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sec-Butylpropylacetamide (SPD), a New Amide Derivative of Valproic Acid for the Treatment of Neuropathic and Inflammatory Pain

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Abstract

Chronic pain is a multifactorial disease comprised of both inflammatory and neuropathic components that affect ~20% of the world's population. *sec*-Butylpropylacetamide (SPD) is a novel amide analogue of valproic acid (VPA) previously shown to possess a broad spectrum of anticonvulsant activity. In this study we defined the pharmacokinetic parameters of SPD in rat and mouse, and then evaluated its antinociceptive potential in neuropathic and acute inflammatory pain models. In the sciatic nerve ligation (SNL) model of neuropathic pain, SPD was equipotent to gabapentin and more potent than its parent compound VPA. SPD also showed either higher or equal potency to VPA in the formalin, carrageenan and writhing tests of inflammatory pain. SPD showed no effects on compound action potential properties in a sciatic nerve preparation,

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DK, PJW, MDS and HSW designed the experiments; DK performed the experiments; DK, MB, MD, KCB, HSW performed analysis and interpretation of the experiments; DK, PJW, MDS, MB, BY, MD, KCB, and HSW wrote the manuscript.

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suggesting that its mechanism of action is distinct from local anesthetics and membrane stabilizing drugs. SPD's activity in both neuropathic and inflammatory pain warrants its development as a potential broad-spectrum anti-nociceptive drug.

Graphical abstract

Keywords

sec-Butylpropylacetamide (SPD); pharmacokinetics; Chung model; compound action potentials; formalin test; carrageenan test; writhing test

1. Introduction

Chronic pain affects 10–55% [1] of the world's population, causing significant morbidity [2]. Pharmacotherapy for chronic pain is only partially effective and suffers from severe side effects and abuse potential [2, 3]. Neuropathic pain is caused by traumatic, inflammatory or dysmetabolic lesions to the central or peripheral nervous system [4]. Traditionally, changes in neuronal or synaptic physiology have been advanced as mechanisms of neuropathic pain [5], however many studies indicate that the traditional focus on these factors provides an incomplete picture [6–9]. Neuropathic pain is typically treated with membrane stabilizing agents (e.g. antiepileptic drugs (AEDs)) or local anesthetics (e.g. lidocaine), that aim to modulate ion channel activity [10, 11]. However, this strategy has met with limited success, suggesting the involvement of other, possibly inflammatory, mediators in chronic pain states [5, 7, 9]. Inflammatory mechanisms are known to contribute significantly to the development of chronic neuropathic pain [8, 10, 12]. That said, non-steroidal anti-inflammatory drugs (NSAIDs) have limited efficacy for neuropathic pain conditions [13] and are not included in the pain treatment guidelines [14, 15]. Opioids are indicated for both inflammatory and neuropathic pain, although their efficacy in neuropathic pain is still controversial [16] and they carry the risk of potential tolerance and have a high addiction liability [17]. This supports the need for further development of more tolerable broad spectrum pain medications that target both the inflammatory and neuropathic components of chronic pain.

sec-Butylpropylacetamide (SPD) (Fig. 1) is a second generation amide derivative of valproic acid (VPA, Fig. 1), previously found to be active in numerous animal seizure models [18,

19]. Epilepsy and neuropathic pain are thought to share some aspects of their underlying pathophysiology [11], and some AEDs are used for the treatment of neuropathic pain (e.g. gabapentin) [20, 21]. Owing to its broad-spectrum and promising effect as an anticonvulsant compound we evaluated SPD's potential in both neuropathic and acute inflammatory pain models. In this work we show SPD to be active in the spinal nerve ligation (SNL) model for neuropathic pain and three models of acute inflammatory pain. Compared to its parent compound VPA, SPD had higher potency in the SNL model and a higher or equivalent potency in the inflammatory pain models. Its effects suggest a different pharmacological profile than either conventional membrane stabilizers or NSAIDs. We also evaluated SPD's pharmacokinetic profile to demonstrate its favorable PK profile and to explore its pharmacokinetic (PK) – pharmacodynamics (PD) correlation. Our results support further evaluation of SPD for the treatment of refractory pain.

2. Materials and Methods

All experiments, analysis, and reporting were performed according to ARRIVE criteria [22].

2.1. Animals

All acute inflammatory pain experiments were conducted using male CF-1 mice (20–30g, Charles River laboratories Raleigh, NC). Neuropathic pain models were performed on male Sprague-Dawley rats (200–250g, Harlan laboratories, Jerusalem). The Electrophysiology recordings were done *in vitro* on acutely dissected sciatic nerves from male Sprague Dawley rats (300–450g). Animals were housed in plexiglass cages in an Institutional Animal Care and Use Committee (IACUC) approved animal facility and were allowed free access to food (product 8640 Harlan Teklad, WI, USA) and water. All animals were maintained in a temperature, humidity and 12:12h light:dark cycle controlled environment. Every attempt was made to minimize the pain experienced by experimental animals. All experiments were approved by the University of Jerusalem and University of Utah's IACUC.

2.2. Materials used

sec-Butylpropylacetamide (SPD), N-methyl tetramethylcyclopropyl acetamide (MTMCD) used as a positive control in the SNL model, and N-methyl valnoctamide (N-methyl VCD) used as internal standard in the PK studies were synthesized according to a previously published procedure. [19].

