Pharmacokinetics and Pharmacodynamics of the Glutamate Carboxypeptidase (GCP) II Inhibitor 2-MPPA Show Prolonged Alleviation of Neuropathic Pain Through an Indirect Mechanism

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Total text pages: 24 (incl refs, fig legends, tables etc)  
Number of Tables: 1  
Number of Figures: 7  
Number of References: 45 (60 max)  
Number of words in Abstract: 240 (250 max)  
Number of words in Introduction: 406 (750 max)  
Number of words in Discussion: 1492 (1500 max)  

Abbreviations:  
GCP II is Glutamate Carboxypeptidase-II;  
NAAG is N-acetyl-aspartyl glutamate;  
2-MPPA is (2-(3-mercaptopropyl) pentanedioic acid);

Running title:  
Pharmacokinetics/Pharmacodynamics of GCP II Inhibitor 2-MPPA
Abstract

Glutamate Carboxypeptidase II (GCP II) is a therapeutic target in neurological disorders associated with excessive activation of glutamatergic systems. The potent, orally bioavailable GCP II inhibitor 2-MPPA is effective in preclinical models of diseases where excess glutamate release is implicated, including neuropathic pain and was the first GCPII inhibitor to be administered to man. The relationship between dosing regimen, pharmacokinetics and analgesia in a neuropathic pain model were examined in rats to aid development of clinical dosing. The efficacy of oral 2MPPA in the chronic constrictive injury (CCI) model was not simply related to plasma concentrations. Even though maximal concentrations were observed within one hour of dosing, the analgesic effect took at least 8 days of daily dosing to become significant. The delay was not due to tissue drug accumulation since inhibitory concentrations of the drug were achieved in the nerve within one hour of dosing. There was also no accumulation of drug in plasma or tissue after multiple daily dosing. Effects were dependent on reaching a threshold concentration since dividing daily dose led to a loss of effect. The analgesic effect outlasted plasma exposure and was maintained for days even after daily dosing was halted. The delayed onset, dependence on threshold plasma concentration, and sustained effects after exposure support the hypothesis that an indirect, long-lived mechanism of action exists. While these longer lasting secondary mechanisms are not yet identified, daily clinical dosing of a GCP II inhibitor appears justified.
INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the central and peripheral nervous system. Excessive activation of glutamatergic systems has been implicated in number of neurological disorders including neuropathy and neuropathic pain (Carozzi et al., 2008a; Yogeeswari et al., 2009). The neuropeptide N-acetyl-aspartyl glutamate (NAAG) is abundant in both brain and peripheral nervous system where it appears to serve as a neurotransmitter both as a dipeptide and as a precursor of glutamate. As a dipeptide, it appears to act through mGluR3 receptors to decrease glutamate release (Neale et al., 2011; Barinka et al., 2012).

Glutamate Carboxypeptidase II (GCP-II, also termed NAALADase and NAAG peptidase) is a 94 kD membrane bound zinc metalloenzyme that catalyzes the hydrolysis of neuropeptide NAAG to N-acetyl-aspartate and glutamate (Slusher et al., 1990). GCP-II acts to terminate the neurotransmitter activity of NAAG at mGluR3 receptors resulting both in increased synaptic glutamate release (Wroblewska et al., 1993; Coyle, 1997; Slusher et al., 1999) and liberation of glutamate from NAAG that can further activate various glutamate receptors. Inhibition of GCP-II therefore attenuates glutamatergic effects both by causing an increase in NAAG’s inhibition of glutamate release and simultaneously decreasing glutamate levels.

