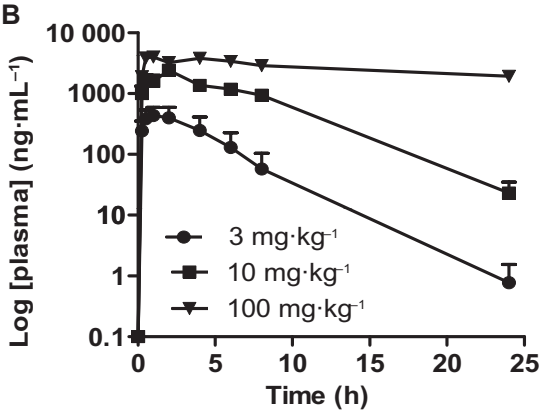


C

Route/dose/tissue (mg·kg^{-1})	*C*max (ng·mL^{-1})	*T*max (h)	$T_{1/2}$ (h)	AUC-inf (hr*ng·mL^{-1})
p.o. / 3 / plasma	449 ± 297	1.3	1.8	2368 ± 2537
p.o. / 10 / plasma	2419 ± 778	2.0	3.0	19 506 ± 5821
p.o. / 100 / plasma	4645 ± 883	1.8	17.0	105 799 ± 58 221

Inf; infinity

D

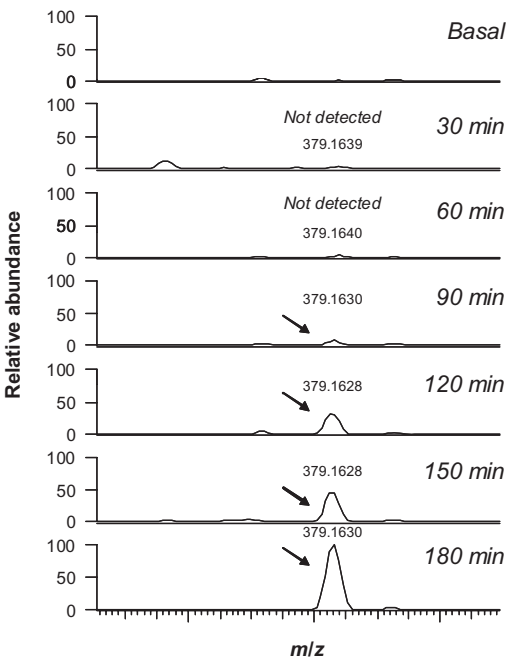
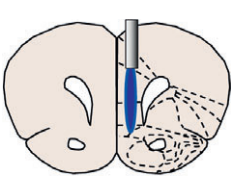


Figure 1 Chemical structure and pharmacokinetic profile of WAY-318068. (A) Chemical structure of WAY-318068. (B) WAY-318068 showed dose-dependent levels of plasma exposure following oral administration. (C) Summary table of pharmacokinetic characteristics for WAY-318068. (D) WAY-318068 was detected in the mPFC of rats using *in vivo* microdialysis/mass spectrometry.

A



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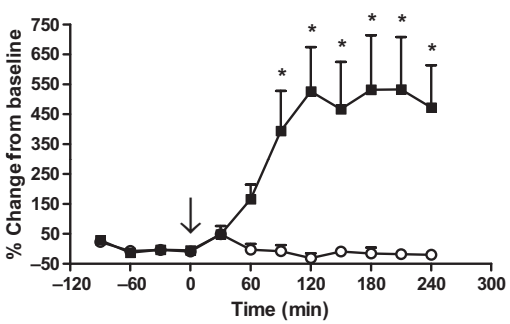

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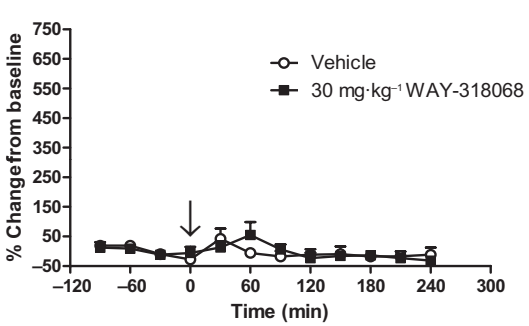

Figure 2 Measurements of monoamine neurotransmitters in the mPFC of rats using *in vivo* microdialysis. (A) Digram showing microdialysis probe placement in the mPFC. (B) Oral WAY-318068 increased levels of noradrenaline, but not 5-HT levels (C) Arrow indicates time of WAY-318068 administration. *$P < 0.05$ versus vehicle.