Formalin solution, 37% v/v in water (10%–15% in methanol), glacial acetic acid, methyl cellulose (MC, 4000 Centipoise), λ -carrageenan, Ibuprofen sodium salt, sodium valproate and sodium pentobarbital were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Lidocaine hydrochloride was purchased from Spectrum Chemicals Mfg. Corp. (Gardena, CA, USA). Both SPD and VPA were suspended in 0.5% MC before they were injected i.p. to mice, at doses lower than their TD₅₀ values previously evaluated (88 mg/kg and 391 mg/kg i.p. for SPD and VPA respectively [18]).

2.3. Spinal nerve ligation (SNL) model for neuropathic pain

Prior to surgery rats were evaluated twice for their tactile allodynia threshold, 1 and 2 days before surgery. All rats included in the experiment had pain threshold >15g in both hind paws before surgery. The surgical procedure used to produce allodynia was previously described by Kim and Chung [23]. Briefly, rats were anesthetized following i.p. administration of 85 mg/kg ketamine and 15 mg/kg xylazine. With the rats in prone position the paraspinal muscles on the left were carefully separated from the L4 to the S2 transverse processes followed by removal of the L6 transverse process in order to visualize the L5–L6 spinal nerves. These were tightly ligated with a 5-0 silk thread and cut distal to the ligature. The paraspinal muscles were closed with sutures and then the skin was closed with Michel clips. A bacteriostatic powder was then applied topically followed by intramuscular administration of ampicillin. Recovery after surgery lasted 5 days before commencement of the behavioral tests. The rat's foot withdrawal in response to tactile stimulus was used to detect tactile allodynia using a set of nine nylon von-Frey filaments (VFF). VFF used produced an initial bending force of (in mN): 5.8, 13.7, 19.6, 39.2, 58.7, 78.3, 97.9, 146.9, and 254.5, equivalent to a mass of (in grams): 0.6, 1.4, 2, 4, 6, 8, 10, 15, and 26. The same set was calibrated and used in all experiments. SPD was administered at 40, 60, and 80 mg/kg vs. negative and positive controls (MC and MTMCD respectively) intraperitoneally (i.p.) at 7, 14 and 21 days post-surgery using a Latin square design protocol where the experimenter who performed the behavioral tests was not aware of the dose or substance given to the animals tested. The VFF were applied briefly just before and 30, 60, 120, 180, 240 minutes after injection at 1–2 seconds interval to the mid plantar skin of the hind paw. Stimulation began with the 0.6g VFF, using a perpendicular force to the skin that was just sufficient to bend the monofilament. If the animal failed to respond with a brief paw withdrawal to at least 3 out of 5 stimuli the next monofilament was tested using an ascending staircase protocol. The response threshold was set as the average of the minimal force required to obtain a criterion response on the two repeats. Rats were considered "protected" from allodynia if they failed to respond to the 15g VFF (considered 100% protection).

2.4. Formalin test

Formalin test was performed according to a previous published paradigm [24, 25]. A total of ten groups, each having eight mice per group, were used in the study; a control group (MC) was paired with each of the five dose groups. Mice were placed in cylindrical plexiglass chambers (6" tall x 4" diameter) for a 15 min acclimation period followed by i.p. injection of either MC or test compound. SPD and VPA were administered i.p. to mice at 35 and 70 mg/kg for SPD and 70, 100 and 300 mg/kg for VPA. Fifteen minutes after i.p. injection, (anti-seizure time to peak effect (TPE) for both SPD and VPA [18]), 20 µl of formalin solution (5% v/v solution in double-distilled water) was injected into the plantar region of each mouse's left paw. Time spent licking or biting their injected paw was measured for the first two minutes of each five minute interval over a total observation period of forty minutes following formalin injection. Two animals were observed in each trial and a total of eight animals were tested per dose. Licking time was quantified as the area under the curve (AUC) divided in two phases for analysis: the acute (0–10 min) and inflammatory (10–40 min)

phases, normally seen in this test [26]. Results are reported as % control AUC for each phase. The experimenter who performed the procedure was blinded to the treatment groups.

2.5. Carrageenan test

Heat hyperalgesia was evaluated for control, SPD and VPA treated mice 3h following subcutaneous (s.c.) plantar injection of carrageenan using the Hargreaves test [27]. Four doses were chosen for evaluation of SPD's activity (10, 30, 50, and 70 mg/kg; n=14 in the 10, 30 mg/kg SPD; n=24 in the 50, 70 mg/kg SPD). VPA was evaluated at 100 mg/kg (n=23). Animals were acclimated to the hot plate (maintained at 30°C) for one hour before carrageenan injection. Each mouse was injected with 25 µl of Carrageenan solution (20 mg/ml w/v, in saline) s.c. into the plantar area of their left hind paw and were returned to the warmed plate for an additional 2h before evaluation of heat hyperalgesia. Two control groups were used: a negative control group in which mice received a plantar injection of saline followed by an i.p. injection of MC (Sal/MC, n=30); a positive control group in which mice received a plantar injection of carrageenan followed by i.p. injection of MC (Carr/MC, n=40). Fifteen minutes before evaluation of heat hyperalgesia (corresponding to their TPE [18]), mice received an i.p. injection of either MC, SPD or VPA and were returned to the hot plate apparatus. A light beam set to 35% intensity (Hargreaves Plantar test model 400, IITC Life Sciences, Woodland Hills, CA) was aimed at the center of the hind paw and the latency of the injected hind paw to flinch from the radiant heat source was observed and recorded. This was defined as the withdrawal latency of the measured hind paw. The flinch was often accompanied by licking of the injected hind paw. Latency to flinch was measured twice in both the injected (ipsilateral) and un-injected (contralateral) paws and the mean of two consecutive recordings spaced 5 min apart, served as a representative mean withdrawal time for each paw. In each experiment, two groups of up to eight animals per group were tested sequentially over a period of 30 min. The normalized mean withdrawal time for each treatment was calculated by subtracting the mean withdrawal latency of the un-injected paw from the mean withdrawal latency of the injected paw. One hour following commencement of testing, paw thickness for both hind paws was recorded as a measure of edema using a caliper. The experimenter was blinded to the treatment groups.

