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δ -Thiolactones as prodrugs of thiol-based glutamate carboxypeptidase II (GCP II) inhibitors

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Abstract

δ -Thiolactones derived from thiol-based glutamate carboxypeptidase II (GCP II) inhibitors were evaluated as prodrugs. In rat liver microsomes, 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA, **1**) was gradually produced from 3-(2-oxotetrahydro-thiopyran-3-yl)propionic acid (**5**), a thiolactone derived from **1**. Compound **1** was detected in plasma at concentrations well above its IC₅₀ value for GCP II following oral administration of **5** in rats. Consistent with the oral plasma pharmacokinetics, thiolactone **5** exhibited efficacy in a rat model of neuropathic pain following oral administration.

INTRODUCTION

Glutamate carboxypeptidase II (GCP II) is a membrane-bound binuclear zinc metalloproteinase that cleaves N-acetylaspartylglutamate (NAAG) into N-acetylaspartate (NAA) and glutamate (Glu) in the extracellular space of the nervous system. Inhibition of GCP II has gained considerable attention as an alternative therapeutic approach to blocking postsynaptic glutamate receptors for treating neurodegenerative disorders associated with glutamate excitotoxicity.

Among a variety of GCP II inhibitors reported to date,^{1–3} thiol-based inhibitors have shown promising pharmacological profiles in preclinical studies (Figure 1). For example, 2-(3-mercaptopropyl)pentanedioic acid **1** (2-MPPA) represents the first orally active GCP II inhibitor with an IC₅₀ value of 90 nM.⁴ Compound **1** showed efficacy in a variety of preclinical animal models by oral administration.⁵ Further structural optimization studies revealed that GCP II is more tolerant of structurally diverse scaffolds presented by the thiol-based compounds than other series. For instance, rigorous SAR studies of thiol-based GCP II inhibitors led to the discovery of 3-(2-mercaptoethyl)-biphenyl-2,3'-dicarboxylic acid **2** (E2072) containing a biphenyl scaffold distinct from that of compound **1**.⁶ Compound **2** was

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Author Contributions

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found to inhibit GCPH with much higher potency ($IC_{50} = 2$ nM) than compound **1**. Compound **2** showed significantly improved potency over **1** in a preclinical model of neuropathic pain following oral administration, presumably due to its enhanced GCPH inhibitory potency coupled with the improved oral pharmacokinetic properties.⁷

From a drug development perspective, however, there has been a reluctance to pursue thiol-containing compounds as therapeutic agents. Unlike other zinc-binding groups, the thiol group is relatively nucleophilic and prone to oxidation. These chemical properties compromise the metabolic stability and increase the risk of inducing immune reactions when conjugates are formed with endogenous proteins. Indeed, some of the adverse reactions reported for captopril are believed to be due in large part to its thiol group.⁸ In addition, a more immediate concern lies with the complexity involved in the development of consistent processes to produce thiol compounds of high quality free from the corresponding homo-disulfide impurities. Furthermore, the instability of thiol-containing compounds often presents a challenge to identifying a stable formulation with an acceptable shelf life.

One approach to circumventing some of the issues associated with thiol-containing drugs is to explore prodrugs in which the thiol group is protected in the form of a metabolically cleavable thioester. For instance, M100240 (compound **3**) is a thioacetyl derivative of MDL 100,173 (compound **4**), a dual angiotensin-converting enzyme (ACE)/neutral endopeptidase (NEP) inhibitor (Figure 1). Oral administration of **3** to healthy subjects resulted in the substantial plasma exposure to **4** while significantly lower plasma levels of **3** were detected,⁹ suggesting rapid in vivo hydrolysis of the thioester moiety of **3**.

A common structural feature shared by nearly all potent thiol-based GCPH inhibitors is the presence of a 5-mercaptopentanoic acid backbone. This feature allows us to explore δ -thiolactones as potential prodrugs of thiol-based GCPH inhibitors. Such an approach may offer more stable forms of the drugs by temporally masking a reactive thiol group yet rapidly generating the parent compounds in vivo.

