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Characterization of the anticonvulsant profile and enantioselective pharmacokinetics of the chiral valproylamide propylisopropyl acetamide in rodents

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1 Propylisopropyl acetamide (PID) is a new chiral amide derivative of valproic acid. The purpose of this study was to evaluate the anticonvulsant activity of PID in rodent models of partial, secondarily generalized and sound-induced generalized seizures which focus on different methods of seizure induction, both acute stimuli, and following short-term plastic changes as a result of kindling, and to assess enantioselectivity and enantiomer–enantiomer interactions in the pharmacokinetics and pharmacodynamics of racemic PID and its pure enantiomers in rodents.

2 Anticonvulsant activity of (S)-PID, (R)-PID and racemic PID was evaluated in the 6 Hz psychomotor seizure model in mice, in the hippocampal kindled rat, and in the Frings audiogenic seizure susceptible mouse. The pharmacokinetics of (S)-PID and (R)-PID was studied in mice and rats.

3 In mice (S)-PID, (R)-PID and racemic PID were effective in preventing the 6 Hz seizures with (R)-PID being significantly ($P<0.05$) more potent (ED_{50} values 11 mg kg^{-1}, 46 mg kg^{-1} and 57 mg kg^{-1} at stimulation intensities of 22, 32 and 44 mA, respectively) than (S)-PID (ED_{50} values 20 mg kg^{-1}, 73 mg kg^{-1} and 81 mg kg^{-1} at stimulation intensities of 22, 32 and 44 mA, respectively). (S)-PID, (R)-PID and racemic PID also blocked generalized seizures in the Frings mice (ED_{50} values 16 mg kg^{-1}, 20 mg kg^{-1} and 19 mg kg^{-1} respectively).

4 In the hippocampal kindled rat a dose of 40 mg kg^{-1} of (R)- and (S)-PID prevented the secondarily generalized seizure, whereas racemic PID also blocked the expression of partial seizures following an i.p. dose of 40 mg kg^{-1}. Racemic PID also significantly increased the seizure threshold in this model.

5 Mechanistic studies showed that PID did not affect voltage-sensitive sodium channels or kainate-, GABA- or NMDA- evoked currents.

6 The pharmacokinetics of PID was enantioselective following i.p. administration of individual enantiomers to mice, with (R)-PID having lower clearance and longer half-life than (S)-PID. In rats and mice, no enantioselectivity in the pharmacokinetics of PID was observed following administration of the racemate, which may be due to enantiomer–enantiomer interaction.

7 This study demonstrated that PID has both enantioselective pharmacokinetics and pharmacodynamics. The better anticonvulsant potency of (R)-PID in comparison to (S)-PID may be due to its more favorable pharmacokinetic profile. The enhanced efficacy of the racemate over the individual enantiomers in the kindled rat may be explained by a pharmacokinetic enantiomer–enantiomer interaction in rats. This study also showed the importance of studying the pharmacokinetics and pharmacodynamics of chiral drugs following administration of the individual enantiomers as well as the racemic mixture.

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Abbreviations: AED, antiepileptic drug; AGS, audiogenic seizure susceptible; AUC, area under the drug concentration time curve; CL, clearance; DID, di-isopropylacetamide; ED_{50}, median effective dose; MES, maximal electroshock seizure; PI, protective index; PID, propylisopropyl acetamide; s.c., Met-subcutaneous metrazole seizures; SI, stereoselective index; TD_{50}, median dose causing minimal motor impairment; $t_{1/2}$, half life; VPA, valproic acid; V_{ss}, volume of distribution at steady-state

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Introduction

Valproic acid (VPA, Figure 1) displays a broad spectrum anticonvulsant profile in both animal models and in patients

with epilepsy. In humans, it is utilized for the treatment of partial and generalized seizures. However, its clinical use is limited by two rare, but severe, side effects; hepatotoxicity and teratogenicity. The frequency of hepatotoxicity is highest (1 : 600) in patients under the age of two years who are on polytherapy (Dreifuss, 1995). Teratogenicity, including structural malformations, has been observed in 10% of infants exposed in utero to VPA (Kaneko *et al*., 1999).

Numerous derivatives of VPA have been designed in an attempt to develop a non-teratogenic and non-hepatotoxic CNS-active VPA-derivative, with better anticonvulsant activity than the parent compound (Loscher & Nau, 1985; Haj-Yehia & Bialer, 1990; Nau *et al*., 1991; Bialer, 1999). Valpromide (VPD, Figure 1), the primary amide of VPA, is 4–10 times more potent than VPA as an antiepileptic drug (AED) in rodents and is not teratogenic in animal models (Nau & Scott, 1986; Bialer, 1991; Radatz *et al*., 1998). However, the better anticonvulsant potency of VPD, compared to VPA, and the lack of teratogenicity have little clinical implications, because in humans unlike rats and dogs, VPD acts as a prodrug to VPA (Bialer, 1991). Therefore, there is a substantial need to develop 'metabolically stable' VPD analogues, which unlike VPD, will not undergo amide-acid biotransformation to their corresponding (inactive or less active and potentially teratogenic) acid (Bialer, 1999).

Propylisopropyl acetamide (PID, Figure 1) is a VPD isomer that possesses one chiral carbon in its structure. PID was developed in a series of structure–pharmacokinetic–pharmacodynamic relationship (SPPR) studies of valproylamides, aimed at developing a CNS-active VPD analogue that would not undergo metabolic hydrolysis to its corresponding acid (Haj-Yehia & Bialer, 1989; Haj-Yehia *et al*., 1992). Racemic PID demonstrated anticonvulsant activity in two models of generalized seizures in rats (i.e., the maximal electroshock test (MES) and the subcutaneous metrazole (s.c. Met) model) at doses devoid of behavioural impairment and in mice at doses causing minimal motor impairment (Spiegelstein *et al*., 1999a). Following oral administration to rats, (R)-PID (Figure 1) was found to be significantly more potent ($ED_{50} = 16$ mg kg^{-1}) in the MES test than (S)-PID ($ED_{50} = 25$ mg kg^{-1}), but in mice no enantioselectivity was observed in the MES or s.c. Met tests. Furthermore, neither racemic PID nor one of its enantiomers was teratogenic in mice (Spiegelstein *et al*., 1999a, b) and PID enantiomers did not undergo metabolic hydrolysis to the corresponding acid. Based on these findings, it would appear that PID represents a better candidate than VPD as a potential CNS-active follow-up compound of VPA. As such, there is a continuing interest in studying its anticonvulsant profile in models of partial and secondarily generalized seizures evoked by different stimuli, and in investigating the pharmacokinetic–pharmacodynamic relationships of the PID enantiomers.

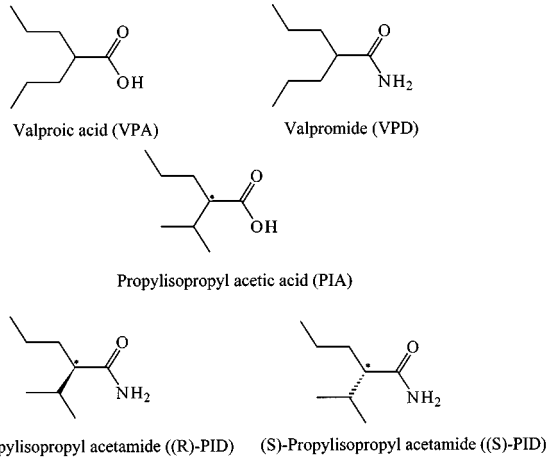

Figure 1 Chemical structures of valproic acid (VPA), valpromide (VPD), propylisopropyl acetic acid (PIA), (S)-propylisopropyl acetamide ((S)-PID) and (R)-propylisopropyl acetamide ((R)-PID).* indicates the chiral centre.

