Selective cathepsin S inhibition with MIV-247 attenuates mechanical allodynia and enhances the anti-allodynic effects of gabapentin and pregabalin in a mouse model of neuropathic pain

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Text pages:
Tables: 4
Figures: 7
References: 35
Abstract: 249 words
Introduction: 554 words
Discussion: 1498 words
Section recommendation: Neuropharmacology
Non-standard abbreviations:
ANOVA: Analysis of variance; AMC: 7-amino-4-methylcoumarin; AUC: Area under the curve; CX3CR1: Chemokine (C-X3-C-motif) receptor 1; DMSO: Dimethyl sulfoxide; DTT: Dithiotreitol; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; PAR: Protease activated receptor; PEG: Polyethylene glycol; PWT: paw withdrawal threshold
ABSTRACT

Cathepsin S inhibitors attenuate mechanical allodynia in preclinical neuropathic pain models. The current study evaluated the effects when combining the selective cathepsin S inhibitor MIV-247 with gabapentin or pregabalin in a mouse model of neuropathic pain. Mice were rendered neuropathic by partial sciatic nerve ligation. MIV-247, gabapentin or pregabalin were administered alone or in combination via oral gavage. Mechanical allodynia was assessed using von Frey hairs. Neurobehavioural side effects were evaluated by assessing beam walking. MIV-247, gabapentin and pregabalin concentrations in various tissues were measured. Oral administration of MIV-247 (100-200 µmol/kg) dose-dependently attenuated mechanical allodynia by up to approximately 50% reversal when given as a single dose or when given twice daily for 5 days. No behavioural deficits were observed at any dose of MIV-247 tested. Gabapentin (58-350 µmol/kg) and pregabalin (63-377 µmol/kg) also inhibited mechanical allodynia with virtually complete reversal at the highest doses tested. The minimum effective dose of MIV-247 (100 µmol/kg) in combination with the minimum effective dose of pregabalin (75 µmol/kg) or gabapentin (146 µmol/kg) resulted in enhanced anti-allodynic efficacy without augmenting side effects. A sub-effective dose of MIV-247 (50 µmol/kg) in combination with a sub-effective dose of pregabalin (38 µmol/kg) or gabapentin (73 µmol/kg) also resulted in substantial efficacy. Plasma levels of MIV-247, gabapentin and pregabalin were similar when given in combination as to when given alone. Cathepsin S inhibition with MIV-247 exerts significant anti-allodynic efficacy alone and also enhances the effect of gabapentin and pregabalin without increasing side effects or inducing pharmacokinetic interactions.
INTRODUCTION

Neuropathic pain is likely to be multi-factorial and preclinical data from animal models of neuropathic pain suggest that in addition to spinal neuronal excitability, spinal microglial cells are also activated. Microglia cells are recognised as resident macrophages in the central nervous system and can release neuromodulatory transmitters that influence neuronal firing. Peripheral nerve injury has been shown to increase the density of spinal microglia cells and alter microglial gene expression (McMahon and Malcangio, 2009). These changes occur in response to the increased peripheral sensory afferent input into the dorsal horn (Hathway et al., 2009; Suter et al., 2009). Changes in microglial density and phenotype correlate with tactile allodynic behaviour in rats subjected to partial sciatic nerve injury (Coyle, 1998) or spared nerve injury (Suter et al., 2009) and in mice subjected to partial sciatic nerve ligation (Staniland et al., 2010). Taken together, the data suggest that inhibiting microglial activation may be beneficial in the treatment of neuropathic pain.

The cysteine protease cathepsin S plays a role in maintaining microglia activity in pain states (Clark and Malcangio, 2012). Cathepsin S is expressed by spinal microglia and becomes more abundant in response to nerve injury since the number of microglia increases (Clark et al., 2007). Cathepsin S is released from microglia in response to nociceptive neurotransmitters such as ATP (Clark et al., 2010) and inhibitors of cathepsin S reverse mechanical allodynia and hyperalgesia in rodent models of neuropathic pain (Clark et al., 2007; Irie et al., 2008). The proposed target substrate for cathepsin S in the spinal cord is neuronal fractalkine. Dorsal horn preparations ex vivo show that liberation of soluble fractalkine is dependent upon cathepsin S (Clark et al., 2009) and mechanical allodynia evoked by spinal administration of exogenous cathepsin S is abolished in mice lacking the fractalkine CX3CR1 receptor (Clark et al., 2007).
Gabapentin and pregabalin exert analgesic effects in neuropathic pain states in humans such as post-herpetic neuralgia (Stacey and Glanzman, 2003; Gore et al., 2007) and diabetic neuropathy (Backonja et al., 1998). The effects of gabapentin and pregabalin do not appear to be mediated by GABA receptors, but rather via inhibition of calcium currents mediated by calcium channels containing the α2δ-1 subunit (Fink et al., 2002; Sutton et al., 2002). Blockade of these channels leads to reduced release of pro-nociceptive neurotransmitters and attenuation of postsynaptic excitability in the spinal dorsal horn (Fehrenbacher et al., 2003; Takasusuki et al., 2011).

Despite the efficacy displayed on clinical conditions, gabapentin and pregabalin have several drawbacks. Common side effects of gabapentin/pregabalin treatment include dizziness and sedation (Dworkin et al., 2010) and gabapentin/pregabalin provide substantial benefit in a minority of patients only (Moore et al., 2011). Since neuropathic pain is multifactorial due to multiple pathways, enhanced pain relief is more likely to be achieved by combining compounds that affect pain transmission via different mechanisms of action (Raffa et al., 2010).

MIV-247 is a selective, orally active inhibitor of cathepsin S designed for the treatment of neuropathic pain. MIV-247 data has previously been presented in abstract form at the International Association for the Study of Pain congress 2014 (Buenos Aires) and the Special Interest Group on Neuropathic Pain 2015 (Nice). The current study characterizes MIV-247 and evaluates the effect of combining MIV-247 with gabapentin or pregabalin in a mouse model of mechanical allodynia evoked by peripheral nerve injury.
MATERIALS AND METHODS

Enzyme assays

Cathepsin S from all species and cathepsin K were recombinant human enzymes expressed in Baculovirus, purified and activated in-house. Purified human Cathepsin L, trypsin, chymotrypsin and human neutrophil elastase were obtained from Calbiochem. Purified human cathepsin B and H were obtained from Athens Research Technology. Purified human cathepsin V was obtained from R & D Systems.