2.6. Carrageenan induced paw edema (24h)

SPD (70 mg/kg) was evaluated for its effect on carrageenan induced paw edema vs. Ibuprofen (100 mg/kg) as a positive control [28]. Twenty male CF-1 mice (20–35 g) were used in this study equally and randomly divided to four groups: two drug treated (SPD/Carr, Ibu/Carr) and two control groups (MC/Carr, MC/Saline). SPD and Ibuprofen vs. MC were injected i.p. two hours before intra-plantar injection of 25 µl λ-carrageenan. A negative control group i.p. injected with MC and intraplantar injection of saline (MC/Saline) was evaluated as well. Paw thickness of both the injected and un-injected paws were recorded using a caliper over a 24h period immediately before (baseline) and after plantar injection of either carrageenan or saline at eight time points: 0.5, 1, 2, 3, 4, 6, 8 and 24 hours. Percent change in paw edema was calculated as paw thickness measured in each time point divided by baseline paw thickness.

2.7. Acetic acid-induced abdominal constriction (Writhing) test

SPD (10, 30, 50 and 70 mg/kg) and VPA (10, 50 and 100 mg/kg) were evaluated for their ability to block acetic-acid induced abdominal constriction [29]. Eleven male CF-1 mice (20–30g) were used in each of the groups. Before administration of either control or test substance, the mice were allowed to acclimatize for 15 minutes in a Plexiglass chamber (6" tall x 4" diameter). Each of the compounds tested were mixed with 1.5 mg/ml of methylene blue in order to verify the injection site. Each mouse received an i.p. injection of either control or test compound. Fifteen minutes later, a contralateral injection of 0.6% v/v acetic acid solution in saline was injected into the peritoneal cavity at a volume of 0.1 ml for 10g body weight, corresponding to the compound's TPE [18]. Mice were placed in the transparent round Plexiglas chambers and were observed for the occurrence of abdominal constriction (writhing). A writhing was defined as a contraction of the abdominal muscles accompanied by elongation of the body and extension of the hind limbs. The individual writhing response was considered terminated when the animal returned to normal posture. The number of writhes in a five minute epoch was recorded for a total of 30 minutes. The experimenter was blinded to the treatment group tested.

2.8. Sciatic nerve recording

Compound action potentials (CAPs) were recorded using a modified sucrose gap technique [30, 31]. Male naïve Sprague Dawley rats (300–450 g) were deeply anesthetized by 60 mg/kg, i.p. sodium pentobarbital and sacrificed by decapitation. The sciatic nerve was removed from its proximal spinal bifurcation of the L4 and L5 branches to its distal trifurcation to the tibial sural and common peroneal branches, while constantly maintaining a hydrated environment using HEPES buffered artificial cerebrospinal fluid (ACSF) (NaCl 130 mM, KCl 3 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, HEPES 10 mM, D(±)-Glucose 20 mM). The excised sciatic nerve was mounted on a Sylgard chamber divided by vasoline gaps and perfused with ACSF at a temperature of 20–25°C. Low and high frequency stimulation protocols were evaluated and stimulation was provided via an A395 linear stimulus isolator (WPI, FL) using platinum/iridium wire electrodes. Recordings were made with a P55 differential A/C preamplifier (Grass Instruments, RI) with bandpass filter settings of 1 Hz and 1 kHz. Supramaximal stimulation (50 V) was delivered using a low frequency stimulation protocol (tonic stimulation: 1ms duration, 0.2 Hz frequency) or a high frequency stimulation protocol (phasic stimulation: 0.1ms, 40 Hz train frequency, 700 ms train duration). A 15 min baseline recording was obtained followed by a 15 min perfusion with 1 mM or 3 mM of SPD for tonic stimulations and a 25 min perfusion for phasic stimulations. All experiments were concluded by administering a 15 or 25 min perfusion of 1mM lidocaine as a positive control in the tonic and phasic stimulation protocols, respectively. With the exception of 3mM SPD all compounds were dissolved in ACSF, 3mM SPD was dissolved in 1% ethanol in ACSF. CAPs for the A and C fibers were determined based on the difference in their conduction velocity and stimulation thresholds. Peak to peak area ratio was calculated for the A and C fibers in the tonic stimulation protocol, and for the first and last CAPs seen in the phasic stimulation protocol for baseline and following each treatment. Percent change in amplitude between first and last CAPs in the phasic stimulation protocol (f) was calculated using the following formula:

Equation

An average of 10 consecutive traces was calculated at the end of each treatment and was normalized to baseline for comparison. All experiments were performed at room temperatures i.e., 20–25°C.