Despite the knowledge that enantiomers often exhibit pronounced differences in their pharmacokinetic as well as in their pharmacodynamic properties (Hutt & Tan, 1996), a number of racemic drugs are still marketed (e.g. warfarin, propranolol, verapamil and omeprazole). Very little is known about pharmacokinetic or pharmacodynamic interactions between the enantiomers of racemic drugs. Enantioselective pharmacokinetics can lead to enantioselective pharmacodynamics in terms of efficacy, toxicity, and clinical outcome. In previous studies, a pharmacokinetic interaction was found between the PID enantiomers in dogs and the pharmacokinetics of PID enantiomers was found to be enantioselective following administration of individual enantiomers (Spiegelstein *et al*., 1999a). In this study, the possible pharmacokinetic and/or pharmacodynamic enantiomer–enantiomer interaction of the PID enantiomers was investigated in established rodent models employed in the search for novel anticonvulsant compounds. Additional studies evaluated pharmacokinetic–pharmacodynamic relationships between the racemic mixture of PID and its enantiomers. This study was designed to evaluate the anticonvulsant activity of PID in models of difficult to treat epilepsies and to investigate the possible mechanisms underlying the enantioselective pharmacokinetics and pharmacodynamics of PID.

The objective of the present study was to characterize the spectrum of anticonvulsant activity of racemic PID and its enantiomers in models of partial, secondarily generalized and sound-evoked generalized seizures in rodents and to compare these results to previous results in models of generalized seizures. Pharmacokinetic studies were undertaken in mice and rats in order to assess the contribution of enantioselective pharmacokinetics and pharmacokinetic enantiomer–enantiomer interaction to the anticonvulsant activity of racemic PID and PID enantiomers in rodents. *In vitro* electrophysiological studies were also undertaken in an attempt to identify a potential mechanism of action of PID.

Methods

Chemicals

Racemic PID, enantiomerically pure (R)-PID and (S)-PID (enantiomeric excess >99%) and di-isopropylacetamide (DID) were synthesized by previously described procedures (Haj-Yehia & Bialer, 1990; Spiegelstein *et al*., 1999b). All solvents were analytical, and all chemicals were reagent grade.

The animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Utah

and Texas A&M University system and the ethics committee of the Hebrew University of Jerusalem. All animal experiments adhered to the principles of laboratory animal care. Adult male CF#1 albino mice were obtained from Charles River, Wilmington, MA, U.S.A. and Sprague Dawley rats from Simonsen, Gilroy, CA, U.S.A. The SWV mice were obtained from a breeding colony at the Laboratory Animal Resources and Research Facility at the College of Veterinary Medicine, Texas A&M University. Frings mice were obtained from an in-house colony maintained by the Animal Resource Center at the University of Utah.

Anticonvulsant activity

Test solutions For all the anticonvulsant activity studies, the compounds were suspended in 0.5% methylcellulose and administered intraperitoneally (i.p.) to mice in a volume of 0.01 ml g^{-1} body weight and in a volume of 0.004 ml g^{-1} body weight either orally (p.o.), or i.p. to rats.

Electrically induced seizures Racemic PID and the individual PID enantiomers were tested for their ability to block 6Hz seizures following i.p. administration to male CF#1 mice (18–27 g). The psychomotor seizures characterized by stun, forelimb clonus, twitching of the vibrissae and straub tail, were induced according to a previously described procedure (Brown *et al*., 1953; Barton *et al*., 2001). Animals in which none of these characteristics of the psychomotor seizures were observed were considered protected. Ten minutes before the electrical stimulation a drop of 0.5% tetracaine in saline was applied to the eyes of the test animals. In mice, the time of peak effect for each of the test compounds was determined by injecting an effective dosage of the drug in several groups of mice (4–8 individuals for each time point) and testing each group once after drug administration at time points 15, 30, 60, 120 and 240 min. At the time of peak effect (15 min), a current of 22 mA, 32 mA or 44 mA at 6 Hz for 3 s was delivered through corneal electrodes. The median effective dose (ED_{50}) was determined at these three different current intensities.

The compounds were also tested for their ability to prevent partial seizures in the hippocampal-kindled rats. For hippocampal kindling, a bipolar stimulating electrode was stereotactically implanted in the ventral hippocampus (AP -5.2, ML 4.9, DV -5.0 from dura, flat skull, Paxinos & Watson, 1997) of adult male Sprague–Dawley rats (250–300 g) under ketamine–xylazine anesthesia. The rats were kindled according to previously described procedures (Lothman *et al*., 1988). Briefly, one week after implantation of the electrodes, the rats were stimulated with suprathreshold trains (200 μA for 10 s, 50 Hz) every 30 min for 6 h on alternate days until the animals were fully kindled. Animals were considered fully kindled when they displayed stable Stage 5 seizures as defined by Racine, 1972. The behavioural seizures were scored according to the following criteria as originally described by Racine: Stage 1, mouth and facial clonus; stage 2, stage 1 plus head nodding; stage 3, stage 2 plus forelimb clonus; stage 4, stage 3 plus rearing; stage 5, stage 4 plus repeated rearing and falling (Racine, 1972). At least a week after the fully kindled state was reached, a single dose of one of the PID enantiomers or racemic PID was administered i.p. in a randomized parallel design and their effect on behavioural seizure score (BSS) and afterdischarge duration (ADD) following 200 μA stimulation was assessed 75, 45 and 15 min before drug administration and 15, 45, 75, 105 and 135 min after drug administration. Animals not displaying stage 4 or 5 seizures were considered protected from seizure generalization. The ADD was determined from the EEG recording.

The effect of racemic PID and the PID enantiomers on the afterdischarge threshold in hippocampal kindled rats was evaluated in rats kindled according to the above described procedure. On the day of the test, the afterdischarge threshold of each rat was determined by increasing the current intensity stepwise until the rat displayed an electrographic afterdischarge with a duration of at least 4 s. The initial stimulation was conducted at 20 μA and increased in 10 μA increments every 1–2 min until an afterdischarge was elicited. The individual rat's afterdischarge threshold was redetermined at 0.5, 1, 2 and 4 h after i.p. dosing of one of the test compounds. The ADD and BSS were recorded at each time point and compared to the control values obtained before drug administration. The criteria for seizure scoring was as described above for kindled seizures.

Audiogenic seizures

To further characterize the anticonvulsant profile of racemic PID, (R)-PID and (S)-PID, they were evaluated for their ability to prevent sound-induced generalized seizures in Frings audiogenic seizure (AGS)-susceptible mice. Audiogenic seizures, characterized by wild running, clonus and forelimb and hindlimb tonic extension, were induced by exposure to 110 decibels (11 kHz, 20 s) sound stimulus. Animals not displaying hindlimb tonic extension were considered protected. The severity of the seizures was evaluated 15 min after drug administration (time of peak effect) and the seizure severity quantified by assigning a numerical score to the response according to following criteria: Stage 0, no response; Stage 1, wild running for <10 s; stage 2, wild running for >10 s; stage 3, clonic seizure; stage 4, forelimb extension and hindlimb flexion; stage 5, tonic seizure.

Determination of acute motor impairment

Minimal motor impairment was determined in CF#1 mice following i.p. administration by the rotorod procedure as described previously (Swinyard *et al*., 1989). In rats the endpoint for behavioural impairment was ataxia, defined by the gait and stance test. The PID enantiomers and racemic PID were tested for behavioural impairment after p.o. dosing of the test compound.

Determination of the median effective dose or behaviourally impairing dose

For the determination of the ED_{50} (or TD_{50}) by the respective anticonvulsant procedures, doses of the PID enantiomers or racemic PID were varied until at least four points were established between the dose level of 0% protection and 100% protection. These data were then subjected to probit analysis (Finney, 1971), and the ED_{50} and 95% confidence interval were calculated.

The protective index was calculated from the quotient of TD_{50}/ED_{50} (same species, vehicle and route of administration). The stereoselective index was determined as the ratio between the high enantiomer ED_{50} and the low enantiomer ED_{50}.

Electrophysiology

Tissue culture Dissociated cortical neurons obtained from Swiss Webster mouse fetuses (E18) were maintained at 37°C in primary culture using standard techniques as previously described (Skeen *et al*., 1994; Otto *et al*., 2002). Cortical cells were used 2–3 weeks after plating on poly-L-lysine coated coverslips. The N1E-115 neuroblastoma cell line (obtained from American Type Culture Collection, VA, U.S.A.) was maintained at 35°C in Dulbecco's modified Eagles Medium supplemented with 5% foetal calf serum, 20 mM HEPES, 80 μg ml^{-1} gentamicin and 4 mM glutamine. Prior to electrophysiological studies, cells were plated on poly-L-lysine coated coverslips and incubated for 24–36 h in a differentiation medium similar to above with reduced (2.5%) foetal calf serum and 2% DMSO.