The first potent and selective GCP-II inhibitor identified was 2-PMPA (2-(phosphonomethyl) pentanedioic acid) (Jackson et al., 2001). 2-PMPA was shown to possess therapeutic efficacy in various preclinical models including ischemia (Slusher et al., 1999), spinal cord injury (Long et al., 2005), neuropathic pain (Jackson et al., 2001; Wozniak et al., 2012), cocaine addiction (Xi et al., 2010a; Xi et al., 2010b) and schizophrenia (Olszewski et al., 2008; Zuo et al., 2012). Thus, GCP-II inhibition may provide broad therapeutic utility in neurological diseases especially where excess glutamate is presumed pathogenic (Rojas et al.,
2011; Barinka et al., 2012). Although potent and selective, 2-PMPA is not orally available perhaps due to its extreme hydrophilic nature and limited permeability through the gastrointestinal tract.

Replacement of the phosphonomethyl group with a thioalkyl group led to discovery of 2-MPPA (2-(3-mercaptopropyl) pentanedioic acid) (Majer et al., 2003) which was found to be orally available and was the first GCP II inhibitor to be administered to man (van der Post et al., 2005) where it was well tolerated and reached exposure levels similar to those required for therapeutic effects in animal models. In order to develop a dosing regimen for clinical use, the pharmacokinetics and pharmacodynamics were examined in more detail in animals to determine the required concentration and duration of exposure.

METHODS

Animals: Male Sprague-Dawley rats (Charles River) weighing 200-250g were used. Animals were housed in suspended polycarbonate cages under a 12h light/dark cycle. Food (Harlan/Teklab) and water (filtered and delivered via an automatic watering system) were provided ad libitum. All procedures were conducted in compliance with the laws, regulations and guidelines of the National Institutes of Health (NIH/PHS) and with approval from the local Animal Care and Use committee.

Drugs: 2-MPPA was dissolved in 50mM HEPES buffered saline and brought to neutral pH with NaOH. Dosing solution was prepared fresh every 2-3 days and stored under refrigeration (2° - 8°C).

Pharmacokinetic Studies: 2MPPA was administered to rats as a solution via the oral gavage or intravenous (caudal vein) route, at doses of 1,3,10, 30, 50, 100, 500 or 1000 mg/kg. At various time points after dosing, rats were sacrificed. At each time point, plasma was derived from
whole blood collected and centrifuged at 4 °C in plasma separator tubes for 10 min. In some studies tissue was also removed. All samples were stored at -80 °C until subsequent analysis.

Quantification of 2-MPPA using HPLC with tandem mass spectrometry (LC-MS/MS) was performed as described in bioanalytical method described below.

**Bioanalysis of 2-MPPA:** 2-MPPA was quantified in plasma and tissue samples following derivatization with N-ethylmaleimide and analyzed via LC/MS/MS as described previously (Rais et al., 2012). Briefly, 180 µL plasma sample (or 160 µL of matrix blank and 20 µL of stock for standards) was reacted with 10 µL of 100 mM NEM for 30 min along with structurally similar internal standard at 5µg/mL in acetonitrile. The derivatized samples were extracted with methanol followed by vortexing and centrifugation at 10,000 rpm for 5 min. The supernatant (50 µL) was transferred to LC vial and a volume of 5 µL injected on LC/MS/MS. The tissue samples were processed in a similar manner to plasma. Briefly, Tissue samples were weighed, followed by addition of PBS buffer (also containing 10 µL of 100 mM NEM), and volume adjusted to obtain all samples equal per gram tissue. The samples were homogenized, vortexed and extracted following the same procedure as described for plasma. The calibration range was 1-4000 ng/mL for 2-MPPA in plasma and 50-50,000 ng/mL in tissue.

Samples were analyzed on an Agilent 1100 HPLC, (Agilent Technologies, Santa Clara, CA) coupled to API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) Derivatized -2-MPPA was separated on a Luna C18 (2 mm) 30 x 4.60 mm 5 µM column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (A), and 0.1% formic acid in MilliQ water (B). Separation was achieved using a gradient run, with the 40% (A) going to 90% (A) over a period of 4 min, maintaining at 90% for 3 min, then re-equilibrating over 3 min (40% A) at a flow rate of 350 µL/min and total run time of 10 min. MS instrument
was operated in a negative ion mode. The multiple reaction monitoring (MRM) transition of derivatized 2-MPPA was 330>205 (Q1/Q3) and for the internal standard was 645.3>323.3 with a declustering potential of 40 V, entrance potential 10 V, and collision energy of 21 V. The curtain gas, ion-spray voltage, temperature, nebulizer gas (GS1), and auxiliary gas (GS2) were set at 8 psi, 5500 V, 350°C, 8 psi, and 4 psi, respectively, and the interface heater was on.