2.9. Pharmacokinetic (PK) study

SPD Plasma concentrations vs. time were evaluated following i.p. injection of 80 mg/kg SPD to both mice and rats. SPD was suspended in 0.5% MC and i.p. injected to twenty eight mice, and twenty four rats. Four mice and four rats were used for each time points. The choice of dose was based on the maximal effective dose for SPD. Following i.p. administration of the compound, 0.5–1.0 ml blood was withdrawn from the aorta into heparinized tubes in mice, and 5 ml was withdrawn via cardiac puncture in rats, immediately following sacrifice of the animal. Blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 2.5 and 4 hours in mice and 0.25, 0.5, 1, 1.5, 2, and 4 hours in rats after administration of the compound, and were centrifuged at 3000g for 10 minutes. Plasma was separated from blood and stored at –80°C until analyzed.

2.10. Determination of median effective dose (ED₅₀)

The ED₅₀ value was defined as the dose that produced antinociception in 50% of the animals tested. The Lichtfield & Wilcoxon II procedure was used to calculate the ED₅₀ value from the corresponding quantal dose-response curve [32]. The percent responders were calculated for each dose as the number of animals which displayed 50% or more change in behavior compared to control animals thereby permitting the establishment of dose-response curves. The data was subjected to probit analysis [33] and the 95% confidence interval (CI) was calculated for both SPD and VPA.

2.11. SPD plasma concentration analysis

SPD plasma concentrations were analyzed following development of a bioanalytical assay and using gas chromatograph mass spectrometry (GC/MS). Plasma (100 µl) was added to tubes containing 5 µl internal standard solution (N-methyl VCD in methanol 50 µg/ml). Analyte was extracted from plasma samples by adding 0.3 ml of chloroform and vigorously vortex-mixing the samples for 30s. Following brief centrifugation to separate the phases, the chloroform layer was removed and evaporated to dryness under a stream of nitrogen at ambient temperature. The sample residues were reconstituted with 0.1 ml of chloroform of which 2 µl was injected into an Agilent 7000 GC/MS-MS instrument. The GC-MS was equipped with an Agilent 7693 autosampler, and a 7890A Gas Chromatograph. The column used was an Agilent J&W DB-1MS (30m x 0.25mm I.D. 0.1 micron film thickness). Samples were injected using the pulsed splitless mode at 225°C using Helium as a carrier gas. The oven was held at 60°C for 1 min then heated at 30°C/min to 200°C followed immediately by heating to 325°C at 40°C/min and held for 1 minute.

A weighted quadratic calibration model was used to calculate sample concentrations over the concentration range assayed (from 1–100 µg/ml). This analytical method was based on the published guidelines [34].

2.12. Determination of PK parameters

A non-compartmental analysis was employed in order to obtain the PK parameters using the PK solutions software version 2 (Summit research services, Mentrose, CO, USA). The half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$ where λ_z is the terminal slope of the concentration-time curve. AUC corresponding to the area under the SPD concentration vs. time curve was calculated using the trapezoidal rule and extrapolated to infinity. MRT (mean residence time) was calculated as $AUMC/AUC$, where AUMC is the area under the concentration-time product vs. time curve and extrapolated to infinity. Extravascular or oral clearance (CL/F) was calculated from the quotient Dose/AUC, F is the absolute bioavailability following i.p. administration. The apparent volume of distribution (V/F) or estimated volume of distribution was calculated as CL/F divided by the linear terminal slope. V_{ss}/F was calculated as Dose times AUMC divided by AUC^2 .

2.13. Statistics

All data, except the electrophysiology experiments, are presented as mean $\pm$ s.e.m. (standard error of the mean). In the electrophysiology experiments data are presented as mean $\pm$ s.d. (standard deviation). In all experiments a $p < 0.05$ is considered significant. In the formalin test, data is presented as % control AUC $\pm$ s.e.m. in both the acute and inflammatory phases. ID_{50} values in the formalin test (The dose reducing the nociception response by 50% compared to control) were determined by linear regression from individual experiments vs. each dose level converted to logarithmic scale. The different doses in each test were compared using ANOVA followed by a Tukey multiple comparison test. Comparison between ED_{50} values was done using a t-test analysis. Dunnet's test was used to evaluate differences between baseline and treatment in the electrophysiological tests.

3. Results

3.1. SNL neuropathic pain model

All rats post-surgery displayed a significant reduction in pain threshold for the ipsilateral paw compared to pre-surgery: 1.5 ± 0.5 , 1.5 ± 0.5 , 1.4 ± 0.5 and 3.7 ± 1.1 for the MC, 40, 60 and 80 mg/kg groups respectively (Fig. 2, $p < 0.05$). Contralateral paw had a pain threshold of $>15g$ in all groups which was similar to the tactile allodynia threshold before surgery ($p > 0.05$). A positive control, MTMCD, was used in the study as a comparator to SPD's antiallodynic activity. MTMCD is a VPA analogue previously reported to possess antiallodynic activity equipotent to gabapentin [35]. In this work MTMCD at 80 mg/kg produced an antiallodynic effect significantly different than MC and 40 mg/kg SPD but not 60 mg/kg or 80 mg/kg SPD. SPD had a dose-dependent antiallodynic effect 0.5–4 hours after dosing (Fig. 2). The TPE for antiallodynia for each experimental group was defined as the earliest time at which mean response threshold reached its highest value during the observation period. Both the 60 and 80 mg/kg significantly reduced tactile allodynia compared to control ($p < 0.05$ Fig. 2). The ED_{50} calculated for SPD at TPE (60 min) was 49

mg/kg (95% CI of 9–61 mg/kg) which was equipotent to the ED₅₀ of gabapentin (32 mg/kg) and more than five times higher than the ED₅₀ of VPA (269 mg/kg) evaluated using the same paradigm [19, 36].