To this end, herein we report the synthesis and pharmacological evaluations of δ -thiolactones **5** and **6** derived from two structurally distinct thiol-based GCPH inhibitors, **1** and **2** (Figure 1).

RESULTS

As illustrated in Scheme 1, δ -thiolactone **5** was synthesized by refluxing a solution of **1** in the presence of *p*-toluenesulfonic acid using Dean-Stark apparatus. All attempts to synthesize δ -thiolactone **6** from **2** via acid-catalyzed condensation failed, with only unreacted starting material recovered. While searching for alternative methods to form δ -thiolactones, we came across an unusual route to thiolactones through *S*-benzyl sulfide intermediates.^{10, 11} To test this procedure, **2** was converted into the corresponding *S*-benzyl sulfide **7**. Cyclization of **7** in refluxing trifluoroacetic anhydride successfully provided δ -thiolactone **6**. This cyclization reaction presumably proceeds through an initial formation of the corresponding anhydride **8**, followed by its conversion to the sulfinium intermediate **9**. Subsequent rearrangement of **9** through a six-membered transition state produces δ -thiolactone **6** and benzyl trifluoroacetate **10**. Indeed, careful analysis of the reaction mixture by ¹H and ¹³C NMR confirmed the formation of **10** as a by-product.

In the enzyme assay using N-acetyl-L-aspartyl-[³H]-L-glutamate as a substrate and purified human recombinant GCPH,¹² both compounds **5** and **6** showed substantially weaker inhibitory potency compared to the parent compounds with IC_{50} values of 2 μ M and 20 μ M, respectively. The reduced potency can be attributed to the loss of the zinc-binding thiol

group and the key α -carboxylate group interacting with the glutamate recognition site of GCPII.

In order to assess the ability of **5** and **6** to serve as prodrugs, release of the parent compounds was investigated in simulated gastric fluid (pH 1.2) and rat liver microsomes. Neither **5** nor **6** generated the parent compounds even after an extended period of incubation (48 h) in simulated gastric fluid at 37 °C. As shown in Figure 2, however, time-dependent formation of **1** from **5** was observed in rat liver microsomes. Due to the weak intensity of the parent ion, a reliable bioanalytical method to quantify **5** was not successfully developed. Consequently, we could not determine the stoichiometric balance between prodrug **5** and the parent compound **1**. Thus, the possibility of **5** forming other metabolites besides **1** cannot be ruled out. Nevertheless, gradual generation of **1** in rat liver microsomes indicates the potential of δ -thiolactone **5** to act as a prodrug of **1**. In contrast, δ -thiolactone **6** was completely stable in the rat liver microsomes over a period of 1.5 h (data not shown). Although speculative, it is conceivable that a sterically hindered environment around the thioester moiety of **6** renders it resistant to hepatic esterases.

In light of the rat liver microsomal stability data, we chose to conduct preliminary in vivo pharmacokinetics study (n = 2, four time points) of δ -thiolactone **5** in rats to determine if it serves as a prodrug of compound **1**. As shown in Figure 3, following oral administration of δ -thiolactone **5**, compound **1** was detected in plasma at concentrations well above its IC₅₀ value for GCPII. Among four time points (t = 0.25, 0.5, 1, and 3 h), plasma levels of compound **1** were highest at the earliest time point. Although its levels were nearly 7-fold lower than plasma levels of compound **1** at the same time point following oral administration of **1**, the two curves become nearly identical after 1 h.

δ -Thiolactone **5** was subsequently tested for its antinociceptive effects following oral administration (10 mg/kg/day) using the rat chronic constriction injury model of neuropathic pain.¹³ As shown in Figure 4, oral administration of **5** resulted in significant reduction of thermal hyperalgesia relative to the vehicle-treated control on days 11, 16, and 18.

DISCUSSION AND CONCLUSION

While both δ -thiolactones **5** and **6** were completely stable in simulated gastric fluid, the two compounds showed different degrees of microsomal stability, presumably due to the preference of hepatic esterases for **5** over the sterically hindered thiolactone **6**.