**Pharmacokinetic Analysis:** Initial pharmacokinetic studies with intense sampling were conducted to determine the plasma concentration time profile of 2-MPPA. Subsequent studies were conducted using time points from the tissue distribution study described above and from single plasma samples obtained from animals evaluated in the chronic constrictive injury model. The entire dataset was analyzed using a two-compartment model with first order absorption. A population pharmacokinetic model was developed utilizing both intense and sparse sampling paradigms (WinNonmix, Version 2.0.1, Pharsight Corporation). AUC and mean peak plasma concentration (Cmax) for each animal were the primary outputs for the population pharmacokinetic analysis.

Pharmacokinetic parameters following single doses of 2-MPPA to rats were calculated using non-compartmental methods (WinNonlin, Version 5.1, Pharsight Corporation). Bioavailability was calculated using the following equation:

\[
F\% = \frac{\text{Mean AUC (Oral)}}{\text{Mean AUC (i.v.)}} \times \frac{\text{Dose (i.v.)}}{\text{Dose (Oral)}} \times 100
\]

**Chronic Constrictive Injury Model:** The methods used were as previously described (Bennett and Xie, 1988) using male Sprague Dawley rats. In brief, the common sciatic nerve was exposed
and 4 ligatures (4.0 chromic gut) were tied loosely around it with 1mm spacing. Hyperalgesia testing was initiated 10 days post-surgery. Thermal sensitivity was assessed by determining withdrawal latencies to a constant infrared stimulus to the plantar surface of the hind paw using a Basile Plantar apparatus (Ugo Basile, Vaarese, Italy) according to the method described by Hargreaves et al (Hargreaves et al., 1988). Withdrawal latency was measured as the time taken for the rat to withdraw its paw from the heat source to the nearest 0.1 sec. The “difference score” was calculated by subtracting the average latency of the non-ligated versus ligated side.

Statistical analyses were conducted using the student’s t-test (when 2 groups including vehicle were compared) or ANOVA with post hoc Dunnett’s test (when 3 or more groups were compared).

**Open field locomotion:** Animals were dosed with vehicle or 10, 50 or 100 mg/kg 2-MPPA po and placed in open field locomotor counter monitoring infrared beam interruptions (Columbus Instruments, Ohio). Locomotion was counted for 5 min intervals for one hour starting from one hour after administration of vehicle or 2-MPPA.

**Once Daily Dose-response Studies:** 2-MPPA or vehicle at various doses was given daily by oral gavage in a solution volume of 1 ml/kg. Testing was initiated from the day before treatment onset and then on test days from Day 1 of treatment, twice a week and one hour after dosing. In follow-up studies investigating the efficacy time course, testing was conducted at 1, 4 or 16 h post dose.

**Dose Fractionation Studies:** 2-MPPA or vehicle was administered once, twice or three times daily by oral gavage. The 5 mg/kg dose was administered twice daily with a 5h interval between the two administrations. The 3.3 mg/kg dose was administered three times a day with 4 hour
intervals between the administrations. Behavioral testing was always conducted one hour after
the final daily dose, regardless of the regimen.

RESULTS

Pharmacokinetics of 2-MPPA in rats

Following an IV bolus of 10 mg/kg, $C_{\text{max}}$ (extrapolated to zero) was 27.0 μg/ml, which
declined in a biphasic manner, with the initial rapid decline within the first hour. Average
clearance, terminal elimination half-life ($T_{1/2\text{elim}}$) and mean residence time were 4.0 L/hr/kg, 1.0
hour and 0.2 hours respectively. Likewise, the oral absorption following a 10 mg/kg dose was
rapid with a similar biphasic decline (Figure 1).

