A rodent model of HIV protease inhibitor indinavir induced peripheral neuropathy

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Abstract:
HIV-associated sensory neuropathy (HIV-SN) is the most frequent manifestation of HIV disease. It often presents with significant neuropathic pain and is associated with previous exposure to neurotoxic nucleoside reverse transcriptase inhibitors. However, HIV-SN prevalence remains high even in resource-rich settings where these drugs are no longer used. Previous evidence suggests that exposure to indinavir, a protease inhibitor commonly used in antiretroviral therapy, may link to elevated HIV-SN risk. Here we investigated whether indinavir treatment was associated with the development of a "dying back" axonal neuropathy and changes in pain-relevant limb withdrawal and thigmotactic behaviours. Following two intravenous injections of indinavir (50 mg/kg, 4 days apart), adult rats developed hindpaw mechanical hypersensitivity, which peaked around 2 weeks post first injection (44% reduction from baseline). At this time, animals also had 1) significantly changed thigmotactic behaviour (62% reduction in central zone entries) comparing to the controls and 2) a significant reduction (45%) in hindpaw intraepidermal nerve fibre density. Treatment with gabapentin, but not amitriptyline, was associated with a complete attenuation of hindpaw mechanical hypersensitivity observed with indinavir treatment. Furthermore, we found a small but significant increase in microglia with the effector morphology in the lumbar spinal dorsal horn in indinavir-treated animals, coupled with significantly increased expression of phospho-p38 in microglia. In summary, we have reported neuropathic pain-related sensory and behavioural changes accompanied by a significant loss of hindpaw skin sensory innervation in a rat model of indinavir-induced peripheral neuropathy that is suitable for further pathophysiological investigation and preclinical evaluation of novel analgesics.
5th September 2016

Prof Francis J. Keefe,
Editor-in-Chief, Pain
111 Queen Anne Ave N.,
Suite 510, Seattle,
WA 98109-4955, USA

Re: PAIN-D-14-13965R2

Dear Prof Keefe,

We are submitting our revised manuscript characterizing a rodent model of HIV protease inhibitor indinavir induced peripheral neuropathy.

We are pleased to hear that you, the Section Editor, and the reviewer #1 have considered that our manuscript is suitable for publication in PAIN pending minor revisions. We have addressed the reviewer’s minor concerns (highlighted in red color in the manuscript) as follows:

1. Concern 1: “The authors should indicate the concentrations of each antibodies they used.”
   **Response:** We have included antibody concentrations in the result section as suggested by the reviewer (page 8, second paragraph; page 9, second paragraph).

2. Concern 2: “There are also some spelling mistakes that need to be fix. For example, in the abstract: phospho-p38 instead of "phopspho-p38", p12 line 3-4 cold hypersensiitivity instead of mechanical hypersensitivity.”
   **Response:** We have corrected the spelling mistakes (page 2, line 16; page 12, line 3).

We feel that the above changes have addressed the minor concerns of the reviewer. We thank you for considering our submission for publication in Pain.

Yours Sincerely,

Dr. Wenlong Huang
Abstract

HIV-associated sensory neuropathy (HIV-SN) is the most frequent manifestation of HIV disease. It often presents with significant neuropathic pain and is associated with previous exposure to neurotoxic nucleoside reverse transcriptase inhibitors. However, HIV-SN prevalence remains high even in resource-rich settings where these drugs are no longer used. Previous evidence suggests that exposure to indinavir, a protease inhibitor commonly used in antiretroviral therapy, may link to elevated HIV-SN risk. Here we investigated whether indinavir treatment was associated with the development of a “dying back” axonal neuropathy and changes in pain-relevant limb withdrawal and thigmotactic behaviours. Following two intravenous injections of indinavir (50 mg/kg, 4 days apart), adult rats developed hindpaw mechanical hypersensitivity, which peaked around 2 weeks post first injection (44% reduction from baseline). At this time, animals also had 1) significantly changed thigmotactic behaviour (62% reduction in central zone entries) comparing to the controls and 2) a significant reduction (45%) in hindpaw intraepidermal nerve fibre density. Treatment with gabapentin, but not amitriptyline, was associated with a complete attenuation of hindpaw mechanical hypersensitivity observed with indinavir treatment. Furthermore, we found a small but significant increase in microglia with the effector morphology in the lumbar spinal dorsal horn in indinavir-treated animals, coupled with significantly increased expression of phospho-p38 in microglia. In summary, we have reported neuropathic pain-related sensory and behavioural changes accompanied by a significant loss of hindpaw skin sensory innervation in a rat model of indinavir-induced peripheral neuropathy that is suitable for further pathophysiological investigation and preclinical evaluation of novel analgesics.
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A rodent model of HIV protease inhibitor indinavir induced peripheral neuropathy

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Abstract

HIV-associated sensory neuropathy (HIV-SN) is the most frequent manifestation of HIV disease. It often presents with significant neuropathic pain and is associated with previous exposure to neurotoxic nucleoside reverse transcriptase inhibitors. However, HIV-SN prevalence remains high even in resource-rich settings where these drugs are no longer used. Previous evidence suggests that exposure to indinavir, a protease inhibitor commonly used in antiretroviral therapy, may link to elevated HIV-SN risk. Here we investigated whether indinavir treatment was associated with the development of a “dying back” axonal neuropathy and changes in pain-relevant limb withdrawal and thigmotactic behaviours. Following two intravenous injections of indinavir (50 mg/kg, 4 days apart), adult rats developed hindpaw mechanical hypersensitivity, which peaked around 2 weeks post first injection (44% reduction from baseline). At this time, animals also had 1) significantly changed thigmotactic behaviour (62% reduction in central zone entries) comparing to the controls and 2) a significant reduction (45%) in hindpaw intraepidermal nerve fibre density. Treatment with gabapentin, but not amitriptyline, was associated with a complete attenuation of hindpaw mechanical hypersensitivity observed with indinavir treatment. Furthermore, we found a small but significant increase in microglia with the effector morphology in the lumbar spinal dorsal horn in indinavir-treated animals, coupled with significantly increased expression of phosho-p38 in microglia. In summary, we have reported neuropathic pain-related sensory and behavioural changes accompanied by a significant loss of hindpaw skin sensory innervation in a rat model of indinavir-induced peripheral neuropathy that is suitable for further pathophysiological investigation and preclinical evaluation of novel analgesics.