Although the lack of plasma data of **5** precludes in-depth interpretation of the given data, the lower plasma levels of **1** at the early time points in the rat in vivo pharmacokinetics study may reflect the gradual conversion of **5** to **1** in liver as indicated by the microsomal stability test. However, lower plasma levels of **1** also raise the possibility that δ -thiolactone **5** is poorly bioavailable and that only a portion hydrolyzed to **1** enters the circulatory system. After 1 h, there is little difference in plasma levels, if any, of **1** between oral administration of **5** and **1**, indicative of complete loss of δ -thiolactone in plasma by this time point. Since δ -thiolactone **5** is a racemic mixture, it is also possible that only one of the enantiomers is preferentially hydrolyzed, resulting in lower plasma levels of **1** following oral administration of **5**.

The degree of antinociceptive effect appears less than that following oral treatment with compound **1** (10 mg/kg) reported previously.⁴ The inferior efficacy of δ -thiolactone **5** could be attributed to the significantly lower plasma exposure to compound **1** at the early time points when compound **5** is given orally as shown in Figure 3. This is in a good agreement with the previously reported PK/PD analysis, which suggests that C_{max} rather than AUC is

the key determinant of in vivo efficacy in this particular preclinical model.¹⁴ As mentioned above, the animals might be exposed to an enantiomerically enriched form of **1** due to enantioselective hydrolysis of δ -thiolactones **5**. This, however, should have little implication for efficacy since the two enantiomers of **1** were found to show similar degree of in vivo efficacy in this animal model.¹⁵

Although δ -thiolactone **5** did not offer any particular pharmacological advantages over the parent compound **1**, the δ -thiolactone-based prodrug approach should represent an attractive option from chemistry, manufacturing and controls (CMC) perspective as it alleviates issues associated with handling of thiol-containing drug substances. While the δ -thiolactone-based prodrug approach appears promising, one must be aware of the potential safety risk associated with thiolactones given the well-documented reactivity of homocysteine thiolactone with endogenous proteins.¹⁶ The striking difference in stability between **5** and **6** suggests varying degrees of reactivity within δ -thiolactones. Selecting the most suitable thiol-containing parent compound is, therefore, the crucial step in this particular prodrug approach. It is worth noting that the 5-mercaptopentanoic acid scaffold has been embedded in a variety of thiol-based GCPII inhibitors,^{4, 6, 17, 18} which can be exploited in search for δ -thiolactone prodrugs with superior pharmacological properties.

EXPERIMENTAL SECTION

General

All solvents were reagent grade or HPLC grade. Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. All reactions were performed under nitrogen. The analytical HPLC conditions used a gradient of 5% ACN/95% H₂O (both containing 0.1% formic acid) for 0.25 minutes followed by an increase to 40% ACN/60% H₂O over 1.75 minutes and continuation of 40% ACN/60% H₂O (containing 0.1% formic acid) until 4 minutes (detection at 220 nm) with a Luna C18 column (5 micron, 150 $\times$ 4.6 mm). All solvents were reagent grade or HPLC grade. Melting points were obtained on a Mel-Temp apparatus and are uncorrected. ¹H NMR spectra were recorded at 400 MHz. ¹³C NMR spectra were recorded at 100 MHz. Elemental analyses were obtained from Atlantic Microlabs, Norcross, GA. The purity of test compounds was confirmed by elemental analysis (within $\pm 0.4\%$ of the calculated value).

