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Inhibition of human gamma-glutamyl transpeptidase: development of more potent, physiologically relevant, uncompetitive inhibitors

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SYNOPSIS

Gamma-glutamyl transpeptidase (GGT) is an essential enzyme for maintaining cysteine homeostasis, leukotriene synthesis, metabolism of glutathione-conjugates and catabolism of extracellular glutathione. Overexpression of GGT has been implicated in many pathologies, and clinical inhibitors of GGT are under development for use in the treatment of asthma, cancer and other diseases. Inhibitors are generally characterized using synthetic GGT substrates. This study of uncompetitive inhibitors of GGT, has revealed that the potency with which compounds inhibit GGT activity in the standard biochemical assay does not correlate with the potency with which they inhibit the physiological reaction catalyzed by GGT. Kinetic studies provided insight into the mechanism of inhibition. Modifications to the sulfobenzene or distal benzene ring of the uncompetitive inhibitor, OU749, affected activity. One of the most potent inhibitors was identified among a novel group of analogs with an amine group *para* on the benzosulfonamide ring. New, more potent uncompetitive inhibitors of the physiological GGT reaction were found to be less toxic than the glutamine-analogs that have been tested clinically. Development of non-toxic inhibitors is essential for exploiting GGT as a therapeutic target.

Keywords

Glutathione; gamma-glutamyl transferase; OU749

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AUTHOR CONTRIBUTIONS

Each author made a substantive contribution to this work. Stephanie Wickham contributed to the study design, preformed the biochemical and cell culture studies with the inhibitors, analyzed and interpreted the data, and wrote the paper. Nicholas Regan and Justin Thai synthesized and purified the novel analogs, and wrote the methods sections for the synthesis of the compounds. Matthew B. West expressed and isolated the GGT used in these studies and contributed to the interpretation of the data. Paul F. Cook contributed to the study design, analysis and interpretation of data. Simon S. Terzyan contributed the figure of the homology model of human GGT showing the proposed binding site of OU749. Pui Kai Li designed the OU749 analogs and supervised their synthesis. Marie H. Hanigan is the corresponding author. She contributed to the study design, supervised the experiments and data analysis carried out Stephanie Wickham and Matthew West and edited the manuscript.

INTRODUCTION

Gamma-glutamyl transpeptidase (GGT, P19440, EC 2.3.2.2) is a cell surface enzyme that cleaves gamma-glutamyl bonds of extracellular substrates including glutathione (GSH), GSH *S*-conjugates and leukotriene C₄ [1–3]. Catabolism of GSH by GGT affects intracellular redox levels and cysteine homeostasis [4, 5]. GGT cleavage of GSH *S*-conjugates alters drug toxicity and inflammation [6–8]. Overexpression of GGT has been implicated in pulmonary disease, cardiovascular disease and cancer [9–11]. Therefore, development of potent inhibitors of human GGT (hGGT) for clinical use is predicted to have broad therapeutic impact. Currently, the most potent GGT inhibitors are glutamine analogs [12, 13]. Glutamine analogs that inhibit GGT and have been evaluated in humans are too toxic for clinical use [14–16]. We have recently identified a new class of uncompetitive inhibitors of hGGT that are considerably less toxic than the glutamine analogs [17, 18].

The reaction catalyzed by GGT *in vivo* is the hydrolysis of the gamma-glutamyl bond. GGT hydrolyzes GSH into glutamate and cysteinylglycine [1, 2]. The gamma-glutamyl substrate (donor substrate) binds to GGT, which initiates a nucleophilic attack on the gamma-glutamyl bond (Fig. 1A). An acyl-bond is formed between the oxygen on the side-chain of Thr-381 of hGGT and the gamma-glutamyl group of the substrate, creating the gamma-glutamyl enzyme intermediate (F-form of the enzyme) [19]. The acyl-bond is hydrolyzed by water to release glutamate and cysteinyl-glycine. The reaction is a modified ping-pong reaction [1]. The addition of high concentrations of dipeptide or amino acid acceptor molecules (second substrate for the ping-pong reaction) results in a transpeptidation reaction (Fig. 1B). The free alpha-amine on the acceptor attacks the acyl-bond, transferring the gamma-glutamyl group to the acceptor, thereby forming a new gamma-glutamyl compound [20].

The most commonly used assay for GGT activity monitors the transpeptidation reaction with the synthetic compound L-gamma-glutamyl *para*-nitroanilide (L-GpNA) as the donor substrate and glycylglycine (GlyGly) as the acceptor. L-GpNA can also serve as a weak acceptor and therefore cannot be used in the absence of GlyGly to measure the hydrolysis reaction. D-gamma-glutamyl *para*-nitroanilide (D-GpNA), a stereoisomer of L-GpNA, cannot serve as an acceptor and is therefore used as a substrate to measure the hydrolysis reaction [21, 22]. However, the D-isomer of glutamate is not a physiological compound and there have been concerns as to the relevance of D-GpNA as a substrate. OU749 and its structural analogs are the only known uncompetitive inhibitors of the GGT transpeptidation reaction and the hydrolysis of D-GpNA [17, 18]. Uncompetitive inhibitors bind the gamma-glutamyl intermediate (F-form) of the enzyme (Fig. 1). In the transpeptidation reaction, the inhibitors exhibited competitive inhibition with the dipeptide acceptor, GlyGly, indicating that they have a shared or overlapping binding site with GlyGly on the enzyme [17]. We have developed a novel assay (L-Glutamate Release Assay) that monitors GGT hydrolysis of GSH and other physiological substrates [3].

OU749 was identified by high-throughput screening of small molecules as inhibitors of the transpeptidation reaction [17]. In this study, we compare the potency and mechanism with which OU749 and its analogs inhibit GGT activity as measured by the transpeptidation reaction with the synthetic substrate, L-GpNA, the hydrolysis reaction with the synthetic substrate, D-GpNA, and the physiological hydrolysis reaction with GSH as the substrate. We found that there were dramatic differences in the potency of the inhibitors dependent on the substrate used to measure hGGT activity. We also report several novel compounds that are more potent inhibitors of hGGT with the physiological substrate and evaluate the toxicity of these compounds.

EXPERIMENTAL

Synthesis of Compounds

The syntheses of OU749, Compounds 2–4, 6–11 and 13–20 were described previously [18]. The syntheses of Compound 21 to Compound 27 are described below.

General procedure A—2 mmol of the appropriate phenyl acetic acid and 2 mmol of thiosemicarbazide were dissolved in 1 mL of POCl₃ and refluxed for 45 minutes. The reaction was then cooled to room temperature and 3 mL of water were added carefully. The solution was then refluxed for 4 hours. The reaction mixture was filtered hot and the solid was washed with warm water. The filtrate was basified with saturated KOH and the solid was isolated by filtration. The solid was recrystallized from ethanol.