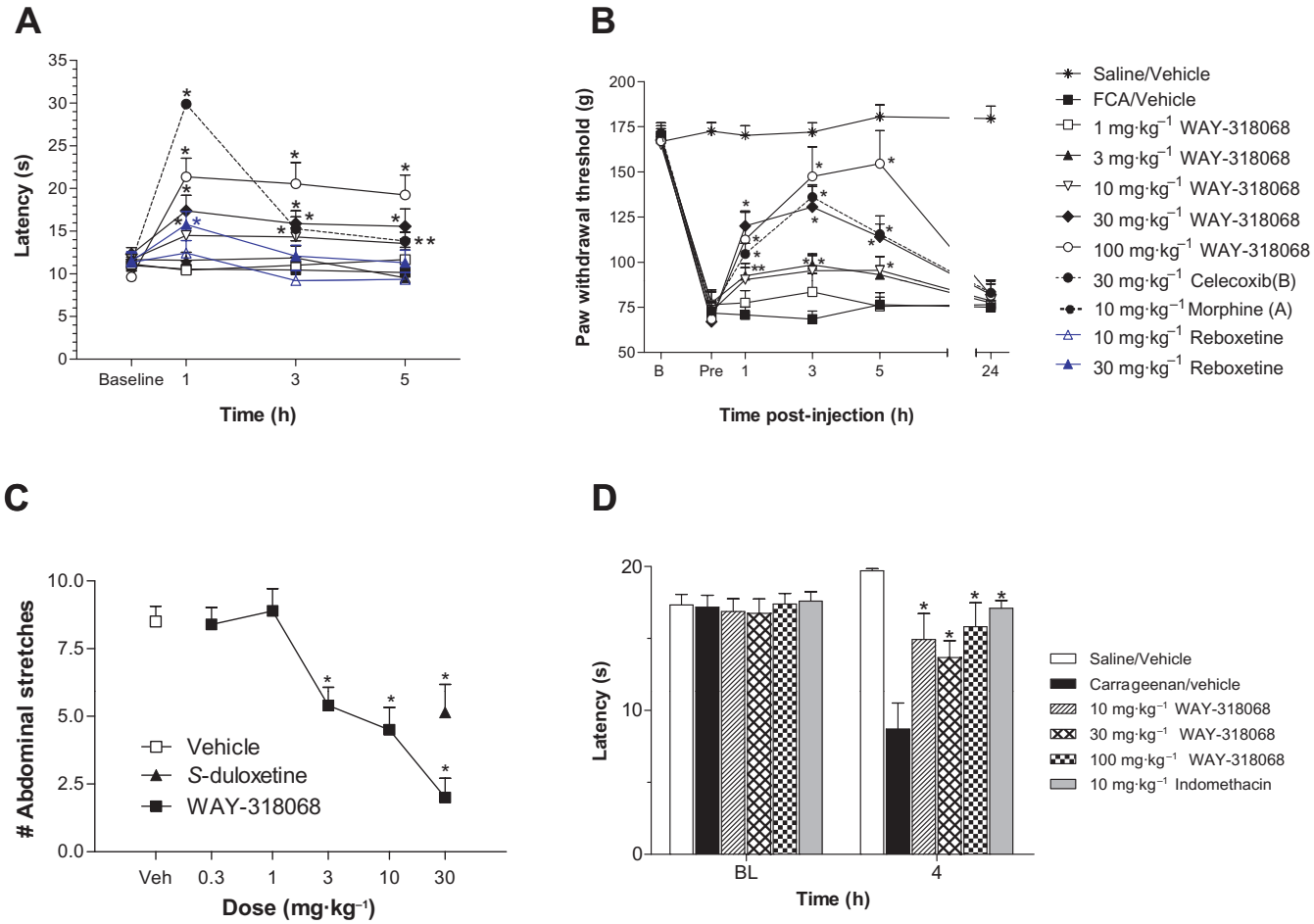

Figure 3 Evaluation of WAY-318068 in acute and inflammatory pain models. (A) WAY-318068 and reboxetine produced dose-dependent analgesia in the hot plate assay (n = 10–20 per group). (B) WAY-318068 dose-dependently reversed FCA-induced mechanical hyperalgesia (n = 10–20 per group). (C) WAY-318068 blocked PPQ-induced visceral pain (n = 10 per group) and (D) reversed carrageenan-induced sensitivity (BL, baseline) to radiant heat (n = 10 per group). *P < 0.05 versus vehicle.