3.2. Formalin test

The AUC for the formalin test, measured in both acute and chronic phases after 70 mg/kg SPD was significantly lower than control vehicle ($p < 0.01$) and showed a significant 55% and 74% reduction in the acute and inflammatory phases respectively (Fig. 3). Similar effects (46% and 75% reduction vs. control in the acute and inflammatory phases, respectively; $p > 0.05$ (Fig. 3))) were only seen for VPA at a 300 mg/kg dose. Lower VPA doses were either unable to reduce the licking time significantly (70 mg/kg) or showed reduction in only one of the two phases evaluated (100 mg/kg) (Fig. 3). The ID₅₀ values (The dose reducing the nociceptive response by 50% compared to control) for both SPD and VPA were calculated to be 39 and 40 mg/kg for SPD and 136 and 113 mg/kg for VPA, in the acute and inflammatory phases, respectively. SPD and VPA at their effective doses (70 mg/kg SPD, 300 mg/kg VPA) showed a similar percent reduction in nociception in the acute phase to be 23% and in the inflammatory phase to be 40% for both compounds. However SPD's effective dose was more than 4 times lower than VPA indicating an increased potency. Compared to the TD₅₀ values of the compounds previously mentioned (White et al., 2012), the protective indices (PI) for SPD and VPA (calculated as the ratio between their ID₅₀ and TD₅₀) were 2.2 and 2.5, respectively.

3.3. Carrageenan test

SPD produced a dose-dependent effect on carrageenan induced heat hyperalgesia at its TPE (Fig. 4). Increasing doses of SPD caused a comparable increase in normalized mean withdrawal time, indicating that SPD was able to alleviate heat-induced hyperalgesia (Fig. 4). Only the 70 mg/kg dose of SPD was statistically different from the Carr/MC group (negative control), whereas the 30, 50 and 70 mg/kg SPD doses were all statistically similar to Sal/MC (positive control). Compared to SPD, 100 mg/kg VPA was statistically different from Sal/MC but similar to Carr/MC indicating that VPA at 100 mg/kg was not able to reduce heat hyperalgesia in the animals tested and was less potent than SPD. SPD's ED₅₀ value in the carrageenan model was 49 mg/kg (95% CI 29.3–110). The ipsilateral paw was significantly thicker than the contralateral paw ($p < 0.05$) one hour after testing, confirming carrageenan induced inflammation in all carrageenan injected groups but not in the Sal/MC group (Fig. 5A). Neither SPD nor VPA decreased paw thickness an hour following administration (Fig. 5A).

3.4 Paw edema evaluation

70 mg/kg SPD, the highest dose evaluated in this work, was evaluated for its effect on carrageenan induced paw edema vs. 100 mg/kg Ibuprofen as a positive control (Fig. 5B) up to 24 hours post carrageenan injection, similar to a previous published paradigm [28]. Plantar injection of carrageenan significantly increased paw thickness compared to injection of saline up to 72 hours post injection (data not shown). SPD did not decrease percent paw thickness up to 24 hours following carrageenan injection (Fig. 5B). Ibuprofen decreased percent paw thickness which was significant after 4 hours ($p < 0.05$). Plantar injection of

saline did not produce edema as witnessed by a statistically similar percent paw thickness over time.

3.4. Acetic acid induced abdominal constriction test (writhing) test

The mean number of writhes was significantly reduced after 30, 50 and 70 mg/kg doses of SPD ($p < 0.001$) (Fig. 6A). VPA significantly reduced the number of writhes at doses of 50 and 100 mg/kg (Fig. 6B). The control groups in both experiments had a similar mean number of writhes (43 ± 5.3 and 48 ± 4.0 for the SPD control and VPA control respectively). SPD and VPA showed similar dose response curves (Fig. 6) and potency in this model with ED_{50} values of 40 mg/kg (95% CI 24.3–67 mg/kg) and 44 mg/kg (95% CI 18–61 mg/kg) for SPD and VPA, respectively. Due to its higher TD_{50} , VPA had a higher PI at 8.8 vs. 2.2 for SPD.