General procedure for the reduction of Compounds 21–30 to the amine—The appropriate nitro-Compound (0.25 mmol) and SnCl₂·H₂O (8 g/g of Compound) were dissolved in 15 mL of ethanol dichloromethane (DCM) (1:1) and heated to 40°C. Concentrated HCl (0.2 mL) was added and reaction was stirred for 2 hr. The reaction mixture was poured into H₂O and filtered through celite. The filtrate was neutralized with solid NaHCO₃ and extracted with ethylacetate (EtOAc). The organic layer was dried with Na₂SO₄ and concentrated *in vacu*. **Compound 21:** 89.5 mg (97%), off white crystals, mp 120–121°C; ¹H NMR (300 MHz, DMSO) 3.74 (s, 3H), 4.08 (s, 2H), 5.89 (s, 2H), 6.54 (d, *J* = 8.6 Hz, 2H), 6.91 (d, *J* = 8.6 Hz, 2H), 7.23 (d, *J* = 8.5 Hz, 2H), 7.35 (d, *J* = 8.6 Hz, 2H), 13.71 (s, 1H). MS(*m/z*) = 399.06 [M+Na]⁺. **Compound 25:** 85 mg (98%), white solid, mp 94–96°C. ¹H NMR (300 MHz, DMSO) 4.16 (s, 2H), 5.91 (s, 2H), 6.54 (d, *J* = 8.3 Hz, 2H), 7.39–7.27 (m, 7H). MS(*m/z*) = 369.02 [M+Na]⁺. **Compound 29:** 86 mg (83%), orange solid, mp 186–188°C. ¹H NMR (300 MHz, DMSO) 4.20 (s, 2H), 5.94 (s, 2H), 6.56 (d, *J* = 8.6 Hz, 2H), 7.32 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.38 (d, *J* = 8.6 Hz, 2H), 7.60 (t, *J* = 8.2 Hz, 1H), 7.64 (d, *J* = 1.7 Hz, 1H). MS(*m/z*) = 436.90 [M+Na]⁺. **Compound 28:** 89 mg (93%), white solid, mp 198–202°C. ¹H NMR (300 MHz, DMSO) 4.16 (s, 2H), 5.87 (s, 2H), 6.54 (d, *J* = 8.4 Hz, 2H), 7.44–7.30 (m, 6H). MS(*m/z*) = 403.02 [M+Na]⁺. **Compound 22:** 85 mg (94%), white solid, mp 136–139°C. ¹H NMR (300 MHz, DMSO) 2.28 (s, 3H), 4.10 (s, 2H), 5.91 (s, 2H), 6.54 (d, *J* = 8.4 Hz, 2H), 7.29–7.10 (m, 4H), 7.42 (ddd, *J* = 20.3, 8.4, 5.7 Hz, 4H). MS(*m/z*) = 383.03 [M+Na]⁺. **Compound 26:** 72 mg (79%), orange solid, mp 124–126°C. ¹H NMR (300 MHz, DMSO) 4.16 (s, 2H), 5.90 (s, 2H), 6.54 (d, *J* = 8.6 Hz, 2H), 7.18 (t, *J* = 9.2 Hz, 2H), 7.48–7.29 (m, 5H). MS(*m/z*) = 386.98 [M+Na]⁺. **Compound 23:** 57 mg (63%), yellow solid, mp 125–127°C. ¹H NMR (300 MHz, DMSO) 3.93 (s, 2H), 5.11 (s, 2H), 5.93 (s, 2H), 6.53 (dd, *J* = 8.2, 3.5 Hz, 4H), 6.94 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.1 Hz, 2H). MS(*m/z*) = 384.01 [M+Na]⁺. **Compound 27:** 78 mg (80%), orange solid, mp 125–127°C. ¹H NMR (300 MHz, DMSO) 2.88 (s, 6H), 4.01 (s, 2H), 5.93 (s, 2H), 6.54 (d, *J* = 8.4 Hz, 2H), 6.70 (d, *J* = 8.1 Hz, 2H), 7.11 (d, *J* = 8.2 Hz, 2H), 7.35 (d, *J* = 8.3 Hz, 2H). MS(*m/z*) = 412.07 [M+Na]⁺. **Compound 24:** 87 mg (97%), white solid, mp 194–196°C. ¹H NMR (300 MHz, DMSO) 2.29 (s, 3H), 4.11 (s, 2H), 5.92 (s, 2H), 6.54 (d, *J* = 8.6 Hz, 2H), 7.14–7.08 (m, 3H), 7.27–7.22 (m, 1H), 7.36 (d, *J* = 8.6 Hz, 2H). MS(*m/z*) = 383.06 [M+Na]⁺. **Compound 30:** ¹H NMR (300 MHz, DMSO) 3.76 (s, 3H), 4.18 (s, 2H), 6.91 (d, *J* = 8.6 Hz, 2H), 7.26 (d, *J* = 8.6 Hz, 2H), 7.49–7.62 (m, 2H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.83–8.05 (m, 3H), 8.17 (s, 1H), 9.00 (s, 1H).

Enzyme Isolation

hGGT (P19440), lacking the transmembrane domain, was expressed in *Pichia pastoris* and isolated as previously described [17]. The specific activity of the purified GGT was 400 units/mg. One unit of GGT activity was defined as the amount of enzyme that released 1

μmol of paranitroaniline/min at 37°C at pH 7.4 in the transpeptidation reaction with L-GpNA.

L-Glutamate Release Assay (Hydrolysis of GSH)

This assay measures the production of glutamate from the hydrolysis of GSH by GGT enzyme and has been described in detail previously [3]. The concentration of the substrate, GSH, was varied from 5 μM to 20 μM. The concentration of the inhibitors, OU749 and its analogs, were varied from 15.6 μM to 250 μM. The reaction was initiated with the addition of 10 mU hGGT. The reaction was incubated at 37°C and monitored continuously at 490 nm by a Bio-Rad model 680 microplate reader with Microplate Manager 5 software (Bio-Rad, Hercules, CA). All compounds were also evaluated as inhibitors of glutamate dehydrogenase, the enzyme in the second half of the L-glutamate release assay. None of the compounds in this study inhibited glutamate dehydrogenase.

L-GpNA Transpeptidation Assay

The assay has been described previously [18]. The concentration of the substrate for the transpeptidation reaction, L-GpNA (Sigma, St. Louis, MO) was varied from 0.25 mM to 3 mM in the presence of 40 mM GlyGly (Sigma) as the acceptor. The concentration of L-GpNA was 3 mM for experiments in which the concentration of GlyGly was varied. To initiate the transpeptidation reaction, 4 mU GGT were added. A comparison of the activity of GGT among assays showed that the amount of hGGT that had 10 mU activity in the L-GpNA transpeptidation reaction had 0.868 mU of hGGT activity in the L-Glutamate Release Assay.

D-GpNA Hydrolysis Assay

The assay was carried out as previously described [18]. Briefly, the concentration of the D-gamma-glutamyl paranitroanilide (D-GpNA, Bachem, Torrance, CA) substrate was varied from 0.125 mM to 1 mM D-GpNA. The reaction was initiated with the addition of 19 mU GGT as determined by the L-GpNA transpeptidation assay. The reaction was incubated at 37°C and monitored continuously at 405 nm.

Cell Lines

786-O cells (ATCC CRL-1932), a human renal adenocarcinoma cell line, were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (50 units/ml, 50 μg/ml). NIH3T3 cells (ATCC CRL 1658) which are GGT-negative and NIH3T3 cells expressing hGGT [described previously [23]] were grown in DMEM/F12 media supplemented with 5% FBS and 0.2 mg/ml G418.