A single-dose, dose-proportionality study was conducted in non-fasted, male and female
Dawley rats with 2-MPPA at oral doses of 1, 3, 10, 30, 50, 100, 500 and 1000 mg/kg (Table 1).
After oral administration, plasma pharmacokinetic profiles were biphasic with terminal $t_{1/2}$
ranging from 2.4 to 9.2 hours. Although $C_{\text{max}}$ increased proportionally approximately with dose,
AUC increased supra-proportionally with dose over the range of 1 to 1000 mg/kg.
Bioavailability was high (82%) based on the 10 mg/kg IV and PO administrations.

Daily Administration of 2-MPPA Attenuates Thermal Hyperalgesia

2-MPPA administered at doses of 10 and 30 mg/kg/day PO attenuated thermal
hyperalgesia compared to vehicle-treated controls reaching significance on days 8 through to 15
of dosing (Figures 2A and B). Repeated daily dosing was required for the effect to develop.
Animals receiving 3 mg/kg/day 2-MPPA PO displayed a strong trend towards reduction of
hyperalgesia although the effect did not achieve statistical significance (Figure 2C). Animals
receiving 1 mg/kg/day PO 2-MPPA daily showed no drug effect (Figure 2D). The sensitivity to thermal stimulation on the non-ligated (i.e., normal) paw was unaltered by 2-MPPA treatment at all doses tested. As representative, Figure 2E depicts the sham-operated side responses after the two highest doses evaluated, 10 and 30 mg/kg.

**Administration of 2-MPPA has no effect on open-field locomotor activity**

2-MPPA administered to rats at doses of 30, 50 and 100 mg/kg PO had no significant effect on locomotion measured in an open-field compared to vehicle-treated controls (Figure 3), suggesting no direct suppression of motor function.

**2-MPPA does not accumulate in tissue following multiple dosing**

The observation that the speed of onset is dependent on daily exposure can be explained either by an indirect pharmacological mechanism in which effect accumulates over time or a deep compartment where drug accumulates even though there is no accumulation in plasma of this rapidly eliminated drug. In order to investigate whether this effect was due to tissue accumulation, a single- and multiple-dose oral tissue distribution study was performed.

2-MPPA (10 mg/kg/day PO) was distributed throughout the body within 5 minutes after dosing on day 1. The $C_{\text{max}}$ in sciatic nerve was 0.018 $\mu$g/g, equivalent to 87 nM based on wet weight of tissue, above the $K_i$ for GCPII inhibition by 2-MPPA of 30 nM. The $C_{\text{max}}$ on both days 5 and 11 was achieved within 30 minutes in sciatic nerve without evidence of accumulation. Data for plasma and sciatic nerve on days 1 and 5 are shown in Figure 4.

The time course of enzyme inhibition on each day of dosing was determined by measuring enzyme activity in sciatic nerve 30 minutes, 6 or 12 hours following dosing. In sciatic
nerve removed from vehicle treated animals, GCP II activity was 2230±198 fmoles/mg prot/hr (mean ± SEM). As predicted by tissue concentrations, enzyme was significantly inhibited after 30 minutes (1363±120 fmoles/mg prot/hr, p<0.05), returning to baseline at 6 hrs (2312±283 fmoles/mg prot/hr, n.s.). This suggests that while enzyme is rapidly inhibited in a concentration dependent manner, the behavioral effects on hyperalgesia are achieved more gradually.

**2-MPPA efficacy reduced by divided dosing**

To confirm that the slower behavioral effect was due to daily brief inhibition of GCPII and not a loading of a deep tissue compartment, the effect of divided doses was examined. If the effect is dependent on maximal exposure (e.g., Cmax) or a time above a threshold concentration, then divided doses would be less effective. If, on the other hand, total exposure (e.g., AUC) were occurring, then overall exposure would be more important and divided doses would be just as effective.

