The Orally Active Glutamate Carboxypeptidase II Inhibitor E2072 Exhibits Sustained Nerve Exposure and Attenuates Peripheral Neuropathy

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ABSTRACT

Peripheral neuropathy from nerve trauma is a significant problem in the human population and often constitutes a dose-limiting toxicity in patients receiving chemotherapy. (3–2-Mercaptoethyl)biphenyl-2,3-dicarboxylic acid (E2072) is a potent (K_i = 10 nM), selective, and orally available inhibitor of glutamate carboxypeptidase II (GCPII). Here, we report that E2072 attenuates hyperalgesia and nerve conduction velocity deficits in preclinical rodent models of neuropathic pain and oxaliplatin-induced neuropathy. In the chronic constrictive injury model, orally administered E2072 reversed pre-existing thermal hyperalgesia in rats in a dose-dependent fashion with a minimally effective dose of 0.1 mg/kg/day. It is noteworthy that multiple days of dosing of E2072 were required before analgesia was realized even though GCPII inhibitory exposures were achieved on the first day of dosing. In addition, analgesia was found to persist for up to 7 days after cessation of dosing, consistent with E2072’s pharmacokinetic profile and sustained exposure. Furthermore, in a chronic oxaliplatin-induced neuropathy model (6 mg/kg i.p. oxaliplatin twice weekly for 4 weeks), female BALB/c mice receiving daily oral E2072 at 1.0 and 0.1 mg/kg displayed no deficits in either caudal or digital velocity compared with significant deficits observed in mice treated with oxaliplatin alone (12 ± 3 and 9 ± 2%, respectively). Similar findings were seen with oxaliplatin-induced digital and caudal amplitude deficits. It is noteworthy that E2072 showed no interference with the antineoplastic efficacy of oxaliplatin in mice bearing leukemia (L1210), even at doses 100 times its neuroprotective/analgesic dose, indicating a selective effect on neuropathy. These data support the therapeutic utility of GCPII inhibitors in neuropathy and neuropathic pain.

Introduction

Glutamate carboxypeptidase II (GCPII) (EC 3.4.17.21) is a membrane-bound, 94-kDa Zn^{++} metalloprotease abundant in both the central and peripheral nervous systems (Robinson et al., 1987; Slusher et al., 1992; Carozzi et al., 2008; Neale et al., 2011; Barinka et al., 2012). GCPII hydrolyzes the neuropeptide N-acetyl-aspartyl glutamate (NAAG) to N-acetyl-aspartate and glutamate (Robinson et al., 1987; Slusher et al., 1990). NAAG acts as a potent agonist at group II metabotropic glutamate receptors that inhibit glutamate release (Wroblewska et al., 1997) and exhibits mixed agonist/antagonist activity at N-methyl-D-aspartate receptors, albeit at lower affinities (Bergeron et al., 2005). In general, GCPII is thought to terminate the neurotransmitter activity of NAAG, resulting in higher levels of synaptic glutamate (Coyle, 1997).

Altered glutamate neurotransmission has been implicated in several central and peripheral nervous system diseases

Abbreviations

GCPII, glutamate carboxypeptidase II; E2072, (3–2-mercaptopoethyl)biphenyl-2,3-dicarboxylic acid; NAAG, N-acetyl-aspartyl glutamate; 2-MPPA, 2-(3-mercaptopropyl)pentanedioic acid; 2-PMPA, 2-(phosphonomethyl pentanedioic acid); CCl, chronic constrictive injury; NGV, nerve conduction velocity; mGiUR, metabotropic glutamate receptor; LC, liquid chromatography; MS/MS, tandem mass spectrometry; LY341495, 2-amino-2-(2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoic acid; APDC, (2R,4R)-aminopyrrolidine 2,4-dicarboxylate; ZJ17, (S)-2-(3-(S)-1-carboxy-2-(4-hydroxyphenyl)ethyl)ureido)pentanedioic acid, SLX-3095–1, (S)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6 dicarboxylic acid; ZJ11, (S)-2-(3-(S)-1-carboxy-3-(methylthio)propyl)ureido)pentanedioic acid; ZJ43, (S)-2-(3-(S)-1-carboxy-3-methylbutyl)ureido)pentanedioic acid; Veh, vehicle; Oxali, oxaliplatin.
including stroke, amyotrophic lateral sclerosis, Alzheimer’s disease, multiple sclerosis, diabetic neuropathy, and neuro-pathic pain (Coyle and Puttfarcken, 1993; Lau and Tymianski, 2010). Consistent with the hypothesis that GCPII regulates glutamate transmission, GCPII inhibitors have been shown to possess therapeutic utility in many preclinical models of neurodegenerative diseases with presumed underlying glutamate aberrations (Neale et al., 2005; Barinka et al., 2012). In addition, abnormal NAAG and GCPII levels have been detected in tissue samples from preclinical animal models and patients with neurodegenerative disorders, including amyotrophic lateral sclerosis (Tsai et al., 1991; Plaitakis and Constantakakis, 1993; Ghadge et al., 2003), epilepsy (Meyerhoff et al., 1992; Pacheco Otalora et al., 2007), schizophrenia (Ghose et al., 2004; Guijarre et al., 2008; Profaci et al., 2011), and Huntington’s disease (Passani et al., 1997).