Receptor-gated channels Whole-cell voltage-clamp recordings from murine cortical neurons in primary culture were carried out at room temperature (23°C) according to previously described techniques (Hamill *et al*., 1981) in an external solution containing NaCl 142, KCl 1.5, $CaCl_2$ 1, HEPES 10, glucose 10 and sucrose 20 (320 mOsm, pH 7.4). The bath solution also contained 500 nM tetrodotoxin to block voltage-gated sodium channels. Whole cell voltage clamp recordings were obtained with an EPC-7 amplifier (List Instruments) using patch electrodes (2–3 MΩ) filled with an internal solution containing (mM): CsCl 153, EGTA 10, HEPES 10 and $MgCl_2$ 4 (290 mOsm, pH 7.4). Currents were filtered at 5 kHz, digitally sampled at 10 kHz, and acquired on a computer using PCLAMP6 software (Axon Instruments).

Cortical neurons were voltage clamped at −70 mV. A gravity-fed, three-barrelled glass flowpipe was positioned 200–400 μM from the cell through which agonist, agonist plus PID (100 μM) and wash solution flowed continuously. A computer-controlled piezo-electric stepper system was employed to determine which solution flowed past the neurons. $GABA_A$ receptor currents were evoked using 5.0 μM GABA, whereas ionotropic glutamate currents were evoked using 100 μM kainate or 10 μM NMDA and 1 μM glycine. Agonists were applied for 1–2 s multiple times and separated by a 10–15 s wash period. Recordings were terminated if a substantial change in either series resistance or holding current were noted. The agonist-evoked current values were averaged for each condition and measured using Clampfit software (Axon Instruments).

Voltage-gated Na^+ channels The effect of PID on voltage-gated Na^+ channels was assessed using N1E-115 murine neuroblastoma cells. Recordings were carried out at room temperature in an external solution containing (mM): NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 5, HEPES 5. To block voltage-gated Ca^{2+} and K^+ channels, 0.1 mM $CdCl_2$ and 25 mM tetraethylammonium chloride were added to the bath. Whole cell recordings were obtained using patch electrodes (1–2 MΩ) filled with the intracellular solution and coated with sylgard. The currents were filtered at 10 kHz and the series resistance and capacitive currents were compensated using the internal amplifier circuitry. The series resistance was 3–5 MΩ, and 40–50% of the series resistance was compensated.

Cells were voltage clamped at −60 mV, unless otherwise noted, and PID was applied using the perfusion system described above. To activate voltage-gated sodium channels, cells were hyperpolarized to −90 mV for a minimum of 90 ms and then depolarized to 0 mV for five trials in control solution, and then again in the solution containing test compound. This protocol was then repeated, but from a holding potential of −60 mV instead of −90 mV.

For these studies, PID was dissolved in DMSO (50 mM stock) and diluted to make a final concentration of 100 μM. Final DMSO concentration was <1%.

Pharmacokinetic studies

Mice experiments Male SWV mice weighing 22–30 grams were randomly assigned to one of the following treatments: 400 mg kg^{-1} racemic PID; 200 mg kg^{-1} of (S)-PID or 200 mg kg^{-1} (R)-PID. The compounds were dissolved in 25% Cremophor EL aqueous solution and were administered by a single i.p. injection (10 μl g^{-1} body weight). Three mice were sacrificed per time point and blood samples (600–700 μl) were collected *via* the retro-orbital sinus at 10, 20, 40, 60, 90, 120, 180, 240, 300 and 360 min after dosing. The blood was centrifuged at 3000 *g* for 5 min; the plasma (150–400 μl) separated and stored at −20°C until analysed.

Rat experiments Male Sabra rats weighing 280–300 g were kept in the laboratory three days for acclimatization. Each rat received a single i.v. injection of 20 mg racemic PID (69 mg kg^{-1}), dissolved in 0.4 ml of propylene glycol-ethanol-saline mixture (2:5:3). Food was withheld after dosing, and water was supplied *ad libitum*. At 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, 600 and 720 min after dosing the rats (four rats for each time point) were anaesthetized with ether and blood samples (7 ml) were collected from the ascending vena cava. Plasma was immediately separated by centrifugation at 3000 g for 15 min and stored at −20°C until analysed. From the same rats, brain and liver were quickly removed; snap freezed in liquid nitrogen and stored at −70°C until analysed.

Analysis of PID concentrations in plasma, brain and liver

Mouse plasma The analysis was performed as previously described (Spiegelstein *et al*., 1999a). Two μg (for concentrations <100 mg l^{-1}) or 10 μg (for concentrations >100 mg l^{-1}) of DID (internal standard) were added to 100 μl of plasma. To the plasma, 5 ml of tert-butyl-methyl ether were added, the organic phase was separated and dried under reduced pressure using a vortex evaporator (Buchler Instruments, Lenexa, KS, U.S.A.). The organic residue was reconstituted in 35 μl (concentrations <100 mg l^{-1}) or 100 μl (concentrations >100 mg l^{-1}) of chloroform and 2 μl were injected into the GC apparatus (Hewlett Packard 5890 Series II GC equipped with flame ionization detector, HP6890 autosampler and 3396A integrator).

Rat plasma, brain and liver Three μg of DID (internal standard) were added to 0.5 ml of rat plasma or to 0.5 g of rat tissue (brain or liver). The plasma was acidified by 0.5 ml of Na-acetate -perchloric acid solution (0.2 N, pH 5.2). Brain and liver samples were homogenized with 3 ml of the same solution. The sample mixture was briefly vortexed prior to being extracted twice with 2 ml of chloroform. For plasma samples, the combined organic extractions were evaporated under nitrogen, the dry residue was dissolved in 30 μl of chloroform and 2 μl was injected into the GC apparatus. For tissue samples the combined organic extractions were loaded on silica columns (500 mg silica gel 60 for chromatography 230–400 mesh, E. Merck Dramstadt, F.D. Germany) preconditioned with 2 ml of chloroform. After washing the column with 2 ml of chloroform, the analyte was eluted by 2 ml of 10% methanol in chloroform. The organic phase was evaporated to dryness under nitrogen, the dry residue was reconstituted in 30 μl of chloroform, and 2 μl were injected into the GC apparatus.

Chromatographic conditions An HP5890 series II Gas Chromatograph equipped with capillary split inlet, HP 7673 autosampler, flame ionization detector and HP 3396A integrator was used for the analysis. Enantioselective separation was achieved using capillary column (10 m, 0.25 mm i.d., 0.25 μm) coated with Heptakis-(2,3-di-o-methyl-6-o-tert-butyl-dimethyl-silyl)- γ-cyclodextrin as stationary phase. The column head pressure was 50 kPa, split ratio 1 : 25, injection port temperature 280°C and detector temperature was 300°C. The temperature program was started at 110°C for 12 min followed by 30°C min^{-1} rise to 150°C for additional 7 min. For rat brain and liver samples 170°C was used as the final temperature for 10 min.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by non-compartmental methods, based on the statistical moment theory (Gibaldi & Perrier, 1982). The area under the plasma concentration *versus* time curve (AUC) was calculated by the linear trapezoidal method with extrapolation to infinity. For each time point of plasma and tissue levels the median result obtained from four rats was used. For the plasma concentrations in mice, each point in the concentration-time curve is the median of three mice. The mean residence time (MRT) was calculated from the quotient AUMC/AUC, where AUMC is the area under the concentration time product *versus* time curve from zero to infinity. The clearance (CL) was calculated from the quotient Dose/AUC and the volume of distribution at steady-state (Vss) from the product of CL*MRT. The Vss following i.p. administration was calculated with an assumption that the bioavailability of the drug was 100%. The linear terminal slope (β) of natural logarithm of PID plasma, liver or brain concentrations vs t (time) curve was calculated by the method of least squares. The terminal half-life was calculated from the quotient ln2 β^{-1}.

The stereoselective index was determined as the parameter ratio between the high enantiomer value and the low enantiomer value.