10 mg/kg was chosen as the dose since it was the minimally effective daily dose of 2-MPPA in the CCI model. As shown in Figure 5, drug effect was completely lost when this single daily dose was divided into two doses of 5 mg/kg or three doses of 3.3 mg/kg each. This suggests that Cmax not AUC determines the pharmacodynamic effect, i.e. there is a minimal plasma concentration threshold of 2-MPPA that must be achieved each day for attenuation of hyperalgesia.

**2-MPPA Efficacy Persists After Daily Dosing Is Stopped**

In order to confirm that daily exposure acted through a long lasting mechanism, the time course of effect related to plasma concentration was further examined. The duration of
neuropathic pain alleviation following administration of 2-MPPA was tested 1 hour, 4 hours and 
16 hours after a 10 mg/kg 2-MPPA. As shown in Figure 6, on day 11 animals tested one hour 
after the daily dose showed a significant reduction in thermal pain perception. A strong trend was 
also observed at 4 or 16 hours, but did not attain statistical significance. After 15 days of dosing, 
significant improvement was also observed 4 or 16 hours after the daily dose of 10 mg/kg 2-
MPPA. These data suggest that once effective neuropathic pain alleviation is observed in 
animals, the effect can be maintained for up to 16 hours following drug administration.

Maintenance of effect was further examined by cessation of dosing after analgesic effect 
was well established after 15 days of daily dosing. As shown in Figure 7, significant attenuation 
of hyperalgesia continued to be observed for an additional 17 days without further administration 
of 2-MPPA confirming that enzyme inhibition is not necessary once the reduction in 
hyperalgesia is established.

DISCUSSION

Neuropathic pain is thought to be due to aberrant neuronal responses along pain pathways 
from the dorsal root ganglion (DRG) to spinal cord, thalamus and cortex with both central and 
peripheral mechanisms likely involved (Zhuo et al., 2011).

2-MPPA is a potent and specific inhibitor of GCP II (Majer et al., 2003) that has safely 
been administered to man (van der Post et al., 2005). We show that 2-MPPA efficacy in the CCI 
model of neuropathic pain is dependent upon, but not simply correlated with, dose and tissue 
exposure. Lack of general sedative effects of 2-MPPA at doses up to 100 mg/kg acutely on 
locomotor activity, as well as unaltered sensitivity to thermal stimulation on non-ligated (i.e., 
normal) paws in CCI-rats after multiple days of treatment suggest a specific effect of GCPII
inhibition on decreasing nociceptive input and not decreasing motor outflow. Similar non-sedative effects have been reported for other GCPII inhibitors (Lukawski et al., 2008; Olszewski et al., 2008). The analgesic effects took at least 8 days of daily dosing to become significant even though inhibitory concentrations of the drug were achieved in the nerve rapidly on the first day of dosing and there was no accumulation of drug in plasma or tissue after multiple daily dosing. Divided doses lost effectiveness, suggesting the effect is dependent more upon $C_{\text{max}}$ than AUC. Thus the effect is not simply due to total daily exposure, consistent with an indirect mechanism of action caused by daily enzyme inhibition rather than tissue loading with drug. In addition, the half-life of 2-MPPA in plasma was short, yet the analgesic effect once established was sustained. The analgesia outlasted the short plasma exposure on testing days and was maintained for days even after dosing was halted. This lack of direct correlation of pharmacodynamic with pharmacokinetic time profile is also typical of an indirect pharmacological effect.

It is noteworthy that while we observed a delayed analgesia onset in our model of neuropathic pain, more rapid effects have been observed in other peripheral pain models such as those induced by carrageenan and formalin (Yamamoto et al., 2007). The time course of action may depend on site of injury, participation of central mechanisms, or degree of GCP II inhibition. There may also be two analgesic mechanisms, one acute and one chronic involving plasticity in pain response pathways.