did not affect tail flick latency at 1, 3 or 5 h following administration of doses up to 100 mg·kg^{-1} (data not shown). In contrast, WAY-318068 produced a significant main effect on hot plate latencies ($F_{4,45}$ = 7.08 P = 0.0002) that was dose dependent in nature and significant at 10, 30 and 100 mg·kg^{-1} 1, 3 and 5 h post-administration (Figure 3A). Similarly, reboxetine did not affect tail flick latencies while significantly elevating hot plate latencies at 30 mg·kg^{-1} 1 h post-dosing with a magnitude of effect less than that achieved by the same dose of WAY-318068 (Figure 3A). In contrast, the positive control morphine (10 mg·kg^{-1}, s.c.) significantly increased tail flick latencies 1 and 3 h post-administration, and hot plate latencies 1, 3 and 5 h post-administration. Intraplantar injection of 50 μL FCA into the hind paw of rats resulted in development of mechanical hyperalgesia as indicated by a decreased PWT to a noxious mechanical stimulus (Figure 3B). Oral administration of WAY-318068 produced a dose-dependent reduction in mechanical hyperalgesia ($F_{7,112}$ = 34.26 P < 0.0001) post-administration (Figure 3B). At the 1 and 3 h time-points, statistically significant increases in PWT were seen following the 3, 10, 30 and 100 mg·kg^{-1} doses (Figure 3B). At the 5 h time-point, statistically significant increases in PWT were seen at the 10, 30 and 100 mg·kg^{-1} doses (Figure 3B). The maximum percent reversal (89 ± 19%) was achieved 3 h following the 30 mg·kg^{-1} dose. Oral administration of celecoxib (30 mg·kg^{-1}) also produced a statistically significant reversal of hyperalgesia 1, 3 and 5 h post-administration (Figure 3B).

Intraperitoneal injection of PPQ resulted in the development of visceral pain as indicated by the occurrence of abdominal stretches that were dose-dependently blocked by WAY-318068 (Figure 3C, $F_{6,75}$ = 11.09, P < 0.0001). Further *post hoc* analysis revealed a significant reduction after 3, 10 and 30 mg·kg^{-1} as compared to vehicle-treated controls (P < 0.01). The greatest blockade (77 ± 8%) was observed at 30 mg·kg^{-1}. Intraplantar injection of carrageenan resulted in the development of thermal hyperalgesia within 4 h that was reduced by WAY-318068 (Figure 3D, $F_{5,123}$ = 7.04, P < 0.0001). Further *post hoc* analysis revealed a significant reduction after all three doses as compared to vehicle-treated controls (P < 0.01). The maximum reversal (84 ± 20%) was observed at 100 mg·kg^{-1}. These results demonstrate that WAY-318068 is an effective analgesic in reducing pain due to chemical and inflammatory insult.