Glutamate has also been widely implicated in pain perception, so consequently there has been interest in the development of potent orally available GCPII inhibitors as therapeutics for chronic pain states (Wozniak et al., 2012). The initial characterization of early GCPII inhibitors showed alleviation of allodynia and thermal hyperalgesia in neuropathic pain models (Chen et al., 2002; Majer et al., 2003) and attenuation of thermal hyperalgesia, nerve conduction velocity (NCV) deficits, and neuropathological changes in models of diabetic neuropathy (Zhang et al., 2002, 2006). These early inhibitors, however, were not orally available and/or did not readily penetrate into nervous tissues, thus requiring large doses or local/intrathecal administration for efficacy.

Chemotherapy-induced peripheral neuropathy represents a major dose-limiting side effect in cancer treatment, causing not only a significant decline in the quality of life for affected patients but also limiting doses of life-saving therapies (Cavalletti and Marmiroli, 2004). Platinum-based chemotherapy agents such as oxaliplatin are heavy metals that bind to DNA, disrupting base pair linkage, although other mechanisms, including dorsal root ganglia neuron apoptosis, have also been implicated for their neurotoxicity (Gill and Windbank, 1998). Apoptosis prevention with putative neuroprotectants, as well as pain alleviation, would have major clinical benefits for patients requiring these kinds of chemotherapy. Consequently, the development of novel analgesic and/or neuroprotective therapies, such as those targeting GCPII, is advocated.

The design of initial GCPII inhibitors focused on a series of phosphonic and phosphinic acid derivatives (Jackson et al., 2001). Several potent urea-based compounds were also synthesized (Kozikowski et al., 2004). Although these compounds were potent inhibitors, they were also very polar and possessed poor tissue penetration and in vivo potency. To circumvent this, thiol-based inhibitors were designed that possessed poor tissue penetration and in vivo potency (K_{50} = 20 nM), enhanced chemical stability, and higher melting point (Stoermer et al., 2012) (Table 1).

We now report, for the first time, on the dose-dependent analgesic efficacy of E2072 against established hyperalgesia in chronic constrictive injury neuropathy and demonstrate that its efficacy correlates with its long-lived pharmacokinetic profile. We also report on the significant neuroprotective effects of E2072 in preventing chronic oxaliplatin-induced deficits in nerve conduction without adversely affecting oxaliplatin’s antitumor efficacy. These data support the selective utility of GCPII inhibition as a therapeutic intervention for chemotherapy-induced neuropathy and neuropathic pain.

## Materials and Methods

All studies involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health and SoBran, Inc. (Fairfax, VA).

### Pharmacokinetic Studies

**Animals.** Male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), weighing approximately 200 to 250 g, were housed in suspended polycarbonate cages under a 12-h light/dark cycle. Food (Harlan, Indianapolis, IN) and water were provided ad libitum.

**Preparation of Dosing Solution.** E2072 was dissolved in vehicle (degassed 50 mM HEPES-buffered saline) by using sonication for approximately 10 min and brought to neutral pH with NaOH (pH 6–7).