Drug accumulation in a deep tissue compartment can sometimes explain a lack of direct temporal correlation between plasma concentration and analgesic effect. In cases of deep compartment loading, slow onset of effect occurs as drug slowly enters an effect compartment and the loss of effect occurs over time due to slow efflux of drug. Such a site would have to be
hydrophilic due to the nature of 2-MPPA and of small volume to explain lack of accumulation in tissue samples.

Our measurements of enzyme inhibition in tissue exclude tissue accumulation as an explanation for the delayed onset and persistent effects of daily administration. The concentration of 2-MPPA in sciatic nerve, a possible site of action, reached levels consistent with GCP II inhibition (greater than 0.018 μg/g for at least 8 hrs). This was confirmed by measurement of enzyme activity in tissue, further excluding the possibility of persistent enzyme inhibition. We also excluded accumulation of an active metabolite by measuring the time course of enzyme inhibition in tissue and showing direct correlation to plasma drug levels. Since enzyme inhibition was well predicted by tissue drug levels, we conclude that after an oral dose, enzyme is rapidly inhibited but the analgesic effects outlast the period of enzyme inhibition. Thus the magnitude of the pharmacodynamic effect is dependent on daily exposure but not directly correlated with its time course.

GCP II has been localized to both the PNS and CNS. In the periphery, GCP II is found in satellite cells of the dorsal root ganglia, the cytoplasm of Schwann cells and the surface of nerve fibers (Berger and Lassner, 1994; Carozzi et al., 2008a; Carozzi et al., 2008b). Enzymatic activity can be found at nerve endings in human skin (Rojas et al., 2011). In the brain and spinal cord, GCP II has been localized to astrocytes by both in situ hybridization (Ghose et al., 2004) and immunochemistry (Berger et al., 1999; Sacha et al., 2007). Overall, this localization is typical of an enzyme involved in neuronal and glial signaling and is not consistent with the presence of a deep anatomical compartment to explain delayed onset and prolonged effect. In addition, NAAG is expressed in millimolar levels in the spinal cord (Fuhrman et al., 1994) and intrathecal administration of GCPII inhibitors induces an analgesic response to inflammatory
pain in the hindlimb (Yamamoto et al., 2001). Similarly, introduction of GCPII inhibitors directly into the ipsilateral lateral ventricle reduced responses to footpad inflammation (Yamamoto et al., 2008). GCPII Inhibition has also been shown to reduce induction of contralateral hindlimb allodynia 24h after an inflammatory insult (Adedoyin et al., 2010).

Overall, the underlying pharmacological mechanism involved in GCPII inhibition attenuating pain is not established. Analgesia induced by systemically administered GCPII inhibitors appears to be mediated both via spinal cord and brain. GCP II inhibitors have effects both in the peripheral nervous system, decreasing ectopic discharges from injured nerves (Chen et al., 2002) and in the central nervous system, reversing central sensitization in the spinal cord (Yamamoto et al., 2001; Carpenter et al., 2003; Yamamoto et al., 2004).