(Word count = 250)

Key words: HIV; peripheral; neuropathy; neuropathic pain; rat; indinavir; thigmotaxis
Introduction

HIV-associated sensory neuropathy (HIV-SN) is the most frequent neurological manifestation of HIV disease and is seen in 40-50% of patients whose HIV disease is otherwise well controlled by antiretroviral therapy (ART) [22; 24; 47]. HIV-SN is a distal symmetrical, predominantly sensory, polyneuropathy. The symptoms of HIV-SN present with a characteristic gloves and socks distribution and it is associated with significant neuropathic pain [1; 12; 31; 45; 48]. HIV-SN has been hitherto thought to result from two clinically indistinguishable neuropathies with distinct pathogenesis: a distal axonal degeneration caused by interaction of sensory neurones with HIV proteins e.g. HIV glycoprotein gp120 [3; 16; 23; 32; 38; 47; 49] and ART-induced toxic neuropathy associated with nucleoside reverse transcriptase inhibitors (NRTIs) [22; 24; 50].

Since NRTI introduction, the morbidity and mortality of HIV infection have been markedly reduced [22]. Whilst certain d-NRTIs such as zalcitabine (ddC) and stavudine (d4T) [18; 50] are undoubtedly neurotoxic, the prevalence of HIV-SN in resource-rich settings did not decline in patients who have never been exposed to these drugs [9; 22], suggesting that alternative or additional factors may underlie HIV-SN in the clinical setting. Protease inhibitors are regularly used a part of combinational ART. A number of studies have linked exposure to protease inhibitor medication to HIV-SN risk [8; 29; 39; 46], including a demonstration of indinavir potentiating the neurotoxicity of HIV in a transgenic rat model using cultured dorsal root ganglia (DRG) [39][52]. Thus, HIV-infected DRG cultures exposed to indinavir showed significant neuronal atrophy, neurite retraction, and process loss, compared with controls. However, this association between protease inhibitors and HIV-SN also remains far from clear. A review of adults initiating combinational ART in AIDS Clinical Trials Group (ACTG) studies found HIV-SN risk was only increased by protease inhibitor use if the patient was also using at least one neurotoxic d-NRTI [13]. Further analysis of patients involved in the US-based CHARTER (CNS HIV Anti-Retroviral Treatment

Rat models have been used to understand the pathogenesis of HIV-SN and to develop novel therapeutics for HIV-SN [17; 19-21; 23; 49-52]. Here we hypothesised that systemic indinavir treatment in rats would produce signs of peripheral neuropathy and neuropathic pain-like behaviours. Initially, we validated the approach in behavioural studies, which showed that indinavir-treated rats developed hindpaw mechanical and cold, but not heat, hypersensitivity and pain-related aberrations in complex, ethologically relevant thigmotactic behaviour [17; 49; 50]. We then elucidated the clinical diagnostic feature of a length dependent “dying back” small fibre axonal neuropathy by demonstrating loss of epidermal innervation following indinavir treatment.

(Word count = 435)
Materials and Methods

**Ethical statement**

Animal experiments were conducted in accordance with United Kingdom law (Animal Scientific Procedures Act 1986; Project License PPL70/7162) and IASP guidelines [54]. The ARRIVE reporting guidelines were followed [25]. An ARRIVE checklist is provided in the supplementary materials.

**Experimental animals**

Temperature-controlled standard rat IVC cages (21°C, 2-3 per cage) with corncob bedding were used for housing the animals (male adult Wistar rats; 200–300 g; Charles River, UK). We did not use environment enrichment. Rats were kept on a 12:12 h light–dark cycle. Normal rat chow (RM1 pellets; Special Diet Services, Essex, UK) and tap water *ad libitum* were provided. Animals were allowed to acclimatise for 48 h following delivery.

**Study design**

In order to reduce experimental bias, we followed major domains of Good Laboratory Practice [30; 43] (Supplementary Table 1). Behavioural experiments were carried out in the light phase in the behavioural laboratory, and intravenous (i.v.) and intraperitoneal (i.p.) injection procedures were conducted in the surgical laboratory, all at Imperial College London (Chelsea & Westminster Campus). We used batches of subset experiments (normally 2-3 animals per group) for thigmotaxis. Sequences of A-B-C then C-B-A (letters assigned to mask the cage labels during testing) were used to select animals.
Indinavir administration

Under general anaesthesia [1-2% isoflurane (Abbott, UK) in O₂ and N₂O ratio 1:1], indinavir (0.5 ml; 50 mg/kg in sterile saline; donated by Pfizer Ltd.) was administered via a tail vein. Four days later, a second injection of indinavir was carried out at the same dose and volume. Control animals were given sterile saline at equivalent volumes. Previous animal data with other antiretroviral drugs have shown that oral gavage and i.v. routes result in comparable nocifensive behavioural profiles [19]. Therefore, we decided to use the i.v. route, which would minimise handling stress caused by oral gavage. The dose and treatment regime were chosen based on previous studies with ddC and d4T [17; 19; 50].

Hindpaw mechanical hypersensitivity

The procedure to assess hindpaw withdrawal to mechanical stimuli was the same as in our previous study [50]. An electronic “von Frey” device (0.5 mm² probe tip area; Somedic Sales AB, Sweden) was used to measure the withdrawal threshold in response to punctate static mechanical stimulation. We carried out 2 habituation sessions (40-50 min each) and then 2 baseline tests. Animals were placed in plexiglass boxes (23 x 18 x 14 cm) with 0.8 cm diameter mesh flooring for acclimatisation. When exploratory behaviour ceased, the probe was used to deliver an increasing force (rate of 8-15 g/s) and was applied to the mid-plantar until the animal actively withdrew the paw. This was repeated 4 times at 1 min interval between each application.

Hindpaw cold hypersensitivity

Cold hypersensitivity was assessed using the acetone drop method [5]. Animals were placed in plexiglass boxes (23 x 18 x 14 cm) with 0.8 cm diameter mesh flooring and allowed to acclimatise for 15 min or until exploratory behaviour ceased. The cooling stimulus was a single bubble of acetone applied to the mid plantar surface of each hindpaw delivered from the tip of a 1 ml syringe. A positive response was recorded when the rat withdrew its paw following the acetone application.
For each measurement, five acetone drop applications were delivered and a mean limb withdrawal rate calculated. At least 3 min were allowed to elapse between each test.

**Hindpaw response to noxious heat**

Hypersensitivity to noxious heat was assessed by measuring the limb withdrawal time following application of an infrared heat stimulus (Plantar test, Ugo Basile, Italy, Hargreaves et al., 1988). Briefly, animals were placed in a clear plexiglass box (23 x 18 x 14 cm) with a dry glass floor and allowed to acclimatise for 15 min or until exploratory behaviour ceased. A focused infrared beam (46°C, wavelength 50 nm) was delivered to the plantar surface of the hindpaw. The paw withdrawal latency (s) to this stimulus was tested three times at intervals of not less than 3 min and a mean withdrawal latency calculated. To avoid thermal injury, an automatic cut-off time of 21 s was set.