3.5. Electrophysiology recordings

A characteristic CAP response in the low and high frequency protocols is shown in Fig. 7. In the tonic stimulation protocol, the peak-to-peak area ratio (PAR) of both A and C fibers (determined based on their conduction velocities) was constant during baseline recording. Vehicle perfusion (ACSF with 1% ethanol) did not produce any significant effect on CAPs recorded (data not shown). Following perfusion with 1 or 3 mM SPD, no significant difference was seen in the normalized PAR value compared to baseline recordings of CAPs in both the A and C fibers. In contrast, perfusion with 1mM lidocaine (after a 15 min SPD washout period) significantly decreased the PAR value of both A and C fibers (Fig. 8A). In the phasic stimulation protocol, CAPs were recorded following high frequency stimulation (40 Hz) producing 28 CAPs per epoch. The ratio between last and first CAP (f) following perfusion with the highest concentration of SPD, 3mM, were similar to baseline (96.5% vs. 96% for baseline and 3mM SPD respectively, Fig. 8B). However, following administration of 1mM lidocaine, f values were significantly decreased (38.5% for lidocaine $p < 0.001$). 3mM SPD did not decrease PAR of the first and last CAP (100% vs. 90% for the first and last CAP following baseline and 3mM SPD respectively, Fig. 8B). Following perfusion with lidocaine, a significant decrease in both the first and last CAP was observed (100% vs. 22.5% in the first CAP and 100% vs. 14.7% for the last CAP of baseline and lidocaine, respectively, Fig. 8B).

3.6. PK results and PK-PD correlation

The plasma concentration vs. time curve following i.p. administration of 80 mg/kg SPD is depicted in Fig. 9. SPD's peak plasma concentration (C_{max}) was 77.2 mg/L in mice and 66.2 mg/L in rats. C_{max} was observed 15 min following administration in both species. PK parameters were estimated utilizing a non-compartmental approach. In mice and rats, SPD had the following PK parameters respectively: $t_{1/2}$ (half-life) of 19 min (0.3 hr) and 47 min (0.8 hr), CL/F 1.7 L/hr/kg and 0.9 L/hr/kg, volume of distribution 0.9 L/kg and 1.1 L/kg (Fig. 9). SPD's t_{max} was 15 min as determined by its time to peak effect in behavioral testing, and in previous anticonvulsant screening tests [18].

4. Discussion

Accumulating evidence has indicated that the cellular changes which occur in injured nociceptors following injury are not solely responsible for the chronic pain state. Inflammatory mediators released from neurons and glia alter neuronal function in both the peripheral and central nervous system and both are thought to contribute to the development of a chronic pain state following tissue injury [10]. This suggests that compounds proposed for use in chronic pain should possess activity in both the neuropathic and inflammatory pain models. SPD is a novel amide derivative of VPA, developed in an attempt to design a better and more tolerable CNS drug that will be a second generation compound to VPA. SPD previously showed the highest potency (out of thirty four novel compounds) and a broad spectrum profile in numerous animal seizure models and was active in two benzodiazepine-resistant models for status epilepticus [18, 19]. Epilepsy and neuropathic pain share similar underlying mechanism of action [11] and some AEDs (e.g. gabapentin, pregabalin) are regarded the mainstay of neuropathic pain treatment [38, 39]. With this in mind, we assessed SPD's antinociceptive activity in both the neuropathic and acute inflammatory pain models presented in this study and compared them to the activity of SPD's parent compound VPA.

4.1 SPD is potent and effective in multiple pain models

SPD's ED₅₀ in the SNL model was initially estimated in a previous work [19]. In this work we report the complete dose response in the SNL model. We find that SPD has five times higher potency than VPA and is equipotent to gabapentin. Compared to VPA, SPD also showed increased potency in the formalin and carrageenan models. In the formalin test, anti-inflammatory drugs such as the NSAIDs indomethacin and naproxen, have been shown to exert their effect only in the second phase [24, 26]. In contrast, centrally acting drugs, including morphine, codeine, and orphenadrine, exert their effect on both phases of the test [24]. VPA and SPD were found to be active in both phases of the test, similar to centrally acting drugs, distinguishing them from conventional anti-inflammatory drugs. SPD had no effect on carrageenan induced paw edema at the highest dose tested unlike the NSAID ibuprofen and previous reports for indomethacin [40]. These further suggest a different antinociceptive profile for SPD compared to marketed NSAIDs. Moreover, SPD's potency in the formalin test is lower (70 mg/kg) than morphine (5–10 mg/kg) but comparable to codeine (25–50 mg/kg) evaluated in the same test [24].

We used the writhing test as a useful model for visceral pain [41, 42]. Visceral pain has different clinical features from that of somatic pain [43], which might explain why some CNS drugs (e.g. topiramate) exert their effect in the animal writhing test and not in the formalin test [44, 45]. We evaluated SPD's potential in this test and observed a maximum efficacy at a dose of 30 mg/kg. VPA was active in the writhing test at lower doses than those seen in the other pain models, suggesting a possibly different mode of action, and validating the use of this test in addition to the other models. In contrast to the SNL, formalin, and carrageenan models, VPA and SPD were equipotent in the writhing test, as indicated by their identical ED₅₀ values. It is possible that SPD saturates an active site (yet to be determined) responsible for the effect which was not seen in the formalin and carrageenan tests.

However, VPA's higher TD_{50} makes its PI value higher compared to SPD in this test. The ED_{50} of both SPD and VPA are either better or comparable to the commercially available NSAID's evaluated in this model (e.g. aspirin - 82–228 mg/kg, naproxen – 13 mg/kg, ibuprofen – 33 mg/kg, piroxicam – 100 mg/kg [46, 47]). On the contrary SPD's potency in the writhing test was lower than morphine [48].

