Title: PNU-120596, a positive allosteric modulator of α7 nicotinic acetylcholine receptors, reverses a sub-chronic phencyclidine-induced cognitive deficit in the attentional set-shifting task in female rats

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Abstract

The α7 nicotinic acetylcholine receptors (nAChRs) have been highlighted as a target for cognitive enhancement in schizophrenia. Adult female hooded-Lister rats received sub-chronic PCP (2 mg/kg) or vehicle i.p. twice daily for seven days, followed by 7-days washout. PCP-treated rats then received PNU-120596 (10 mg/kg; s.c.) or saline and were tested in the attentional set-shifting task. Sub-chronic PCP produced a significant cognitive deficit in the extra-dimensional shift (EDS) phase of the task ($P<0.001$, compared with vehicle). PNU-120596 significantly improved performance of PCP-treated rats in the EDS phase of the attentional set-shifting task ($P<0.001$). In conclusion, these data demonstrate that PNU-120596 improves cognitive dysfunction in our animal model of cognitive dysfunction in schizophrenia, most likely via modulation of α7 nACh receptors.

Keywords: Cognition, Schizophrenia, Attentional set-shifting; α7 nACh receptors; Phencyclidine; Female rat

Introduction

Cognitive deficits in schizophrenia have an important impact on outcome for patients and remain largely untreated by current medication (Keefe *et al.,* 2007). There is thus a clear need for improvements in therapy of this aspect of the illness. We have demonstrated robust impairments in set-shifting ability measured by impaired performance in the IED task using the CANTAB in first episode psychosis patients (Saleem *et al.,* 2010). This task assesses rule acquisition and reversal learning, based on visual discrimination of shapes and lines. Our results are supported by other studies showing that patients with schizophrenia show impaired set-shifting ability

(Haut *et al.,* 1996; Kolb and Wilshaw, 1983; Pantelis *et al.,* 1999). The rodent version of the attentional set-shifting task was adapted by Birrell and Brown (2000) and is a useful analogue of the Wisconsin Card Sorting Test (Berg, 1948). The TURNS initiative has determined that this rodent test can be used in preclinical tests to evaluate new therapeutic strategies (www.turns.ucla.edu). Initial studies using the perceptual attentional set-shifting task showed that lesions of the medial prefrontal cortex (mPFC) produce a selective deficit in the extra-dimensional shift (EDS) phase (Birrell and Brown, 2000). In our laboratory we have shown a selective deficit in female rats in the EDS phase following our 7 day sub-chronic phencyclidine (PCP) treatment regime; this deficit was attenuated by clozapine and risperidone, but was unaffected by haloperidol (McLean *et al.,* 2008). This PCP model in female rats has been found to induce robust cognitive deficits and neurobiological changes of relevance to schizophrenia and shows efficacy of a range of novel targets (see Neill *et al.,* 2010 for review).

The most prevalent nicotinic acetylcholine receptors (nAChRs) are the α4β2 and α7 subtypes, both of which show reduced numbers in schizophrenia (Freedman *et al.,* 1995; Breese *et al.,* 2000). Both of these receptor subtypes play a role in cognitive processing (Chan *et al.,* 2007; Gray and Roth, 2007; Schreiber *et al.,* 2002). See Leiser *et al.,* 2009 for a recent review of the role of α7 nAChRs in cognitive processing of relevance to schizophrenia. We have recently shown that the α7 nAChR full agonist, PNU-282987, reverses a sub-chronic PCP-induced deficit in two cognitive tests; novel object recognition and operant reversal learning (McLean *et al.,* 2011). It has been suggested that full nicotinic receptor agonists may provide sub-optimal benefits due to sustained activation and/or desensitisation at the target receptor (Quick and Lester, 2002; Smith *et al.,* 2002; Harris *et al.,* 2004; White and

Levin, 2004); this dilemma poses a pharmacological problem with respect to long-term therapy. However, we showed that the full α7 nAChR agonist, PNU-282987, was effective in reversing the PCP-induced deficit in novel object recognition following once daily administration for 15 days, suggesting that repeated activation of the target over a 15 day period does not evoke tolerance. However, this is relatively short-term and does not necessarily reflect the clinical situation, in which patients may have received a specific treatment for months or years.