**Thigmotactic behaviour**

The rationale of thigmotaxis as a predator avoidance ethologically-relevant behavioural outcome measure in rodent pain studies has been previously described [17]. At PID 15, the rats were introduced for the first time to the 100 x 100 cm open field arena, which was lit to a light intensity of 12 lux. Locomotor activity was then recorded for 15 min using a high-sensitivity Sanyo camera (VCB 3372, Japan). EthoVision software (v.4.1, Tracksys Ltd., UK) was used to track the movement of animals in the arena, and to calculate the frequency of entry and time spent in the virtual central zone (40 x 40 cm) as well as the total distance travelled in the whole open field arena.

**Pharmacological validation**

Animals received i.p. injections of either analgesic drugs or vehicle solutions twice per day (b.d.) between PID 12 and 15, during which the hindpaw withdrawal thresholds were measured in response to punctate static mechanical stimulation once per day at 1.5–2 h after the first injection. We chose to test gabapentin (0.5 ml; 30 mg/kg in sterile saline; a gift from Pfizer Ltd.) and
amitriptyline (0.5 ml; 10 mg/kg in sterile saline; Sigma, UK;), which was based on previous studies and clinical trials [15; 41; 50].

**Immunohistochemistry and quantitative analysis**

The procedures for tissue processing, immunohistochemistry and quantitative analysis were the same as previously described [17]. Briefly, at PID 14, we terminally anaesthetised some animals with sodium pentobarbital, and then transcardially perfused them using 4% paraformaldehyde. Following perfusion, we removed L5 spinal cord, L5 DRGs, and glabrous hindpaw skin, and then post fixed the tissue in 4% paraformaldehyde overnight. We then used 30% sucrose in PBS to cryoprotect the tissue for 72 h. Cryostat sections of OCT-embedded tissue were cut (spinal cords at 20 µm, DRG at 10 µm, skin at 14 µm) and collected on superfrost slides. Sections were incubated with 10% normal donkey serum for 60 min followed by overnight incubation with the following appropriate primary antibodies: rabbit anti-GFAP (1:1000; Dako, UK), rabbit anti-CGRP (1:2000; Sigma, UK), rabbit anti-Iba1 (1:1000; WAKO, Japan), rabbit PGP 9.5 (1:1000; Ultraclone Ltd., UK). Following 3 PBS washes, sections were incubated with appropriate secondary donkey anti-rabbit Cy3 or FITC antibodies (1: 400; Stratech, UK) for 2 h. Biotin-conjugated isoelectin B4 (IB4; 0.5 mg/mL used at 1:50; Sigma, UK) and ExAvidin–fluorescein isothiocyanate (1:400; Sigma, UK) were used to detect nonpeptidergic C-fibres in the skin. Following 3 PBS washes, slides were cover-slipped with Vectashield mounting medium (Vector Laboratories, UK) and visualised under a Zeiss Axioplan 2 fluorescent microscope (Zeiss, U.K).

The experimenter who performed quantitative analysis was blind to treatment groups. We used 4 areas (50,000 µm² each) in the superficial dorsal horn and 4 areas (1,500 µm² each) in the DRG from 5–7 randomly selected sections per rat to quantitatively analyse Iba1 immunoreactive cell numbers. We classified Iba1 immunoreactive cells in the dorsal horn as having “effector” morphology when their process lengths were less than double the soma diameter. In contrast, we
classified Iba1 immunoreactive cells in the dorsal horn as having “resting” morphology when their process lengths were double the soma diameter. We used 6–8 randomly selected sections from L5 dorsal horn per rat to analyse the intensity of GFAP immunoreactivity that was expressed in arbitrary units. We also measured the intensity of IB4 or CGRP immunoreactivity in laminae I and II. We used 6-8 DRG sections per rat to analyse IB4/CGRP expression. The number of IB4+ or CGRP+ cells was expressed as a percentage of the total DRG cells. We only sampled DRG cells with visible nucleus and distinctly delineated borders. We live counted PGP9.5+ epidermal fibres at 40X objective magnification using the method described previously [28]. Thus, we only counted single fibres crossing the dermal-epidermal junction without secondary branches. Then the epidermal innervation density (IENFD/mm) was calculated based on the epidermis length measure by Image J software 1.45 (NIH).

For investigating the expression of phospho-p38 (pp-38) in microglia, spinal cord sections were incubated overnight with the primary antibody rabbit anti-pp-38 (1:100; Cell Signalling, USA), which was then amplified using a TSA™ Biotin System (Perkin Elmer, UK). The slides were then incubated with rabbit anti-Iba1 (1:1000) primary antibody, followed by corresponding secondary antibody (1:400) solution. Double staining images were taken at 20x objective magnification using a Leica DM R light microscope (Leica Microsystems, Germany). The number of cells immunoreactive for both pp-38 and Iba1 in the superficial dorsal horn was counted using Photoshop CS5 (Adobe, USA) and expressed as a percentage of Iba1 immunoreactive cells.

**Statistics**

Statistical analysis for the behavioural study data was performed using IBM SPSS Statistics Version 23. For the mechanical, cold, and heat hypersensitivity as well as the pharmacological validation study, two-way ANOVA was used to examine the main effects of treatments, times, and interaction between treatments and times where appropriate. The Tukey-Kramer post hoc multi-comparison
adjustment was used to determine if there was any significant difference between treatment groups, i.e. vehicle vs indinavir, and saline vs gabapentin or amitriptyline. For the thigmotaxis study, one-way ANOVA followed by Tukey-Kramer post hoc multi-comparison adjustment was used to evaluate if there was a main treatment effect and if significant differences between groups (indinavir, vehicle and naive) existed. In addition, we included the total distance moved in the open field arena as a secondary outcome for the thigmotactic analysis. The box and scatter plots for the thigmotaxis data were made using OriginPro 2016 (OriginLab, USA). All measurements are expressed as mean value±standard error of the mean (SEM) in the Result section. In addition, behavioural data are presented using 95% confidence levels in the Supplementary Table 3. P<0.05 was considered as statistically significant.

For histological analysis, differences between vehicle- and indinavir-treated animals were determined using a non-parametric test, the Wilcoxon-Mann-Whitney Test. We report our data as mean values±SEM and consider P<0.05 as statistically significant.
Results

No animals were excluded from the current study according to the inclusion and exclusion criteria set in the Supplementary Table 1. A summary of group sizes and primary outcome measures is in Supplementary Table 2.