3-(2-Oxotetrahydro-2H-thiopyran-3-yl)propanoic acid (**5**)

A solution of **1** (0.530 g, 2.57 mmol) and 10-camporsulfonic acid (0.120 g, 0.52 mmol) in toluene (60 mL) was stirred at the refluxing temperature. The water formed in the reaction was removed by Dean-Stark apparatus. After 6 h, excess solvent was removed and the residual oil was purified by silica gel chromatography (hexanes/EtOAc, 4:1) to give 0.187 g of **5** as a white solid (35% yield): mp 80–82 °C; ¹H NMR (DMSO-*d*₆) δ 1.48–1.65 (m, 2H), 1.86–2.07 (m, 4H), 2.26 (t, *J* = 7.5 Hz, 2H), 2.59–2.70 (m, 1H), 3.08–3.26 (m, 2H); ¹³C NMR (CD₃OD) δ 23.34, 27.47, 29.34, 31.29, 32.32, 50.05, 177.13, 206.5. Anal calcd. For C₈H₁₂O₃S: C, 51.04; H, 6.43; S, 17.03. Found: C, 50.77, H, 6.35; S, 17.25.

3-(1-Oxoisothiochroman-8-yl)benzoic acid (**6**)

To a solution of **2** (200 mg, 0.66 mmol) in ethanol (10 mL) were added a 4% solution of NaOH (3 mL) and benzyl bromide (120 mg, 0.69 mmol) at 0 °C. The mixture was stirred at rt for 3h. The solvent was removed under reduced pressure and the residue was partitioned between EtOAc (20 mL) and 1N HCl (15 mL). The organic layer was dried over MgSO₄ and concentrated to give **7** as off-white foam. ¹H NMR (CDCl₃) δ 2.73–2.88 (m, 2H) 3.03 (m, 2H) 3.79 (s, 2H) 7.23–7.40 (m, 8H) 7.42–7.51 (m, 1H) 7.52–7.60 (m, 1H), 7.70 (dq, *J* = 7.7, 1.1 Hz, 1H), 8.00 (dt, *J* = 7.8, 1.4 Hz, 1H), 8.26–8.33 (brs, 1H). The resulting foam was

dissolved in of trifluoroacetic anhydride (4.5 mL) and refluxed at 60°C for 2 h. Excess solvent was removed and the residue was quenched with saturated NaHCO₃ at 0 °C. This was followed by acidification with 1 N HCl and extraction with EtOAc (20 mL × 2). The combined extracts were dried over MgSO₄ and concentrated. The residual material was purified by silica gel chromatography (EtOAc/hexanes/AcOH, 1/1/0.02) to give 55 mg of **6** as a white solid (30% yield): mp 210–212 °C; ¹H NMR (DMSO-*d*₆) δ 3.21–3.29 (m, 2H), 3.37–3.44 (m, 2H), 7.30 (d, *J* = 7.6 Hz, 1H), 7.44–7.54 (m, 3H), 7.60 (t, *J* = 7.6 Hz, 1H), 7.76 (s, 1H), 7.89 (d, *J* = 8.3 Hz, 1H), 13.01 (brs, 1H); ¹³C NMR (DMSO-*d*₆) δ 28.75, 31.55, 127.66, 128.39, 128.83, 129.02, 130.51, 130.73, 130.82, 132.27, 132.74, 140.25, 142.03, 143.03, 167.24, 190.16. Anal. Calcd for C₁₆H₁₂O₃S·0.25H₂O: C, 66.53; H, 4.36; S, 11.10. Found: C, 66.51; H, 4.40; S, 10.97.

Chemical stability studies in simulated gastric fluid

Solutions of compounds **5** (4.7 mg/mL in H₂O) and **6** (1.4 mg/mL in DMSO) were prepared. A 50 µL aliquot of each of these solutions was added to 1 mL of simulated gastric fluid (pH = 1.2, Ricca Chemical 7108-16). These mixtures were incubated at 37°C and 50 µL aliquots were taken at 30 min, 1h, 4h and 48h. The samples were analyzed by HPLC in order to quantify the remaining thiolactones and the hydrolyzed compounds.