Statistical analysis

Results are presented as either the ED_{50} and 95% confidence intervals, or as mean ± standard deviation. Differences between the potency of the enantiomers was evaluated by use of the one-tailed Z-test. The upper and lower limit of the 95% confidence interval of the log transforms provided by the probit analysis were used to calculate the standard error for the ED_{50} values and the ED_{50} values were then compared to determine statistical significance. Reduction in the kindled rat behavioural seizure score as compared to the predosing control value was evaluated using two-tailed Mann-Whitney test. Difference in the afterdischarge threshold and ADD were evaluated using a two-tailed *t*-test. For the electrophysiological data, results of individual cells were averaged, the standard error of mean was calculated and paired Student's *t*-test was used to analyse differences. The variances of the AUC values were calculated using the previously described method (Bailer, 1988; Yuan, 1993) and the significance of the differences between the AUCs was determined by two-tailed Z-test. A *P*-value <0.05 was considered significant except with the electrophysiological data where a *P*-value <0.01 was considered significant.

Results

Anticonvulsant activity and behavioural toxicity (minimal motor impairment)

The ability of racemic PID and the individual PID enantiomers to suppress partial seizures was evaluated in the 6 Hz test in mice. The obtained results, together with the minimal motor impairment data and the Frings audiogenic seizure-susceptible mice data, are summarized in Table 1. Racemic PID, as well as each of the PID enantiomers, were efficacious in the 6 Hz seizure model, with (R)-PID being significantly more potent ($P<0.05$) than (S)-PID at all stimulation intensities. A shift to the right in the dose response curves was observed for all compounds when

Table 1 Anticonvulsant activity of racemic PID, (S)-PID and (R)-PID in mice following i.p. administration.

	Frings mice	*6Hz 22mA*	*6Hz 32mA*	*6Hz 44mA*	*Minimal motor impairment*
	ED_{50} (mg kg^{-1}) (95% confidence interval) {protective index}				
PID (racemic)	20 (16–25) {5.6}	23 (15–32) {4.9}	44 (35–57) {2.5}	73 (60–100) {1.5}	112 (106–118)
(S)-PID	16 (14–19) {6.1}	20 (14–27) {4.9}	73 (56–92) {1.3}	81 (70–95) {1.2}	97 (89–137)
(R)-PID	20 (11–76) {5.6}	11* (5–17) {10.1}	46* (31–65) {2.4}	57* (41–69) {1.9}	111 (92–122)
SI†	1.3	1.8	1.6	1.4	1.1

*Significantly more potent than the corresponding enantiomer. †The ratio between the high enantiomer ED50 and the low enantiomer ED_{50}.

stimulation intensity was increased from 22 mA to 32 mA and for the racemate when stimulation intensity was increased from 32 mA to 44 mA. The dose response curves together with the dose dependant minimal motor impairment are depicted in Figure 2. The stereoselective index (SI, the ratio between the high enantiomer ED_{50} and low enantiomer ED_{50}) decreased with increasing stimulation intensity from 1.8 at 22 mA to 1.4 at 44 mA (Table 1). Since the TD_{50} value of (R)-PID in these mice was slightly higher than the TD_{50} of (S)-PID, the protective index, a measure of the safety margin, of the (R)-enantiomer was greater than that of (S)-PID.

The efficacy of (S)-PID, (R)-PID and racemic PID in blocking sensory-evoked generalized seizures was studied in Frings audiogenic seizure susceptible mice (Table 1). In this model, no enantioselectivity in the anticonvulsant activity of the enantiomers was observed (SI = 1.3) and racemic PID was equipotent with both enantiomers. The dose dependent effect of racemic PID, (S)-PID and (R)-PID on the behavioural seizure score in the Frings mice is shown in Figure 3.

The ability of (R)-PID, (S)-PID and racemic PID to block partial seizures and to prevent seizure generalization was assessed in the hippocampal-kindled rat following suprathreshold stimulation of 200 μA and at threshold stimulation. The time courses of the anticonvulsant effect are presented in Tables 2 and 3. Table 2 summarizes the effect of racemic PID and its enantiomers on suprathreshold seizures whereas Table 3 shows the effect of racemic PID and the individual enantiomers on the hippocampal-kindled afterdischarge threshold, on behavioural seizure score and on afterdischarge duration at threshold stimulation. (R)-PID and (S)-PID significantly attenuated the secondarily generalized seizure ($P<0.05$) in hippocampal kindled rat with a suprathreshold current in a time-dependent manner (Table 2). Racemic PID also blocked the partial seizures following a dose of 40 mg kg^{-1} and was more efficacious than either one of the individual enantiomers in this model. In addition, racemic PID had a longer duration of action than the individual enantiomers. In contrast to the racemate, both (R)-PID and (S)-PID significantly reduced the afterdischarge duration when stimulated at a suprathreshold stimulus at a single time point.

Racemic PID was more efficacious than either one of the enantiomers when administered at equivalent dose in elevating afterdischarge threshold (Table 3). Racemic PID significantly increased the afterdischarge threshold in fully hippocampal kindled rats when administered at a dose of 40 mg kg^{-1} and effectively prevented the expression of partial seizures. In contrast, individual enantiomers did not exert any effect on afterdischarge threshold in the hippocampal kindled rats and the individual enantiomers only blocked the secondary generalization of the seizures. None of the compounds affected the afterdischarge duration at threshold stimulation.

Behavioural impairment in rats was assessed following i.p. administration to the hippocampal kindled rats and after p.o. administration to neurologically intact rats (data not shown). In neurologically intact rats (R)-PID produced greater behavioural impairment than (S)-PID. (R)-PID displayed a TD_{50} value of 55 mg kg^{-1} (95% confidence interval 39 – 76)

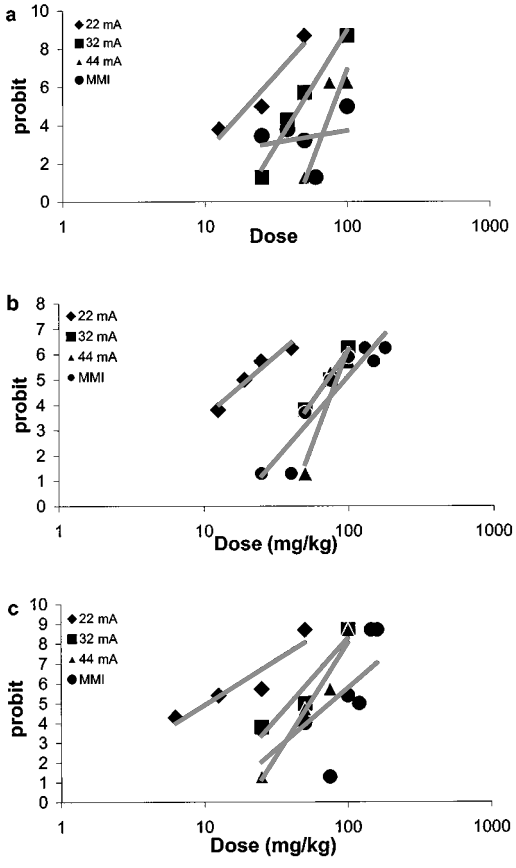

Figure 2 Dose response curves for racemic PID (a), for (S)-PID (b) and for (R)-PID (c) in the 6 Hz model at the three different stimulation intensities (22 mA, 32 mA, 44 mA) together with the dose response effect on minimal motor impairment (MMI) in the same mice.

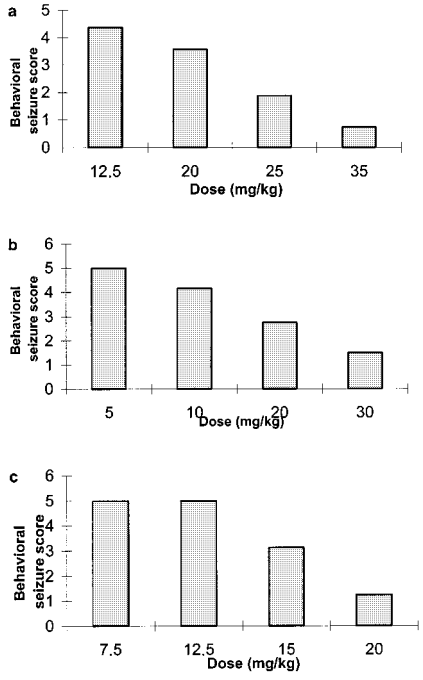

Figure 3 Effect of four different doses of racemic PID (a), (R)-PID (b) and (S)-PID (c) on seizure severity in the Frings audiogenic seizure susceptible mice following i.p. administration.