**Indinavir treatment results in hindpaw hypersensitivity to mechanical stimuli**

To investigate the effect of indinavir treatment in mechanical sensitivity of rats we tested animals that have and have not received the drug for up to 42 days using an electronic ‘von Frey’ device. We observed that rats treated with indinavir (n=6) developed bilateral hindpaw mechanical hypersensitivity, which occurred from PID 4 and reached a peak at PID 14 (Figure 1). Withdrawal thresholds were changed from the baseline at -26%, -33%, -39%, -44%, -36% and -26% for PID 4, 7, 11, 14, 28 and 35 respectively. Here, we did not observe significant difference in mechanical hypersensitivity between the left and right hindpaws (data not shown). Therefore, we pooled the withdrawal thresholds from both hindpaws. The hindpaw mechanical hypersensitivity following indinavir treatment was maintained until PID 42, and then the thresholds showed a trend returning to the baseline. The statistical analysis revealed significant effects of treatments, times and the interaction between treatments and times on hindpaw mechanical hypersensitivity development (for treatment: P=0.0001, df=1, F=944.05; for time: P=0.0001, df=7, F=62.170; for interaction between treatment and time: P=0.0001, df=7, F=55.844).

**Indinavir treatment results in hindpaw hypersensitivity to cold stimuli**

To examine if indinavir treatment could lead to changes in cold sensitivity we tested treated versus untreated animals using the acetone test. We observed that rats treated with indinavir (n=6) developed bilateral hindpaw cold hypersensitivity, which occurred from PID 6, plateaued between PID 13 and 31, and then maintained between PID 41 and 45 (Figure 2). Mean percentage changes
of the response to cold stimuli from the baseline were -14%, 286%, 515%, 529%, 650%, 487%, 501%, 258% and 258% for PID 6, 9, 13, 17, 21, 24, 31, 41 and 45 respectively. We did not observe significant difference in cold hypersensitivity between the left and right hindpaws (data not shown). Therefore, we pooled the thresholds from both hindpaws. The statistical analysis revealed significant effects of treatments, times and the interaction between treatments and times on hindpaw cold hypersensitivity development (for treatment: P=0.0001, df=1, F=1212.65; for time: P=0.0001, df=9, F=16.95; for interaction between treatment and time: P=0.0001, df=9, F=17.28).

**Indinavir treatment does not induce heat hypersensitivity**

We next tested if indinavir treatment results in changes in heat sensitivity. In contrast to the development of hindpaw mechanical and cold hypersensitivity following indinavir treatment, we did not observe increased hindpaw responses to noxious thermal stimuli using the Hargreaves’s device in indinavir-treated animals (n=6) as compared to the baseline and that of the vehicle-treated animals (n=6) (Figure 3). The statistical analysis revealed no significant effects of treatments, times and the interaction between treatments and times on hindpaw heat hypersensitivity development (for treatment: P=0.474, df=1, F=0.554; for time: P=0.935, df=5, F=0.255; for interaction between treatment and time: P=0.911, df=5, F=0.299).

**Thigmotaxis was increased following indinavir treatment**

An open field apparatus was used to assess the impact of indinavir treatment on thigmotactic behaviour. Such behaviour has been previously shown to be associated with pain behaviour in animal models of nerve trauma, varicella zoster virus, HIV gp120, and antiretroviral drugs [15; 17; 49-52]. Here we showed that animals treated with indinavir (n=8) established hindpaw mechanical hypersensitivity at PID 14 (Indinavir: baseline=44.6±0.7 vs PID 14=25.8±0.6, P<0.05; Vehicle: baseline=45.5±0.6 vs PID 14=45.3±0.7, P>0.05). Then at PID 15 we showed a significant treatment effect on thigmotaxis behaviour using statistical analysis (P=0.021, df=2, F=4.671). Thus, at PID
15, track pattern analysis using entry number and time spent in the virtual inner zone demonstrated a significant effect of indinavir on spontaneous explorations. Animals received indinavir treatment had much lower number of entry (4.0±1.3) and less time spent (5.0±1.7 s) in the virtual zone in contrast to the naïve (n=8, 11.4±1.7 and 12.8±1.9 s; P<0.05, ANOVA/Tukey-Kramer post hoc test) and vehicle-treated (n=8, 10.5±1.9 and 11.4±1.5 s; P<0.05, ANOVA/Tukey-Kramer post hoc test) animals (Figure 4). We did not observe any difference in the total distance travelled in the open field among the groups at PID 15 (indinavir 7326.5±12.8cm vs naïve 7602.1±23.6 or vehicle 7513.9±18.5cm, P>0.05), which is in line with our previous data [17; 49; 50].

**Effects of analgesic drugs on hindpaw hypersensitivity**

We examined the pharmacological validity of our model using analgesic drugs, which have been shown either effective or not effective in the clinic for treating HIV-SN [41]. First we demonstrated that amitriptyline was not effective in reversing hindpaw mechanical hypersensitivity in animals treated with indinavir (Figure 5). The statistical analysis revealed no significant effects of treatments (amitriptyline/saline), times and the interaction between treatments and times on hindpaw mechanical hypersensitivity development (for treatment: P=0.179, df=1, F=2.087; for time: P=0.201, df=3, F=1.875; for interaction between treatment and time: P=0.538, df=3, F=0.738). In contrast systemic administration of gabapentin (Figure 5) was associated with a complete attenuation of hindpaw mechanical hypersensitivity observed with indinavir treatment. The statistical analysis revealed significant effects of treatments (gabapentin/saline), times and the interaction between treatments and times on hindpaw mechanical hypersensitivity (for treatment: P=0.0001, df=1, F=14486.08; for time: P=0.002, df=3, F=6.395; for interaction between treatment and time: P=0.0001, df=3, F=29.05). We did not observe any effects of vehicle administration. By PID 18, hindpaw mechanical hypersensitivity was re-established in the gabapentin group, suggesting a return of the neuropathic state.
Indinavir treatment results in reduced epidermal innervation of hindpaw skin

We applied PGP 9.5 immunostaining to visualise unmyelinated fibres in the hindpaw skin. Following indinavir treatment (n=6) at PID 14, there was a significant reduction in intraepidermal nerve fibre density (IENFD) suggesting a withdrawal of unmyelinated axons from the epidermis when compared to that of the vehicle-treated animals (n=6) (Wilcoxon-Mann-Whitney Test, P=0.02; Figure 6).