For cathepsins S and V the substrate used was boc-Val-Leu-Lys-AMC, for non-rodent cathepsin K the substrate was H-D-Ala-Leu-AMC, for mouse cathepsin K the substrate was Z-Leu-Arg-AMC, for cathepsin L the substrate was H-D-Val-Leu-Lys-AMC, for cathepsin B the substrate was Z-Arg-Arg-AMC, for cathepsin H the substrate was H-Arg-AMC, for trypsin the substrate was Boc-Gln-Gly-Arg-AMC, for chymotrypsin the substrate was Succ-Ala-Ala-Pro-Phe-AMC, for human neutrophil elastase the substrate was Succ-Ala-Ala-Pro-Val-AMC. All substrates were from Bachem.

For cathepsin S the buffer was 100 mmol/L sodium phosphate, 100 mmol/L NaCl, 1 mmol/L DTT, 0.1% PEG 4000, pH 6.5. For cathepsin K the buffer was 100 mmol/L sodium phosphate, 5 mmol/L EDTA, 1 mmol/L DTT, 0.1% PEG 4000, pH 6.5. For cathepsin L the buffer was 100 mmol/L sodium acetate, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% PEG 4000, pH 5.5. For cathepsin B the buffer was 50 mmol/L sodium phosphate, 1 mmol/L EDTA, pH 6.25. For cathepsin H the buffer was 100 mmol/L tris-acetate, 1% PEG4000, pH 7.5. For cathepsin V the buffer was 25 mmol/L sodium acetate, 2.5 mmol/L EDTA, pH 5.5. For trypsin, chymotrypsin and human neutrophil elastase the buffer was 50 mmol/L HEPES, 100 mmol/L NaCl, 10 mmol/L CaCl₂, 0.1% CHAPS, pH 8.0.
Assays were carried out in white polystyrene 96-well plates in a final volume of 100 µL. Substrate concentrations were 10 – 100 µmol/L and enzyme concentrations were 0.1 – 5 nmol/L. Compounds were added in DMSO in the range 1 nmol/L to 100 µmol/L at a final DMSO concentration of 1%.

Plates were read in a Fluoroskan Ascent (Thermo Labsystems, Helsinki) in kinetic mode, with excitation and emission filters of 390 nm and 460 nm, respectively. Rates were determined by linear regression of the fluorescence/time data in Excel. Rates were fitted by non-linear regression to either the competitive inhibition equation, with the substrate concentration fixed at the value in the assay and the $K_M$ fixed to the value previously determined, or the $IC_{50}$ equation using GraphPad Prism (version 6, GraphPad Software) to obtain $K_i$ or $IC_{50}$ respectively.

In one set of experiments, the reversibility of MIV-247 binding to human recombinant cathepsin S was evaluated using the dilution method. Cathepsin S activity (20 nmol/L) was evaluated in the presence of 200 nmol/L MIV-247 (full inhibition expected). A 100-fold dilution of 20 nmol/L cathepsin S and 200 nmol/L MIV-247 into a cathepsin S enzyme assay was performed and the steady-state measured. This was compared to the steady-state rate by adding 0.2 nmol/L cathepsin S to an assay containing 2 nmol/L MIV-247. A comparable steady state rate in the dilution experiments would indicate full reversibility.

**Selectivity**

The selectivity of MIV-247 was also tested against a panel of 273 receptors, ion channels, transporters and enzymes at Eurofins PanLabs (Taipei, Taiwan). MIV-247 was evaluated at a concentration of 10 µmol/L. Responses were defined as significant if more than 50% inhibition occurred.

**Animals**
All experiments were carried out in accordance with UK Home Office Regulations (Animal Scientific Procedures Act, 1986). Male C57/BL6 mice (25-30 g), obtained from Harlan UK, were used in partial nerve ligation and beam walking experiments. The animals were housed in groups of 5 under a 12 h light/dark cycle (lights on at 7.00 a.m.) with food and water *ad libitum*. Animals were allowed to habituate for at least 5 days prior to surgery or experiments.

Male C57/BL6 mice (20-30 g), obtained from Taconic, Denmark, were used in the pharmacokinetic studies. The animals were housed (max 5 animals/cage) with food and water *ad libitum*. Fluorescent lighting with 12 h light and 12 h dark cycle was used, and the animals were allowed to habituate for at least 5 days prior to the experiments.

*Surgery – partial nerve ligation*

Mice were anaesthetised with isoflurane 2% O₂ mixture maintained during surgery via a nose cone. After surgical preparation the common sciatic nerve was exposed at the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatic trifurcation, about 7 mm of nerve was freed of adhering tissue and one third to one half of the dorsal aspect of the nerve was ligated (5-0 silk) (according to Seltzer et al., 1990, as adapted to mice by Malmberg and Basbaum, 1998), then the incision was closed in layers.

*Assessment of mechanical withdrawal thresholds*

Mechanical thresholds of the ipsilateral (left) paw and contralateral (right) paw were assessed before surgery and on days 3, 5, 7 and 10 after surgery to ascertain the development of mechanical allodynia (assessed as the difference in thresholds between contra and ipsilateral paws). Animals were transferred to the experimental room on the test day and allowed to acclimatise in acrylic cubicles (8 x 5 x 10 cm) atop a wire mesh grid for up to 60 min prior to testing. Static mechanical withdrawal thresholds were assessed by applying calibrated von Frey hairs (flexible nylon fibres of increasing diameter that exert defined levels of force;
Touch Test; Stoelting, USA) to the plantar surface of the hind paw until the fibre bent. The hairs were held in place for 3 s or until the paw was withdrawn, the latter defining a positive response. Starting with a stimulus strength of 0.07 g, hairs were applied according to the ‘up-down’ method within a range of 0.008 g – 1.0 g and from this 50% paw withdrawal thresholds (PWT) were calculated (Dixon, 1980; Chaplan et al., 1994). All experiments were carried out by an observer blinded to drug treatments.

Animals were considered allodynic when they displayed a response of 0.1 g or less, normal responses are from 0.6 g - 1 g. From day 11 after surgery approximately 80% of the mice developed allodynia and were randomised into groups. Paw withdrawal thresholds were assessed before and at 1, 3 and 6 h post dosing of compound or vehicle.