**Experimental Procedures.** In the tissue distribution study, rats were dosed by oral gavage with 10 mg/kg E2072 for either 1 or 5 days and sacrificed at 0.25, 0.5, 1, 2, 4, 6, and 8 h after the last dose, and terminal blood was taken by cardiac puncture. Sciatic nerves and whole brains were also dissected and frozen at approximately –80°C until analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS). In the 7-day pharmacokinetic study rats were dosed with E2072 at 10 mg/kg i.v. and sacrificed at time points of 1, 2, 3, 5, 7, 8, 24, 26, 28, 48, 51, 54, 72, 76, and 79 h and 4, 6, and 7 days postadministration. Terminal blood samples were taken by cardiac puncture and analyzed as above. In both experiments five rats were used per time point.

**Bioanalysis of E2072 and Its Homodisulfide.** E2072 was analyzed by derivatization with N-ethyldimaleimide (Giustarini et al., 2011) followed by LC/MS/MS. For quantification of E2072 in the plasma samples, the stocks for standards were prepared fresh. In
brief, 180 μl of plasma sample (or 160 μl of matrix blank and 20 μl of stock for standards) with 10 μl of 100 mM N-ethylmaleimide were allowed to react at room temperature for 30 min followed by the addition of 10 μl of 0.1 N HCl and 20-μl internal standard, 5 μg/ml in acetonitrile/water (1:1, v/v). The tubes were vortexed, and samples were extracted with 400 μl of methanol, followed by vortexing and centrifugation at 10,000 rpm for 5 min. Fifty microliters of supernatant was transferred to LC vials, and 5 μl was injected on the LC/MS/MS system. The tissue samples were homogenized and then extracted following the same procedure as described for plasma. For each tissue, the same matrix was used for the preparation of the standard curve. The calibration range was 1 to 5000 ng/ml for E2072 in plasma. For concentrations >5000 ng/ml samples were diluted with blank matrix before extraction for quantitation. Chromatographic analysis was performed on a high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) coupled with a API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Derivatized E2072 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 by using a gradient run, with the organic composition changing from 50 to 90% over a period of 3 min, maintained at 90% for 4 min, then re-equilibrated to 50% over 3 min at a flow rate of 350 μl/min and total run time of 10 min. The MS instrument was operated in a negative ion mode. The multiple reaction monitoring transition of derivatized E2072 was 426.25 – 301.25 (Q1/Q3), and for the internal standard it was 323.25. For the caudal and digital nerves. Differences in measures across groups were determined by quantitative analysis of wave form.

### Oxaliplatin-Induced Neuropathy

**Animals.** Female BALB/c mice (Harlan), weighing approximately 20 g, were housed in suspended polycarbonate cages and maintained under a 12-h light/dark cycle. Food (Harlan) and water were provided ad libitum.

**Preparation of Dosing Solutions.** E2072 was prepared as described above. Oxaliplatin was made fresh on days of dosing and solubilized in 5% dextrose in water.

**Experimental Procedures.** Mice were dosed with oxaliplatin (6 mg/kg i.p.) twice per week for 4 weeks and daily oral E2072 (0.01, 0.1, or 1 mg/kg) or vehicle (HEPES-buffered saline). Dosing of E2072 or vehicle started on the same day as the first dose of oxaliplatin, and on cotreatment days they were given 60 min before oxaliplatin. Mice were assessed for changes in caudal and digital NCV at baseline and after treatment as described previously (Wozniak et al., 2011). Weight was monitored three times a week throughout the study, and any mouse losing 20% or more of its starting body weight was euthanized.

In brief, the mice were anesthetized with isoflurane and placed in a prone position for recording; respiration was maintained within normal physiological limits. Throughout recording, the mice were positioned on a heated pad, and rectal temperature was maintained at 37 ± 1°C.

Subdermal needle electrodes (platinum) were used for both recording and stimulation; locations were determined with reference to anatomical landmarks as detailed below to facilitate consistent electrode placement. Supramaximal stimulation was achieved by using a constant voltage square pulse (0.02- to 0.10-ms duration) isolated from the ground. Latency measurements were scored to the nearest 0.01 ms, and amplitude measurements were scored to the nearest 0.01 μV for the caudal and digital nerves. Differences in measures across groups were determined by quantitative analysis of wave form.
Characteristics and direct statistical comparisons of measures at each time point, using standard multivariate inferential procedures (analysis of variance with post hoc Tukey's test) with significance levels set at 0.05.