It is likely that the slow onset and prolonged effect of 2-MPPA in the CCI model is due to an indirect effect at the site of injury or on the function of pain pathways. Neuropathic pain itself is thought to be dependent on neuroplasticity and persistent changes in pain pathway structure and function (Besson, 1999; Woolf, 2011). Therefore it is possible that GCP II inhibition reverses neuropathic pain-inducing mechanisms in the periphery and/or the brain and spinal cord and that the effects outlast inhibition of the enzyme. Since GCP II inhibition attenuates excessive glutamatergic activity in a number of pathological models, the effect might be produced at multiple points at which excessive glutamatergic activity triggers long-term changes in pain sensitivity. This might occur in a manner analogous to the delayed onset of effect of antidepressants that depend on neuroplasticity in brain circuits (Danzer, 2012; Quide et al., 2012; Schloesser et al., 2012). In addition, GCP II inhibition might act at the site of nerve injury. This is suggested by finding that GCP II inhibition prevents or reverses damage in models of diabetic (Zhang et al., 2006) and chemotherapy induced peripheral neuropathy (Carozzi et al., 2008a).
Consistent with the expression of NAAG-immunoreactivity in large and some mid-size spinal sensory neurons (Cangro et al., 1987), expression of mGluR3 by these neurons (Carlton and Hargett, 2007), and the analgesic efficacy of Group II mGluR agonists on peripheral neurites (Yang and Gereau, 2003), NAAG and GCPII inhibitors were shown to be analgesic when injected directly into the hindpaw prior to induction of an inflammatory insult. In each of these studies, analgesia induced by peptidase inhibition was blocked by co-administration of the group II mGluR3 antagonist, LY 341495. In addition, analgesic effects of locally administered GCPII inhibitors were also attenuated by mGluR3 antagonism (Yamamoto et al., 2007), supporting the conclusion that the process is mediated by NAAG activation of mGluR3. The slow onset and prolonged effect of inhibition might be due to effects on pain generating mechanisms at the site of injury. This would be consistent with the observation that hyperalgesia did not return for days even after dosing was halted once analgesia had been achieved.

Although we have interpreted the delayed analgesic effect of GCPII inhibition as indirect pharmacology, alternative possibilities exist. The CCI ligation model has temporal alterations in its mechanisms including an initial neuroinflammatory component followed by more central plastic changes (Bennett and Xie, 1988). It is possible, for example, that new mechanisms of neuropathic pain exquisitely sensitive to GCII inhibition might be emerging after the initiation of the treatment at 10 days. These developing neuropathic mechanisms may be responsible, in part, for the delayed onset of effect. Further experiments exploring the exact timing of effect would be necessary to evaluate this alternative hypothesis.

The prolonged effects of 2-MPPA suggest that GCP II inhibition would be a valuable addition to currently available pain therapies. Such a therapy would be long lasting and with a mechanism distinct from available analgesics, it would be useful for combination approaches. In
the clinic, 2-MPPA was well tolerated in man at doses achieving plasma exposures equivalent to those shown to be effective in the current animal models (van der Post et al., 2005). These experiments suggest that GCP II inhibition may have therapeutic utility in neuropathic pain and that efficacy might not be expected in acute administration but rather might take multiple administrations for effect and have be sustained for some time even without further drug administration. Thus clinical trials could explore once per day dosing even for a short half-life compound.

Acknowledgements.
This work was supported and performed at Eisai Inc. Baltimore MD 21224 and Johns Hopkins Brain Science Institute, Baltimore MD 21205

Authorship contributions.
Participated in research design: Slusher, Vornov, Wozniak and Rojas
Conducted experiments: Wu and Wozniak
Performed data analysis: Wu, Rais and Wozniak
Wrote or contributed to the writing of the manuscript: Vornov, Wozniak, Rais and Slusher
REFERENCES


Carozzi V, Marmiroli P and Cavaletti G (2008a) Focus on the role of Glutamate in the pathology of the peripheral nervous system. CNS & neurological disorders drug targets 7:348-360.


FOOTNOTES

This work was supported and performed at Eisai Inc. Baltimore MD 21224 and Johns Hopkins Brain Science Institute, Baltimore MD 21205
FIGURE LEGENDS

**Figure 1**: Pharmacokinetic Profile of 2-MPPA after IV and Oral Administration in rats. Plot of mean plasma concentration vs time following an oral gavage or intravenous (caudal vein) administration of 10 mg/kg.