The raw 50% PWT data was used for statistical analysis (see below). For simplicity and comparison, effects of compounds were expressed as % reversal using the following formula:

\[
\% \text{ reversal} = \frac{\text{Ipsilateral 50\% PWT (compound)} - \text{Ipsilateral 50\% PWT (vehicle)}}{\text{Contralateral 50\% PWT (compound)} - \text{Ipsilateral 50\% PWT (vehicle)}}
\]

**Beam walking**

The risk of pregabalin, gabapentin or MIV-247 exerting possible dizziness or ataxia behaviour was assessed in normal (non-injured) C57BL/6 mice using a beam walking test (Stanley et al., 2005). The beam walking apparatus consisted of a 1.5 m long wooden beam which was elevated 1 m above the floor and the test was conducted in a light attenuated room. A switch-activated source of bright light (60 W tungsten bulb) was located at the start-end of the beam and served as avoidance stimuli (approximately 520 LUX) while a dark box at the other end represented a goal box to reach (approximately 18 LUX). Animals were acclimatised to the
beam for five days with two trials per day. On the test day, animals that crossed the beam in less than 10 s, and with no more than 1 foot slip were selected. The animals were then randomly divided in sub-groups. Animals were assessed before and at 1, 3 and 6 h post dosing. A maximum score of 20 s and 10 foot slips was assigned to those mice that did not cross or fell off the beam.

Pharmacokinetic data

Pharmacokinetic data was generated in two separate studies where C57BL/6 male mice were administered MIV-247, pregabalin, gabapentin, a combination of MIV-247 and pregabalin or a combination of MIV-247 and gabapentin. Three mice were included in each group and the doses were 100 µmol/kg, 75 µmol/kg and 146 µmol/kg for MIV-247, pregabalin and gabapentin, respectively. The dose volume was 5 mL/kg for each compound.

Compounds

MIV-247 was synthesised by Medivir AB (Huddinge, Sweden), formulated in 20% hydroxypropyl-β-cyclodextrin in water and given via oral gavage at a dose volume of 5 mL/kg at doses up to 200 µmol/kg. The molecular structure of MIV-247 is shown in Figure 1. The synthesis description of MIV-247 can be found in Supplemental file 1.

Gabapentin was purchased from Toronto Research Chemicals Inc., dissolved in distilled water, and given via oral gavage at a dose volume of 5 mL/kg at doses up to 584 µmol/kg.

Pregabalin was purchased from Tocris Bioscience, dissolved in distilled water, and given via oral gavage at a dose volume of 5 mL/kg at doses up to 377 µmol/kg.

Measurement of MIV-247, gabapentin and pregabalin concentrations
In some cases, blood was withdrawn from euthanized neuropathic mice immediately after the experiments (i.e. ~6 h post-dose) by heart puncture into pre-chilled heparinised tubes. Blood samples were immediately put on ice prior to centrifugation. Plasma was prepared by centrifugation for 10 min at approximately 1,000 g at +4 ºC within 10 min from sampling. Plasma samples were stored at -20 ºC prior to analysis. Brain and spinal cord were dissected and snap-frozen in liquid nitrogen and stored at -20 ºC prior to analysis.

In the pharmacokinetic studies, seven blood samples (~20 µL) were drawn from the lateral saphenous vein of each mouse at 15 min, 30 min, 1 h, 2 h, 3 h, 5 h and 7 h post dose. The blood was collected into lithium heparin coated tubes, placed on wet ice and protected from light prior to centrifugation (3600 rpm, Eppendorf rotor A-4-44) at approximately 4 ºC within 30 min of collection. Plasma samples were stored at -20 ºC prior to analysis. In addition, in some pharmacokinetic experiments, brain and spinal cord were dissected out at 3-3.5 h post-dose and snap-frozen in liquid nitrogen and stored at -20 ºC prior to analysis.

Determination of compound concentrations in plasma and tissue homogenates were performed using LC-MS/MS. Brain samples were homogenised in 0.9% NaCl in a volume 4 times the frozen weight using Ultra Turrax (13,500 rpm/min) for 20 s. Spinal cord samples were homogenised in 0.9% NaCl in a volume 4 times the frozen weight using a TissueLyser (50 oscillations/min) for 60 s. The homogenised samples were stored at -20 ºC until further analysis.

Extraction of MIV-247 from brain and spinal cord tissue samples was performed by the addition of 4 parts acetonitrile to 1 part homogenate, mixing and sonication for 15 min. The samples were centrifuged (for 10 min at 20,000 g and 10 ºC) and the supernatants were injected into the analytical column (Kinetex C18, 2.1 x 50 mm, 2.6 µ). Plasma was de-proteinised by adding 4 parts acetonitrile to 1 part plasma, mixing and centrifugation (for 10
min at 20,000 g and 10°C). The supernatants were injected into the analytical column. Mass analysis was carried out using Agilent Technologies 6460 Triple Quad LC/MS with electrospray ionisation in a positive mode. MIV-247 was monitored by using transition m/z 438 to m/z 418 at a fragmentor voltage of 135 V and collision energy of 5 V. The limit of detection for MIV-247 was 0.025 µmol/L.

Extraction of gabapentin from brain and spinal tissue homogenate was performed by the addition of 5 parts 70% methanol to 1 part homogenate, sonication of the homogenate for 15 min and centrifugation (for 10 min at 20,000 g and 10°C). The supernatants were filtered through filter cups (Amicon Ultrafree MC, 30 000 NMWL) for 20 min at 5,000 g and the ultrafiltrates were injected into the analytical column (Zorbax SB-Phenyl, 3 x 150 mm, 3.5 µ). Plasma was de-proteinised by addition of 1 part acetonitrile to 1 part plasma, mixing and centrifugation (for 10 min at 20,000 g and 10°C). The supernatants were diluted with 2 parts of water prior to injection into the analytical column. Mass analysis was carried out using Agilent Technologies 6460 Triple Quad LC/MS with electrospray ionisation in a positive mode. Gabapentin was monitored by using transition m/z 172 to m/z 154 at a fragmentor voltage of 100 V and collision energy of 10 V. The limit of detection for gabapentin was 0.025 µmol/L.

Extraction of pregabalin from brain and spinal tissue homogenate was performed by the addition of 5 parts 70% methanol to 1 part homogenate, sonication for 15 min and centrifugation (for 10 min at 20,000 g and 10°C). Plasma was de-proteinised by addition of 5 parts 70 % methanol to 1 part plasma, mixing and centrifugation (for 10 min at 20,000 g and 10°C). The remaining supernatants of the tissue and plasma were injected into the analytical column (Synergi 4u Polar, 4.6 x 100 mm). Mass analysis was carried out using Agilent Technologies 6460 Triple Quad LC/MS with electrospray ionisation in a positive mode.
Pregabalin was monitored using transition m/z 160 to m/z 142 at a fragmentor voltage of 80 V and collision energy of 5 V. The limit of detection for pregabalin was 0.025 µmol/L.

**Pharmacokinetic calculations**

The pharmacokinetic (PK) data analysis was performed using the software WinNonlin, version 5.3 (Pharsight Corporation, CA, USA). The PK data of MIV-247, pregabalin and gabapentin was analyzed using non-compartmental methodology.