Interaction Studies with Oxaliplatin

Animals. Female DBA2 mice (Charles River Laboratories, Inc.), which were 6 to 7 weeks old at the onset of the experiment, were used. Animals were fed rodent diet [PicoLab Rodent Diet 20 (PMI Nutrition International, St. Louis, MO)] and water ad libitum. Mice were observed for clinical signs daily, and any found to be in obvious distress or in a moribund condition were euthanized. Weight loss in excess of 20% from the start of study was considered unacceptable and resulted in appropriate euthanasia.

Dosing Solutions and Treatment. E2072 and oxaliplatin were prepared as described above. E2072 was dosed orally for 14 days as a single agent (30 mg/kg) and in combination (at 10 and 30 mg/kg) with oxaliplatin daily for 14 days. Oxaliplatin was dosed at 8 mg/kg i.v. every 4 days for a total of two treatments.

Experimental Procedures. L1210 murine leukemia cells were isolated from euthanized donor animals by injecting 3 ml of chilled bovine serum albumin solution into the abdomen of the animals with obviously progressive L1210 leukemia ascites (showing distended abdomens) as described previously (Skipper et al., 1965). The ascites suspension containing leukemia cells was then withdrawn, and the cells were counted with a hemocytometer. The ascites was then diluted with 2% bovine serum albumin solution to achieve a final cell suspension containing $1 \times 10^5$ cells/ml. Ten thousand leukemia cells in a 100-μl volume were injected intraperitoneally into each mouse by using a 27-g needle on day 0. Drug treatments (administered in 0.2 ml/20 g) began on day 3.

The primary end points were percentage of the median life spans of the treated/control groups, percentage increase in life span, and body weight loss. All animals were observed for clinical signs at least once daily and weighed on each day of treatment and at least three times weekly thereafter. Animals with treatment-related weight loss in excess of 20% were sacrificed. Differences in survival curves were evaluated by the application of a log rank survival analysis (Kaplan-Meir). A pairwise multiple comparison analysis was used to determine significance between selected groups.

Results

$K_i$ Determination. The rate of hydrolysis of $[^3H]$NAAG as measured by the appearance of $[^3H]$glutamate is shown in Fig. 1. $K_i$ was determined to be 10 nM. Maximal rate ($V_{max}$) remained constant as the concentration of inhibitor was increased, while the apparent binding constant ($K_{app}$) increased, this being the hallmark of competitive inhibition.

Selectivity Evaluation. E2072 was evaluated in the general profiling Spectrum Screen Panel conducted by MDS Pharma Services (recently acquired by Ricerca Biosciences LLC). E2072 (10 μM) showed no significant activity (<50%) in 129 of 130 targets tested (see Supplemental Table 1), comprising diverse enzymes and receptors (Ricerca Biosciences LLC). The single assay in which the compound showed appreciable activity (85% enhancement of radioligand displacement at 10 μM) was the prostaglandin E$_1$ receptor binding assay. However, no binding activity was apparent at lower concentrations of E2072 (100 and 1 nM). In addition, a functional in vitro assay of activity at prostaglandin E$_1$ receptors in isolated guinea pig ileum (Ricerca Biosciences LLC) demonstrated no activity at concentrations up to 30 μM. In addition, E2072 was evaluated in an mGluR3 agonist activity assay by Genscript Inc. using a human cell line (human embryonic kidney 923/EAAC1/mGluR3/G16). There was no significant agonist activity at 1 or 10 μM.

Pharmacokinetics. E2072 was present in plasma samples for up to 7 days after a single administration of 10 mg/kg i.v. (Fig. 2A). E2072 exhibited a biphasic decline in plasma concentration over time with an initial distribution $t_{1/2,a}$ of 0.87 ± 0.15 h and a long elimination $t_{1/2,b}$ of 105 ± 40 h.