Catabolism of Extracellular GSH

786-O and NIH3T3 cells were plated in 96-well plates at a density of 5×10^3 cells/well. After 24 hours, the media was changed to 100 μl cysteine-free DMEM/F12 supplemented with either 20 μM GSH or 20 μM GSH and 100 μM compound 6. After one hour, 50 μl of media was removed and immediately acidified with 5 μl ice-cold 43.1% 5-sulfosalicylic acid. After 10 minutes on ice, the solution was neutralized with 15 μl of 1 M NaOH. The GSH concentration was determined by the method of Tietze [24].

Cytotoxicity Assays

786-O cells were plated at 10^3 cells/well in a 96-well plate in DMEM supplemented with 10% FBS and penicillin/streptomycin (50 units/ml, 50 μg/ml). After 24 hours, the media was changed to fresh media containing OU749 analogs. A 100 mM stock of OU749 and each of its analogs were made in DMSO then diluted in DMEM. Control wells were treated

with equivalent concentrations of DMSO. Cell viability was determined 72 hours after the addition of the inhibitors by the MTT assay [25].

Data Analysis

In each experiment the assays were run in triplicate. Each inhibitor was evaluated in two or more independent experiments. Double reciprocal plots were generated to assess data quality and determine the correct rate equation for data fitting. Data were fitted using the proper rate equation and the Marquardt-Levenberg algorithm supplied with the Enzfitter program (BIOFITTER, Cambridge, UK). Kinetic parameters with standard errors (S.E.) were estimated using a simple weighting method.

Data adhering to Michaelis-Menten Kinetics were fitted to eq. 1. Data for competitive, uncompetitive, and noncompetitive inhibition were fitted to eq. 2, 3, and 4, respectively. Data for the dependence of V_{max} and V_{max}/K_{D-GpNA} on the concentration of activator were fitted using eq. 4 [26].

$$v = \frac{V_{max}A}{K_a + A} \quad (1)$$

$$v = \frac{V_{max}A}{K_a \left(1 + \frac{I}{K_{is}}\right) + A} \quad (2)$$

$$v = \frac{V_{max}A}{K_a + A \left(1 + \frac{I}{K_{ii}}\right)} \quad (3)$$

$$v = \frac{V_{max}A}{K_a \left(1 + \frac{I}{K_{is}}\right) + A \left(1 + \frac{I}{K_{ii}}\right)} \quad (4)$$

$$y = \frac{a + \frac{X}{K_{IN}}}{1 + \frac{X}{K_{ID}}} \quad (5)$$

In eqs 1–4, v and V_{max} are the initial and maximum rates, respectively, K_a is the Michaelis constant for the varied substrate, and K_{is} and K_{ii} are slope and intercept inhibition constants. In eq. 5, a is the value of V_{max} or V_{max}/K_{D-GpNA} at zero activator/inhibitor, K_{ID} is the activation or inhibition constant, and K_{IN} is a constant that causes the parameter to go to a constant value at infinite concentrations of X , the activator/inhibitor. The product $a(K_{ID}/K_{IN})$ is the value of V_{max} or V_{max}/K_{D-GpNA} at infinite activator/inhibitor [26].

For the toxicity of the OU749 and its analogs in 786-O cells all determinations were done in triplicate. The LD₅₀ of each compound and the standard deviation (S.D.) were calculated with a Prism log (inhibitor) versus normalized response (variable slope) curve fit (Prism, GraphPad Software Inc., San Diego, CA).

Structural Model of OU749 Interaction with hGGT

A refined structural model of hGGT described previously and based on crystallographic templates of bacterial GGT was used [27]. The model of OU749 was built using the

program Insight II and manually fitted into the structural model of hGGT. Then the complex structure was energy minimized using the CNS program suite [28]. The value of the total energy after 300 cycles decreased from 113350 to 11866 calories.

RESULTS

hGGT can catalyze both a hydrolysis reaction and a transpeptidation reaction (Fig. 1). When present at high concentrations, L-amino acids and dipeptides can serve as acceptors in the transpeptidation reaction. GSH has an L-gamma-glutamyl moiety, and the free amino group on the alpha carbon might serve as an acceptor. Our initial studies were designed to clarify whether GSH serves as both a donor substrate and an acceptor. The alpha carbon of D-amino acids is unable to act as an acceptor, therefore, the substrate D-GpNA functions as a donor substrate but cannot function as an acceptor [21]. Kinetic analyses of D-GpNA cleavage in the presence of GSH showed that GSH was competitive with D-GpNA. These data reveal that GSH competes for the gamma-glutamyl donor binding site but did not function as an acceptor with a D-gamma-glutamyl donor (Fig. 2A). The K_m of D-GpNA was $160 \pm 20 \mu\text{M}$. The K_{is} of GSH was $10 \pm 1.2 \mu\text{M}$. Kinetic analyses were also performed with L-GpNA in the presence of GSH. Analysis of the GSH inhibition of the transpeptidation reaction with L-GpNA and the acceptor GlyGly show that GSH is a noncompetitive inhibitor of both (Fig. 2B and C). The K_m of L-GpNA in the transpeptidation reaction is $1.2 \pm 0.1 \text{ mM}$. In the presence of 40 mM GlyGly, GSH inhibits L-GpNA in the transpeptidation reaction with $K_{ij} = 1.70 \pm 0.02 \text{ mM}$ and $K_{is} = 1.40 \pm 0.01 \text{ mM}$. When evaluated with GlyGly in the presence of 3 mM L-GpNA, GSH was a noncompetitive inhibitor of the transpeptidation reaction with $K_{ij} = 3 \pm 0.5 \text{ mM}$ and $K_{is} = 0.8 \pm 0.1 \text{ mM}$ (Fig. 2C). This noncompetitive inhibition profile indicates that GSH binds both the free enzyme and the F-form, competing with both the donor (L-GpNA), and the acceptor (GlyGly) substrate when GSH is present at high (millimolar) concentrations.

We then analyzed the interaction between GSH and L-GpNA in the absence of GlyGly. Both GSH and L-GpNA are donor substrates. They both have an L-glutamate as the gamma-glutamyl group and, thus, can also serve as acceptors. The complex data obtained from these experiments were most clearly presented as a Dixon plot (Fig. 2D). Initial velocity analysis of GSH inhibition of the L-GpNA cleavage is shown for two concentrations of L-GpNA (0.25 mM and 3 mM). In the presence of 3 mM L-GpNA, the Dixon plot was linear with GSH acting as a very weak inhibitor of L-GpNA as a donor substrate (Fig. 2D). The K_m of L-GpNA in these studies was $0.83 \pm 0.06 \text{ mM}$. The K_{is} of GSH is $0.073 \pm 0.006 \text{ mM}$. However, the biphasic nature of the Dixon plot at 0.25 mM L-GpNA indicates GSH has two modes of action under these reaction conditions (Fig. 2D). At concentrations up to 40 μM , GSH is a competitive inhibitor of L-GpNA (Fig. 2E). At concentrations of GSH above 0.31 mM, the Dixon plot shows a second phase, in which GSH is competitive with both L-GpNA as a donor substrate and with L-GpNA as an acceptor. Thus, at high concentrations GSH can act as an acceptor substrate, confirming data from Abbott and colleagues [29]. In the L-Glutamate Release Assay, GSH is used as a substrate over the range of 5–20 μM . Under these conditions GSH is functioning solely as a donor substrate. Therefore, data from the L-Glutamate Release Assay is a direct measurement of the hydrolysis of the gamma-glutamyl bond of glutathione.