An alternative approach to enhance α7 nAChR function is by augmenting effects of acetylcholine (ACh) via positive allosteric modulation (PAM) that can reinforce the endogenous cholinergic tone without directly activating α7 nAChRs (see Maelicke, 2000). Various molecules have been reported to positively modulate α7 nAChRs, including PNU-120596 (Hurst *et al.,* 2005), ivermectin (Krause *et al.,* 1998) and galantamine (Zhao *et al.,* 2006). PNU-120596 represents a novel positive allosteric modulator at the α7 nAChRs, which can increase the maximal agonist-evoked current and slow the decay of the currents in the continued presence of an agonist. The aim of this study was to investigate the efficacy of the selective α7 nAChR positive allosteric modulator, PNU-120596, in a model of relevance to cognitive deficits in schizophrenia, specifically PCP-induced deficits in the perceptual attentional set-shifting task.

Materials and Methods

Subjects and housing conditions

Thirty adult female hooded-Lister rats (Harlan, UK) housed in groups of five were used as subjects. Animals initially weighing 200-220 g were maintained under standard laboratory conditions at a temperature of 21°C (±2°C) and humidity of 40–

50%. They were maintained on a 12 h/12 h light/dark cycle (lights on at 0700 h) and experimental procedures were performed during the light phase. Rats were gradually food deprived to approximately 90% of free-feeding body weight; reduced body weight was maintained by restricting the amount of food (standard laboratory chow, Special Diet Services, Essex, UK) given to each rat per day (12 g/day). The availability of water was not restricted. Experiments were conducted in accordance with the Animals (Scientific Procedures) Act UK (1986), and approved by the University of Bradford ethical review process.

Attentional set-shifting

Rats were trained and tested in the attentional set-shifting procedure as described in detail in McLean *et al.* (2008), except rats were counterbalanced equally on odour and digging medium initially in simple discrimination. In all cases, rats were tested in the attentional set-shifting procedure 24 h after training. The first stage of testing was the SD, which was identical to the SD in the training session on the previous day, except new exemplars were used. Testing continued until the rat reached a criterion of six consecutive correct responses. For the compound discrimination (CD), a second dimension was introduced (odour or medium), but the correct and incorrect exemplars remained the same. For the reversals, the exemplars and relevant dimensions remained the same, but the rats had to learn that the previously baited odour was now incorrect and the other odour was now the correct one. New exemplars were used for the ID and ED shifts. The specific exemplars used are shown in Table 1; odours were aromatic oils (Bodyshop, UK) and were selected based on a pilot study showing that the oils used did not induce aversive behaviours. For the ED shift, the previously irrelevant parameter (odour or medium) was now relevant.

Drugs

Rats were pre-treated with 2.0 mg/kg PCP or vehicle (0.9% saline) by the intraperitoneal (i.p.) route twice daily for seven days. Dosing with sub-chronic PCP or vehicle was followed by a washout period of a further seven days. PCP hydrochloride (Sigma, UK) was dissolved in 0.9% saline. PCP-treated rats were then administered either PNU-120596 (supplied by Johnson and Johnson, Beerse, Belgium) or vehicle (PBS + 5% DMSO + 5% solutol) 30 min prior to testing. All treatment groups were n=10. PNU-120596 (N-[5-chloro-2,4-dimethoxyphenyl]-N'-[5-methyl-3-isoxazo lyl]-urea) was dissolved in PBS containing 5% DMSO (Sigma, UK) and 5% solutol (Johnson and Johnson, Beerse, Belgium), and was given in 1 ml/kg volume via the s.c. route and tested at one dose only of 10 mg/kg. The dose was selected based on efficacy to reverse a sub-chronic PCP deficit in the novel object recognition task in our laboratory, which tested doses of 5, 10 and 20 mg/kg of PNU-120596 and showed greatest efficacy at 10 mg/kg and some efficacy at 20 mg/kg (Idris et al, unpublished observations).