In tissue distribution studies after 10 mg/kg p.o. E2072, the maximal levels of E2072 in sciatic nerve were achieved at approximately 1 h (~200 ng/g) and remained 20-fold above the GCPPI $K_i$ (approximately 70 ng/g or 250 nM) for at least 8 h (Fig. 2B). The levels of E2072 in both plasma and sciatic nerve were similar after 1 or 5 days of dosing (Fig. 2B), suggesting no compound accumulation. Based on these data, doses of 0.01 to 10 mg/kg E2072 were chosen for efficacy studies.

CCI Studies. Loose constriction of the sciatic nerve produced thermal hyperalgesia beginning 10 days after surgery, as evidenced by a 3-s decrease in response latency on the ligated side (Fig. 3). In vehicle-treated rats, hyperalgesia persisted throughout the experiment, whereas rats receiving 10, 1, or 0.1 mg/kg E2072 orally daily showed attenuation of the hyperalgesia (Fig. 3, A-C). Rats receiving 0.01 mg/kg E2072 showed no effect (Fig. 3D). As shown in Fig. 3E, E2072's analgesic effect continued for at least 7 days after cessation of 10 mg/kg E2072 treatment. Similar results were...
Nerve conduction velocity in oxaliplatin-treated mice (averaged E2072 at 0.01 to 1 mg/kg/day (Fig. 4A). The digital of control; p < 0.05) with 1 and 0.1 mg/kg/day significantly preventing both of these deficits (Fig. 4, B and D).

Interactions with Efficacy of Chemotherapy. To evaluate whether E2072’s attenuation of neuropathy was associated with an alteration of the beneficial antineoplastic effects of the chemotherapy, we tested the impact of E2072 on oxaliplatin in both in vitro and in vivo models. E2072 at concentrations up to 125 μM (which is >100-fold its IC_{50} for GCPII inhibition) had no effect on the antiproliferative potency of oxaliplatin in SW480 human colon adenocarcinoma cells (IC_{50} of 601 nM for oxaliplatin alone versus 640 nM with 125 μM E2072; data not shown). In addition, no effect was observed when E2072 (up to 30 mg/kg/day) was administered with oxaliplatin (8 mg/kg i.v. every four days × 2) in an in vivo L1210 survival model (Fig. 5). It is noteworthy that this dose of E2072 (30 mg/kg/day) is more than 100 times the minimally effective dose in the CCI and oxaliplatin efficacy models described.

Discussion

E2072 is a potent and orally bioavailable inhibitor of the GCPII enzyme with a K_{i} of 10 nM (Fig. 1) and an oral bioavailability in rats of 38% (Table 1; Fig. 2). In a diverse selectivity screen of more than 125 targets, E2072 was found to be selective for GCPII, displaying no significant activity at 10 μM in the other assays, including mGluR3. Unexpectedly, E2072 was also found to possess a long plasma terminal t_{1/2} of approximately 105 h in rats after administration (Fig. 2). The long t_{1/2} of E2072 is probably the result of the formation of a long-lived reversible homodimer, as has been described for other thiol-containing drugs such as captopril and penicillamine (Drummer and Jarrott, 1984; Pilkington and Waring, 1988). Characterization of the pharmacokinetic profile of E2072 and its homodimer is the subject of a separate study by our group (R.R., unpublished work). In addition to having a long terminal half-life, E2072 was found to penetrate peripheral nerve tissue at levels exceeding its K_{i} for GCPII for more than 8 h (Fig. 2).

In preclinical efficacy studies, daily oral administration of E2072 significantly attenuated pre-existing hyperalgesia produced by loose sciatic nerve ligation, with a minimally effective dose of 0.1 mg/kg (Fig. 3C). Although E2072 was found to penetrate into the peripheral nerve within 1 h of administration at concentrations that inhibit GCPII (Fig. 2B), its analgesic effect required several days of dosing. At all doses tested the hyperalgesic response was significantly reduced from the eighth day of treatment, with no clear dose response observed. The reason for this pharmacokinetic/pharmacodynamic disconnect is unclear. It is unlikely that this is merely a drug concentration requirement, especially because multiple-day dosing did not increase drug concentration in the peripheral nerve (Fig. 2B). It is possible that GCPII inhibition acts via an indirect mechanism involving long-term physiological or structural changes in pain pathways requiring chronic inhibition as has been described for other drugs, including gabapentin (Kuner, 2010). We are investigating this possibility. E2072’s analgesic effect, once present, however, was maintained for up to 7 days after

![Graph](image_url)
cessation of dosing. This was not unexpected in view of the compound’s long elimination $t_{1/2}$ of 105 ± 40 h.