Metabolic stability studies in the rat liver microsomes

Test compound (10 µM) was incubated in 100 mM potassium phosphate buffer pH 7.4 containing rat liver microsomes (0.5 mg/mL). The reaction was run in triplicate and at predetermined times (0, 30, 60 and 90 min) aliquots of the mixture were removed and the reaction quenched by addition of two times the volume of ice cold acetonitrile spiked with the internal standard (losartan). Compound appearance/disappearance over time was monitored using a liquid chromatography and tandem mass spectrometry (LC/MS/MS) method. Chromatographic separation was performed on an Agilent Eclipse Plus C18 column (1.8 µm, 100 × 2.1 mm) with a flow rate of 0.4 mL/min. The solvent system consisted of distilled water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). 10% B was used from 0 to 0.2 min followed by a linear gradient of 10% to 70% B over 1.6 min. Detection was achieved under negative ion electrospray using the [M-H][−] peaks. The multiple reaction monitoring transitions used for quantification of **1** were m/z 205.501 → 187.432 and m/z 205.501 → 171.492. For quantification of the internal standard (losartan), m/z 421.71 → 179.414 and m/z 421.71 → 127.297 were used.

In vivo Pharmacokinetics and Pharmacology

Pharmacokinetics studies in rats and the subsequent plasma bioanalysis of compound **1** were conducted as previously described.¹⁴ The chronic constrictive injury model of neuropathic pain was performed following the procedure reported by Bennett's group.¹³ A more detailed procedure is described in the recent article.¹⁴