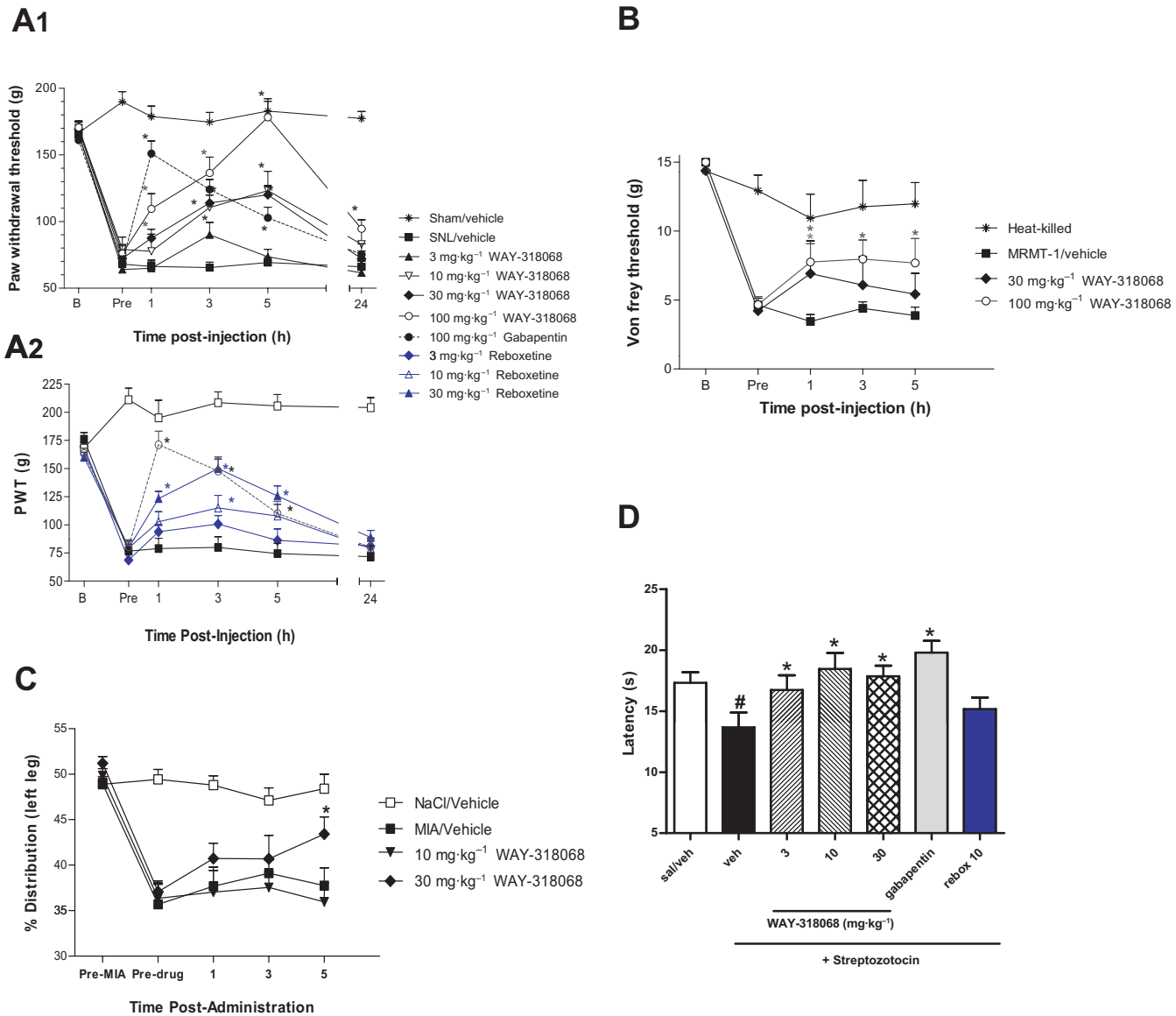

Figure 4 Evaluation of WAY-318068 in neuropathic, osteoarthritis and bone cancer pain models. (A) WAY-318068 and reboxetine produced dose-dependent reversal of mechanical hyperalgesia induced by SNL (n = 10–20 per group), and (B) reversed tactile allodynia induced by intratibial injection of MRMT-1 cells (n = 10 per group). *P < 0.05 versus SNL/vehicle or MRMT-1/vehicle. (C) WAY-318068 partially normalized weight bearing in a model of osteoarthritis (n = 10 per group). *P < 0.05 versus pre-MIA; #P < 0.05 versus pre-WAY-318068. (D) WAY-318068 reverses hyperalgesia in a model of STZ-induced diabetic neuropathy 3 h post-dosing (n = 10 per group). Rebox; reboxetine. #P < 0.05 versus saline/vehicle; *P < 0.05 versus STZ/vehicle.

WAY-318068 reduces pain associated with neuropathy, bone cancer and osteoarthritis

Ligation of the L5 spinal nerve resulted in the development of mechanical hyperalgesia within 3 weeks of surgery. Oral administration of WAY-318068 3 weeks after surgery produced a dose-dependent reduction in mechanical hyperalgesia ($F_{7,116}$ = 35.18, P < 0.0001 (Figure 4A1). At the 1 h time-point, statistically significant increases in PWT were seen only at the 30 and 100 mg·kg^{-1} doses, while at the 3 h time-point, statistically significant increases in PWT were seen at 3, 10, 30 and 100 mg·kg^{-1} (Figure 4A1). At the 5 h time-point, statistically significant increases in PWT were seen at the 10, 30 and 100 mg·kg^{-1} doses. Twenty-four hours post-administration, a statistically significant increase in PWT was observed for only the 100 mg·kg^{-1} dose in line with the sustained plasma levels at this dose. The maximum reversal (118 ± 22%) was achieved 5 h following the 100 mg·kg^{-1} dose. Similarly, reboxetine dose-dependently reduced mechanical hyperalgesia with statistically significant reversals 3 h after 10 mg·kg^{-1}, and 1, 3 and 5 h following 30 mg·kg^{-1} with a magnitude of effect comparable to that achieved with WAY-318068 (Figure 4A2). Intraperitoneal administration of gabapentin (100 mg·kg^{-1}) also produced a statistically significant reversal of hyperalgesia 1, 3 and 5 h post-administration (Figure 4A1,A2). In the bone cancer pain model, intratibial injection of MRMT-1 cells resulted in the development of tactile allodynia within 3 weeks that was reversed by oral administration of WAY-318068 ($F_{3,30}$ = 18.60, P < 0.0001 (Figure 4B). At the 1 h