Table 2 Effect of racemic PID, (R)-PID and (S)-PID on behavioural seizure score (BSS) and afterdischarge duration (ADD) at tested time points in hippocampal kindled rats at suprathreshold stimulation (200 μA)

	Time of test (min)					
	−15	*+15*	*+45*	*+75*	*+105*	*+135*
			Racemic PID (40 mg kg^{-1})			
BSS(protected/ tested)	5.0±0.0 (0/6)	1.7±1.8* (5/6)	3.2±2.1* (2/6)	3.3±1.5* (2/6)	3.8±0.8* (2/6)	3.3±1.6* (1/6)
ADD (sec)	64±18	40±32	44±30	64±32	61±18	46±20
			(S)-PID (40 mg kg^{-1})			
BSS(protected/ tested)	5.0±0.0 (0/6)	2.8±0.8* (3/6)	4.5±0.2 (0/6)	4.5±0.2 (0/6)	3.7±0.6* (1/6)	4.2±0.2* (0/6)
ADD (sec)	65±8	15±4*	55±9	56±10	48±9	46±8
			(R)-PID (40 mg kg^{-1})			
BSS(protected/ tested)	5.0±0.0 (0/6)	4.7±0.2 (0/6)	2.8±0.7* (3/6)	4.2±0.2* (0/6)	4.2±0.3* (1/6)	4.5±0.2 (0/6)
ADD (sec)	55±2	34±11	41±4*	57±8	47±12	50±10
			0.5% methyl cellulose			
BSS	4.8±0.2	5.0±0.0	4.7±0.2	4.8±0.2	5.0±0.0	4.7±0.2
ADD (sec)	78±10	71±9	86±9	86±9	88±9	88±10
			Ethosuximide†			
BSS	4.9±0.1	4.3±0.4	4.4±0.4	4.7±0.2	4.8±0.1	4.8±0.1
ADD (sec)	56±5	46±9	35±6	43±6	39±3	49±9

*Significantly different from pre-dosing value ($P<0.05$). †Unpublished data of the Anticonvulsant Screening Project, University of Utah

Table 3 Effect of racemic PID, (S)-PID and (R)-PID on afterdischarge (AD) threshold, behavioural seizure score (BSS) and AD duration in the hippocampal kindled rats following i.p. administration (40 mg kg^{-1}) of the test compounds at threshold stimulation.

	Time of test (min)				
	−30	*+30*	*+60*	*+120*	*+240*
			Racemic PID (40 mg kg^{-1})		
AD threshold (μA)	92±13	183±42	210±44*	167±41	143±37
ADD (sec)	59±6	32±15	28±15	55±13	56±10
BSS (protected/tested)	5±0 (0/6)	0.6±0.3* (6/6)	1.3±0.7* (5/6)	3.6±0.8* (2/6)	4.8±0.2 (0/6)
			(R)-PID (40 mg kg^{-1})		
AD threshold (μA)	71±10	74±11	86±18	76±15	76±15
ADD (sec)	61±8	59±11	50±9	67±13	60±7
BSS (protected/tested)	5±0 (0/7)	3.6±0.6* (3/7)	3.3±0.8* (3/7)	4±0.4 (1/7)	5±0 (0/7)
			(S)-PID (40 mg kg^{-1})		
AD threshold (μA)	90±8	107±12	114±18	109±16	99±13
ADD (sec)	72±9	55±11	76±12	69±5	58±5
BSS (protected/tested)	5±0 (0/7)	3.5±0.6 (3/7)	4.7±0.2 (0/7)	4.4±0.4 (1/7)	5±0 (0/7)

*Significantly different from the 30 min pre-dosing value ($P<0.05$)

whereas (S)-PID did not cause any signs of motor impairment at a dose of 63 mg kg^{-1}. In the kindled rat (S)-PID produced minimal motor impairment in all tested animals following a dose of 40 mg kg^{-1}, whereas following administration of (R)-PID only four out of the 13 tested animals showed signs of behavioural impairment after i.p. dose of 40 mg kg^{-1}.

Electrophysiology

Electrophysiological studies were undertaken in order to evaluate the potential mechanism of action of PID. Whole cell patch clamp experiments determined that coapplication of racemic PID (100 μM) did not significantly affect current flow through ionotropic glutamate receptors. Kainate-induced inward currents in cultured cortical neurons in the presence of racemic PID (100 μM) were 99%±1.0% of currents elicited by kainate alone ($n=11$). In addition, in the presence of racemic PID (100 μM), NMDA-induced currents in these cells were 87±4.0% of currents elicited by NMDA alone ($n=7$). This 13% reduction in the NMDA-induced current approached significance ($P<0.05$), but, due to variability between cells, did not meet our criterion of $P<0.01$. Finally, application of PID with 5 μM GABA resulted in currents that were 96%±2% of currents elicited by GABA alone ($P<0.05$). As was the case for NMDA induced currents, the weak effect of PID on GABA approached significance, but did not meet our criterion of $P<0.01$. The effect of racemic PID on NMDA and GABA currents is shown in Figure 4.

Application of racemic PID (100 μM) did not have a significant effect on voltage-dependent sodium currents elicited in mouse N1E-115 neuroblastoma cells. In the presence of PID, depolarizing step pulses to 0 mV from a holding potential of −60 mV resulted in sodium currents, which were 95±2% of control ($n=7$). From a holding potential of −90 mV ($n=8$), a step pulse to 0 mV elicited sodium currents that were 98±1% of control currents in the presence of PID.

Pharmacokinetics

The enantioselectivity and possible enantiomer–enantiomer interaction in the pharmacokinetics of PID enantiomers in

rodents was studied following i.p. administration of racemic PID and (R)- and (S)- PID to mice and after administration of racemic PID to rats. The plasma concentration *versus* time curves of (R)- and (S)-PID following administration of the racemate (400 mg kg^{-1}) or individual enantiomers (200 mg kg^{-1}) to mice are presented in Figure 5. The obtained pharmacokinetic parameters in mice are summarized in Table 4. The AUC values of (R)- and (S)-PID were significantly different both following racemate administration ($0.01 > P > 0.005$) and administration of the individual enantiomers ($P < 0.001$). Following i.p. administration of the PID enantiomers separately, (S)-PID had a higher CL (SI = 1.8) and Vss (SI = 1.3) values than (R)-PID. As a result of its lower CL, (R)-PID had a longer half-life (SI = 1.5) than (S)-PID. A stereoselective index (SI) that is equal to or above 1.2 is considered an indication of enantioselectivity in the pharmacokinetic parameter (Levy & Boddy, 1991). Concomitant administration of the two enantiomers to mice abolished all the differences in the major pharmacokinetic parameters despite the still significant difference in the AUC values, and similar pharmacokinetic parameters were obtained for both enantiomers (SI value about unity, Table 4). However, large differences in the pharmacokinetic parameters were observed for each of the enantiomers following their administration individually *versus* in the racemic mixture. The CL value obtained for both enantiomers following the administration of racemic mixture was 2–3 times lower than that obtained after the administration of the individual enantiomers. Also, the $t_{1/2}$, Cmax and MRT values obtained for both enantiomers following administration of racemic mixture were approximately 50% higher than those obtained after administration of equivalent doses of the individual enantiomers. Altogether, these differences in the major pharmacokinetic parameters between administration of a racemate and individual enantiomers to mice led to higher plasma concentrations of each enantiomer over the whole time course following racemate administration when compared to administration of individual enantiomers.

The enantioselectivity in brain and liver partitioning and in the pharmacokinetics in the site of action (brain) was studied in rats after i.v. administration. The plasma, brain and liver concentration *versus* time curves of PID enantiomers following i.v. administration of racemic PID to rats are depicted in Figure 6. The calculated pharmacokinetic parameters are presented in Table 5. (R)-PID had a significantly higher AUC than (S)-PID in brain ($0.002 > P > 0.001$), plasma ($P < 0.001$) and liver ($P < 0.001$) and consequently, it had a lower plasma CL than (S)-PID (SI-value 1.3) and longer plasma MRT (SI = 1.2). No enantio-selectivity was observed in the other major pharmacokinetic parameters calculated following i.v. administration of racemic PID to rats. The liver AUC was higher for both enantiomers than the brain and plasma AUC and the brain-to-plasma AUC ratio for (R)-PID was 1.1 and for (S)-PID 1.3 indicating good brain penetration for these amides and showing that plasma concentration measurements effectively reflect the brain concentrations for the whole time course of the pharmacodynamic effect.