Systemic indinavir induces a minimal spinal microglial response and no inflammatory cell response in the DRG

We examined if a glial cell response in the dorsal horn could be induced by systemic treatment of indinavir. We stained L5 sections with the microglial marker Iba1, and then counted the number of cells in the superficial dorsal horn exhibiting “effector” morphology, i.e. cell body hypertrophy and process retraction. We found that indinavir-treated rats had significantly increased numbers of microglia with “effector” morphology (indinavir: 7.66±0.61 vs vehicle: 2.80±0.31 cells per 50,000 μm², n=5 per group, P=0.0001, Wilcoxon-Mann-Whitney Test; Figure 7). There appeared no difference in Iba1 immunoreactivity in other areas of the spinal cord (Supplementary Figures 1 and 2). Then we assessed microglial activation by looking at pp-38 expression. Double immunostaining showed a significant increase in the number of microglia positive for pp-38 in animals treated with indinavir in comparison to the controls at PID 14 (indinavir: 41.41±7.68% vs vehicle: 6.65±2.15%; n=5, Wilcoxon-Mann-Whitney Test, P=0.008; Figure 8). The level of this increase is much less in contrast to that following spinal nerve ligation (Figure 8). We also investigated the astrocytic response to indinavir and found that there was no difference in GFAP immunoreactivity in the dorsal horn between the indinavir-treated and control groups at PID 14 (indinavir 91.39±14.27 vs vehicle 100.00±14.42, n=5 per group, P=0.68, Wilcoxon-Mann-Whitney Test; Figure 7). Nerve injury recruits macrophages into the DRG. Therefore, we examined if such macrophage infiltration accompanied the painful peripheral neuropathy induced by indinavir treatment. We found that the
number of Iba-1 immunoreactive cells in L5 DRG did not increase following indinavir treatment (n=5, vehicle 6.5±0.45 vs indinavir 7.52±0.97, P=0.36, Wilcoxon-Mann-Whitney Test; Figure 9).

Systemic indinavir does not alter IB4 and CGRP expression of lumbar spinal cord and DRG

We examined the expression of neurochemical markers for different DRG cell populations following indinavir treatment. Nonpeptidergic and peptidergic small-diameter DRG cells can be labelled with IB4 and CGRP respectively. After nerve trauma, the two markers are down-regulated. In contrast, here we found no change in the percentages of IB4+ DRG cells and CGRP+ DRG cells following indinavir treatment in the lumbar spinal cord (n=5, IB4: vehicle 100.00±8.12% vs indinavir 108.55±13.51%, P=0.75, CGRP: vehicle 100.00±12.13% vs indinavir 90.34±19.15%, P=0.47, Wilcoxon-Mann-Whitney Test in both cases; Figure 7). A similar finding was found in the lumbar DRG (n=5, IB4: vehicle 33.62±1.13% vs indinavir 33.98±0.50%, P=0.76, CGRP: vehicle 31.52±1.05% vs indinavir 31.82±1.19%, P=0.86, Wilcoxon-Mann-Whitney Test in both cases; Figure 9).
Pharmacological agents such as drugs inhibit the protease activity of HIV (e.g. indinavir) are key components of drug therapy for HIV patients. However, limited clinical observations and one in vitro study suggest that indinavir exposure might increase the risk for HIV-SN [11; 29; 39; 41; 46]. We, and others, have previously extensively described the neurotoxicity associated with the d-NTRI group of antiretroviral drugs. However, we are the first who have comprehensively documented in vivo the neurotoxicity associated with wholly different class of antiretroviral drugs, the protease inhibitors, which were hitherto hinted at the above mentioned limited evidence, but in the main were not suspected of being neurotoxic. Our study provides the first in vivo evidence of an indinavir induced peripheral neuropathy in a rodent model. Here, we have shown a persistent painful peripheral sensory neuropathy developed in indinavir-treated animals that had no motor deficits, resembling a major clinical problem in HIV management. In accordance with the clinical presentation, we have shown not only simple reflex pain behaviour, which was sensitive to pharmacological perturbation, but also a complex thigmotactic behaviour associated with pain. Furthermore, we have shown, using histology, that our model is characterised by a retraction of epidermal axons, which is an established clinical diagnostic technique for HIV-SN [42] and other peripheral neuropathies that have a small fibre component [26; 34]. We have also demonstrated a small but significant microglial response in indinavir-treated animals at the time of peak hindpaw mechanical hypersensitivity. Prominently, this neurotoxicity seen in our model happens independently of HIV infection. The latter is a difficult confound to dissect in patients, because not only the two conditions co-exist, but also HIV and sensory neurons interaction can cause painful neuropathy [40; 50].

Here we have observed both mechanical and cold, but not heat, hypersensitivity in the hindpaws of indinavir-treated animals, contrasting to animal models of NRTIs-induced peripheral neuropathy,
which shows only hindpaw mechanical hypersensitivity [17; 19; 50]. This finding is also in contrast with early clinical data showing that patients with HIV-related painful neuropathy do not usually present with thermal hypersensitivity [33]. Our recent study involving a cohort of HIV-infected patients with and without HIV-SN, most of whom had received combinational ART including indinavir, has shown that the most frequent sensory abnormalities demonstrated in the HIV-SN group are loss of mechanical and vibration detection thresholds followed by a significant loss of cold and warm detection thresholds and heat pain threshold when compared to those of neuropathy free HIV positive patients and healthy volunteers, demonstrated by quantitative sensory testing [40]. The same study has also shown that whilst the presence of gain-of-sensory function is rare across all groups, a small minority of patients has features of mechanical wind-up ratio in HIV-SN patients. Subgroup analysis of a randomized controlled trial of pregabalin in HIV-SN has reported a small group of patients with signs of mechanical sensory gain [44]. Furthermore, by using the Neuropathic Pain Symptom Inventory it has been revealed that 42% of participants experiencing painful HIV-SN report symptoms of moderate and severe cold evoked pain, although this is not detected with sensory profiling [40]. Here, we have shown that indinavir-treated rats display increased thigmotaxis in the open field at the time of peak hindpaw mechanical hypersensitivity. This suggests the presence of pain-driven alterations in affect, which may be representative of ongoing pain and/or pain-related affective co-morbidities, which are known to be a feature of neuropathic pain in humans [14; 35; 36]. Indeed, our recent study has shown that participants with painful HIV-SN have reduced quality of life, a higher incidence of insomnia, and increased depression, anxiety, and catastrophizing, when compared to participants without HIV-SN [40].