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ABBREVIATIONS

GCPII	glutamate carboxypeptidase II
AUC	area under the curve

REFERENCES

1. Zhou J, Neale JH, Pomper MG, Kozikowski AP. NAAG peptidase inhibitors and their potential for diagnosis and therapy. *Nat. Rev. Drug Discov.* 2005; 4:1015–1026. [PubMed: 16341066]
2. Tsukamoto T, Wozniak KM, Slusher BS. Progress in the discovery and development of glutamate carboxypeptidase II inhibitors. *Drug Discov. Today.* 2007; 12:767–776. [PubMed: 17826690]
3. Ferraris DV, Shukla K, Tsukamoto T. Structure-activity relationships of glutamate carboxypeptidase II (GCP II) inhibitors. *Curr. Med. Chem.* 2012; 19:1282–1294. [PubMed: 22304717]
4. Majer P, Jackson PF, Delahanty G, Grella BS, Ko YS, Li W, Liu Q, Maclin KM, Polakova J, Shaffer KA, Stoermer D, Vitharana D, Wang EY, Zakrzewski A, Rojas C, Slusher BS, Wozniak KM, Burak E, Limsakun T, Tsukamoto T. Synthesis and biological evaluation of thiol-based inhibitors of glutamate carboxypeptidase II: discovery of an orally active GCP II inhibitor. *J. Med. Chem.* 2003; 46:1989–1996. [PubMed: 12723961]
5. Barinka C, Rojas C, Slusher B, Pomper M. Glutamate carboxypeptidase II in diagnosis and treatment of neurologic disorders and prostate cancer. *Curr. Med. Chem.* 2012; 19:856–870. [PubMed: 22214450]
6. Stoermer D, Vitharana D, Hin N, Delahanty G, Duvall B, Ferraris DV, Grella BS, Hoover R, Rojas C, Shanholtz MK, Smith KP, Stathis M, Wu Y, Wozniak KM, Slusher BS, Tsukamoto T. Design, Synthesis, and Pharmacological Evaluation of Glutamate Carboxypeptidase II (GCP II) Inhibitors Based on Thioalkylbenzoic Acid Scaffolds. *J. Med. Chem.* 2012; 55:5922–5932. [PubMed: 22642259]
7. Wozniak KM, Wu Y, Vornov JJ, Lapidus R, Rais R, Rojas C, Tsukamoto T, Slusher BS. The orally active glutamate carboxypeptidase II inhibitor E2072 exhibits sustained nerve exposure and attenuates peripheral neuropathy. *J. Pharmacol. Exp. Ther.* 2012; 343:746–754. [PubMed: 22988061]
8. Jaffe IA. Adverse effects profile of sulfhydryl compounds in man. *Am. J. Med.* 1986; 80:471–476. [PubMed: 2937293]
9. Emmons GT, Argenti R, Martin LL, Martin NE, Jensen BK. Pharmacokinetics of M100240 and MDL 100,173, a dual angiotensin-converting enzyme/neutral endopeptidase inhibitor, in healthy young and elderly volunteers. *J. Clin. Pharmacol.* 2004; 44:901–905. [PubMed: 15286094]
10. Lumma WC, Dutra GA, Voeker CA. Synthesis of two benzothiacyclanones via a novel two-carbon ring expansion of thiolactones with vinylolithium. *J. Org. Chem.* 1970; 35:3442–3444.
11. Levine JA, Ferrendelli JA, Covey DF. Alkyl-substituted thio-, thiono-, and dithio-gamma-butyrolactones: new classes of convulsant and anticonvulsant agents. *J. Med. Chem.* 1986; 29:1996–1999. [PubMed: 3761317]
12. Rojas C, Frazier ST, Flanary J, Slusher BS. Kinetics and inhibition of glutamate carboxypeptidase II using a microplate assay. *Anal. Biochem.* 2002; 310:50–54. [PubMed: 12413472]
13. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain.* 1988; 33:87–107. [PubMed: 2837713]
14. Vornov J, Wozniak K, Wu Y, Rojas C, Rais R, Slusher B. Pharmacokinetics and Pharmacodynamics of the Glutamate Carboxypeptidase (GCP) II Inhibitor 2-MPPA Show Prolonged Alleviation of Neuropathic Pain through an Indirect Mechanism. *J. Pharmacol. Exp. Ther.* 2013; 346:406–413. [PubMed: 23776202]
15. Tsukamoto T, Majer P, Vitharana D, Ni C, Hin B, Lu XC, Thomas AG, Wozniak KM, Calvin DC, Wu Y, Slusher BS, Scarpetti D, Bonneville GW. Enantiospecificity of glutamate carboxypeptidase II inhibition. *J Med Chem.* 2005; 48:2319–2324. [PubMed: 15801825]
16. Jakubowski H, Glowacki R. Chemical biology of homocysteine thiolactone and related metabolites. *Adv Clin Chem.* 2011; 55:81–103. [PubMed: 22126025]
17. Majer P, Hin B, Stoermer D, Adams J, Xu W, Duvall BR, Delahanty G, Liu Q, Stathis MJ, Wozniak KM, Slusher BS, Tsukamoto T. Structural Optimization of Thiol-Based Inhibitors of Glutamate Carboxypeptidase II by Modification of the P1' Side Chain. *J. Med. Chem.* 2006; 49:2876–2885. [PubMed: 16686531]
18. Grella B, Adams J, Berry JF, Delahanty G, Ferraris DV, Majer P, Ni C, Shukla K, Shuler SA, Slusher BS, Stathis M, Tsukamoto T. The discovery and structure-activity relationships of in-dole-

based inhibitors of glutamate carboxypeptidase II. *Bioorg. Med. Chem. Lett.* 2010; 20:7222–7225. [PubMed: 21074428]

Figure 1.
Chemical structures of compounds 1–6.

Figure 2. Formation of **1** from **5** in rat liver microsomes. Values and error bars represent the mean and standard deviation of triplicate measurements.

Figure 3. Plasma concentration versus time profiles of compound **1** (□) in rats following oral administration of δ -thiolactone **5** (10 mg/kg). Plasma concentration versus time curve of compound **1** (Δ) following oral administration of **1** (10 mg/kg) is superimposed for comparison.

Figure 4.

Antinociceptive effects of compound **5** in the rat chronic constriction injury (CCI) model of neuropathic pain. Oral administration of **5** at $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ significantly attenuated CCI-induced hyperalgesic state relative to the vehicle-treated control (*, $p < 0.05$; **, $p < 0.01$).

Scheme 1. Synthesis of δ -thiolactones 5 and 6a

^aReagents and conditions: (a) PTSA, toluene, reflux; (b) BnBr, 4% NaOH, EtOH, rt; (c) TFAA, reflux.