4.2 Possible mechanisms of action

In order to gain an insight into SPD's mechanism of action, we investigated whether SPD's antinociceptive effect is associated with modulation of peripheral neuronal activity. Lidocaine, a local anesthetic blocker of voltage dependent sodium channels, modifies CAPs in peripheral nerves [49, 50] and was used as a positive control in this study [51–53]. Unlike lidocaine, SPD (up to 3mM) did not produce a significant change in conduction properties after either tonic or phasic stimulation. This concentration was six fold higher than SPD's C_{max} at the highest effective dose in-vivo (80 mg/kg). Thus SPD is not likely to act on peripheral voltage-dependent sodium channels. A possible beneficial correlate is that SPD may not suffer from adverse side effects such as vertigo, somnolence and nausea associated with membrane stabilizing drugs [11], but this remains to be determined. NSAIDs although shown to suppress conduction properties in the CAP model have not been useful for the treatment of chronic neuropathic pain and also suffer from long term side effects such as gastrointestinal bleeding. Since SPD is not a membrane stabilizing drug and does not act as an anti-inflammatory agent per se, we conclude that SPD likely exerts its effect through a combination of mechanisms that are yet to be fully defined. A previous study evaluated SPD's mechanism of action *in-vitro* in primary cultures of dissociated cortical neurons to indicate that SPD enhanced GABA induced currents [54]. Although some clinically used drugs, e.g. benzodiazepines, were shown to have antinociceptive effects in animals and humans, possibly due to their effect on GABA receptors [55], it is still unknown whether SPD's antinociceptive properties described herein are attributed to its potential GABAergic mechanism of action.

4.3. Pharmacokinetics

In the PK study, SPD's t_{max} was identical to its TPE in behavioral tests and in previously reported anticonvulsant tests [18]. This suggests rapid distribution to all body compartments following i.p. administration. The high dose given, 80 mg/kg, was not associated with any signs of toxicity except sedation in both species for the first 15 minutes after dosing, which was not expected to affect SPD's PK. SPD's $t_{1/2}$ in rats (0.8 hr) is similar to the $t_{1/2}$ previously reported for SPD using a lower dose (60 mg/kg, $t_{1/2}$ =0.84 hr) [18]. In contrast, both the CL/F and Vss/F were half of what was reported in the previous study (0.9 vs. 1.9 for CL/F and 1.1 vs. 2.3 for Vss/F). Since both these PK parameters are dependent on bioavailability when given extravascularly, this two-fold reduction might be explained by different bioavailability (F) due to the different parenteral vehicles (MC suspension in the current study vs. a multisol solution in the previous study) which can affect SPD's absolute bioavailability (F).

Compared to VPA, in mice SPD has a shorter $t_{1/2}$ and a higher CL/F values [36, 37, 56]. Both SPD and VPA have been shown to be primarily eliminated by metabolism, thereby

indicating that the higher CL/F value for SPD is due to higher affinity to liver enzymes. Since $t_{1/2}$ is directly proportional to V and inversely proportional to CL, this indicates that SPD's shorter half-life is governed mainly by increased metabolic (hepatic) clearance. In humans, drugs with shorter half-lives can reach plasma steady state levels quicker thereby making those good candidates for emergency or acute care use. However it is known that drug's half-life in rodents is shorter than humans (the smaller the animal the shorter is the half life) [57], therefore one may expect a longer half-life for SPD in humans like in the case of SPD's eight carbon homologue VCD [58].

5. Conclusion

The novel VPA amide derivative, SPD, was active in three animal models for acute inflammatory pain as well as in chronic neuropathic pain, and in most tests was more potent than VPA. Moreover, its rapid PK may enable it to be used in acute care. Although SPD's mechanism of action remains to be elucidated, the results of this study support the hypothesis that SPD does not directly modulate neuronal conduction properties, and is not an anti-inflammatory agent per se. Further pharmacological studies to ascertain SPD's novel pharmacological profile, and potential as a future analgesic and anti-seizure drug are warranted.

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Abbreviations

SPD	<i>sec</i> -Butylpropylacetamide
VPA	valproic acid
CAPs	compound action potentials
AEDs	antiepileptic drugs
SNL	spinal nerve ligation
PK	pharmacokinetic
PD	pharmacodynamics
VFF	von-Frey filaments
MC	methyl cellulose
TPE	time to peak effect
ACSF	artificial cerebrospinal fluid

PAR	peak-to-peak area ratio
ED₅₀	median effective dose
TD₅₀	median toxic dose
PI	protective index
AUC	area under the SPD plasma concentration vs. time curve
CI	confidence interval
MRT	mean residence time
CL/F	oral or extravascular clearance
V/F	apparent volume of distribution
AUMC	area under the first moment (concentration-time product) curve

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Figure 1.
Chemical structures of valproic acid (VPA) and *sec*-butylpropylacetamide (SPD). Asterisks denote chiral carbons.

Figure 2.