Previously it has been shown that in cultures of CD4 and CCR5 expressing rat DRG neurons infected with HIV-1 with subsequent treatment by indinavir, there is a marked reduction of neurites numbers and lengths, suggesting additive neurotoxic effects by indinavir [39]. Indinavir-treated DRG cultures also showed numerous TUNEL (an apoptosis marker)-positive nuclei in cells that
were ED-1 (a macrophage marker) immunoreactive, suggesting that resident DRG macrophages may be targets of indinavir toxicity. In the current study no DRG abnormalities were observed following indinavir administration, i.e. no change in CGRP+ or IB4+ neurons or in macrophage infiltration, which markedly contrasts to nerve trauma models. Furthermore, no changes were found in the central projections of primary afferents after indinavir treatment. Thus, we did not observe reduced CGRP expression and IB4 binding in L5 dorsal horn, where primary afferents from hindpaws end. This finding is in contrast to significant reductions in CGRP/IB4 immunoreactivity in L5 spinal dorsal horn seen in animals treated with d4T [17]. Activation of the innate immune system in the dorsal horn of the spinal cord is key in the development of pain after nerve injury [2; 6]. However in the case of indinavir treatment we only observed a small but significant increase (1.7 folds) in microgliosis; we observed an increased microglial expression of pp-38, which is known to promote the microglial proinflammatory responses to produce mediators such as COX-2, IL-1β, BDNF and iNOS, contributing to neuropathic pain development and maintenance [6]. We did not observe any evidence of astrocyte response to indinavir treatment. Our observation of a minimal glial and immune response in the spinal cord and DRG following indinavir treatment is comparable to those reported in chemotherapy or metabolic agents induced chronic painful neuropathy [4; 6; 17; 53], highlighting the need for relevant animal models to address particular clinical scenarios.

Importantly here indinavir administration resulted in a reduction in hindpaw IENFD, which is also manifested in many other painful neuropathies [27], such as SN resulted from ddC [50] and d4T [17] treatment, and direct neurotoxicity mediated by HIV virus [49]. Reduced IENFD, a key clinical diagnostic tool for HIV-SN, correlates inversely with neuropathic pain progress [40; 42]. There is evidence suggesting that protease inhibitors are associated with insulin resistance and resultant diabetic complications in HIV patients [7], which is thought to be mediated through the inhibition of insulin-regulated glucose transporter [10]. Therefore, it is possible that such diabetic
complications could also result in the development of diabetic peripheral sensory neuropathy with a characteristic loss of IENFD on skin biopsy [37]. In the current study, we found no significant difference in blood glucose levels and body weights at PID 19 and PID 45 between indinavir-treated and vehicle-treated animals (data not shown), suggesting that the dose regime of indinavir in our study did not cause insulin resistance.

Our study could explain the well-documented persistence of painful peripheral neuropathy in patients who have not been exposed to d-NRTIs or who were not susceptible to d-NRTI-neurotoxicity, which had hitherto been assumed to be the main cause of neurotoxicity. Our study also highlights the importance of using animal models to study cause of the neurotoxicity of protease inhibitors in isolation, since there are too many confounds in patients because they have concomitant HIV disease and also take a plethora of other drugs, including a combination antiretroviral drugs. We chose to explore indinavir, one of the prototypical protease inhibitors, as it is representative of the class. However, we acknowledge that in well-resourced settings it has been replaced by new generation protease inhibitors, and it has also been replaced by other protease inhibitors on the World Health Organization's List of Essential Medicines. However, there is still a huge clinical legacy of patients who had been exposed to it and who have persistent peripheral neuropathy that requires clinical management. We do not know whether the neurotoxicity which we demonstrated as being associated with indinavir is a PI class effect or unique to indinavir - that is for further studies. Here we have not studied the dose effect for indinavir, as the purpose of this report is to test whether indinavir can produce neurotoxicity in isolation rather than mimic the patient living with HIV. The dose of 50 mg/kg was chosen to keep consistency with our previous ddC and d4T studies and is likely to under estimate the human dose if converted using surface area dosage conversion. However, we agree that a dose effect study is important to expand the testing of our hypothesis and will be included in future studies along with ddC and d4T. We also agree that
electron microscopy studies might be useful in yielding further information, but are outside the scope of the current study.

In summary, we have established a rodent model of painful SN mediated by systemic indinavir treatment. Our model mimics a number of clinical features and reveals important mechanistic differences when compared to the previously reported d4T model (Table 1). HIV-SN continues to be one of the most prevalent morbidities experienced by people living with HIV in both high- and low-resource settings. Our model offers an important tool to better comprehend the pathogenesis, develop preventive strategies, and discover effective drugs for HIV-SN.

(Word count = 1469)
Acknowledgements

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Conflicts of interest

The authors have no conflicts of interest to declare.
Reference


Figure Captions

Figure 1: The development of hindpaw hypersensitivity to punctate mechanical stimuli following indinavir treatment. We measured the withdrawal thresholds at the baseline (BL) and after indinavir (50 mg/kg, twice at 4 days apart) or vehicle administration. Two-way ANOVA with Tukey-Kramer post hoc multiple comparisons were used to determine the difference between treatment groups. Asterisk=significant difference from the vehicle group (P<0.05); ‡=significant difference from the baseline (P<0.05).

Figure 2: The development of hindpaw hypersensitivity to cold stimuli following indinavir treatment. We measured withdrawal responses at the baseline (BL) and after indinavir (50 mg/kg, twice at 4 days apart) or vehicle administration using an acetone drop. Two-way ANOVA with Tukey-Kramer post hoc multiple comparisons were used to determine the difference between treatment groups. Asterisk=significant difference from the vehicle group (P<0.05); ‡=significant difference from the baseline (P<0.05).

Figure 3: No hindpaw hypersensitivity to thermal stimuli following indinavir treatment. We measured withdrawal responses at the baseline (BL) and after indinavir (50 mg/kg, twice at 4 days apart) or vehicle administration using a noxious thermal stimulus (Hargreaves’s device). Two-way ANOVA with Tukey-Kramer post hoc multiple comparisons were used to determine the difference between treatment groups. Asterisk=significant difference from the vehicle group (P<0.05); ‡=significant difference from the baseline (P<0.05).
Figure 4: Thigmotactic changes in the open field arena following indinavir treatment at PID 15. (A) Virtual inner zone (dotted square in C, 40 x 40 cm²) entry number and (B) the time spent in the virtual inner zone were assessed in the naïve, vehicle-treated, and indinavir-treated (50 mg/kg, i.v., twice at 4 days apart) animals. (C) Illustration of movement of (i) naïve, (ii) vehicle-treated, and (iii) indinavir-treated animals in the arena. The statistical significance of differences between the indinavir group and its relevant control (*P<0.05) was determined by a one-way ANOVA with Tukey-Kramer post hoc multiple comparisons. (A-B) Data were displayed using box and scatter plots. Each box represents mean±SEM. Bars above and below each box represents standard deviations. The line and the circle within the box represents median and mean respectively.