E2072’s ability to reduce established thermal hyperalgesia after loose sciatic nerve ligation is in agreement with previous reports of GCPII inhibition in other pain models. For example, spinal delivery of the GCPII inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (Jackson et al., 2001) was found to attenuate noiception in the hot plate and formalin pain test (Yamamoto et al., 2001). Intrapertoneal 2-PMPA was found to attenuate allodynia and accompanying increased ectopic discharges after partial sciatic nerve ligation in a model of neuropathic pain (Chen et al., 2002), as well as reduced mechanical allodynia in rats receiving carrageenan (Yamamoto et al., 2001) or unilateral sciatic nerve ligation (Nagel et al., 2006). 2-PMPA markedly inhibits noxious evoked dorsal horn activity in normal and inflamed rats (Carpenter et al., 2003). In a model of excitotoxic spinal cord injury, 2-PMPA inhibited dynorphin-induced elevations in cerebrospinal fluid glutamate levels and improved histological scores (Long et al., 2005). These effects seem common with effects on hyperalgesia, nerve conduction, and morphology associated with long-term diabetes (Zhang et al., 2002) with similar results obtained when using the orally available inhibitor 2-MPPA (Zhang et al., 2006). Various urea-based GCPII inhibitors ([ZJ11, (S)-2-((3-((S)-1-carboxy-3-(methylthio) propyl)ureido)pentanedioic acid; ZJ17, (S)-2-((3-((S)-1-carboxy-2-(4-hydroxyphenyl)ethyl)ureido)pentanedioc acid; and ZJ43, (S)-2-((3-((S)-1-carboxy-3-methylbutyl)ureido)pentanedioic acid]]

Fig. 3 E2072 treatment in rats with chronic constrictive injury-induced neuropathy. The data shown represent the mean difference in paw withdrawal latencies to a thermal stimulus, noted as difference in scores as determined by subtracting the mean nonligated paw latency from ligated paw response in seconds at baseline (pretreatment) versus after E2072 treatment of 10 mg/kg/day (A), 1 mg/kg/day (B), 0.1 mg/kg (C), and 0.01 mg/kg (D). *p < 0.05, versus vehicle. Blue, vehicle; red, E2072. E, time course of E2072’s (10 mg/kg/day) effects after cessation of dosing showing the persistence of analgesia for up to 7 days ($n = 10$ rats per dose group).
acid]) have also been reported to be effective in pain models (Yamamoto et al., 2004, 2007). The first orally bioavailable GCPII inhibitor, 2-MPPA, also demonstrated analgesic efficacy in the rat CCI model of neuropathic pain (Majer et al., 2003, 2006). In all cases, GCPII inhibitors were found to significantly reduce injury-related, but not alter, normal pain sensation, implying a specific effect on hyperalgesia.

In addition to attenuating neuropathic pain, we report E2072’s ability to reduce oxaliplatin-induced peripheral neuropathy without affecting its chemotherapeutic efficacy. Nerve conduction velocity deficits induced by oxaliplatin treatment were prevented completely by coadministration of 0.01 to 1 mg/kg/day E2072 (Fig. 4). These data add support to previously reported effects of GCPII inhibition in cisplatin-, paclitaxel-, and bortezomib-induced models of peripheral neuropathy (Carozzi et al., 2010). In this context, it is especially important to note that E2072 did not interfere with chemotherapy antineoplastic efficacy at up to 3000 times its minimally effective neuroprotective dose.

The mechanisms by which GCPII inhibitors alleviate pain and protect nerves in models of chemotherapeutic neuropathy are not well understood. Glutamate has been broadly implicated in the generation and maintenance of neuropathic pain either directly or indirectly via spinal wind-up mechanisms. GCPII inhibitors have been shown to reduce excitotoxic glutamate (Slusher et al., 1999), reduce ectopic discharges from injured afferents (Chen et al., 2002), and reduce aspects of spinal wind-up mechanisms (Carpenter et al., 2003). In addition, there is evidence for a down-regulation of glial glutamate transporters (which play a major role in maintaining extracellular glutamate homeostasis) being induced by paclitaxel administration in preclinical models of chemotherapy-induced hyperalgesia (Weng et al., 2005). Functional down-regulation of transporters might be expected to cause increased glutamate and thereby implicate a potential mechanism of action for GCPII inhibition.