Previous studies had shown that OU749 was an uncompetitive inhibitor of hGGT, binding to the gamma-glutamyl-enzyme intermediate (the F-form of the enzyme), while competing with the acceptor in the transpeptidation reaction. We assayed a large series of OU749 analogs and evaluated their effect on the hydrolysis of the acyl bond of the L-gamma-glutamyl-enzyme intermediate (GSH as substrate), the hydrolysis of the acyl bond of the D-gamma-glutamyl-enzyme intermediate (D-GpNA as a substrate) and the transfer of the L-

gamma-glutamyl-enzyme moiety to an acceptor (L-GpNA as the substrate, GlyGly as the acceptor).

OU749 and a series of its analogs bearing *para*-substitutions on the benzosulfonamide ring were the first set of compounds that were analyzed (Table 1). All of the compounds were uncompetitive inhibitors of GSH. They were also uncompetitive inhibitors of D-GpNA and L-GpNA, but competitive inhibitors of GlyGly as previously reported for OU749 [17, 18]. Substituting an amine in the *para* position increased the potency of inhibition of GSH hydrolysis, while substituting a methyl group at this position decreased the potency. Bulky groups at the *para*-position also impeded the compound's function as an inhibitor of GSH hydrolysis. A comparison of the inhibition of the hydrolysis of D-GpNA by these same compounds showed that they were more potent inhibitors of the hydrolysis of the D-gamma-glutamyl enzyme acyl bond (D-GpNA substrate) than the L-gamma-glutamyl enzyme acyl bond (GSH substrate), but the rank order of potency remained the same (Table 1). Even though each of these compounds was an uncompetitive inhibitor of L-GpNA in the transpeptidation reaction, their potency correlated more directly with their competitive inhibition of the acceptor GlyGly than their inhibition of the cleavage of the L-gamma-glutamyl acyl bond (GSH hydrolysis). This may be due to the fact that acyl transfer to an acceptor is more rapid than hydrolysis of the acyl bond [19]. Therefore, competing with GlyGly would have a more potent effect on the rate of the reaction than inhibition of hydrolysis of the acyl bond. The methyl-substituted OU749 analog, Compound 2, was previously identified as a more potent inhibitor of hGGT based on its inhibition of the L-GpNA transpeptidation reaction [18]. However, Compound 2 was 1.3-fold less potent than OU749 in inhibiting the hGGT-mediated hydrolysis of GSH, the physiological reaction catalyzed by hGGT (Table 1). In contrast to Compound 2 and the other *para*-substituted OU749 analogs, Compound 21, with a *para*-amine, was 3.6-fold more potent than OU749 in inhibiting the catabolism of GSH. Therefore, we classified Compound 21 as a new lead compound from among this series of OU749 analogs (Table 1). These findings demonstrate that data from the standard L-GpNA transpeptidation assay can be misleading when optimizing inhibitors of hGGT for use *in vivo*.

Based on the improved potency of Compound 21 compared with OU749 in inhibiting the hydrolysis of GSH, we synthesized a series of structural analogs of Compound 21 in which the distal benzene ring was modified (Table 2). All of these compounds were found to be uncompetitive inhibitors of the hydrolysis of GSH, D-GpNA and L-GpNA but competitive inhibitors of GlyGly. The kinetic data for Compound 22 are shown in Fig. 3. As uncompetitive inhibitors, these compounds bind the gamma-glutamyl-enzyme intermediate (F-form) with all three donor substrates. The rank order of potency with which these compounds inhibit the hydrolysis of GSH versus D-GpNA differs (Table 2). These data indicate that there are differences in the F-form of the enzyme dependent on the stereochemistry of the bound glutamate group (L-glutamate in GSH and D-glutamate in D-GpNA) and that these differences affect the potency with which this class of compounds inhibits the reaction. Compound 22 and its analogs are uncompetitive inhibitors of L-GpNA (Fig. 3C). The F-form of the enzyme is the same with either GSH or L-GpNA as substrates, because both have an L-gamma-glutamyl group. However, as observed for the compounds in Table 1, the K_{ij} s for this group of compounds with GSH versus L-GpNA as a substrate do not correlate in part as result of L-GpNA binding as an acceptor and perhaps affecting the rate of hydrolysis. The most potent competitive inhibitors of GlyGly (Compounds 29, 22 and 28) are also the most potent inhibitors of the transpeptidation reaction with L-GpNA, again demonstrating the influence of the acceptor on the kinetics of the cleavage of the acyl bond. Among this group of compounds, Compound 22 and Compound 23 were more potent inhibitors of the hydrolysis of GSH by hGGT than the progenitor compound, Compound 21 (Tables 1 and 2). Compound 22 has a methyl group in the *para* position on the distal

benzene ring. Compound 23, has an amine group in the *para* position. OU749 analogs with a small neutral or slightly charged group on the distal benzene ring paired with an amine group *para* on the benzosulfonamide ring are more potent inhibitors of the catabolism of GSH by hGGT than Compound 21.

In this study, we also evaluated a series of OU749 analogs with chlorine or benzene substitutions on the sulfobenzene ring as inhibitors of GSH hydrolysis (Table 3). These compounds had previously been evaluated as inhibitors of D-GpNA hydrolysis and the transpeptidation reaction with L-GpNA and had been shown to be potent inhibitors hGGT1 transpeptidation [18]. Compound 3, which has a chlorine in the *meta* position on the benzosulfonamide ring, was 5.6-fold more potent than OU749 in inhibiting GSH hydrolysis (K_{ii} $67 \pm 6 \mu\text{M}$ and $376 \pm 50 \mu\text{M}$, respectively). Interestingly, this same substitution had no effect on the inhibition of the cleavage of D-GpNA relative to that observed for the unsubstituted OU749 core (Compound 3 K_{ii} $75 \pm 2 \mu\text{M}$ and OU749 K_{ii} $73 \pm 9 \mu\text{M}$). Similar to OU749, all of the compounds in Table 3 were uncompetitive inhibitors of the three donor substrates (GSH, D-GpNA and L-GpNA) and competitive inhibitors of GlyGly. The kinetic data for Compound 3 is shown in Fig. 4. Remarkably, shifting the chlorine substituent to the *para* position on the benzosulfonamide ring (Compound 10) virtually abolished the inhibitory potency of the OU749 core structure in the hydrolysis of GSH. This result is in marked contrast to the enhanced GSH inhibition observed when an amine group was substituted at this position (OU749 and Compound 21, Table 1). When chlorine was present at both the *meta* and *para* positions, the *para* chlorine substitution counteracted the enhanced potency gained by a chlorine in the *meta* position (Compounds 3 and 8, Table 3). Addition and relative placement of the chlorines on the sulfobenzene ring (Table 3: Compounds 3, 4, 8 and 10) had the same relative effect on the hydrolysis of the acyl bond in the D-gamma-glutamyl enzyme intermediate (D-GpNA substrate) as on the L-gamma-glutamyl enzyme intermediate (GSH substrate). However, there was a dramatically different effect of the chlorine substitutions on the transpeptidation reaction. All three compounds with chlorines in the *para* position were potent inhibitors of the transpeptidation reaction (L-GpNA and GlyGly), while weak to ineffective inhibitors of the hydrolysis of GSH (Table 3: Compounds 4, 8, 10). Compound 3 is the most potent inhibitor of hGGT among all the compounds in Table 3 and among all of the OU749 analogs we have tested to date