The following PK parameters were reported:

- The area under the plasma concentration vs. time curve (AUC0-t) from time zero to time t was calculated by the log/linear trapezoidal method. The last sampling time-point was 7 h post dose. AUC0-t was extrapolated to infinity (AUC0-inf) by adding Clast/λz, where Clast is the last measurable plasma concentration and λz is the terminal elimination rate constant.

- Maximal plasma concentration (C_max)

- Time at maximal concentration (T_max)

- The terminal half-life (t_1/2)

The half-life was calculated as ln2/λz, where λz is the elimination rate constant. The half-life was only calculated if at least 3 data-points could be included in the regression and the r^2 for the regression was >0.80.

**Statistical analysis**

Mechanical thresholds in neuropathic pain studies were expressed as 50% paw withdrawal threshold (50% PWT) in grams (g) and statistically analysed by Repeated Measure (RM) Two-way ANOVA followed by Tukey test or Dunnett’s test. Beam-walking data were
expressed as foot slips (number), and were statistically analysed by Repeated Measure (RM) Two-way ANOVA followed by Tukey test. In partial nerve ligation studies, data are given as mean ± SEM, while pharmacokinetic data are given as mean ± SD with n reflecting the number of individual mice. In enzyme studies in vitro, Ki values are given as geometric mean ± confidence interval.
RESULTS

Characterization of MIV-247 pharmacology in vitro

MIV-247 had a mean $K_i$ (dissociation constant) value of 2.1 nmol/L for human cathepsin S and was highly selective vs. other related cathepsins (Table 1). MIV-247 was also a potent inhibitor of mouse and cynomolgus monkey cathepsin S with $K_i$ values of 4.2 nmol/L and 7.5 nmol/L, respectively (Table 1), while having moderate potency against mini-pig and dog cathepsin S. In the rat there is an important difference in the S2 pocket between strains. In human, cathepsin S residue 137 is a glycine, while this residue is a cysteine (G137C) in wild-type Sprague-Dawley and Wistar rat strains commonly used in preclinical research (Irie et al., 2008). This probably explains the 10,000-fold loss in potency on rat cathepsin S (Table 1).

The reversibility of MIV-247 binding to human cathepsin S was evaluated. A 100-fold dilution of 20 nmol/L cathepsin S and 200 nmol/L MIV-247 into a cathepsin S enzyme assay resulted in a steady-state rate of 1.16 $\Delta F/s$ (change in fluorescence per second) which is close to the steady-state rate of 1.18 $\Delta F/s$ obtained by adding 0.2 nmol/L cathepsin S to an assay containing 2 nmol/L MIV-247. Thus, the dilution experiments conclude that inhibition of cathepsin S by MIV-247 was rapid and reversible.

The selectivity of MIV-247 was also tested against a panel of receptors, ion channels, transporters and enzymes. No significant responses were noted for MIV-247 against 273 different targets at a concentration of 10 µmol/L (Medivir, data on file).

Taken together, the data generated in vitro suggest that MIV-247 is a potent, selective and reversible human cathepsin S inhibitor with similar potency against mouse cathepsin S while displaying 10,000-lower potency against rat cathepsin S. Hence, mice were the chosen species for in vivo pharmacology.
Effect of MIV-247 on PNL-evoked allodynia

Single oral dosing of MIV-247 attenuated mechanical allodynia in mice in a dose-dependent manner from 100 to 200 µmol/kg (Fig. 2A). A dose of 100 µmol/kg MIV-247 transiently attenuated mechanical allodynia while a dose of 200 µmol/kg MIV-247 resulted in sustained significant efficacy lasting at least 6 h. The maximal anti-allodynic effect of 200 µmol/kg MIV-247 (52% reversal of alldynia, p < 0.01) was reached at 6 h post dose. A higher dose of 500 µmol/kg MIV-247 resulted in similar, but not higher, anti-allodynic efficacy (data not shown). Contralateral thresholds were not affected by MIV-247 at any dose (Fig. 2B). Repeated oral dosing of MIV-247 (twice daily for 5 days) also attenuated mechanical allodynia in a dose-dependent manner when measured at 3 h post-dose in the morning (Fig. 2C). Significant reversal (ranging between 36-49%) was observed compared to vehicle on all days following oral administration of MIV-247 at the dose of 200 µmol/kg. The lower dose of 100 µmol/kg MIV-247 also affected mechanical allodynia and a significant effect was recorded on day 3 of the study (32% reversal, p < 0.05).

Effect of pregabalin and gabapentin on PNL-evoked allodynia

Single oral dosing of pregabalin and gabapentin attenuated mechanical allodynia in a dose-dependent manner (Figs. 3A and B). Doses of 188 and 377 µmol/kg (equivalent to 30 and 60 mg/kg) pregabalin resulted in sustained efficacy lasting at least 6 h (Fig. 3A), but a dose of 63 µmol/kg (10 mg/kg) was without effect. A dose of 350 µmol/kg gabapentin (60 mg/kg) resulted in sustained efficacy, while a dose of 175 µmol/kg (30 mg/kg) gabapentin resulted in transient efficacy lasting 1 h (Fig. 3B). The lowest dose of gabapentin (58 µmol/kg, 10 mg/kg) was without effect. The contralateral thresholds were not affected by pregabalin or gabapentin (data not shown).

Effect of MIV-247 and pregabalin in combination on PNL-evoked allodynia
Minimum effective doses of MIV-247 and pregabalin were evaluated alone and in combination (Fig. 4A). MIV-247 (100 µmol/kg p.o.) and pregabalin (75 µmol/kg p.o.), when given alone, transiently attenuated mechanical allodynia. The effect of MIV-247 was non-significant (22% and 29% reversal at 1 h and 3 h, respectively) while the effect of pregabalin was significant at 1 h post-dose (28% reversal, p < 0.05) but not at 3 h post dose (32% reversal, p > 0.05). When combining these compounds at the indicated doses, significant and prolonged anti-allodynic efficacy was observed with 51%, 81% and 33% reversal at 1 h, 3 h and 6 h post-dose, respectively.

Sub-effective doses of MIV-247 and pregabalin were evaluated alone and in combination (Fig. 4B). Neither MIV-247 (50 µmol/kg p.o.) nor pregabalin (38 µmol/kg p.o.) when given alone had any effect on mechanical allodynia. When combining these compounds at the indicated doses, significant anti-alldynmic efficacy with 35% reversal was observed at 3 h post-dose.