Furthermore, multiple studies have shown the effects of GCPII inhibitors in pain are sensitive to attenuation by mGluR3 antagonists such as 2-amino-2-(2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495) (Yamamoto et al., 2004, 2007, 2008) and therefore support mediation of GCPII inhibition effects via increasing NAAG levels and mGluR3 activity. In addition, group II mGluR agonists such
as SLX-3095-1 ([1S]-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6 dicarboxylic acid), NAAG, and APDC ([2R,4R]-aminopyrrolidine 2,4-dicarboxylate) behave similarly to GCPII inhibitors in alleviating pain on local injection in carrageenan and formalin inflammatory pain models (Yamamoto et al., 2007). These data suggest that GCPII inhibition increases NAAG, which in turn mediates its effects predominantly, if not exclusively, via mGluR3 to the resultant alleviation of pain. However, it remains possible that NAAG attenuates the excitability of postsynaptic neurons by interfering with the binding of released glutamate at the N-methyl-D-aspartate receptor as suggested previously (Carpenter et al., 2003), although this effect may be indirect (Zuo et al., 2012) and remains controversial (Losi et al., 2004). It should also be noted that the analgesic effects of NAAG (mediated directly or via GCPII inhibition) may be at least partially caused by interactions with other transmitters in the ascending and descending pathways (Neale et al., 2011). In addition, GCPII inhibition, resultant NAAG increases, and subsequent activation of mGluR group II receptors have been linked to the activation of trophic factors, such as transforming growth factor-β (Bruno et al., 1998; Slusher et al., 1999; Thomas et al., 2001), which may be at least part of the underlying mechanism for the neuroprotective effects seen with GCPII inhibition. Indeed, the release of neurotrophic factors could regulate chloride transporter levels and thereby alter the equilibrium potential for chloride (and γ-aminobutyric acid and glycine receptor effects) or have other actions that influence descending pain regulatory pathways. In addition, the possible effect of increasing levels of N-acetylaspartylglutaminylglycine, a secondary substrate for GCPII, has not yet been established (Neale et al., 2011).

Several studies have suggested the site of action for GCPII inhibitors in alleviating neuropathy is dorsal root ganglia, where both NAAG and mGLuRs are expressed (Cangro et al., 1987; Carlton et al., 2001). However, other reports support a more peripherally mediated action of GCPII inhibition for analgesia. For example, the GCPII inhibitor 2-PMPA reducesafferent discharge in the partial sciatic nerve ligation model when injected systemically (Chen et al., 2002). In addition, 2-PMPA decreases noxious evoked activity but not low threshold Aβ fiber responses in normal rats when dosed spinally (Carpenter et al., 2003), and intrathecal ZJ-17 and 2-PMPA are analgesic in formalin pain (Kozikowski et al., 2004). In addition, GCPII inhibitors seem to reduce inflammatory pain perception by acting directly in the brain and mediating their effects via an action sensitive to group II mGluR antagonists (Yamamoto et al., 2008). Most recently, data have been provided suggestive of GCPII inhibitor-induced increases in NAAG causing a decrease in glutamate release and pain after direct administration to the mouse amygdala (Adedoyin et al., 2010), a brain area thought to be associated with chronic inflammatory pain (Neugebauer et al., 2010), a brain area thought to be associated with chronic inflammatory pain (Neugebauer et al., 2010), and inflammation mediated and local component.

In conclusion, the data presented support a neuroprotective role of GCPII inhibition in models of neuropathic pain and chemotherapy-induced neuropathy and further augment the body of literature advocating the development of a novel inhibitor with which to proceed into clinical development.

**Authorship Contributions**

**Participated in research design:** Wozniak, Lapidus, Rojas, Tsukamoto, and Slusher.

**Conducted experiments:** Wozniak and Wu.

**Performed data analysis:** Wozniak, Wu, and Rais.

**Wrote or contributed to the writing of the manuscript:** Wozniak, Vornov, Rais, Rojas, and Slusher.

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