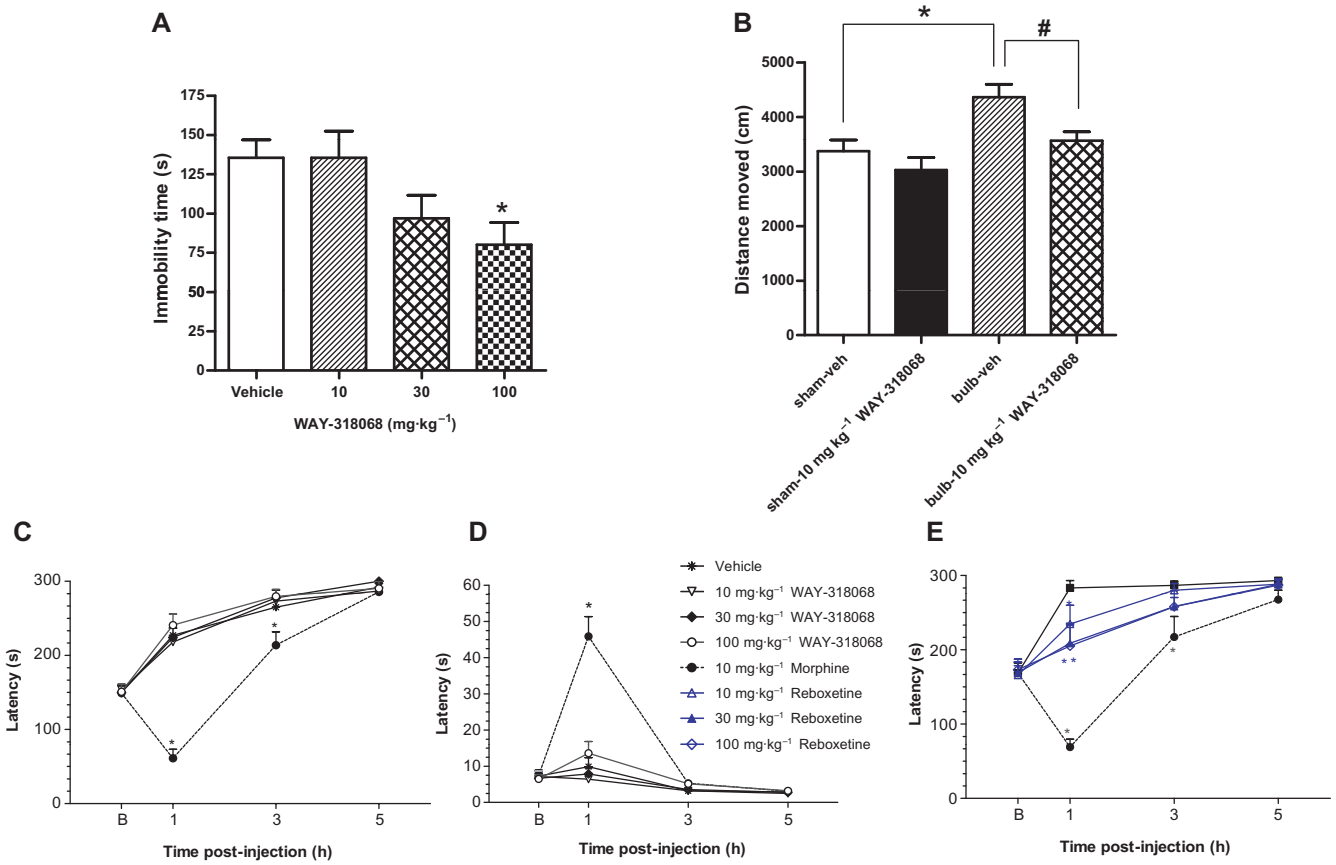

Figure 5 Evaluation of WAY-318068 in models of depression and motor coordination. WAY-318068 produced antidepressant-like effects in the (A) tail suspension and (B) olfactory bulbectomy assays. WAY-318068 did not produce deficits in the (C) rotarod or (D) beam walking assays (n = 10 per group). *P < 0.05 versus vehicle.