**Effects of MIV-247 and gabapentin in combination on PNL-evoked allodynia**

Minimum effective doses of MIV-247 and gabapentin were evaluated alone and in combination (Fig. 5A). MIV-247 (100 µmol/kg p.o.) and gabapentin (146 µmol/kg p.o.), when given alone, transiently attenuated allodynia 1 – 3 h post-dose. The effects of MIV-247 were not statistically significantly different from controls (22% reversal, p < 0.05) while the effect of gabapentin was significant only at the 3 h post-dose time point (34% reversal, p < 0.05). When combining these compounds at the indicated doses, significant anti-allodynic efficacy was observed for up to 8 h post-dose with 48%, 85%, 61% and 35% reversal at 1, 3, 6 and 8 h post-dose, respectively. The effect of the combination had subsided at 24 h post-dose.
Sub-effective doses of MIV-247 and gabapentin were evaluated alone and in combination (Fig. 5B). MIV-247 (50 µmol/kg p.o.) and pregabalin (73 µmol/kg p.o.) had no significant effects on allodynia when given alone as expected. When combining these compounds at the indicated doses, significant anti-allodynic efficacy with 56%, 68%, and 41% reversal at 1, 3, and 6 h post-dose was seen.

Effects of minimum effective doses of MIV-247 and pregabalin on beam walking

As expected, 584 µmol/kg (100 mg/kg) gabapentin p.o., serving as a positive control, induced a significant increase in the number of foot slips at 1 h and 3 h post dose in normal, non-injured mice (Fig. 6A). Minimal effective doses of MIV-247 (100 µmol/kg, p.o.) or pregabalin (75 µmol/kg, p.o.) or their combination did not affect the number of slips compared to vehicle (Fig. 6A). Hence, the enhanced anti-allodynic effects exerted by combining MIV-247 and pregabalin were most likely not associated with behavioural changes consistent with dizziness or ataxia.

Effects of minimum effective doses of MIV-247 and gabapentin on beam walking

As expected, 584 µmol/kg (100 mg/kg) gabapentin p.o., used as a positive control, induced a significant increase in the number of foot slips at 1 h and 3 h post dose (Fig. 6B). Minimal effective doses of MIV-247 (100 µmol/kg, p.o.) or gabapentin (146 µmol/kg, p.o.) or their combination did not affect the number of slips compared to vehicle (Fig. 6B). Hence, the enhanced anti-allodynic effects exerted by combining MIV-247 and gabapentin were most likely not associated with behavioural changes consistent with dizziness or ataxia.

Concentrations of MIV-247, pregabalin and gabapentin when given alone or in combination

The plasma concentrations of MIV-247 and pregabalin when given alone or in combination at minimal effective doses in satellite, non-injured mice are shown in Fig. 7A. The
corresponding values for MIV-247 and gabapentin are shown in Fig. 7B. The data show that combining MIV-247 with either pregabalin or gabapentin does not alter plasma exposures of either compound compared to when the compounds were given separately. The pharmacokinetic parameters for these experiments are given in Table 2.

Although not systematically measured in every experiment, the data in hand suggest that the concentrations of the different compounds in the brain and spinal cord did not appear to differ either when given alone or in combination. Table 3 shows plasma, brain and spinal cord concentrations of MIV-247 and pregabalin at 3.5 h post-dose when given at sub-effective doses to satellite mice. Table 4 shows plasma, brain and spinal cord concentrations of MIV-247 and gabapentin at 3 h post-dose when given at minimum effective doses to satellite mice and concentrations at 6 h post-dose when given to neuropathic mice. Similar concentrations were present when compounds were given separately or in combination. The ratios between brain and plasma or spinal cord and plasma concentrations were similar for all MIV-247, pregabalin and gabapentin when administered alone or in combination.
DISCUSSION

The current study demonstrates in a mouse model of neuropathic pain that cathepsin S inhibition *per se*, using the orally active, highly potent and selective inhibitor MIV-247, attenuates mechanical allodynia without any detectable behavioural side effects in mice. Enhanced anti-allodynic efficacy was observed when combining MIV-247 with gabapentin or pregabalin without evoking additional side effects and without any detectable pharmacokinetic interactions. Hence, combination of the selective cathepsin S inhibitor MIV-247 with pregabalin or gabapentin represents a means of increasing both efficacy and therapeutic window.

MIV-247 was slightly more potent than gabapentin at reversing mechanical allodynia while being slightly less potent than pregabalin when given orally. A dose of 100 µmol/kg MIV-247 was required for a minimum anti-allodynic effect vs. 146 µmol/kg gabapentin and 75 µmol/kg pregabalin. The rank order of potency was different when comparing the plasma levels required for minimal efficacy. Minimal effective doses of MIV-247 gave rise to plasma levels of 8-12 µmol/L x h while the corresponding exposures for gabapentin and pregabalin were 68-69 and 86-92 µmol/L x h, respectively. Interestingly, a $C_{max}$ of approximately 16 µmol/L gabapentin is reached in man at 3 h post-dose in response to 300 mg gabapentin (Chang et al., 2014), while a $C_{max}$ of approximately 40 µmol/L pregabalin is reached in man at 1 h post-dose in response to 200 mg pregabalin (Brodie et al., 2005). This is similar to the plasma levels of gabapentin and pregabalin reached in our study at minimum effective doses (Table 2) suggesting that the exposure of gabapentin in the current study is of clinical relevance. It is thus tempting to speculate that the same degree of efficacy could be reached at lower plasma levels of gabapentin or pregabalin when given together with MIV-247, thereby widening the therapeutic window (see discussion below). However, clinical studies will be needed to prove a role for cathepsin S in neuropathic pain and the mechanical allodynia endpoint used in the
current study is not the only clinical symptom in patients. Assuming that the central nervous compartment is the main site of action then MIV-247 is even more potent in comparison since approximately 10-20% of MIV-247 enters the central nervous system (measured in brain and spinal cord) while approximately 50% of pregabalin/gabapentin reached the CNS. Indeed, Table 4 demonstrates that sub-effective threshold doses of MIV-247 gave rise to 0.1 - 0.2 µmol/L concentrations in the spinal cord and brain while corresponding levels for pregabalin were 2 - 3 µmol/L. This superior potency is perhaps not surprising since MIV-247 has a Ki of 4.2 nmol/L against mouse cathepsin S while gabapentin is estimated at having an affinity of approximately 100 nmol/L (depending on splice variant) against the α2δ-1 subunit of voltage-gated calcium channels (Lana et al., 2014).