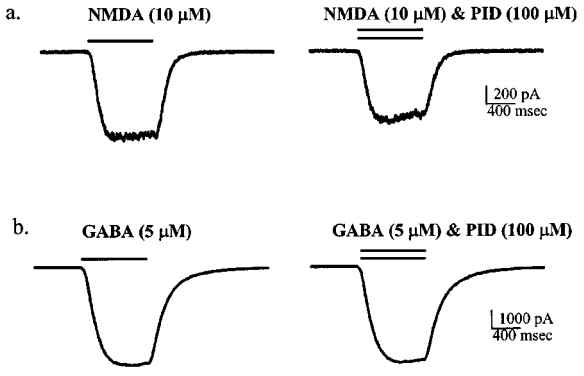

Figure 4 Effect of PID (100 μM) on exogenously evoked (a) NMDA-mediated and (b) GABA-mediated currents in cultured cortical neurons. Agonists were applied as indicated by bars. Traces shown are averaged responses from three consecutive sweeps under each condition. Vm = −70 mV.

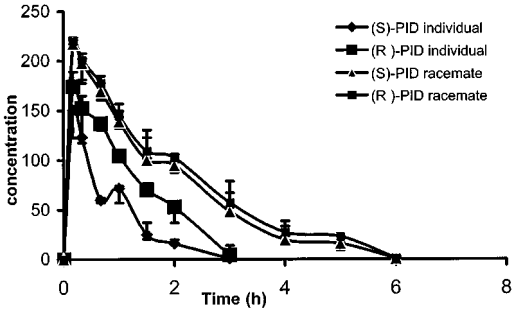

Figure 5 Median (± range) plasma concentration *versus* time curves of (S)-PID and (R)-PID following their i.p. administration either as a racemic mixture (400 mg kg^{-1}) or as individual enantiomers (200 mg kg^{-1}) to SWV mice.

Table 4 Pharmacokinetic parameters of PID enantiomers in mice following i.p. administration (200 mg kg^{-1}) of individual enantiomers or (400 mg kg^{-1}) of racemic PID

Pharmacokinetic parameter	*Administration of individual enantiomers*			*Administration of racemic mixture*			I/R^b	I/R^b
	(R)-PID	(S)-PID	SI[a]	(R)-PID	(S)-PID	SI[a]	(R)-PID	(S)-PID
CL (L h^{-1} kg^{-1})	0.79	1.4	1.8	0.43	0.47	1.1	1.8	3
Vss (L kg^{-1})	0.75	0.96	1.3	0.70	0.71	1.0	1.1	1.4
$t_{1/2}$ (h)	0.54	0.36	1.5	0.83	0.75	1.1	0.7	0.5
C_{max} (mg L^{-1})	199	184	1.1	241	239	1.0	0.8	0.8
MRT (h)	0.96	0.70	1.4	1.64	1.52	1.1	0.6	0.5

a - SI- Stereoselective index: the ratio between the high and the low parameter value of the two enantiomers. b - (I/R)-Parameter ratio between the two modes of administration: individual/racemic.

Discussion

The objective of this study was to evaluate the anticonvulsant activity of racemic PID and the individual PID enantiomers in two rodent models of partial seizures and a genetic model of generalized seizures and to study the pharmacokinetic-pharmacodynamic relationships of PID enantiomers in rodents. In addition, the pharmacokinetic and pharmacodynamic enantiomer–enantiomer interaction was studied and electrophysiological experiments were undertaken in an attempt to identify a potential mechanism of action of PID. A comparison of the anticonvulsant activity data obtained in this study to results obtained previously is presented in Table 6. In mice, the MES and sc Met tests failed to differentiate the anticonvulsant activity and behavioural impairment of the PIDs (individual enantiomers and racemate). In the mouse s.c. Met model, only a mild separation between the TD_{50} and ED_{50} values of PIDs was observed, and in the mouse MES model protection was only observed at doses causing impaired behaviour (Table 6). Therefore, based on mice data, racemic PID and the individual enantiomers were considered to have limited potential as a new AED.

The new AED levetiracetam was completely inactive in the classical MES and sc Met models (Löscher & Honack, 1993), yet highly efficacious in the kindled rat (Löscher *et al*., 2000). In addition, levetiracetam is an effective anticonvulsant in the 6 Hz model and therefore, this model may be considered as a potential new screening model capable of identifying novel AEDs (Barton *et al*., 2001). Furthermore, at stimulus intensity of 44 mA, the model becomes resistant to all tested old and new AEDs except VPA ($ED_{50}=310$ mg kg^{-1}) and LEV ($ED_{50}=1089$ mg kg^{-1}). These findings support the utility of the 6 Hz test as a model for identifying novel therapeutics for the treatment of therapy resistant seizures (Barton *et al*., 2001).

Racemic PID and the pure PID enantiomers were effective in preventing 6 Hz seizures in mice at doses devoid of behavioural impairment. For example, calculated PI values ranged between 1.2 and 10.1 at stimulation intensities between 22 and 44 mA. In addition, the anticonvulsant activity of PID was enantioselective in the 6 Hz model with

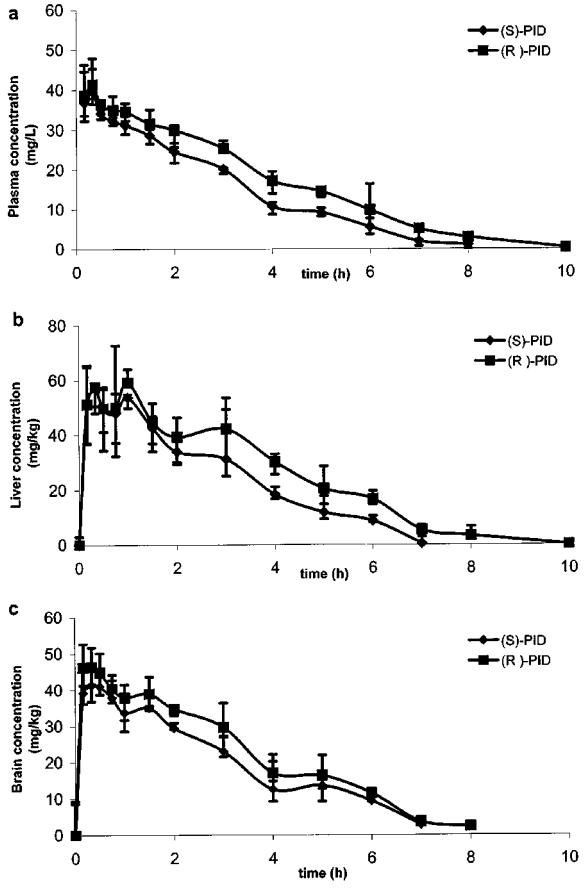

Figure 6 Median (± range) plasma (a), liver (b) and brain (c) concentration *versus* time curves of (S)-PID and (R)-PID following iv administration of racemic PID to rats (69 mg kg^{-1}).

Table 6 Comparison of the anticonvulsant activity (ED_{50} mg kg^{-1}) of racemic PID, (S)-PID and (R)-PID in animal models

	PID (racemic)	*(S)-PID*	*(R)-PID*
		Mice	
MES[a]	122	145	110
sc Met[a]	77	80	67
6Hz 22mA	23	20	11
6Hz 32mA	44	73	46
6Hz 44mA	73	81	57
Frings mice	19	16	20
Minimal motor impairment (TD_{50}, mg kg^{-1})	112	97	111
		Rats	
MES[a]	31	25	16
s.c. Met in rats[a]	37		
Minimal motor impairment (TD_{50}, mg kg^{-1})	62	>63	55

[a]Data from Spiegelstein *et al*., 1999a

Table 5 Pharmacokinetic parameters of PID enantiomers in the plasma, brain and liver following their intravenous administration as racemic mixture (69 mg kg^{-}) to rats.