time-point, statistically significant increases in PWT were seen at both the 30 and 100 mg·kg^{-1} doses, while at the 3 and 5 h time-points, statistically significant increases in PWT were seen only at 100 mg·kg^{-1} (Figure 4B). The maximum reversal (38 ± 18%) was achieved 3 h following the 100 mg·kg^{-1} dose. In the MIA model of osteoarthritis pain, intra-articular injection of MIA resulted in a reduction of weight borne on the injured limb within 3 weeks that was partially reversed by oral administration of WAY-318068 ($F_{3,35}$ = 14.41, P < 0.0001 (Figure 4C). At the 5 h time-point, a statistically significant increase in weight bearing was seen at the 30 mg·kg^{-1} dose as compared to vehicle (Figure 4C). In the STZ model of diabetic neuropathic pain, intraperitoneal injection of STZ resulted in the development of hypersensitivity within 6 weeks as measured by a decreased latency to nocifensive response to thermal stimulation that was reversed by oral administration of WAY-318068 ($F_{5,48}$ = 3.443, P < 0.0076 (Figure 4D). At the 1 h time-point, a statistically significant increase in latency was seen only at the 30 mg·kg^{-1} dose (data not shown), while at the 3 h time-point, a statistically significant increase in latency was seen at 3, 10 and 30 mg·kg^{-1} (Figure 4D). Oral administration of gabapentin (300 mg·kg^{-1}) also produced a statistically significant reversal 1 and 3 h post-dose, while reboxetine (10 mg·kg^{-1}) did not (Figure 4D). These results demonstrate that WAY-318068 is an effective analgesic in reducing nerve injury and diabetic neuropathic pain, as well as bone cancer- and osteoarthritis-related pain.

WAY-318068 displays antidepressant-like effects

WAY-318068 produced a dose-dependent antidepressant-like effect in the mouse TST (Figure 5A, $F_{3,44}$ = 3.661, P < 0.05). Further *post hoc* analysis revealed a significant decrease (41%) in immobility time at 100 mg·kg^{-1} compared to vehicle-treated controls (P < 0.01). Additionally, chronic treatment (14 days) with WAY-318068 produced a complete reversal of olfactory bulbectomy-induced hyperactivity (Figure 5B, $F_{3,31}$ = 7.431, P < 0.001). These results are indicative of a putative antidepressant activity of WAY-318068.

WAY-318068 does not produce motor deficits

A common side effect of CNS active compounds is ataxia, which can confound the interpretation of behavioural assays. We tested rats for motor function using the accelerating rotarod and beam walk assays (Figure 5C–E). Oral administration of WAY-318068 did not affect rotarod performance or latency to cross the beam 1, 3 or 5 h following administration of doses up to 100 mg·kg^{-1} (Figure 5C,D). In contrast, oral administration of reboxetine produced a statistically

significant reduction in latency to fall at all doses tested 1 h post-dosing (Figure 5E). The positive control, morphine (10 mg·kg^{-1}, s.c.), produced a significant decrease in both rotarod performance and beam walking 1 h post-dose (Figure 5C–E), and further deficits in rotarod performance 3 h post-administration (Figure 5C,E).

Discussion and conclusions

Both 5-HT and noradrenaline may contribute to antidepressant and anti-nociceptive activity. Clinically, both conditions are co-morbid and may involve substantial interplay. In general, for analgesic activity, 5-HT is thought to modulate spinal pathways, while noradrenaline activates supraspinal descending inhibitory pathways (Millan, 2002; Ren and Dubner, 2002), although it has been suggested that chronic pain may, in part, result from reduced levels of noradrenaline and 5-HT activity at both spinal and supraspinal levels (Ren and Dubner, 2002). Consequently, it is presumed that noradrenaline and 5-HT re-uptake inhibitors attenuate pain by preventing pre-synaptic re-uptake of noradrenaline and 5-HT, leading to increased post-synaptic levels, potentially in the LC, RVM and in the spinal cord, and subsequent sustained activation of descending inhibitory pathways (Burgess *et al.*, 2002). Furthermore, the actions of 5-HT and noradrenaline in these pathways may produce synergistic effects (Zhuo and Gebhart, 1991). To examine if elevation of noradrenaline alone was sufficient for antidepressant and analgesic activity, the present series of experiments evaluated the novel NRI, WAY-318068 (McComas *et al.*, 2008; Zhang *et al.*, 2009a), a potent and efficacious inhibitor of NET without significant binding to the dopamine transporter. WAY-318068 has only weak activity at SERT, resulting in a 2500-fold functional selectivity for NET. The compound is orally bioavailable with ready access to the CNS, and causes a robust and significant increase in rat cortical noradrenaline levels without effect on 5-HT levels. WAY-318068 is efficacious in models of acute, visceral, inflammatory, osteoarthritic, neuropathic, diabetic and bone cancer pain, as well as in models of depression. Importantly, effects were observed firstly at doses that did not cause motor disruption, which can confound interpretation of efficacy from behavioural assays and secondly, at times consistent with the pharmacokinetic profile of this compound. As such, these data demonstrate that increased noradrenaline in the absence of effects on 5-HT is sufficient for antidepressant and analgesic activity. This pre-clinical profile is comparable to other potent and selective compounds from this class and series that have been profiled, albeit to a lesser extent, in pre-clinical pain models (Vu *et al.*, 2009; Zhang *et al.*, 2009b). The minimum effective dose and degree of reversal achieved with WAY-318068 differed across the *in vivo* models employed. This is not surprising given that noradrenaline is unlikely to contribute equally to different pain and depression states, and that some pain states, such as bone-related pain, are known to be more intractable. We did rule out the possibility that differences in plasma exposure between species could contribute (comparable plasma exposures in rats vs. mice; data not shown). It would therefore be interesting to correlate local effects of WAY-318068 in distinct brain regions with efficacy; this could be achieved using ultra-slow-flow microdialysis or receptor occupancy studies coupled with low doses of WAY-318068.