While MIV-247 was more potent (based on plasma levels) in this model of neuropathic pain, gabapentin and pregabalin displayed higher maximal efficacy. Gabapentin and pregabalin completely reversed mechanical allodynia while MIV-247 resulted in a maximal 50% reversal which was not further increased by raising the dose or by repeated dosing twice daily for 5 days. However, the doses of gabapentin and pregabalin required for maximal efficacy were close to doses associated with side effects which could confound anti-allodynic effects. Indeed, in separate beam walking experiments, 350 µmol/kg gabapentin treatment significantly reduced time to cross the beam indicating signs of dizziness (data not shown). By contrast, although 200 µmol/kg MIV-247 reversed allodynia by only 50%, this was achieved without any obvious side effects. MIV-247 has been run in the neurobehavioural Irwin test battery in mouse at up to 1000 µmol/kg without any side effects being noted (Medivir, data on file).

It is unknown why an approximate 50% reversal appears to be the maximal attainable effect in response to cathepsin S inhibition in this model. Interestingly, this is virtually identical to the results from others who have demonstrated that cathepsin S inhibitors produced
approximately 50% reversal of mechanical hyperalgesia in a similar neuropathic pain model in rats (Clark et al., 2007; Irie et al., 2008). The maximal effect in the current study was probably not due to saturable pharmacokinetic limitations since gradually higher doses of MIV-247 did give rise to higher exposures in the central nervous system and plasma without increasing efficacy. Hence, it appears as if at least a part of the developed mechanical allodynia in this model is independent of cathepsin S.

The first set of experiments demonstrated that gabapentin and pregabalin are agents with low potency (high plasma and CNS levels required) and a narrow therapeutic window but with an apparent high maximal efficacy. By contrast, MIV-247 was a highly potent agent with a wide therapeutic window while reaching a half-maximal effect. In addition, the agents most likely have different mechanisms of action with gabapentin/pregabalin reducing the release of nociceptive transmitters from the central terminals of primary afferent fibres whereas MIV-247 is presumed to reduce the facilitation of nociceptive transmission indirectly in the dorsal horn by inhibiting microglia activity. Considering these two distinct profiles, it was of interest to combine the two agents.

The magnitude of anti-allodynic efficacy was markedly increased when combining minimum effective doses of gabapentin/pregabalin and MIV-247 compared to when either agent was given alone. No significant effects on beam walking were detected in response to the combinations. Since we were able to achieve improved efficacy without detectable side effects, we combined the two agents at lower sub-effective doses. Interestingly, despite neither agent having any detectable efficacy when given alone, their combined effect was significant. For the MIV-247:pregabalin combination at sub-effective doses, anti-allodynic efficacy was significant at 3 h post-dose, whereas the sub-effective MIV-247:gabapentin combination resulted in significant efficacy at several time points post dose. Furthermore, the compounds did not seem to achieve their improved efficacy by interfering with each other’s
pharmacokinetics since plasma and CNS exposures after the experiment appeared similar when given in combination as when given alone. Thus, it is highly likely that the improved efficacy is due to positive pharmacological interactions.

When combining two agents in preclinical pharmacological studies as done herein, a frequently asked question is if the combined effect is additive or synergistic. Often, investigators will construct so-called isobolograms by plotting dose levels of the two agents on the x- and y-axes respectively (see Tallarinda, 2006 for a review). Although the question is interesting for instance in a clinical study where only a few approved dosages of each compound can be given, we feel it is not relevant whether given oral doses of agents are synergistic or additive in preclinical studies like the current one. In preclinical studies, endless combinations can be given and the actual given dose is thus not of interest, the question should rather be whether the pharmacological effects per se may cause additive or synergistic effects. For that analysis to be made, one needs to know the actual concentration of test agent reaching the anticipated site of action. Since measuring compound levels in plasma or target tissue is seldom (if ever) measured in preclinical combination studies, a detailed analysis if two pharmacological actions are synergistic is beyond the scope of the current study. Instead, we considered it was more relevant to evaluate if a given combination enhanced efficacy without enhancing side effects or exposures of the agents. This seems to be achievable by combining agents like pregabalin and gabapentin with MIV-247 in the current study.

Preclinical data generated so far suggest that a major substrate for cathepsin S in the dorsal horn of the spinal cord is the chemokine fractalkine. Cathepsin S liberates soluble fractalkine and mechanical allodynia evoked by spinal administration of exogenous cathepsin S is abolished in mice lacking the CX3CR1 receptor which mediates fractalkine signaling (Clark et al., 2007; 2009). However, it is possible that inhibition of cathepsin S in the periphery may also contribute and increase the effects of pregabalin and gabapentin. Recently, cathepsin S
has been shown to activate PAR2 receptors (Reddy et al., 2010; Elmariah et al., 2014; Zhao et al., 2014), and cathepsin S-induced colonic hyperalgesia in the periphery was suggested to be mediated via PAR2 receptors (Cattaruzza et al., 2011). Less is known about PAR2 receptors in the spinal cord, but specific PAR2 receptor agonists cause mechanical and spinal hyperalgesia when administered intrathecally (Alier et al., 2008), possibly via inhibition of inhibitory GABAergic neurotransmission (Huang et al., 2011).

Given the multi-mechanistic nature of neuropathic pain, it is unlikely that one agent will be optimally effective clinically. Although many studies have evaluated anti-nociceptive drugs in combination on pain-related end-points in preclinical models, few have compared therapeutic interactions together with possible pharmacokinetic and adverse effect interactions. The current study shows that MIV-247 exerts anti-allodynic efficacy without any detectable neurobehavioural side effects in a mouse model of neuropathic pain and also enhances the anti-allodynic effect of gabapentin and pregabalin without enhancing side effects or compound exposures. Cathepsin S inhibition offers a new mechanism of action for the treatment of neuropathic pain, either alone or in combination with established therapeutics such as gabapentin and pregabalin.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hewitt, Pitcher, Tunblad, Rizoska, Malcangio, Lindström

Conducted experiments: Pitcher, Henderson, Sahlberg

Contributed new reagents or analytical tools: Grabowska, Classon

Performed data analysis: Pitcher, Tunblad, Henderson, Sahlberg, Lindström

Wrote or contributed to the writing of the manuscript: Hewitt, Pitcher, Rizoska, Tunblad, Edenius, Malcangio, Lindström
REFERENCES


FOOTNOTES

CONFLICT OF INTEREST

EH, BR, KT, IH, BLS, UG, BC, CE were EL employees at Medivir the time of the studies.

TP was sponsored by Medivir. MM was a consultant for Medivir.
LEGENDS FOR FIGURES

Figure 1. Molecular structure of MIV-247.