Pharmacokinetics analysis

The plasma and brain pharmacokinetics of PNU-120596 were studied in satellite groups of female hooded-Lister rats (~200 g). The compound was administered at 10 mg/kg as reported above, using the same formulation and dose volume as the main pharmacological studies. From each individual animal, (n=3), blood and brain samples were collected at 15 and 30 min, 1, 2, 4, 7 and 24 h after dose administration. Samples were placed immediately on melting ice and plasma was obtained following centrifugation at 4°C for 10 min at 1900 *x g*. Tissue samples were homogenized in

water (1/9 w/v or + 3 ml if tissue weight <0.33 g). All samples were shielded from daylight and stored at ≤ -18°C prior to analysis. Plasma and brain samples were analysed using an LC-MS/MS method with a lower limit of quantification (LLOQ) of 1.0 ng/ml in both matrices. A limited pharmacokinetic analysis was performed using WinNonlin™ Professional (Version 5.1).

Data and statistical analysis

Data for trials to criterion were analysed by a repeated measures two-way ANOVA using phase as a within-subjects factor and drug treatment as between-subjects factor and post-hoc Bonferroni multiple comparisons test. Post-hoc paired t-tests were carried out to compare difficulty between phases within the same treatment group.

Results

Attentional set-shifting

A repeated measures two-way ANOVA using phase as a within-subjects factor and drug treatment as between-subjects factor revealed a significant effect of phase [$F_{6,162}$ = 33.23; P<0.001], a significant effect of drug [$F_{2,27}$ = 11.01; P<0.001] and an overall significant interaction [$F_{2,27}$ = 6.59; P<0.01]. A significant increase in trials to criterion in reversal 1 was observed in all treatment groups compared to simple discrimination (P<0.01; fig 1). A significant increase (P<0.05) was also observed in the vehicle group in the EDS phase compared to the IDS phase, indicating that an attentional set had been formed. Post-hoc analysis within the treatment groups showed that PCP significantly increased trials to criterion in the EDS phase (P<0.001) and in reversal 3 (P<0.01). This deficit was significantly attenuated in the EDS phase by PNU-120596 (10 mg/kg, s.c.; P<0.001). There was no effect of treatment on time

taken to complete the trials (data not shown), suggesting there were no motoric effects of the compound.

Active plasma and brain levels

Following s.c. administration of 10 mg/kg PNU-120596 to female hooded-Lister rats, rather low mean plasma levels were observed (C_{max} 14.5 ng/ml,) at 0.8 h post dose. As can be seen in fig 2 and table 2, plasma levels were low yet relatively stable across the first 4 hours post dose. Area under the concentration time curve ($AUC_{0\text{-}last}$) was 167 ng.h/ml. As the compound was administered as a suspension, dissolution at the s.c. injection site was probably rather slow and incomplete. Brain levels were consistent with plasma indicating a rapid absorption and subsequent distribution (brain/plasma ratio ~1).

Discussion

The current study showed that sub-chronic PCP induced a robust cognitive deficit in attentional set-shifting, and the α7 nAChR positive allosteric modulator, PNU-120596, selectively attenuated the deficit in the EDS phase of the attentional set-shifting task. Recent studies in our laboratory and elsewhere show that many targets are effective to improve the PCP-induced deficit in a variety of tasks assessing different aspects of cognitive function (see Neill *et al.*, 2010 and Jones *et al.*, 2011 for recent reviews). For example, in novel object recognition (NOR): several atypical antipsychotics, a D_1 receptor agonist, ampakines, glycine uptake inhibitors, 5-HT_{2A} receptor inverse agonists, a 5-HT_6 receptor antagonist, $GABA_A$ receptor agonists and full agonists at α7 nACh receptors are active to reverse the PCP-induced deficit (see Neill *et al.*, 2010 for further details). Clearly, the key question here is which, if any, are effective in the

clinic and how valuable the positive animal data are. Inspection of the clinical trials on-going at schizophreniaresearchforum.org shows that many of these targets are currently in various stages of clinical testing, with varying degrees of success, with patients not always assessed for cognitive function. It is therefore still early to correlate pre-clinical with clinical efficacy, although some data are emerging. For animal models, it will be important to show efficacy across a range of cognitive domains, in more than one model (e.g. in a genetic and pharmacological and/or neurodevelopmental model) to show that efficacy is maintained following chronic treatment and also demonstrate effects on neurobiological measures such as gamma oscillations and parvalbumin.