We compared the *in vitro* NET selectivity of WAY-318068 to clinically approved re-uptake inhibitors previously classified as NRIs (desipramine, reboxetine), SNRI (duloxetine) and SSRIs (paroxetine and fluoxetine). In contrast to WAY-318068, these inhibitors have a selectivity for NET over SERT ranging from 115- to 0.02-fold (Table 1), in line with previously reported values (Beique *et al.*, 1998; Hajos *et al.*, 2004; Chen and Skolnick, 2007; Leventhal *et al.*, 2007). However, at effective doses *in vivo*, these compounds may affect 5-HT receptors. In contrast, the *S*-enantiomer of reboxetine (*S*,*S*-reboxetine) has been described as having substantially higher selectivity for NET over SERT in binding assays compared to racemic reboxetine (Hajos *et al.*, 2004). However, the *in vivo* activity of this enantiomer in pre-clinical models of depression and pain has not yet been described. Our demonstration of WAY-318068 efficacy in the TST and olfactory bulbectomy models of depression agrees with previous reports of SNRI effects in these models (Hajos *et al.*, 2004; Bymaster *et al.*, 2005). In contrast, literature reports have been mixed on the efficacy of NRI and 5-HT re-uptake inhibitors in pain models. Our findings with WAY-318068 demonstrate that NRI activity alone is sufficient to produce acute analgesia. These findings are in line with studies demonstrating that compounds that inhibit the NET are also active in models of acute pain; specifically, NRIs and SNRIs have been shown to increase hot plate latencies, while SSRIs do not (Bomholt *et al.*, 2005; Jones *et al.*, 2005). The discrepancy between the observed activity in the hot plate and tail flick assays is interesting, but not unexpected as the analgesic efficacy of NRIs is thought to be mediated by activation of supraspinal descending inhibitory pathways (Millan, 2002; Ren and Dubner, 2002). Traditionally, licking or jumping responses in the hot plate assay are considered to be the result of supraspinal sensory integration, while the tail flick response is thought to be a spinally mediated reflex which may be less relevant by the NRI mechanism (Caggiula *et al.*, 1995; Rubinstein *et al.*, 1996). The pattern of robust activity in the hot plate assay without activity in the tail flick assay is present for many compounds of this class (Whiteside and Leventhal, unpubl. obs.). In addition, the mechanism of action underlying the analgesia may be due to activation of the endogenous opioid system; tricyclic antidepressants and imipramine caused a naloxone-sensitive increase in acute pain thresholds (Isenberg and Cicero, 1984; Michael-Titusa and Costentin, 1987). Our laboratory has previously demonstrated that desipramine, reboxetine, fluoxetine and paroxetine were all active in the PPQ model of visceral pain, with a greater magnitude of effect following treatment with selective NRIs (Leventhal *et al.*, 2007). Overall, these data in both acute and visceral pain models suggest that analgesia correlates well with NRI activity.

WAY-318068 was also found to be highly effective in models of inflammatory pain. Others have reported that the NRIs, desipramine and thionisoxetine, as well as the SNRIs, duloxetine and venlafaxine, reversed carrageenan-induced thermal hyperalgesia, while the SSRIs paroxetine, sertraline and fluoxetine were inactive (Jones *et al.*, 2005). Limited published data exist evaluating re-uptake inhibitors in models of chronic

inflammatory pain; as such, our studies, including data from the FCA model, support the role of the noradrenaline system in both acute and chronic inflammatory pain.