Figure 2. Effect of oral MIV-247 on mechanical allodynia in the mouse PNL model. (A): ipsilateral and (B): contralateral paw withdrawal thresholds (50% PWT) after single oral dosing and (C): ipsilateral thresholds (50% PWT) after repeat oral dosing twice daily for 5 days. Data are from 3 h post-dose in the morning. Mean ± SEM, n = 8 in each group. *p<0.05, **p<0.01, ***p<0.001. Repeated Measure Two-way ANOVA followed by Dunnett’s test.

Figure 3. Effect of oral administration of pregabalin and gabapentin on mechanical allodynia in the mouse PNL model. Paw withdrawal thresholds (50% PWT) after treatment with (A): pregabalin and (B): gabapentin. Mean ± SEM, n = 8 in each group. *p<0.05, **p<0.01, ***p<0.001. Repeated Measure Two-way ANOVA followed by Dunnett’s test.

Figure 4. Effect of oral administration of MIV-247 and pregabalin alone or in combination on mechanical allodynia in the mouse PNL model. (A): Paw withdrawal thresholds (50% PWT) in response to 100 µmol/kg MIV-247 or 75 µmol/kg pregabalin or when given in combination. (B): Paw withdrawal thresholds (50% PWT) in response to 50 µmol/kg MIV-247 or 38 µmol/kg pregabalin or when given in combination. Mean ± SEM, n = 8 in each group. *p<0.05, ***p<0.001. Repeated Measure Two-way ANOVA followed by Dunnett’s test.

Figure 5. Effect of oral administration of MIV-247 and gabapentin alone or in combination on mechanical allodynia in the mouse PNL model. (A): Paw withdrawal thresholds (50% PWT) in response to 100 µmol/kg MIV-247 or 146 µmol/kg gabapentin or when given in combination. (B): Paw withdrawal thresholds (50% PWT) in response to 50 µmol/kg MIV-247 or 73 µmol/kg pregabalin or when given in combination. Mean ± SEM, n = 8 in each
group. *p<0.05, **p<0.01, ***p<0.001. Repeated Measure Two-way ANOVA followed by Dunnett’s test.

Figure 6. (A): Effect of oral administration of MIV-247 (100 µmol/kg) and pregabalin (75 µmol/kg) alone or in combination on beam walking (foot slips). (B): Effect of MIV-247 (100 µmol/kg) and gabapentin (146 µmol/kg) alone or in combination on beam walking (foot slips). Mean ± SEM, n = 10 in each group. ***p<0.001. Repeated Measure Two-way ANOVA followed by Tukey’s test. High-dose gabapentin (584 µmol/kg, p.o.) was used as a positive control.

Figure 7. (A): plasma levels of pregabalin when given alone (75 µmol/kg p.o., open circles) or when given together with MIV-247 (100 µmol/kg p.o., closed circles). Plasma levels of MIV-247 when given alone (100 µmol/kg p.o., open squares) or when given together with pregabalin (75 µmol/kg p.o., closed squares). (B): plasma levels of gabapentin when given alone (146 µmol/kg p.o., open circles) or when given together with MIV-247 (100 µmol/kg p.o., closed circles). Plasma levels of MIV-247 when given alone (100 µmol/kg p.o., open squares) or when given together with gabapentin (146 µmol/kg p.o., closed squares). Mean ± SEM, n = 3.
Table 1. Potency of MIV-247 against various enzymes.

<table>
<thead>
<tr>
<th>Recombinant enzyme</th>
<th>Species</th>
<th>Geometric mean $K_i$ (nM)</th>
<th>Geometric 95% confidence</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin S</td>
<td>Human</td>
<td>2.1</td>
<td>1.9 - 2.3</td>
<td>46</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td></td>
<td>420</td>
<td>350 - 500</td>
<td>46</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td></td>
<td>3100</td>
<td>2700 - 3700</td>
<td>46</td>
</tr>
<tr>
<td>Cathepsin V</td>
<td></td>
<td>6300</td>
<td>5100 - 7700</td>
<td>2</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td></td>
<td>14000</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td></td>
<td>&gt;200000</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td>&gt;200000</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Chymotrypsin</td>
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<td>&gt;200000</td>
<td></td>
<td>2</td>
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<tr>
<td>Neutrophil elastase</td>
<td></td>
<td>&gt;200000</td>
<td></td>
<td>2</td>
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<tr>
<td>Cathepsin S</td>
<td>Mouse</td>
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<td>2.3 - 7.7</td>
<td>2</td>
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<tr>
<td>Cathepsin S</td>
<td>Cynomolgus</td>
<td>7.5</td>
<td>1.3 - 44</td>
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<td>Cathepsin S</td>
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<td>60</td>
<td>55 - 65</td>
<td>10</td>
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<td>Cathepsin S</td>
<td>Dog</td>
<td>180</td>
<td>160 - 210</td>
<td>10</td>
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<tr>
<td>Cathepsin S (Gly isoform)</td>
<td>Rat</td>
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<tr>
<td>Cathepsin S (Cys isoform)</td>
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<td>17000 - 30000</td>
<td>2</td>
</tr>
<tr>
<td>Cathepsin K</td>
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<td>9100</td>
<td>7900 - 11000</td>
<td>2</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td></td>
<td>4500</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