Two compounds containing a bulky, hydrophobic group on the benzosulfonamide ring were also synthesized and evaluated as inhibitors of GSH hydrolysis. A 1- or 2-naphthylsulfonamide ring replaced the benzosulfonamide ring (Table 3). Both compounds inhibited the catabolism of GSH by an uncompetitive mechanism, the same mechanism observed with OU749 and its other analogs (Fig. 4). Compound 6, the 1-naphthylsulfonamide derivative, was 4-fold more potent than OU749 in inhibiting the catabolism of GSH (K_{ii} $96 \pm 2 \mu\text{M}$ and $376 \pm 50 \mu\text{M}$, respectively). Compound 30, a 2-naphthylsulfonamide derivative, was evaluated to determine whether shifting the bulky, hydrophobic group to a more *para* position would decrease the potency, as was observed for the chloro-substituted OU749 analogs. Compound 30 was 2.1-fold less potent than Compound 6 in inhibiting the catabolism of GSH ($206 \pm 37 \mu\text{M}$ vs $96 \pm 2 \mu\text{M}$) (Table 3). Compound 30 was also less potent than Compound 6 in inhibiting both hydrolysis and transpeptidation reactions.

We next evaluated a series of nitro-substituted compounds for their effect on the catabolism of GSH by hGGT. Previously, we observed that this series of OU749 derivatives with a nitro substitution in the *para* position on the benzosulfonamide ring were V-type activators of the hydrolysis of D-GpNA and uncompetitive inhibitors of the L-GpNA transpeptidation reaction of hGGT (Table 4, Compounds 11, 13–19) [18]. V-type activators increase the V_{max} of the reaction. Using the L-Glutamate Release Assay, we discovered that these compounds also accelerated the catabolism of GSH (Table 4). The kinetic analysis of the

activation profiles of these compounds revealed that they are V-type activators of the hydrolysis of GSH, as was observed with D-GpNA. The kinetic data for Compound 14 is shown in Fig. 5. The $V_{\max}$ increases with increasing concentration of Compound 14. Concomitantly, the K_m of GSH increases to a greater extent than the $V_{\max}$. An observed increase in the $V_{\max}$ near saturating concentrations of the donor substrate with inhibition of V/K_m correlates with other V-type activators [30]. None of these compounds activated the transpeptidation reaction, instead they inhibited it (Table 4). If the compounds in Table 4 were accelerating the hydrolysis of GSH by acting as acceptors, a decrease in the rate of glutamate release would be observed in the L-Glutamate Release Assay because glutamate would be converted into a new gamma-glutamyl compound prior to release from the enzyme. The accelerated release of glutamate in the presence of Compound 14 demonstrates that Compound 14 is accelerating the hydrolysis of the acyl-bond and not accelerating the reaction by acting as an acceptor. The potency with which the nitro compounds activate the reaction is similar for both the hydrolysis of GSH and D-GpNA, with only minor discrepancies in the rank order with which they accelerate the reactions (Table 4). These data indicate that the nitro compounds accelerate the hydrolysis of the acyl bond in the F-form of the enzyme with similar potency regardless of whether an L-glutamate or D-glutamate is bound in the F-form of the enzyme. Among the nitro compounds, the only compound that inhibited both the hydrolysis reaction and transpeptidation reaction was Compound 20 (Table 4) [18]. Compound 20 was shown previously to inhibit the hydrolysis of D-GpNA as a competitive inhibitor [18]. In our current analyses, we found that it is also acted as a competitive inhibitor of the catabolism of GSH (data not shown), indicating this compound binds differently than the other OU749 analogs, competing with the donor substrate binding to the free enzyme.

Based on these analyses of hGGT inhibition with a physiological substrate (GSH), we identified three new lead compounds (Compounds 3, 6 and 22) from three distinct classes of OU749 structural derivatives that are more potent inhibitors than OU749. To assess their potential clinical utility, we evaluated the toxicity of our inhibitors towards proliferating cells in culture (Table 5). The glutamine analog, acivicin, is a potent inhibitor of hGGT, but it is too toxic for clinical use [16]. We have reported previously that OU749 is less toxic than acivicin [17]. As shown in Table 5, OU749 is almost 100-fold less toxic than acivicin. Data from the current study reveal that, of the three optimized OU749 analogs evaluated, each was considerably less toxic than OU749. Compound 6, which was almost four times more potent as an inhibitor of hGGT hydrolysis relative to OU749 (K_{ii} of $96 \pm 2 \mu\text{M}$ vs. $376 \pm 50 \mu\text{M}$, respectively), was also more than 16-fold less toxic than OU749, with a LD_{50} of $2090 \pm 20 \mu\text{M}$ (Tables 3 and 5). As a result, this 1-naphthylsulfonamide analog of OU749 is our new lead compound for inhibiting hGGT.

All of the kinetic studies were conducted with soluble purified hGGT. To assess inhibition under *in vivo* conditions, we evaluated the ability of Compound 6 to inhibit membrane-bound hGGT expressed on the surface of the hGGT-positive cell line, 786-O. We measured the rate of cleavage of $20 \mu\text{M}$ GSH by determining the concentration of GSH remaining in the media after one hour in the presence or absence of Compound 6 (Fig. 6). Compound 6 ($100 \mu\text{M}$) blocked the hGGT catabolism of GSH by 51% (Fig. 6). To confirm that the reduced catabolism of GSH was due to inhibition of hGGT, we repeated the experiment using a stably transfected hGGT-positive cell line (NIH3T3^{GGT}) and its GGT-negative parental line (NIH3T3). The control GGT-negative NIH3T3 cells were unable to metabolize extracellular GSH (Fig. 6). Constitutive expression of hGGT in NIH3T3^{GGT} cells resulted in a dramatic increase in the cleavage of extracellular GSH (Fig. 6). Compound 6 reduced the ability of NIH3T3^{GGT} to metabolize GSH in the media by 59%, demonstrating that this new lead inhibitor is capable of efficiently blocking hGGT's enzymatic activity in its cellular context.

DISCUSSION

To develop potent inhibitors of hGGT that can be used clinically, it is essential that compounds be evaluated as inhibitors of the physiological hGGT reaction. This can now be performed with GSH as the substrate in our L-Glutamate Release Assay. GSH is the most abundant substrate of hGGT *in vivo*. The L-Glutamate Release Assay allows for kinetic analysis of compounds as inhibitors of hGGT-mediated GSH catabolism. Most studies of hGGT inhibition have been conducted using the L-GpNA transpeptidation assay with millimolar concentrations of the synthetic gamma-glutamyl donor substrate (L-GpNA) and the acceptor (GlyGly). Screening the analogs of OU749 in this study with the transpeptidation assay would have identified Compounds 2–4 and 8 as new lead inhibitors. Our analysis of their inhibition of the hydrolysis of GSH showed that Compounds 2, 4, and 8 were much less potent than OU749. In fact, Compound 8 had no detectible inhibitory activity towards the hydrolysis of GSH. In contrast, Compounds 3, 6, 21–25 and 30 were more potent inhibitors than OU749 of the physiologic reaction catalyzed by hGGT. These data illustrate that the use of synthetic substrates like D- and L-GpNA can result in the misdirected optimization of weak inhibitors of the physiological hGGT GSH reaction.