The effect of i.p. administration of SPD and MTMCD (as a positive control) on tactile allodynia in rats with SNL neuropathy. **A.** Data shows withdrawal threshold for the ipsilateral paw before (0) and 30, 60, 120, 180, and 240 min after treatment. The protective criterion (15g) is indicated by the dashed gray horizontal line. Each time point represents the mean pain threshold $\pm$ s.e.m. for the entire group of rats evaluated. **B.** box whisker plots representing the area under the curve (AUC) for each treatment in A. Both 60 and 80 mg/kg SPD had a significant higher AUC compared to control vehicle. MTMCD as a positive control was significantly different than MC and 40 mg/kg SPD but not 60 or 80 mg/kg SPD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Tukey multiple comparison test ($n = 8$ for MC and 40 mg/kg, $n = 9$ for 60 and 80 mg/kg, $n = 7$ for MTMCD)). Table. The table indicates the ratio of protected rats (not responding to the 15g VFF denoted by the left number) to the total number of rats tested. The 60 minute time point (highlighted) corresponding to SPD's time to peak antiallodynic effect. .

Figure 3.

The effect of SPD and VPA on the licking time in the formalin test following i.p. administration of 35 and 70 mg/kg SPD (**A**), 70, 100 and 300 mg/kg VPA (**B**). The graph shows two phases: acute (0–10 min) and inflammatory (10–40 min). Each point represents mean $\pm$ s.e.m. (* $p < 0.01$ Tukey test for multiple comparisons, $n=8$). **C.** Table indicating the mean AUC values $\pm$ s.e.m. and % reduction vs. control vehicle in the acute and inflammatory phases of both SPD and VPA.

Figure 4.

Effect of SPD and VPA on heat hyperalgesia in the carrageenan model. Each point represents the mean withdrawal latency $\pm$ s.e.m. of the injected paw in all groups tested. (* $p < 0.05$, *** $p < 0.001$ Tukey multiple comparison test vs. Carr/MC group, $n=14$ in the 10, 30 mg/kg, $n=23$ in the 50, 70 mg/kg and VPA groups, $n=30$ and 40 in the Sal/MC and Carr/MC groups respectively).

Figure 5.

A. Paw thickness as measured in the injected and uninjected hind paws in the carrageenan test for all groups tested. Bars represent mean $\pm$ s.e.m. for all animals tested in the study. Ipsi= injected paw, Contra= uninjected paw. (***) $p < 0.001$, Tukey multiple comparison test, $n=14$ in the 10, 30 mg/kg for SPD, $n=23$ in the 50, 70 mg/kg SPD and 100 mg/kg VPA groups, $n=30$ and 40 in the Sal/MC and Carr/MC groups respectively). **B.** Effect of 70 mg/kg SPD (Carr/SPD) vs. 100 mg/kg Ibuprofen (Carr/Ibu) on carrageenan induced paw thickness as measured for 24h following plantar injection of carrageenan. Each point represents mean $\pm$ s.e.m. (* $p < 0.05$, Tukey multiple comparison test, $n=5$ per group).

Figure 6.

The effect of 10, 30, 50 and 70 mg/kg SPD administered i.p. in the writhing test; (**A**) and 10, 50 and 100 mg/kg VPA (**B**) on the mean number of writhes in the writhing test compared to control. Each bar represents the number of writhes $\pm$ s.e.m. (n=11 in each group, * $p < 0.01$ *** $p < 0.001$ Tukey multiple comparison test).

Figure 7.

Representative compound action potential (CAP) recorded following tonic (**A**) and phasic (**B**) stimulation of the rat sciatic nerve in vitro. **A**. The left and right CAPs correspond to the A and C fibers respectively; recorded after 0.2 Hz stimulation frequency (tonic). Bold traces represent CAPs following perfusion with 3 mM SPD (upper panel) or 1 mM lidocaine (lower panel). Each trace represents an average of 10 consecutive traces. **B**. Upper panels represent individual CAPs in the phasic protocol following control (light gray), 3mM SPD and 1mM lidocaine (dark gray). Lower panels represent CAP recorded in 40 Hz stimulation frequency (phasic) following control, 3mM SPD, washout and 1mM lidocaine.

Figure 8.

A. Tonic stimulation. Percent normalized peak to peak area ratio measured from A fibers (right white bar) and C fibers (left gray bar) before treatment (baseline) and following 1 and 3 mM SPD or 1 mM lidocaine. Each bar represents mean area $\pm$ s.d. (** $p < 0.01$ Dunnet's test, $n=5$). Bars represent 92%, 96%, 97%, 95% reduction for the A and C fibers following 1 and 3 mM SPD respectively, and 51% and 39% reduction of CAP from base line for the A and C fibers respectively following 1mM lidocaine. **B. Phasic stimulation.** left: Data presented as percent decrease in amplitude of the last CAP compared to the first CAP (f) at baseline and following perfusion with 3 mM SPD and 1mM lidocaine. Right: Percent peak area ratio normalized to baseline following the first and last CAP measured in the stimulus train. Bars represent mean $\pm$ s.e.m. ($n=5$). (***) $p < 0.001$ one way ANOVA).

Figure 9.

Plasma concentration vs. time curve following i.p. administration of 80 mg/kg SPD in mice (a) and rats (b). Each point represents mean $\pm$ s.e.m. of four animals per time point (n=4). Table represents PK parameters derived from plasma concentration vs. time plot of SPD following i.p. administration of 80 mg/kg to both mice and rats compared to VPA. The units for each PK parameter are shown in brackets.

^a Taken from [37] parameters derived following administration of 50 mg/kg VPA orally (p.o.) to mice.

^b Taken from [36] following administration of 300 mg/kg VPA i.p. to rats.