The present results show that the vehicle-treated group required significantly fewer trials to reach criterion in the IDS phase than in the EDS phase, suggesting that an attentional set was formed. In all treatment groups, rats required more trials to reach criterion in reversal 1 compared to the SD stage of the task, suggesting that all groups found reversal 1 more difficult than the previous stages. There was a significant increase in trials required to reach criterion in PCP-treated rats in the EDS stage, suggesting that sub-chronic PCP causes a deficit in attentional set-shifting ability. This is in agreement with a previous study in our laboratory (McLean *et al.*, 2008). This deficit in also seen in patients with schizophrenia and in our first episode study (Saleem *et al.*, 2010), strengthening the validity of this model in rats. This result is supported by several studies in other laboratories in which sub-chronic PCP also produces deficits in the EDS phase of the attentional set-shifting task, although ours is the only study to use female rats (Rodefer *et al.*, 2005; 2008; Goetghebeur and Dias, 2009; Egerton *et al.*, 2008). In the current study PNU-120596 (10 mg/kg) significantly reversed this PCP-induced deficit in the EDS phase. In addition, PCP

significantly increased trials to criterion in reversal 3. Although PNU-120596 did not significantly improve the PCP-induced deficit in reversal 3, the number of trials was decreased from 13.3 to 9.9, a level of responding similar to that of the vehicle-treated group i.e. 8.5 trials. PK analysis revealed that PNU-120596 at 10 mg/kg reached plasma and brain, albeit at relatively low levels. Ideally the PK analysis should have been carried out in satellite PCP-treated rats rather than drug naive rats. However, as the dose of PCP is low and is followed by a 7 day washout period, it is unlikely to affect the clearance mechanisms of the compound.

Based on an in vitro calcium flux assay (data not shown), the compound shows activity on the human α7 ion channel with an EC50 of 0.16 µM (50 ng/ml). Data for the rodent ion channel is not available so the assumption is that the activity is comparable across species. In vivo a Cmax of 14.8 ng/ml (0.05 µM) and an $AUC_{0\text{-last}}$ of 167 ng.h/ml (0.53 µM.h) were observed. The concentrations observed in vivo are therefore within the range for in vitro activity (assumption human/rat as above). At this point we do not fully understand the PK/PD relationship of this molecule or mechanism, i.e. a maximum plasma concentration or an AUC driving the response. Further studies are clearly required to elucidate the full PK/PD profile and will be the subject of further studies.

In support of our data, a recent study found the α7 nAChR partial agonist, RG3487, improved sub-chronic PCP-induced deficits in attentional set-shifting in male Long-Evans rats (Wallace *et al.,* 2011). In addition, nicotine was found to improve auditory and visual shift trials in male Sprague-Dawley rats (Brown *et al.*, 2010). An α7 nAChR full agonist, compound A ((R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide), has also been shown to improve olfactory working memory in the odour span task in uncompromised rats (Rushforth *et al.*,

2010). We have also found the full agonist, PNU-282987, to reverse sub-chronic PCP-induced deficits in the novel object recognition task (McLean *et al.*, 2011). For a detailed review of the effects of nicotine in animal models of attention see Levin *et al.*, (2011).

Positive allosteric modulators (PAMs) of α7 nAChRs have been classified as either type 1 or type 2 compounds; type 1 compounds predominantly affect the peak current response, whereas type 2 compounds affect both peak current responses and the kinetics of agonist-evoked responses (Bertrand and Gopalakrishnan, 2007). It has been shown that PNU-120596 causes potentiation of agonist-evoked α7 receptor responses by binding within the nAChR transmembrane region, therefore characterising it as a type 2 PAM (Young *et al.*, 2008). The attenuating effects of PNU-120596 in our cognitive task are supported by a study showing that PNU-120596 increased the potency of ACh by ~10-fold and reversed an amphetamine-induced deficit in auditory gating in rats (Hurst *et al.*, 2005). PNU-120596 has also been reported to enhance the increase in dopamine release in the mPFC induced by choline and an α7 nAChR agonist (compound A; (R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide); it was also shown that PNU-120596 (1 mg/kg; s.c.) when administered systemically (but not locally) also produced an increase in dopamine overflow in the mPFC in the absence of an agonist (Livingstone *et al.*, 2009). This is of importance as it has been previously shown that attentional set-shifting performance is dependent upon the mPFC (Birrell and Brown, 2000) and that dopamine plays a pivotal role in cognition (Goldman-Rakic *et al.*, 2004). Indeed, we have found the PCP-induced deficit in novel object recognition memory is accompanied by impaired dopamine neurotransmission in the PFC during the

retention trial of the task (Snigdha *et al.*, 2008). This result was also recently confirmed in a second study in our laboratory (McLean, unpublished observations).