WAY-318068 reversed mechanical and thermal hyperalgesia in nerve injury and diabetic models of neuropathy, respectively, while reboxetine reversed only mechanical hyperalgesia in the nerve injury model. Our laboratory and others have previously reported that the NRIs, reboxetine and desipramine (Leventhal *et al.*, 2007), reversed allodynia in the SNL model; however, mechanistic conclusions from desipramine are compromised due to activity at molecular targets other than the NET (Sanchez and Hyttel, 1999). In contrast, discrepant findings have been reported with the SSRIs, paroxetine and fluoxetine, such that paroxetine reversed allodynia, but fluoxetine did not (Leventhal *et al.*, 2007); this may be due to the fact that paroxetine elevates noradrenaline levels *in vivo* (Owens *et al.*, 2000; Gilmor *et al.*, 2002). It has also been reported that fluoxetine modestly reverses tactile allodynia, while reboxetine is inactive in this assay but is active against thermal hyperalgesia (Pedersen *et al.*, 2005). The SNRIs, duloxetine, venlafaxine and milnacipran, all reverse SNL-induced tactile allodynia. In addition to small molecules, the conopeptide Xen2174, a highly selective NRI, reversed tactile allodynia following intrathecal administration to neuropathic rats (Nielsen *et al.*, 2005). Overall, these data suggest that more selective NRIs, as well as SNRIs, are more effective than SSRIs in nerve injury-related pain. In contrast to our results showing alleviation of thermal pain by WAY-318068 in the STZ diabetic neuropathic model, the SSRI fluoxetine (Anjaneyulu and Chopra, 2004) reversed thermal hyperalgesia in diabetic mice, while intrathecal administration of paroxetine and fluvoxamine reversed tactile allodynia in a comparable model (Ikeda *et al.*, 2009). In addition, intrathecal administration of the SNRI milnacipran reversed tactile allodynia in diabetic mice (Ikeda *et al.*, 2009). Overall, these data suggest that inhibition of the re-uptake of either noradrenaline or 5-HT activity is sufficient to alleviate painful diabetic neuropathy in rodents. It is not clear why this outcome differs from that in nerve injury-related pain, but may relate to a different extent of nerve damage between these respective models.

The potential of NRIs to treat bone-related pain has not been well studied. The present data with WAY-318068 suggest that selective NRIs may ameliorate the pain associated with both bone cancer and osteoarthritis. WAY-318068 modestly reversed tactile allodynia in the bone cancer model at the highest dose tested. Similar to the present findings, it has been previously reported that the NRI, desipramine, and the SNRI, amitriptyline, decrease spontaneous pain behaviour in a murine bone cancer model, while the SSRI, fluoxetine, has limited efficacy (El Mouedden and Meert, 2007); however, none of the compounds reversed mechanical hypersensitivity or affected limb-use impairment. In contrast to our findings with WAY-318068, amitriptyline and desipramine were only effective in these models at doses that also caused sedation. It is noteworthy that WAY-318068 did not cause motor deficits up to 100 $mg \cdot kg^{-1}$ in contrast to reboxetine which caused small, but significant rotarod effects at 10 $mg \cdot kg^{-1}$ and higher doses, indicating that WAY-318068 has a larger pre-clinical therapeutic index (separation between efficacy and side effects) as compared to comparator compounds in these models. Furthermore, given that bone cancer is difficult to treat clinically and refractory to most classes of analgesic compounds, our data showing efficacy of WAY-318068 are promising. Indeed, clinical data suggest that antidepressants may be beneficial in treating cancer pain (Onghena and Vanhoudenhove, 1992). In the MIA model of osteoarthritis, WAY-318068 partially reversed weight-bearing deficits. Similarly, the SNRI, duloxetine, was reported to moderately reverse deficits in grip force in a comparable model (Chandran *et al.*, 2009). These data are supported by clinical findings demonstrating positive outcomes with duloxetine in osteoarthritis patients (Chappell *et al.*, 2009), and suggest a pre-clinical to clinical translation.

In conclusion, we have presented the *in vitro* pharmacological and *in vivo* pharmacokinetic and pharmacological properties of the novel, potent, orally available and CNS-penetrant NET inhibitor, WAY-318068. These properties make WAY-318068 a particularly well-suited tool that can be used to study the role of noradrenaline in physiological and pathological processes. In addition to robust *in vivo* increases in noradrenaline levels and efficacy in models of depression, WAY-318068 was found effective in a wide range of pain models, suggesting NRIs can serve as broad spectrum analgesics.

Acknowledgement

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Conflict of interest

The authors state no conflict of interest.

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