**Figure 2**: Time Course and Dose Response of 2-MPPA Reduction of Hyperalgesia in the rat CCI model. Each panel (A-D) shows the difference in withdrawal latency (in secs) between the ligated and non-ligated sides (mean +/- SEM) as observed over the course of the experiment. Note that the lowest doses were observed over a longer period of time to allow development of analgesia. Statistical analyses were conducted with comparisons made to vehicle treated animals. Significance criteria of p<0.05 were used for all experiments and are noted as “*”. Panel E shows daily dosing of 10 or 30 mg/kg 2-MPPA did not affect the response latency to the thermal stimulus applied to the sham-operated side compared to vehicle.

**Figure 3**: Lack of effect of 2-MPPA on locomotion in open-field at doses up to 100 mg/kg PO. Total ambulatory scores in rats treated with 30, 50, or 100 mg/kg 2-MPPA were not different from vehicle treated rats.

**Figure 4**: Time course of sciatic nerve tissue distribution following single or 5-day administration of 10 mg/kg 2-MPPA p.o. Note that sampling on Day 1 continued to 250 minutes while on Day 5 was limited to 120 minutes but that profiles during that period are similar.
Figure 5: Loss of effect of 2-MPPA with divided dosing. When the minimal effect dose of oral daily 10 mg/kg 2-MPPA was distributed throughout the day by divided dosing as either 5mg/kg BID or 3.3 mg/kg TID, the analgesic effect was lost. In each case, testing was conducted at 1 h following the last 2-MPPA dose of the day. Statistical analyses were conducted with comparisons made to vehicle treated animals. Significance criteria of p<0.05 were used and are noted as “*”.

Figure 6: Analgesic effect of 2-MPPA in the CCI model up to 16 hours following daily dosing. All animals were dosed with 2-MPPA at 10 mg/kg PO once daily. Different groups were subsequently evaluated for thermal withdrawal latency at 1h, 4h or 16h after administration on the days noted. Analgesia was established at day 11 and by day 15 it was maintained for up to 16 hours post dose. Statistical analyses were conducted with comparisons made to vehicle treated animals. Significance criteria of p<0.05 were used and are noted as “*”.

Figure 7: Sustained effect of 2-MPPA on hyperalgesia after cessation of dosing. Once analgesia was achieved following 15 days of dosing, drug administration was stopped. Withdrawal latency measurements, however, were continued. The analgesic efficacy of 2-MPPA persisted up to 17 days after drug administration was halted. Statistical analyses were conducted with comparisons made to vehicle treated animals. Significance criteria of p<0.05 were used and are noted as “*”.
Table 1: Pharmacokinetic parameters following single oral dose of 2-MPPA in rats.

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<td>0.08</td>
<td>231,100± 41,000</td>
<td>2.2± 0.4</td>
<td>6.8± 8.4</td>
<td>181</td>
</tr>
<tr>
<td>1000</td>
<td>203,800± 76,700</td>
<td>0.08</td>
<td>537,000± 160,500</td>
<td>2.0± 0.6</td>
<td>2.4± 0.3</td>
<td>210</td>
</tr>
</tbody>
</table>

All values are mean ±SD. Parameters were derived using non-compartmental analysis. Bioavailability is calculated from the 10 mg/kg IV dose and expressed as a percentage.
Figure: 1
Figure 2
Figure: 3
Figure 4: Graph showing the concentration of 2MPPA in plasma and sciatic nerve over time. The concentration is expressed in ng/mL or ng/g tissue. The data is presented for Day 1 and Day 5 for both plasma and sciatic nerve samples.
Figure 5: Graph showing the difference in scores over days of treatment. The graph compares the effects of Vehicle, 2-MPPA 10 mg/kg once daily, 2-MPPA 5 mg/kg twice daily, and 2-MPPA 3.3 mg/kg three times daily on treatment days 0 to 18. The bars indicate the mean difference in scores with error bars representing the standard error. Significant differences are marked with an asterisk (*) indicating p < 0.05.
Figure 7: Difference scores (Sec.) over days of treatment with Vehicle or 2-MPPA 10 mg/kg po, showing significant changes marked with asterisks.