Many of the compounds assessed in this study inhibited the catabolism of GSH, some accelerated the reaction and some had no effect. There was no consistent correlation between the effect of a compound on the catabolism of GSH and its effect on the hydrolysis of D-GpNA or on the transpeptidation reaction with L-GpNA as the substrate and GlyGly as the acceptor. There are features of each of the three assays which provided unique insights into the mechanism by which these uncompetitive inhibitors alter the rate of the hGGT reaction. The results presented in this study show that at the physiological concentrations of GSH used in the L-Glutamate Release Assay, the reaction catalyzed by hGGT is the hydrolysis of the gamma-glutamyl bond of GSH. The gamma-glutamyl enzyme intermediate has an L-glutamate in the active site bound by an acyl bond to the oxygen on the side chain of Thr 381 [19]. Free glutamate is detected when it is released as the second product of the reaction. In the assay measuring the hydrolysis of D-GpNA, there is a D-gamma-glutamyl enzyme intermediate. In assays with either L- or D-GpNA as substrates, the release of *p*-NA is the product that is quantifiably measured. Use of L-GpNA and GlyGly as the donor and acceptor substrates, respectively, result in a transpeptidation reaction, with the production of a gamma-glutamyl-acceptor compound as the second product. This assay provides insight into the interaction of the test compound with the acceptor binding site. Therefore, data from a L-GpNA transpeptidation reaction can serve a complementary role with data from a GSH hydrolysis reaction to provide information regarding the relative contribution of the acceptor in accelerating acyl transfer or trapping the acyl-enzyme intermediate by the uncompetitive inhibitor.

In our initial report of the discovery of OU749 as an inhibitor of hGGT, we demonstrated that it acts as an uncompetitive inhibitor of the transpeptidation reaction with L-GpNA as a substrate, binding the F-form of the enzyme and competing with the acceptor GlyGly [17]. With the exception of Compounds 19 and 20, all of the OU749 analogs evaluated in this study showed the same mechanism of inhibition of the transpeptidation reaction.

Our primary goal is to enhance the potency of OU749 as an inhibitor of GSH hydrolysis. OU749 is a weaker inhibitor of hydrolysis of the acyl bond of the gamma-glutamyl enzyme intermediate when GSH is the substrate and an L-gamma-glutamyl group occupies the donor site than when D-GpNA is the substrate and a D-gamma-glutamyl group occupies the active site (OU749, Table 1). In the first set of compounds analyzed, addition of substituents in the *para* position on the sulfobenzene ring showed that an amine group enhanced the potency of OU749 as an inhibitor of GSH hydrolysis, while the addition of bulky, uncharged groups

reduced its potency. Further analysis of modifications to the benzosulfonamide ring (Table 3) showed that chlorine in the *meta* position (Compound 3) is preferential to a chlorine in the *para* (Compound 10), *para* and *ortho* (Compound 4), or *para* and *meta* positions (Compound 8). The chlorine in the *para* position could be eliciting an electrostatic repulsion with a charged amino acid or dipoles within the active site, thereby diminishing the affinity of the OU749 analog and its inhibitory potency. The compounds had the same rank order of potency with which they inhibited GSH and D-GpNA hydrolysis, suggesting that, while substituents in the *para* position do not change the orientation of the compound relative to the acyl bond, they either alter the affinity with which the compounds bind the enzyme or alter the electrophilicity of the sulfonamide group. Han and colleagues synthesized and evaluated a series of gamma-glutamyl phosphono diesters as inhibitors of hGGT [12]. They proposed that the mechanism of inhibition by these compounds included the phosphonate diesters reacting covalently with the catalytic Thr residue of GGT. We propose that the sulfonamide of OU749 and its analogs interact electrostatically with the acyl bond of the gamma-glutamyl-enzyme intermediate. Modifications to the sulfobenzene rings, such as addition of chlorines or a benzyl group (Table 3) may alter the strength of interaction between the sulfonamide and the catalytic nucleophile of hGGT and/or the acyl bond. A homology model of hGGT shows the proposed binding site of OU749 (Fig. 7). There is no crystal structure of any eukaryotic GGT. The model of hGGT is based on crystallographic templates of bacterial GGT and includes glutamate bound in the active site forming an acyl bond with Thr 381. Modeling OU749 into the active site of the gamma-glutamyl enzyme intermediate shows the oxygen of the sulfonamide group of OU749 is 3.1 Å from the oxygen of Thr 381, which forms the acyl bond (Fig. 7). The sulfobenzene ring is located in a hydrophobic pocket formed by the side chains of Phe 540, Leu 402, Tyr 403 and Phe 433. The sulfobenzene ring is in close proximity to Glu 432, which may interact with the *para* amine group stabilizing the position of the analogues with an amine *para* on the sulfobenzene ring. Bulky constituents at this *para* position, including chlorine may alter the affinity with which these *para* substituted analogues bind the enzyme.

In contrast, addition of a benzene group to the sulfobenzene ring not only altered the potency of the compounds but also elicited different effects on the hydrolysis of GSH versus D-GpNA (Table 3). Addition of benzene in the *ortho* position (1-naphthylsulfonamide ring, Compound 6) or in the *meta* position (Compound 30) enhanced the inhibition of GSH hydrolysis yet reduced the inhibition of D-GpNA hydrolysis relative to OU749. These data indicate that there is a difference in the orientation of the acyl bond of the D- or L-glutamyl moiety present in the active site. The model of OU749 binding suggests that additional *ortho* or *meta* benzene rings may interact with the side chains of one or more of the neighboring hydrophobic amino acids (i.e. Phe 540, Tyr 403 and Phe 433), causing a shift in the position of the inhibitor relative to the acyl bond.

Addition of a *para*-nitro on the sulfobenzene ring resulted in a compound that accelerated the hydrolysis reaction but inhibited the transpeptidation reaction (Table 4). In the transpeptidation reaction, GlyGly accelerates acyl transfer of the gamma-glutamyl group to the free amine of GlyGly. Therefore, in the transpeptidation reaction OU749 analogs inhibit the transpeptidation reaction by two mechanisms: blocking GlyGly from the acceptor site and limiting access to the L-gamma-glutamyl acyl bond. One possible explanation for this effect is that the nitro-substituted OU749 analogs bind to the GlyGly site, inhibiting the transpeptidation reaction and destabilizing the acyl-bond to allow a more facile attack by water, thus promoting hydrolysis. We have previously shown, GlyGly increases the L-GpNA cleavage rate by 22-fold [18]. Addition of a *para* nitro OU749 analog increased the D-GpNA cleavage rate by only 5-fold [18]. This difference in the rate of acceleration of hydrolysis of the acyl bond, which is observed with GSH and D-GpNA, is insufficient to offset the decreased rate of cleavage of L-GpNA due to the absence of GlyGly, resulting in

an apparent inhibition of the transpeptidation reaction. All of the inhibitors and the activators are competitive inhibitors of GlyGly in the transpeptidation reaction, indicating that the inhibitors are binding at the acceptor site or at a site that overlaps with the acceptor site, thus blocking GlyGly from binding. The binding site of the acceptors is not known.