	Plasma			*Brain*			*Liver*		
Pharmacokinetic parameters	*(R)-PID*	*(S)-PID*	*SI*[a]	*(R)-PID*	*(S)-PID*	*SI*[a]	*(R)-PID*	*(S)-PID*	*SI*[a]
CL (L h-1 kg^{-1})	0.43	0.55	1.3						
Vss (L kg^{-1})	1.2	1.3	1.1						
$t_{1/2}$ (h)	1.2	1.3	1.1	1.5	2.0	1.3	0.9	0.9	1.0
MRT (h)	2.8	2.4	1.2	2.8	3.0	1.1	2.8	2.3	1.2
AUC (mg h L^{-1})	162	126	1.3*	179	144	1.2*	236	179	1.3*

a, SI-stereoselective index: the ratio between the high and the low parameter value of the two enantiomers. *The AUC values were significantly different between the two enantiomers ($P<0.05$, Z-test)

(R)-PID displaying a greater potency ($P<0.05$) and a wider safety margin (larger PI value) at all stimulation intensities. Strikingly, (R)-PID was more potent in the 6 Hz model at the stimulation intensity of 44 mA than it was in the s.c. Met and MES models, suggesting preferential activity against partial seizures.

Certain amide derivatives of VPA, such as valrocemide (TV1901) and N-Methyl-tetramethylcyclopropyl carboxamide (MTMCD) have previously been found to be effective in the 6 Hz model at all of the stimulation intensities tested including 44 mA (Isoherranen *et al*., 2001; 2002). In this study, the 6 Hz test was used to evaluate the anticonvulsant potential of PID, an amide derivative of VPA that failed in the mouse MES and s.c. Met tests but was effective in these tests in rats (Table 6). The results obtained for PID in this study support the validity of the 6 Hz model in mice as a screening model for novel compounds effective against partial seizures. Activity of the PID enantiomers and racemic PID in the 6 Hz model together with previous data obtained with valrocemide and MTMCD suggests that certain amide derivatives of VPA are more potent than VPA in preventing seizures at the highest stimulation intensity in the 6 Hz model.

A majority of the AEDs appear to exert an action on either voltage- or receptor-gated ion channels. As such, electrophysiological studies were undertaken in an attempt to identify a possible mechanism of action of PID. The results from the electrophysiology studies presented here indicate that racemic PID did not significantly block currents evoked *via* kainate application on cultured cortical neurons and it did not decrease the current flow through voltage sensitive sodium channels. A mild effect on NMDA and GABA evoked currents was observed but this was not statistically significant. Consequently, at present, the mechanism of action of PID is unknown and future experiments are needed to assess the full range of potential mechanisms of action of this new compound. This is similar to the results previously obtained with the PID analogue MTMCD, that had a very mild but statistically significant effect on GABA, NMDA and kainate evoked currents (Isoherranen *et al*., 2002). As such, it is likely that PID and probably also MTMCD exert their effects through some yet undetermined mechanism of action. This is of course of interest because it clearly suggests that the mechanism of action of these two amide derivatives of VPA is unique relative to the established and 2nd generation AEDs.

In order to confirm PID's potential in blocking partial and secondarily generalized seizures, its efficacy was also tested in the hippocampal kindled rat. As predicted by the results in the 6 Hz test, racemic PID as well as the individual enantiomers were effective in preventing secondary generalization of a kindled seizure; racemic PID also blocked partial seizures in the hippocampal kindled rat at supramaximal and threshold stimulation. Unlike the 6 Hz model, there was no apparent enantioselectivity in the PID enantiomers' ability to prevent partial and secondarily generalized seizures. In contrast, racemic PID was more efficacious than the individual enantiomers in preventing partial seizures in the hippocampal kindled rat both at supramaximal and threshold stimulation. Racemic PID was the only agent tested that elevated the afterdischarge threshold in the kindled rat. Interestingly, despite their inability to prevent the motor seizures, the individual enantiomers significantly reduced the duration of electrographic seizures as demonstrated by a reduction in the afterdischarge duration.

The enantioselectivity in the anticonvulsant activity in mice in the 6 Hz model and the more favourable profile of racemic PID in the kindled rat could be due to enantioselective pharmacokinetics and/or enantiomer–enantiomer interaction. In mice, following i.p. administration of individual PID enantiomers, the SI-value for the major pharmacokinetic parameters were: 1.8 (CL), 1.3 (Vss) and 1.5 ($t_{\frac{1}{2}}$). Similar SI values (1.4–1.8) obtained for the ED_{50} values in the 6 Hz model suggest that the lower CL of (R)-PID and its longer $t_{\frac{1}{2}}$ contribute to its better (enantioselective) anticonvulsant activity in the 6 Hz model. The plasma concentrations of (R)-PID following administration of a single enantiomer to mice were higher at all time points than those of (S)-PID. It is likely that the significantly higher plasma AUC of (R)-PID leads to a higher brain AUC, and thus contributes to the better anticonvulsant potency. The significant decrease in the CL of both enantiomers after administration of the racemate and the loss of the enantioselectivity may be due to pharmacokinetic enantiomer–enantiomer interaction. The most probable explanation for the 3 fold decrease in the CL of (S)-PID and the 2 fold decrease in the CL of (R)-PID in the presence of their antipodes is inhibition of their (metabolic) CL by the other enantiomer. The different degrees of inhibition observed for the enantiomers may be due to competition on the same metabolizing enzyme(s) and different affinities (K_m or K_i) of each enantiomer to these enzyme(s) (Levy & Trager, 2000).

In the 6 Hz model, racemic PID was less potent than the individual enantiomers at the lowest stimulation intensity tested (22 mA). At 32 mA, racemic PID was more potent than either one of the enantiomers, whereas at the highest stimulation intensity (44 mA) the ED_{50} value of the racemate was in between the two enantiomers. A possible explanation for this inconsistency is that at the low doses, the plasma concentrations of the enantiomers are below their K_i values and no significant enantiomer–enantiomer interaction can be observed, giving rise to plasma concentrations comparable to those obtained after individual enantiomer administration. At the plasma concentration needed to obtain protection against the higher stimulation intensities the K_i values are reached and thus, the racemate has higher total (S + R) plasma concentrations than those obtained following equivalent doses of the individual enantiomers resulting in better potency.

The enantiomer–enantiomer pharmacokinetic interaction (decrease in CL and lack of enantioselectivity) following administration of the racemate can be expanded to rats from the current mice data and previous dog data (Spiegelstein *et al*., 1999a). The better efficacy of racemic PID in the hippocampal kindled rat may be due to a pharmacokinetic interaction between the enantiomers enabling higher total brain concentration of PID following racemate administration. The better efficacy of racemic PID was limited to the hippocampal kindled rat. In the rat MES test racemic PID was less potent than the individual enantiomers, that, similar to the mice, may be due to lack of enantiomer–enantiomer interaction at the doses tested and at the corresponding plasma concentrations (below K_m or K_i of each enantiomer). The results of this study, that showed that (R)-PID had a

significantly higher brain AUC than (S)-PID even after racemate administration, could explain the previously observed greater potency of (R)-PID in the rat MES test.

The excellent efficacy of racemic PID in the hippocampally-kindled rat suggests that PID may be effective against partial and secondarily generalized seizures in epileptic patients, supporting the hypothesis that the 6 Hz model may be a predictive screening method for compounds active against partial seizures. Racemic PID and the PID enantiomers were also found to block generalized seizures in the Frings audiogenic seizure-susceptible mouse. In this model, racemic PID and (S)-PID were found to be equipotent to the mouse 6 Hz psychomotor seizure test (22 mA) whereas (R)-PID was slightly less potent in the Frings mice than in the 6 Hz model at 22 mA (Table 6). This finding when coupled with previous findings in the MES test suggests that the anticonvulsant profile of PID will include activity against generalized seizures. In addition there was no enantioselectivity in the ability of PID to prevent the sound-induced generalized seizures as previously observed against MES and s.c. Met induced generalized seizures (Table 6). This might suggest that different mechanisms are involved in PIDs anticonvulsant activity against generalized and partial seizures and there is a greater enantioselectivity in the mechanism involved in protecting from partial seizures.