In conclusion, PNU-120596 reversed a sub-chronic PCP-induced deficit in attentional set-shifting ability. To our knowledge this is the first study to investigate an α7 nAChR PAM in the attentional set-shifting task. It is suggested from other studies that these compounds may exert their cognitive enhancing effects by potentiating the effects of endogenous acetylcholine and increasing levels of dopamine and acetylcholine in the prefrontal cortex.

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Conflict of Interest

Drs Pemberton, Lesage and Mackie are employees of Johnson & Johnson Pharmaceutical Research and Development.

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Tables and Figures

Table 1: Specific exemplars used (presented in pairs)

Dimension	Pairing 1 (CD)	Pairing 2 (IDS)	Pairing 3 (EDS)
Odour	Rose = O1 White flower = O2	Green Meadow = O3 Coconut = O4	Orange = O5 Almond = O6
Medium	Wood shavings = M1 Cat litter = M2	Small pebbles = M3 Aspen = M4	Fine sawdust = M5 Wood blocks = M6

Table 2: Individual and mean plasma concentrations and some basic pharmacokinetic parameters after single s.c. administration at 10 mg/kg of PNU-120596 in female hooded-Lister rats.

Exposure in rat after subcutaneous administration of PNU-120596

	B1	**B2**	**B3**	**mean SC**		**s.d.**
Body Weights (g)	**195**	**196**	**186**	**192**	±	**5**
Time (h)	**Plasma levels (ng/ml)**			**Plasma levels (ng/ml)**		
0.25	15.8	12.1	10.7	**12.9**	±	**2.6**
0.5	19.8	12.6	10.6	**14.3**	±	**4.8**
1	18.3	12.7	11.1	**14.0**	±	**3.8**
2	19.1	12.6	9.03	**13.6**	±	**5.1**
4	15.8	11.8	10.8	**12.8**	±	**2.6**
7	10.4	9.00	8.13	**9.18**	±	**1.15**
24	2.59	1.97	1.77	**2.11**	±	**0.43**
C_{max} (ng/ml)	19.8	12.7	11.1	**14.5**	±	**4.6**
T_{max} (h)	0.5	1.0	1.0	**0.8**	±	**0.3**
$t_{1/2}$ (h)	7.9	7.7	7.7	**7.8**	±	**0.1**
time points $t_{1/2}$	4-24	4-24	4-24			
$AUC_{0\text{-last}}$ (ng.h/ml)	204	158	138	**167**	±	**34**
last time point AUC	24	24	24			
$AUC_{0\text{-inf}}$ (ng.h/ml)	233	180	158	**190**	±	**39**
MRT (h)	11.6	11.7	11.9	**11.7**	±	**0.2**