Modifications to the distal benzene ring of OU749 decreased the K_{is} up to 11-fold as competitive inhibitors of GlyGly (Compound 29, Table 2). Two of the most potent inhibitors of GlyGly bear chlorines in the *para* position (Compound 28) or the *meta* and *para* positions (Compound 29). However, there was no correlation between the potency of these compounds as competitive inhibitors of GlyGly and their potency of inhibiting the hydrolysis of GSH. We analyzed the effect of modifications to the distal benzene ring of the amine OU749 structural analogs on the inhibition of hydrolysis of GSH. The data revealed that a small neutral (Compound 22 and Compound 24), small positive (electron donating, Compound 23), or small slightly negatively charged group (Compound 21) on the distal benzene ring was favorable for hGGT inhibition, whereas a bulky group (Compounds 26–29) on the distal benzene ring diminished the inhibitory potency of the amine OU749 analogs (Table 2). Our model suggests the presence of a hydrogen bond between Lys 562 and the carboxylate group on the distal benzene ring of OU749 which are separated by a distance of only 3.08 Å (Fig. 7). Hu and colleagues mutated Lys 562 to asparagine or glutamine and found a significant reduction in the hydrolysis and transpeptidation activity of hGGT as well as reduced inhibition by OU749 [31]. Using a model of hGGT based on the crystal structure of *E. coli*, they examined possible binding interactions between GGT and OU749. They also proposed a hydrogen bond between Lys 562 and the carboxylate group on the distal benzene ring of OU749. Our model indicates that the distal benzene ring of OU749 interacts with the side chains of His 81, through pi-stacking on one side and makes a hydrophobic contact with Ile 541 on the other side. Small neutral or small charged groups *para* on the distal benzene ring (Compound 22, Compound 23, and Compound 24) may be interacting with a hydrophilic residue such as Asn 79 thereby stabilizing inhibitor binding. In contrast, bulky groups may shift the orientation of the inhibitor thereby destabilizing its interaction with the acyl bond.

The toxicity of the OU749 analogs was not related to their potency as inhibitors of hGGT activity. Inhibitors of GGT that are glutamine analogs not only inhibit hGGT, but also inhibit other glutamine-dependent enzymes, which leads to the neurotoxicity observed with acivicin [16, 32, 33]. OU749 is not a glutamine analog and has been shown to be less toxic than acivicin [17]. The development of OU749 structural derivatives has led to the discovery of more potent inhibitors that are even less toxic than OU749. Compound 3 was the most potent hGGT inhibitor and was 3-fold less cytotoxic than OU749 with a LD_{50} of 375 μ M (Tables 3 and 6). Compound 6 was the third most potent hGGT inhibitor (approximately four-fold more potent than OU749) and exhibited the least cytotoxicity with a LD_{50} of approximately 2100 μ M (Tables 4 and 6).

The ability to measure inhibition of the extracellular catabolism of GSH by hGGT is crucial for inhibitor optimization, as the physiologic reaction is the hydrolysis of GSH on the cell surface. Our most potent and least cytotoxic OU749 analog, Compound 6, was also found to inhibit hGGT *in vivo*, blocking the cleavage of extracellular GSH. These data validate the approach of identifying inhibitors of the physiologic hGGT GSH hydrolysis reaction using our L-Glutamate Release Assay. The optimization of compounds as potent, non-toxic inhibitors of hGGT will lead to the development of therapeutics for cancer, cardiovascular disease, and pulmonary diseases, such as asthma.

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Abbreviations

GGT	gamma-glutamyl transpeptidase
D/L-GpNA	D/L-gamma-glutamyl <i>para</i> -nitroanilide
GSH	glutathione
GlyGly	glycylglycine

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Fig. 1.

Illustration of the GGT hydrolysis and transpeptidation reactions. The cleavage of GSH is initiated by the nucleophilic attack of the hydroxyl (OH) of the threonine (Thr) on the gamma-glutamyl bond of GSH forming an acyl-bond with the gamma-glutamyl group (γ -glu) and releasing cysteinylglycine (CysGly) of GSH. The acyl-bond can be either (A) hydrolyzed by water releasing glutamate (Glu) in the hydrolysis reaction or (B) transferred a dipeptide acceptor forming a new gamma-glutamyl group in the transpeptidation reaction.

Fig. 2.

Kinetic analysis of the interaction between GSH and other GGT substrates. (A) Double-reciprocal plot of the initial velocity of the hydrolysis of D-GpNA in the presence of 0 (○), 15 μ M (◐), 31.25 μ M (◑), 62.5 μ M (◒), 125 μ M (◔), 250 μ M (◕) GSH showing competitive inhibition between these two gamma-glutamyl donor substrates. (B) Double-reciprocal plot of the initial velocity of the transpeptidation reaction with L-GpNA with 40 mM GlyGly or (C) GlyGly with 3 mM L-GpNA in the presence of 0 (○), 0.3125 mM (◐), 0.6125 mM (◑), 1.25 mM (◒), 2.5 mM (◔), 5 mM (◕) GSH. (D) A Dixon plot of the cleavage of 0.25 mM (○) or 3 mM (◐) L-GpNA versus GSH concentrations (0 to 5 mM). Data for 0.25 mM L-GpNA is biphasic. (E) Double-reciprocal plot of the initial velocity of the cleavage of L-GpNA in the presence of 0 (○), 5 μ M (◐), 10 μ M (◑), 20 μ M (◒), 40 μ M (◔) GSH. Data shown are average triplicate values $\pm$ S.D. For many data points the S.D. is smaller than the symbol.

Fig. 3.

Kinetic analysis of GGT inhibition by Compound 22. (A) Double-reciprocal plot of the initial velocity of the hydrolysis varying GSH or (B) D-GpNA in the presence of 0 (○), 15 mM (◻), 31.25 μ M (◼), 62.5 μ M (◾), 125 μ M (◿), 250 μ M (●) Compound 22. (C) Double reciprocal plot of the initial velocity of the transpeptidation reaction with varying concentrations of L-GpNA and 40 mM GlyGly or (D) varying concentrations of GlyGly with 3 mM L-GpNA in the presence of 0 (○), 15 mM (◻), 31.25 μ M (◼), 62.5 μ M (◾), 125 μ M (◿), 250 μ M (●) Compound 22. Data shown are average triplicate values $\pm$ S.D. For many data points the S.D. is smaller than the symbol.

Fig. 4. Kinetic analysis of GGT inhibition by Compound 6 and Compound 3. (A) Double-reciprocal plot of the initial velocity of the hydrolysis of GSH in the presence of 0 (○), 15 mM (□), 31.25 μ M (△), 62.5 μ M (◇), 125 μ M (▽), 250 μ M (●) Compound 3 or (B) Compound 6. Data shown are average triplicate values $\pm$ S.D. For many data points the S.D. is smaller than the symbol.

Fig. 5.