In conclusion, this study demonstrated that the chiral valproylamide analogue PID has enantioselective pharmacokinetics and pharmacodynamics. It has a broad anticonvulsant spectrum of activity like VPA, suggesting antiepileptic activity against both partial and generalized seizures. The higher anticonvulsant potency of (R)-PID, lower behavioural impairment and more favourable pharmacokinetics (lower CL) make (R)-PID a more promising candidate than (S)-PID for development as a novel AED. This study also showed the importance of studying the pharmacokinetics and pharmacodynamics of chiral drugs following administration of the individual enantiomers as well as the racemic mixture.

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References

BAILER, A.J. (1988). Testing for the equality of area under the curves when using destructive measurement techniques. *J. Pharmacokin. Biopharm.,* **16,** 303 – 309.

BARTON, M.E., KLEIN, B.D., WOLF, H.H. & WHITE, H.S. (2001). Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. *Epilepsy Res.,* **47,** 217 – 227.

BIALER, M. (1991). Clinical pharmacology of valpromide. *Clin. Pharmacokinet.,* **20,** 114 – 122.

BIALER, M. (1999). Pharmacokinetic considerations in the design of better and safer new antiepileptic drugs. *J. Control. Release,* **62,** 187 – 192.

BROWN, W.C., SCHIFFMAN, D.O., SWINYARD, E.A. & GOOGMAN, L.S. (1953). Comparative assay of antiepileptic drugs by 'psychomotor' seizures and minimal electroshock threshold test. *J. Pharmacol. Exp. Ther.,* **107,** 273 – 283.

DREIFUSS, F.E. (1995). Valproic Acid, Toxicity In: *Antiepileptic Drugs,* 4th ed. Levy, R.H., Mattson, R.H. & Meldrum, B.S., eds. New York: Raven Press, pp 641 – 647.

FINNEY, D.J. (1971). *Probit Analysis,* 3rd edn., Cambridge, U.K: Cambridge University Press.

GIBALDI, M. & PERRIER, D. (1982). *Pharmacokinetics 2nd edn.* New York: Marcel Dekker.

HAJ-YEHIA, A. & BIALER, M. (1989). Structure-pharmacokinetic relationships in a series of valpromide derivatives with antiepileptic activity. *Pharm. Res.,* **6,** 683 – 689.

HAJ-YEHIA, A. & BIALER, M. (1990). Structure-pharmacokinetic relationships in a series of short fatty acid amides that possess anticonvulsant activity. *J. Pharm. Sci.,* **79,** 719 – 724.

HAJ-YEHIA, A., HADAD, S. & BIALER, M. (1992). Pharmacokinetic analysis of the structural requirements for forming 'stable' analogues of valpromide. *Pharm. Res.,* **9,** 1058 – 1063.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch clamp technique for high resolution current recording from cells and cell free membrane patches. *Pflugers. Arch.,* **391,** 85 – 100.

HUTT, A.J. & TAN, S.C. (1996). Drug chirality and its clinical significance. *Drugs,* **52 Suppl.,** 1 – 12.

ISOHERRANEN, N., WHITE, H.S., FINNELL, R.H., YAGEN, B., WOODHEAD, J.H., BENNETT, G.D., WILCOX, K.S., BARTON, M. & BIALER, M. (2002). Anticonvulsant profile and teratogenicity of N-methyl-tetramethylcyclopropyl carboxamide: A new antiepileptic drug. *Epilepsia,* **43,** 115 – 126.

ISOHERRANEN, N., WOODHEAD, J.H., WHITE, H.S. & BIALER, M. (2001). Anticonvulsant profile of valrocemide (TV1901): A new antiepileptic drug. *Epilepsia,* **42,** 831 – 836.

KANEKO, S., BATTINO, D., ANDERMANN, E., WADA, K. KAN, R., TAKEDA, A., NAKANE, Y., OGAWA, Y., AVANZINI, G., FUMAROLA, C., GRANATA, T., MOLTENI, F., PARDI, G., MINOTTI, L., CANGER, R., DANSKY, L., OGUNI, M., LOPES-CENDAS, I., SHERWIN, A., ANDERMAN, F., SENI, M.-H., OKADA, M. & TERANISHI, T. (1999). Congenital malformations due to antiepileptic drugs. *Epilepsy Res.,* **33,** 145 – 158.

LEVY, R.H. & BODDY, A.V. (1991). Stereoselectivity in pharmacokinetics: A general theory. *Pharm. Res.,* **8,** 551 – 556.

LEVY, R.H. & TRAGER, W.F. (2000). From *in vitro* to *in vivo*: an academic perspective. In *Metabolic Drug Interactions*. ed. Levy, RH., Thummel, K.E., Trager, W.F., Hansten, P.D. Eichelbaum, M. pp 21 – 29. Philadelphia: Lippincott Williams & Wilkins.

LÖSCHER, W. & HONACK, D. (1993). Profile of ucb LO59, a novel anticonvulsant drug, in models of partial and generalized epilepsy in mice and rats. *Eur. J. Pharmacol.,* **232,** 147 – 158.

LÖSCHER, W. & NAU, H. (1985). Pharmacological evaluation of various metabolites and analogues of valproic acid. *Neuropharmacology,* **24,** 427 – 435.

LÖSCHER, W., REISSMULLER, E. & EBERT, U. (2000). Anticonvulsant efficacy of gabapentin and levetiracetam in phenytoin-resistant kindled rats. *Epilepsy Res.,* **40,** 63 – 77.

LOTHMAN, E.W., SALERNO, R.A., PERLIN, J.B. & KAISER, D.L. (1988). Screening and characterization of antiepileptic drugs with rapidly recurring hippocampal seizures in rats. *Epilepsy Res.,* **2,** 367 – 379.

NAU, H., HAUCK, R-S. & EHLERS, K. (1991). Valproic acid induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanism. *Pharmacol. Toxicol.,* **69,** 310–321.

NAU, H. & SCOTT, W.J. (1986). Weak acids may act as teratogens by accumulating in the basic milieu of the early mammalian embryo. *Nature,* **323,** 276–280.

OTTO, J.F., KIMBALL, M.M. & WILCOX, K.S. (2002). Effects of the anticonvulsant retigabine on cultured cortical neurons: Changes in electroresponsive properties and synaptic transmission. *Mol. Pharmacol.,* **61,** 921–927.

PAXINOS, G. & WATSON, C. (1997). *The Rat Brain in Stereotaxic Coordinates,* 3rd edn. New York: Academic Press.

RACINE, R.J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroenceph. Clin. Neurophysiol.,* **32,** 281–294.

RADATZ, M., EHLERS, K., YAGEN, B., BIALER, M. & NAU, H. (1998). Valnoctamide, valpromide and valnoctic acid are much less teratogenic in mice than valproic acid. *Epilepsy Res.,* **30,** 41–48.

SKEEN, G.A., WHITE, H.S. & TWYMAN, R.E. (1994). The dihydropyridine nitrendipine reduces N-methyl-D-aspartate (NMDA)-evoked currents of rodent cortical neurons through a direct interaction with the NMDA receptor associated ion channel. *J. Pharmacol. Exp. Ther.,* **271,** 30–38.

SPIEGELSTEIN, O., YAGEN, B., LEVY, R.H., FINNELL, R.H., BENNETT, G.D., ROEDER, M., SCHURIG, V. & BIALER, M. (1999a). Stereoselective pharmacokinetics and pharmacodynamics of propylisopropyl acetamide, a CNS-active chiral amide analog of valproic acid. *Pharm. Res.,* **16,** 1582–1588.

SPIEGELSTEIN, O., BIALER, M., RADATZ, M., NAU, H. & YAGEN, B. (1999b). Enantioselective synthesis and teratogenicity of propylisopropyl acetamide, a CNS-active chiral amide analogue of valproic acid. *Chirality,* **11,** 645–650.

SWINYARD, E.A., WOODHEAD, J.H., WHITE, H.S., FRANKLIN, M.R. (1989). General Principles: Experimental selection, quantification and evaluation of antiepileptic drugs. In: *Antiepileptic Drugs* 3rd edn. Levy RH, Mattson RH, Meldrum BS, Penry JK, Dreifuss, FE. eds. New York: Raven Press, pp 85–102.

YUAN, J. (1993). Estimation of variance for AUC in animal studies. *J. Pharm. Sci.,* **82,** 761–763.

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