Figure 1

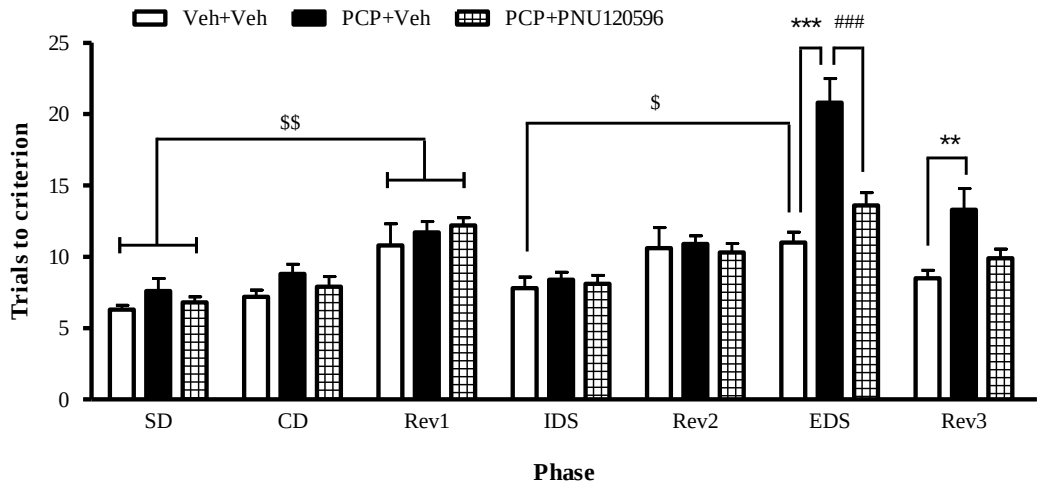
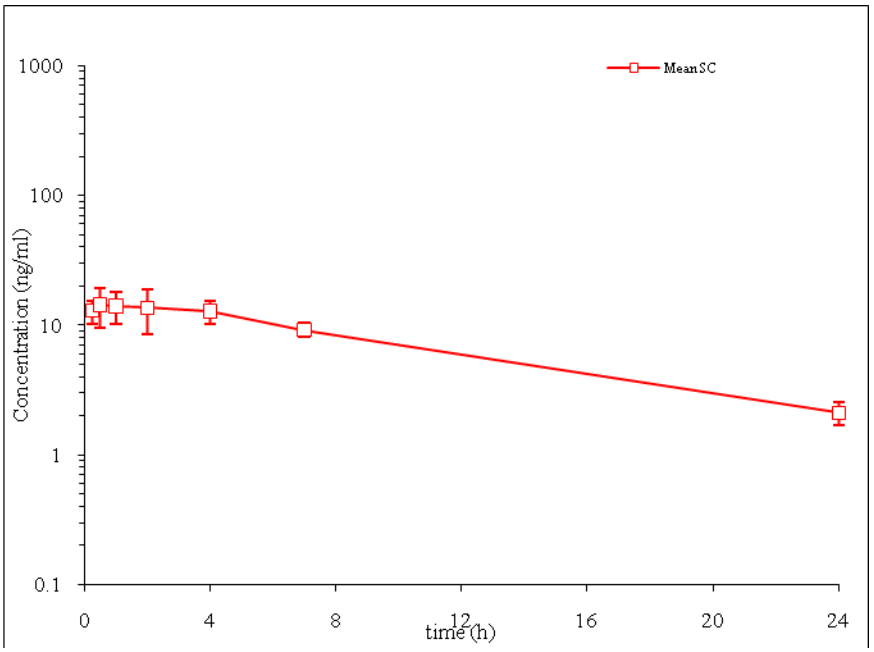


Figure 2



Legends

Table 1: Specific exemplars used in each phase of the attentional set-shifting task. Odours were applied around the rims of digging bowls which were filled with various digging media depending upon the phase being tested. The significance of pairing within a test phase ensures that, for example, rose is always accompanied with white flower within a test trial [O = Odour, M = Medium].

Table 2: Individual (animals B1, B2, B3) and mean plasma concentrations (ng/ml) and some basic pharmacokinetic parameters after single s.c. administration at 10 mg/kg of PNU-120596 in female hooded-Lister rats. Plasma (and brain) samples were collected at different time intervals after compound administration. Concentrations of PNU-120596 were determined as described in materials and methods and plotted as a function of time.

Figure 1: The effect of acute treatment with PNU-120596 (10 mg/kg, s.c.) in sub-chronic PCP (2 mg/kg, twice daily for 7 days, i.p.) treated rats on total trials to reach criterion in the attentional set-shifting task. All data are expressed as mean ± SEM (n=10). Data were analysed by repeated two-way ANOVA and post-hoc Bonferroni multiple comparisons test. $^{\$}P<0.05$-$^{\$\$}P<0.01$; significant increase in trials to criterion between stages of the task analysed by a post-hoc paired t-tests. **P<0.01-***P<0.001; significant increase in trials to criterion in PCP-treated rats. $^{\#\#\#}P<0.001$; significant decrease in trials to criterion in PNU-120596-treated rats.

Figure 2: Mean plasma concentrations of PNU-120596 after s.c. administration at 10 mg/kg in female hooded-Lister rats. Plasma (and brain) was collected at different time intervals after compound administration.