Figure 5: The effect of analgesic drugs on hindpaw mechanical hypersensitivity. (A: gabapentin; B: amitriptyline) The effects of drugs administered around peak change in hindpaw sensitivity to mechanical stimuli in animals received indinavir treatment. Withdrawal thresholds (g) are displayed as prior, during and post each drug treatment (open circle) versus vehicle (filled triangle). A shaded area is used to show drug treatment period. Arrows and arrowheads represent the start and end of drug administration respectively. Two-way ANOVA with Tukey-Kramer post hoc multiple comparisons were used to determine the difference between drug and vehicle threshold values. *P<0.05 vs vehicle.

Figure 6: Reduced IENFD following indinavir treatment at PID 14. (a) An example of PGP9.5 stained skin sections of a vehicle animal. (b) An example of PGP9.5 stained skin sections of indinavir-treated animal. Following indinavir treatment, there is a significant reduction in intraepidermal fibre numbers (Wilcoxon-Mann-Whitney Test, P=0.02), which is demonstrated in the quantitative analysis of IENFD in (c). Scale bar=100 μm.
Figure 7. Systemic indinavir treatment induced microgliosis, but not astrogliosis and changes in IB4 and CGRP expressions, in the lumbar spinal cord at PID 14. (a, b) CGRP expression (green) in laminae I and IIo of L5 dorsal horns. (d, e) IB4 labelling (red) within lamina IIi of L5 dorsal horns. (c, f) Quantitative analyses showing no IB4 and CGRP alterations in L5 dorsal horns between vehicle and indinavir animals. (g, h) GFAP expression in astrocytes in L5 dorsal horns. (i) Quantitative analysis showing no change in GFAP expression in L5 dorsal horns between vehicle and indinavir animals. Indinavir treatment significantly increased the number of microglia with effector morphology (k, l) compared to the vehicle (j). **P<0.01. N=5 per group. Scale bars=200 μm for top two rows (a, b, d, & e); 50 μm for the third row (g & h) and for the bottom row (j & k).

Figure 8: Systemic indinavir treatment increased the level of expression of phospho-p38 in spinal cord microglia. Phospho-p38 (green; e.g. e) is expressed by Iba1 positive spinal microglia (red; e.g. d) within the dorsal horn. This expression is significantly increased at PID 14 following indinavir treatment (50 mg/kg, twice at 4 days apart) (d, e, f) compared to vehicle treatment (a, b, c). N=5 per group. For comparison, the normally observed increase in the proportion of microglia that are immunoreactive for phospho-p38 following SNL is shown in (g). **P<0.01 vs vehicle. Scale bar=50 μm.

Figure 9. Systemic indinavir treatment did not induce neurochemical changes in sensory neurons and macrophage infiltration in L5 DRGs at PID 14. IB4 binding (red) and CGRP expression CGRP (green) were not changed following indinavir treatment (b, e) vs vehicle (a, d), and this was quantified in (c, f). Iba1 immunostaining was used as a marker of macrophages in L5 DRGs following vehicle (g) or indinavir (h) treatment. There was no
change in macrophage numbers in the DRG (quantified in i) following indinavir treatment.

N=5 per group. Scale bars=50 μm for top row (a & b), mid row (d & e), and bottom row (g & h).
Summary

Rats treated with HIV antiretroviral drug indinavir demonstrated alterations in neuropathic pain-related sensory and thigmotactic behaviours accompanied by significant loss of hindpaw skin sensory innervation.
Figure 1

The graph shows the change in paw withdrawal threshold (g) over days post first injection for two groups: Vehicle (open circle) and Indinavir (triangle). The threshold is measured in grams and the days post first injection are marked on the x-axis. The y-axis represents the paw withdrawal threshold. Significant changes are indicated by asterisks (*) on the graph. The group receiving Indinavir shows a decrease in threshold, while the Vehicle group remains relatively stable.
Figure 7

**CGRP**

- **Vehicle**
  - a
- **Indinavir**
  - b

**IB4**

- **Vehicle**
  - d
- **Indinavir**
  - e

**GFAP**

- **Vehicle**
  - g
- **Indinavir**
  - h

**Iba1**

- **Vehicle**
  - j
- **Indinavir**
  - k

Bar charts showing:

- Staining intensity (% of vehicle)
- Iba+ cells with activated morphology/5000 μm²

**Vehicle** and **Indinavir** comparisons.
Table 1. Comparison of key neurochemical markers in the lumbar spinal cord and DRG between indinavir and d4T models at the time of peak hindpaw mechanical hypersensitivity.

<table>
<thead>
<tr>
<th></th>
<th>Indinavir model</th>
<th>D4T model</th>
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<tbody>
<tr>
<td><strong>Spinal cord dorsal horn</strong></td>
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<tr>
<td>IB4 and CGRP expression</td>
<td>No change</td>
<td>Significant reduced in the medial portion</td>
</tr>
<tr>
<td>Microglia with effector morphology</td>
<td>A small but significant increase</td>
<td>A small but significant increase</td>
</tr>
<tr>
<td>Phospho-p38 expression in microglia</td>
<td>A significant increase</td>
<td>No change</td>
</tr>
<tr>
<td>Astrocytes</td>
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<td>No change</td>
</tr>
<tr>
<td><strong>DRG</strong></td>
<td></td>
<td></td>
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<td>IB4 and CGRP expression</td>
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<td>No change</td>
</tr>
<tr>
<td>Macrophages</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>
A rodent model of HIV protease inhibitor indinavir induced peripheral neuropathy

*W. Huang¹, *M. Calvo², T. Pheby³, D.L.H. Bennett⁴, A.S.C. Rice³,⁵

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5. Pain Medicine, Chelsea and Westminster Hospital NHS Foundation Trust, London, UK

*=Joint first authors

**Supplementary materials**

1. Supplementary Table 1. Major domains of good laboratory practice to minimise the effects of experimental bias.

<table>
<thead>
<tr>
<th>Description of procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Size Calculation</strong></td>
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<tr>
<td><strong>Inclusion and Exclusion Criteria</strong></td>
</tr>
<tr>
<td><strong>Randomization</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Allocation Concealment</strong></td>
</tr>
</tbody>
</table>
|                           | This was achieved by the blinding procedure described below, as well as masking cage labels or turning around the cages before each
behavioural assessment session.