Kinetic analysis of GGT activation of GSH hydrolysis by Compound 14. Initial velocity (V) vs Compound 14 concentration (closed symbols) and V/K_m of GSH vs inhibitor concentration (open symbols) plots illustrate that Compound 14 activates GSH hydrolysis as a V-type activator. Data shown are average triplicate values $\pm$ S.D.

Fig. 6.

Compound 6 (Cmpd 6) inhibits the cleavage of GSH by GGT. GGT-positive 786-O cells (black bars), NIH3T3 cells transfected with human GGT (open bars), and GGT-negative NIH3T3 cells (checkered bars) were incubated in media with 20 μ M GSH in the presence or absence 100 μ M Compound 6 for one hour. Cleavage of extracellular GSH by GGT, which is expressed on the surface of the cells, was inhibited by Compound 6. Control was media incubated in the absence of cells. Data shown are average duplicate values $\pm$ S.D. * $p < 0.01$ between Compound 6 treated and untreated cells.

Fig. 7.

Homology model of human GGT showing the proposed binding site of OU749 in the gamma-glutamyl-enzyme intermediate (stereo image). The L-gamma-glutamyl group of the substrate bound in the active site is shown with dot lines. Atoms of OU749 are colored: carbon (gold), sulfur (green), oxygen (red), nitrogen (blue). Select amino acids within the peptide backbone of hGGT are shown as stick figures: T381 (pink), K562 (pale green) and all others (blue). Elements of the secondary structure of the peptide backbone are shown in grey.

Table 1
Inhibition of GGT by OU749 analogs with *para*-substitutions on the benzosulfonamide ring.

Compound #	Substitution	GSH K_{ii} (μ M)	D-GpNA K_{ii} (μ M)	L-GpNA with GlyGly K_{ii} (μ M)	GlyGly with L-GpNA K_{is} (μ M)
21	X = NH ₂	105 $\pm$ 14 ^a	31 $\pm$ 1	36 $\pm$ 1	10 $\pm$ 1
OU749	None	376 $\pm$ 50	73 $\pm$ 9 ^b	68 $\pm$ 9 ^b	54 $\pm$ 4 ^b
2	X = CH ₃	506 $\pm$ 75	73 $\pm$ 4 ^b	23 $\pm$ 1 ^b	10 $\pm$ 0.4 ^b
7	X = C(CH ₃) ₃	NA ^c	342 $\pm$ 6 ^b	150 $\pm$ 13 ^b	143 $\pm$ 9 ^b
9	X = OCH ₃	NA ^c	3800 $\pm$ 729 ^b	149 $\pm$ 23 ^b	133 $\pm$ 10 ^b

^aValues $\pm$ S.E.
^bValues in italics were reported in Wickham et al. [18]. They are included in the table for direct comparison of K_{ii} with GSH to K_{ii} and K_{is} with other substrates.
^cNo GGT inhibition was observed up to 250 μ M inhibitor.

Table 2

Inhibition of human GGT by Compound 21 analogs.

Compound #	Substitution	GSH K _i (μM)	D-GpNA K _i (μM)	L-GpNA with GlyGly K _i (μM)	GlyGly with L-GpNA K _i (μM)
22	Y = CH ₃	82 ± 13 ^a	31 ± 1	30 ± 4	6 ± 1
23	Y = NH ₂	102 ± 16	29 ± 3	181 ± 20	15 ± 1
24	Z = CH ₃	121 ± 5	109 ± 4	129 ± 17	30 ± 5
25	None	242 ± 6	58 ± 6	74 ± 4	42 ± 2
26	Y = F	355 ± 16	24 ± 2	58 ± 4	47 ± 7
27	Y = N(CH ₃) ₂	363 ± 35	180 ± 5	275 ± 12	24 ± 4
28	Y = Cl	419 ± 27	56 ± 0	54 ± 1	7 ± 1
29	Y = Cl Z = Cl	905 ± 53	32 ± 1	34 ± 4	5 ± 1

^aValues ± S.E.

Table 3Inhibition of hGGT by *meta* and *ortho* substituted analogs of OU749.

Compound #	Substitution	GSH K_{ii} (μ M)	D-GpNA K_{ii} (μ M) ^a	L-GpNA with GlyGly K_{ii} (μ M) ^a	GlyGly with L-GpNA K_{is} (μ M) ^a
3	W = Cl	67 ± 6^b	75 ± 2	22 ± 1	12 ± 1
6	WV = Benzene	96 ± 2	161 ± 5	75 ± 4	46 ± 3
30	WX = Benzene	206 ± 37	415 ± 50	364 ± 43	143 ± 20
4	X = Cl; V = Cl	950 ± 242	76 ± 7	16 ± 1	4 ± 1
8	X = Cl; W = Cl	NAC ^c	3160 ± 90	18 ± 1	11 ± 1
10	X = Cl	NAC ^c	5530 ± 1460	33 ± 1	13 ± 1

^aValues in italics were reported in Wickham et al. [18]. The values are included here for direct comparison of K_{ii} with GSH to K_{ii} and K_{is} with other substrates.^bValues $\pm$ S.E.^cNo GGT inhibition was observed up to 250 μ M inhibitor.

Table 4

Activation of GGT Hydrolysis by Compound 11 Analogs.

Compound #	Substitution	GSH K_{Act} (μ M)	D-GpNA K_{Act} (μ M) ^a	L-GpNA with GlyGly K_{ii} (μ M) ^a	GlyGly with L-GpNA K_{is} (μ M) ^a
14	Y = NO ₂	63 ± 8 ^b	52 ± 1	133 ± 6	81 ± 9
13	None	61 ± 8	54 ± 3	112 ± 6	60 ± 4
15	Z = Cl	46 ± 8	39 ± 1	119 ± 7	44 ± 8
11	Y = OCH ₃	41 ± 3	37 ± 6	93 ± 4	96 ± 8
16	Y = Cl	34 ± 4	31 ± 2	62 ± 2	44 ± 3
18	Z = CH ₃	31 ± 7	25 ± 1	122 ± 9	34 ± 1
17	Y = CH ₃	28 ± 7	25 ± 8	113 ± 5	57 ± 8
19	Y = F	28 ± 2	22 ± 2	Slow Onset	32 ± 1

Compound #	Substitution	GSH (μ M) K_{ii}	D-GpNA K_{ii} (μ M) ^a	L-GpNA with GlyGly K_{ii} (μ M) ^a	GlyGly with L-GpNA K_{is} (μ M) ^a
20 ^c	Y = Cl; Z = Cl	859 ± 83	78 ± 2	Slow Onset	10 ± 1

^aValues in italics were reported in Wickham et al. [18]. They are included in the table for direct comparison of K_{ACT} with GSH to K_{ACT}, K_{ii} and K_{is} with other substrates.

^bValues ± S.E.

^cCompound 20 is a competitive inhibitor of GGT with regard to both the gamma-glutamyl substrate (GSH or D/L-GpNA) and the acceptor GlyGly.

Table 5

Toxicity of OU749 analogs toward dividing 786-O cells.

Compound	LD ₅₀ μ M
Acivicin	1.3 $\pm$ 1.4 ^a
OU749	128 $\pm$ 0.04
Compound 3	382 $\pm$ 0.04
Compound 22	727 $\pm$ 0.02
Compound 6	2090 $\pm$ 20

^aValues are LD₅₀ $\pm$ S.E.