*IC$_{50}$

Where no confidence interval is given the replicate values were identical.
Table 2. Pharmacokinetic parameters of MIV-247, pregabalin and gabapentin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Compound assessed</th>
<th>Dose (µmol/kg)</th>
<th>Tmax (h)</th>
<th>Cmax (µmol/L)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µmol/L*h)</th>
<th>AUC&lt;sub&gt;0-inf&lt;/sub&gt; (µmol/L*h)</th>
<th>t½ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIV-247</td>
<td>MIV-247</td>
<td>100</td>
<td>0.50 ± 0.0</td>
<td>8.7 ± 2.9</td>
<td>12 ± 3.7</td>
<td>13 ± 3.7</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>MIV-247 &amp; Pregabalin</td>
<td>MIV-247</td>
<td>100 &amp; 75</td>
<td>0.42 ± 0.1</td>
<td>11 ± 4.9</td>
<td>11 ± 5.2</td>
<td>12 ± 6.3</td>
<td>2.5 ± 1.3</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>Pregabalin</td>
<td>75</td>
<td>0.25 ± 0.0</td>
<td>37 ± 7.9</td>
<td>92 ± 15</td>
<td>93 ± 15</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>MIV-247 &amp; Pregabalin</td>
<td>Pregabalin</td>
<td>100 &amp; 75</td>
<td>0.58 ± 0.4</td>
<td>47 ± 10</td>
<td>86 ± 16</td>
<td>87 ± 14</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>MIV-247</td>
<td>MIV-247</td>
<td>100</td>
<td>0.42 ± 0.1</td>
<td>7.5 ± 0.1</td>
<td>9.4 ± 3.0</td>
<td>11 ± 0.4</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin</td>
<td>MIV-247</td>
<td>100 &amp; 146</td>
<td>0.67 ± 0.3</td>
<td>6.5 ± 0.3</td>
<td>8.8 ± 2.2</td>
<td>9.2 ± 2.4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Gabapentin</td>
<td>146</td>
<td>0.51 ± 0.0</td>
<td>29 ± 4.6</td>
<td>68 ± 7.3</td>
<td>69 ± 7.6</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin</td>
<td>Gabapentin</td>
<td>100 &amp; 146</td>
<td>0.58 ± 0.4</td>
<td>28 ± 6.9</td>
<td>69 ± 8.2</td>
<td>72 ± 7.8</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n=3 per group. Data are from non-injured mice used for pharmacokinetics.
Table 3. Plasma, brain and spinal cord levels of MIV-247 and pregabalin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Compound assessed</th>
<th>Dose (µmol/kg)</th>
<th>Time (h)</th>
<th>Cplasma (µmol/L)</th>
<th>Cbrain (µmol/kg)</th>
<th>Cspinal cord (µmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIV-247</td>
<td>MIV-247</td>
<td>50</td>
<td>3.5</td>
<td>0.88 ± 0.5</td>
<td>0.20 ± 0.2</td>
<td>0.14 ± 0.06</td>
</tr>
<tr>
<td>MIV-247 &amp; Pregabalin</td>
<td>MIV-247</td>
<td>50 &amp; 38</td>
<td>3.5</td>
<td>0.51 ± 0.2</td>
<td>0.10 ± 0.02</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>Pregabalin</td>
<td>38</td>
<td>3.5</td>
<td>5.3 ± 2.1</td>
<td>3.1 ± 0.9</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>MIV-247 &amp; Pregabalin</td>
<td>Pregabalin</td>
<td>50 &amp; 38</td>
<td>3.5</td>
<td>7.0 ± 1.8</td>
<td>3.2 ± 0.5</td>
<td>1.9 ± 0.4</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n=3 per group. Animals are from satellite non-injured animals used for pharmacokinetics.
Table 4. Plasma, brain and spinal cord levels of MIV-247 and gabapentin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Compound assessed</th>
<th>Dose (µmol/kg)</th>
<th>Time (h)</th>
<th>Cplasma (µmol/L)</th>
<th>Cbrain (µmol/kg)</th>
<th>Cspinal cord (µmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIV-247 (satellite)</td>
<td>MIV-247</td>
<td>100</td>
<td>3</td>
<td>1.2 ± 0.3</td>
<td>0.20 ± 0.06</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin (satellite)</td>
<td>MIV-247</td>
<td>100 &amp; 146</td>
<td>3</td>
<td>0.46 ± 0.05</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>MIV-247 (main)</td>
<td>MIV-247</td>
<td>100</td>
<td>6</td>
<td>0.15 ± 0.07</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin (main)</td>
<td>MIV-247</td>
<td>100 &amp; 146</td>
<td>6</td>
<td>0.19 ± 0.1</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Gabapentin (satellite)</td>
<td>Gabapentin</td>
<td>146</td>
<td>3</td>
<td>14 ± 3.3</td>
<td>18 ± 2.6</td>
<td>18 ± 5.0</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin (satellite)</td>
<td>Gabapentin</td>
<td>100 &amp; 146</td>
<td>3</td>
<td>10 ± 2.6</td>
<td>10 ± 2.1</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>Gabapentin (main)</td>
<td>Gabapentin</td>
<td>146</td>
<td>6</td>
<td>3.3 ± 2.0</td>
<td>3.2 ± 0.7</td>
<td>4.2 ± 1.8</td>
</tr>
<tr>
<td>MIV-247 &amp; Gabapentin (main)</td>
<td>Gabapentin</td>
<td>100 &amp; 146</td>
<td>6</td>
<td>2.3 ± 1.4</td>
<td>2.4 ± 1.7</td>
<td>2.7 ± 1.7</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n=3 per group. Satellite: data collected from un-injured mice used in pharmacokinetic experiments. Main: data collected from neuropathic mice after experiment.
Figure 2

A

Vehicle
100 µmol/kg MIV-247
200 µmol/kg MIV-247

50% PWT (g)

Time post dose (h)

B

Vehicle
100 µmol/kg MIV-247
200 µmol/kg MIV-247

50% PWT (g)

Time post dose (h)

C

Vehicle
100 µmol/kg MIV-247
200 µmol/kg MIV-247

50% PWT (g)

Day of dosing

*** ** *
Figure 3

A

- 377 μmol/kg pregabalin
- 188 μmol/kg pregabalin
- 63 μmol/kg pregabalin
- Vehicle

B

- 350 μmol/kg gabapentin
- 175 μmol/kg gabapentin
- 58 μmol/kg gabapentin
- Vehicle

Time post dose (h)

50% PWT (g)
Figure 4

A

- MIV-247+pregabalin
- 75 μmol/kg pregabalin
- 100 μmol/kg MIV-247
- Vehicle

Time post dose (h)

50% PWT (g)

B

- MIV-247+pregabalin
- 38 μmol/kg pregabalin
- 50 μmol/kg MIV-247
- Vehicle

Time post dose (h)

50% PWT (g)
Figure 5

A

- MIV-247 + gabapentin
- 146 μmol/kg gabapentin
- 100 μmol/kg MIV-247
- Vehicle

50% PWT (g)

Time post dose (h)

B

- MIV-247 + gabapentin
- 73 μmol/kg gabapentin
- 50 μmol/kg MIV-247
- Vehicle

50% PWT (g)

Time post dose (h)
Figure 6

A

- 584 µmol/kg gabapentin
- MIV-247 + pregabalin
- 75 µmol/kg pregabalin
- 100 µmol/kg MIV-247
- Vehicle

B

- 584 µmol/kg gabapentin
- MIV-247 + gabapentin
- 146 µmol/kg gabapentin
- 100 µmol/kg MIV-247
- Vehicle
Figure 7

A  
- Pregabalin
- Pregabalin (+ MIV-247)
- MIV-247
- MIV-247 (+ Pregabalin)

Plasma concentration (μmol/L)

Time after dose (h)

B  
- Gabapentin
- Gabapentin (+ MIV-247)
- MIV-247
- MIV-247 + (gabapentin)

Plasma concentration (μmol/L)

Time after dose (h)