<table>
<thead>
<tr>
<th>Reporting of Animals Excluded From Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Any rat showing hunched posture, a marked behavioural change, exudates around wound or sensitivity to palpitation on handling that could be attributable to surgery, the drug, the dosing procedure, infection resulting from surgery or otherwise, was excluded.</td>
</tr>
<tr>
<td>• The details of the number of excluded animals and the reason for exclusion are stated in the results section.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blinded Measurement, Assessment, and Analysis of Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Codes were assigned to different treatments by an independent person and kept in a sealed envelope. The codes were not broken until the analysis had been completed.</td>
</tr>
<tr>
<td>• The experimenter was ‘blinded’ to the treatments received and had no knowledge of the experimental group to which an animal was randomized.</td>
</tr>
</tbody>
</table>

*Treatment here refers to drug administration, i.e. indinavir versus vehicle, and each pharmacological analgesic agent versus vehicle.

2. **Supplementary Table 2.** Details of groups and primary outcomes for behavioural experiments.

<table>
<thead>
<tr>
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<th>Group names</th>
<th>Group sizes</th>
<th>Primary outcomes</th>
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<tr>
<td>MH development</td>
<td>Indinavir/vehicle</td>
<td>6/6</td>
<td>HPW threshold in response to punctate static mechanical stimulus</td>
</tr>
<tr>
<td>CH development</td>
<td>Indinavir/vehicle</td>
<td>6/6</td>
<td>HPW threshold in response to cold stimulus</td>
</tr>
<tr>
<td>HH development</td>
<td>Indinavir/vehicle</td>
<td>6/6</td>
<td>HPW threshold in response to heat stimulus</td>
</tr>
<tr>
<td>Thigmotaxis</td>
<td>Indinavir/vehicle/naive</td>
<td>8/8/8</td>
<td>Frequency of entry and duration in the inner zone</td>
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<td>Pharmacological validation</td>
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<tr>
<td></td>
<td>Amitriptyline/vehicle</td>
<td>6/6</td>
<td>HPW threshold in response to punctate static mechanical stimulus</td>
</tr>
</tbody>
</table>

MH = mechanical hypersensitivity;  
CH = cold hypersensitivity;  
HH = heat hypersensitivity;  
HPW = hindpaw withdrawal
### 3. Supplementary Table 3.

Behavioural data presented with mean and 95% confidence levels (CI).

<table>
<thead>
<tr>
<th></th>
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<td>19.4, 20.4</td>
<td>20.5</td>
<td>20.0, 21.0</td>
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<td>Day 14</td>
<td>19.9</td>
<td>18.9, 20.9</td>
<td>20.2</td>
<td>19.4, 20.9</td>
</tr>
<tr>
<td>Day 15</td>
<td>20.6</td>
<td>20.0, 21.2</td>
<td>20.3</td>
<td>19.5, 21.1</td>
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<th>Mean</th>
<th>95% CI</th>
<th>Mean</th>
<th>95% CI</th>
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<td>44.8</td>
<td>44.0, 45.6</td>
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<td>PID 14</td>
<td>45.7</td>
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<td>Vehicle-treated</td>
<td>12.9</td>
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<tr>
<td>Indinavir-treated</td>
<td>5.3</td>
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MH = mechanical hypersensitivity;  
CH = cold hypersensitivity;  
HH = heat hypersensitivity;
3. Supplementary Figure 1. Iba1 staining of the lumbar spinal cord from vehicle-treated animals at low (10x) objective magnification. (a-e), example images of Iba1 staining of transverse sections of the L5 spinal cord from 5 animals. Scale bar=100μm.

4. Supplementary Figure 2. Iba1 staining of the lumbar spinal cord from indinavir-treated animals at low (10x) objective magnification. (a-e), example images of Iba1 staining of transverse sections of the L5 spinal cord from 5 animals. Scale bar=100μm.
References:


# The ARRIVE Guidelines Checklist

**Animal Research: Reporting In Vivo Experiments**

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¹The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, ²School of Veterinary Science, University of Bristol, Bristol, UK, ³School of Biological Sciences, University of Bristol, Bristol, UK, ⁴National Heart and Lung Institute, Imperial College London, UK, ⁵Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

<table>
<thead>
<tr>
<th>ITEM</th>
<th>RECOMMENDATION</th>
<th>Section/Paragraph</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title</strong></td>
<td>1  Provide as accurate and concise a description of the content of the article as possible.</td>
<td>Page 1</td>
</tr>
<tr>
<td><strong>Abstract</strong></td>
<td>2  Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.</td>
<td>Page 2</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| **Background** | 3  a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale.  
   b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study’s relevance to human biology. | Pages 3-4, Page 4, para. 2 |
| **Objectives** | 4  Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested. | Page 4 |
| **METHODS** |  |  |
| **Ethical statement** | 5  Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research. | Page 5 |
| **Study design** | 6  For each experiment, give brief details of the study design including:  
   a. The number of experimental and control groups.  
   b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when).  
   c. The experimental unit (e.g. a single animal, group or cage of animals).  
   A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out. | Supp. Table 2, Supp. Table 1, Supp. Table 2 |
| **Experimental procedures** | 7  For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example:  
   a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s).  
   b. When (e.g. time of day).  
   c. Where (e.g. home cage, laboratory, water maze).  
   d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used). | Pages 5-10, Page 6, Page 6, Page 6 |
| **Experimental animals** | 8  a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range).  
   b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc. | Page 5, para. 2, Page 5; para. 2 |

Housing and husbandry

Provide details of:

a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).

b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment).

c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.

Sample size

a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group.

b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.

c. Indicate the number of independent replications of each experiment, if relevant.

Allocating animals to experimental groups

a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done.

b. Describe the order in which the animals in the different experimental groups were treated and assessed.

Experimental outcomes

Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).

Statistical methods

a. Provide details of the statistical methods used for each analysis.

b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron).

c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.

RESULTS

Baseline data

For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).

Numbers analysed

a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%).

b. If any animals or data were not included in the analysis, explain why.

Outcomes and estimation

Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).

Adverse events

a. Give details of all important adverse events in each experimental group.

b. Describe any modifications to the experimental protocols made to reduce adverse events.

DISCUSSION

Interpretation/scientific implications

a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature.

b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results.

c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.

Generalisability/translation

Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.

Funding

List all funding sources (including grant number) and the role of the funder(s) in the study.

References:
