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## Divergent Effects of Compounds on the Hydrolysis and Transpeptidation Reactions of Gamma-Glutamyl Transpeptidase

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### Abstract

A novel class of inhibitors of the enzyme gamma-glutamyl transpeptidase (GGT) were evaluated. OU749 was shown previously to be an uncompetitive inhibitor of the GGT transpeptidation reaction. The data in this study show that it is an equally potent uncompetitive inhibitor of the hydrolysis reaction, the primary reaction catalyzed by GGT *in vivo*. A series of structural analogs of OU749 were evaluated. For many of the analogs, the potency of the inhibition differed between the hydrolysis and transpeptidation reactions, providing insight into the malleability of the active site of the enzyme. Analogs with electron withdrawing groups on the benzosulfonamide ring, accelerated the hydrolysis reaction but inhibited the transpeptidation reaction by competing with a dipeptide acceptor. Several of the OU749 analogs inhibited the transpeptidation reaction by slow onset kinetics, similar to acivicin. Further development of inhibitors of the GGT hydrolysis reaction is necessary to provide new therapeutic compounds.

### Keywords

Gamma-glutamyl substrates; OU749; Enzyme; Inhibition

### Introduction

Gamma-glutamyl transpeptidase (GGT) is a cell surface enzyme that catalyzes the cleavage of the  $\gamma$ -glutamyl bond of glutathione (GSH) and other  $\gamma$ -glutamyl compounds (1). In humans, the expression of GGT is restricted predominantly to the apical surface of ducts and glands where fluids leave the body (2). The highest concentration of GGT is on the apical surface of the proximal tubule cells in the kidney where it prevents excretion of GSH into the urine by cleaving GSH present in the glomerular filtrate (2-3). Aberrant expression and localization of GGT is observed in many disease states including cancer (4). Inhibiting GGT would sensitize tumors to chemotherapy and may be therapeutic in other diseases (5-8). However, GGT inhibitors that have been evaluated clinically are glutamine analogs and are neurotoxic (9-13). We have previously reported the discovery of a novel inhibitor of GGT, OU749, which is not a glutamine analog (14).

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GGT can catalyze hydrolysis reaction or a transpeptidation reaction. The first steps in both reactions are the cleavage of the  $\gamma$ -glutamyl bond of the substrate in the enzyme-substrate complex (ES) and the formation of a transient enzyme-glutamyl substrate complex (F-form of the enzyme). As the substrate is cleaved, the  $\gamma$ -glutamyl group forms a transient acyl bond with the enzyme, and the remainder of the substrate is released (Fig. 1) (15-17). In human GGT, the acyl bond forms between the  $\gamma$ -carbon of the  $\gamma$ -glutamyl substrate and the hydroxyl ( $\beta$ -oxygen) on the side chain of Thr-381 (18). In the hydrolysis reaction, water hydrolyzes the acyl bond between the  $\gamma$ -glutamyl group and the nucleophilic residue releasing both glutamate and the enzyme (19-20). In the transpeptidation reaction, the  $\gamma$ -glutamyl group is transferred to the amine of an acceptor, thereby forming a new  $\gamma$ -glutamyl compound (21). The transpeptidation reaction occurs by a modified ping-pong mechanism (16, 22). The pH and amino acid concentrations in extracellular fluids where GGT is localized favor the hydrolysis reaction, and previous studies have indicated that the hydrolysis reaction is the predominant reaction catalyzed by GGT *in vivo* (22-23).

We previously identified OU749, a novel inhibitor of the GGT transpeptidation reaction (14). Studies of its inhibition revealed that it is uncompetitive against L- $\gamma$ -glutamyl paranitroanalide (L-GpNA) in the transpeptidation reaction, binding the enzyme-glutamyl substrate complex (F-form) and competing with the acceptor (14). Our hypothesis is that OU749 and its structural analogs inhibit both the hydrolysis and transpeptidation reactions catalyzed by GGT. In this study, we performed in depth kinetic analyses of the inhibition of both reactions by OU749 and a series of new structural analogs. We evaluated the potency with which the compounds inhibited the reactions and the mechanisms of inhibition. These data were compared to the inhibition by the glutamine analog, acivicin, a slow binding inhibitor with a slow rate of release. Our studies of both the hydrolysis and transpeptidation reactions have been conducted at physiologic pH. The standard GGT assay, used by other investigators, is conducted at pH 8.0 or higher, which may alter the physiologic cleavage mechanism due to decreased protonation of the amino acid side chains within the active site (24). In addition, in the transpeptidation reaction, the presence of high concentrations of acceptor may induce conformational changes in the enzyme similar to the effects of hippurate (24). The data from this study provide insights into the essential features of both the acceptors and inhibitors of the GGT reaction.

## Methods

### Inhibitors

OU749 was purchased from ChemBridge Corp (San Diego, CA). Sodium benzenesulfonamide was purchased from Sigma (St. Louis, MO).

### Synthesis of Compounds 2-20 (refer to Tables 2 and 3 for structures)

**Synthesis of synthetic intermediates TDA1-10 (Fig. 2A)**—Two mmol of the appropriate phenyl acetic acid and 2 mmol of thiosemicarbazide were dissolved in 1mL of POCl<sub>3</sub> and refluxed for 45 minutes. The reaction was then cooled to room temperature, and 3mL of water were added carefully. The solution was then refluxed for 4 hrs. 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. The characteristics of each intermediate is as follows: **TDA-1:** 283mg (64%), off-white crystals, mp 195-197°C; <sup>1</sup>H NMR (300 MHz, DMSO)  $\delta$  3.81 (s, 3H), 4.19 (s, 2H), 5.01 (br s, 2H), 6.88 (d, 2H, 8.4), 7.22 (d, 2H, 8.4). MS(*m/z*) = 244.05 [M+Na]<sup>+</sup>; **TDA-2:** 203mg (53%), off-white crystals, mp 182-184°C; <sup>1</sup>H NMR (300 MHz, DMSO)  $\delta$  4.14 (s, 2H), 7.04 (s, 2H), 7.31 (m, 5H). MS(*m/z*) = 214.04 [M+Na]<sup>+</sup>; **TDA-3:** 317mg (61%), off-white crystals, mp 182°C; <sup>1</sup>H NMR (300 MHz, DMSO)  $\delta$  4.19 (s, 2H),

7.10 (s, 2H), 7.28 (d, 1H, 8.1), 7.58 (s, 1H), 7.60 (d, 1H, 8.1). MS( $m/z$ ) = 281.96 [M+Na]<sup>+</sup>; **TDA-4**: 307mg (68%), off-white crystals, mp 196-198°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.16 (s, 2H), 7.06 (s, 2H), 7.30 (d, 2H, 8.1), 7.39 (d, 2H, 8.1). MS( $m/z$ ) = 248.00 [M+Na]<sup>+</sup>; **TDA-5**: 205mg (50%), off-white crystals, mp 204-206°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 2.27 (s, 3H), 4.09 (s, 2H), 7.02 (s, 2H), 7.14 (s, 4H). MS( $m/z$ ) = 228.06 [M+Na]<sup>+</sup>; **TDA-6**: 217mg (52%), off-white crystals, mp 206-208°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.15 (s, 2H), 7.04 (s, 2H), 7.16 (t, 2H, 8.4) 7.32 (t, 2H, 8.4). MS( $m/z$ ) = 232.03 [M+Na]<sup>+</sup>; **TDA-7**: 368mg (78%), off-white crystals, mp 187°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.34 (s, 2H), 7.12 (s, 2H), 7.57 (d, 2H, 8.7), 8.20 (d, 2H, 8.7). MS( $m/z$ ) = 259.03 [M+Na]<sup>+</sup>; **TDA-8**: 216mg (48%), off-white crystals, mp 193-195°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.18 (s, 2H), 7.07 (s, 2H), 7.25 (d, 1H, 7) 7.34 (m, 3H). MS( $m/z$ ) = 248.00 [M+Na]<sup>+</sup>; **TDA-9**: 194mg (41%), off-white crystals, mp 185-187°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 2.86 (s, 6H), 3.99 (s, 2H), 6.68 (d, 2H, 8.4), 6.97 (s, 2H), 7.07 (2, 2H, 8.4). MS( $m/z$ ) = 257.08 [M+Na]<sup>+</sup>; **TDA-10**: 207mg (50%), off-white crystals, mp 194-196°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 2.26 (s, 3H), 4.10 (s, 2H), 7.02 (s, 2H), 7.06 (m, 3H), 7.21 (t, 1H, 7.5). MS( $m/z$ ) = 228.05 [M+Na]<sup>+</sup>.

**Synthesis of Compounds 2-4 and Compounds 6-20 (Fig. 2B)**—One mmol of the appropriate thiodiazole compound was dissolved in 1 mL of pyridine and cooled to 0°C. The appropriate benzenesulfonyl chloride (1.1 mmol) was then dissolved in 0.5 mL of pyridine and added to the reaction mixture. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was diluted with 10 mL of water and acidified with dilute HCl. The solid was then filtered and washed with water. The product was recrystallized from ethanol. The characteristics of each OU749 analogs is as follows: **OU749**: 153mg (42%), off-white crystals, mp 122-124°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 3.75 (s, 3H), 4.12 (s, 2H), 6.93 (d,  $J$  = 8.6 Hz, 2H), 7.25 (d,  $J$  = 8.5 Hz, 2H), 7.64 – 7.45 (m, 3H), 7.76 (dd,  $J$  = 6.9, 1.5 Hz, 2H), 14.06 (s, 1H). MS( $m/z$ ) = 384.04 [M+Na]<sup>+</sup>; **Compound 2**: 259mg (69%), off-white crystals, mp 143-145°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 2.41 (s, 3H), 3.84 (s, 3H), 4.07 (s, 2H), 6.91 (d,  $J$  = 8.8 Hz, 2H), 7.19 (d,  $J$  = 8.8 Hz, 2H), 7.25 (d,  $J$  = 7.9 Hz, 2H), 7.79 (d,  $J$  = 8.3 Hz, 2H), 11.57 (s, 1H). MS( $m/z$ ) = 398.06 [M+Na]<sup>+</sup>; **Compound 10**: 155mg (39%), off-white crystals, mp 124-126°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.82 (s, 3H), 4.05 (s, 2H), 6.90 (d,  $J$  = 8.6 Hz, 2H), 7.17 (d,  $J$  = 8.4 Hz, 2H), 7.41 (d,  $J$  = 8.4 Hz, 2H), 7.81 (d,  $J$  = 8.4 Hz, 2H), 11.46 (s, 1H). MS( $m/z$ ) = 418.01 [M+Na]<sup>+</sup>; **Compound 8**: 208mg (48%), off-white crystals, mp 135-137°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 3.75 (s, 3H), 4.14 (s, 2H), 6.92 (d,  $J$  = 8.5 Hz, 2H), 7.25 (d,  $J$  = 8.5 Hz, 2H), 7.71 (dd,  $J$  = 8.4, 2.1 Hz, 1H), 7.82 (d,  $J$  = 8.4 Hz, 1H), 7.91 (d,  $J$  = 2.1 Hz, 1H), 14.25 (s, 1H). MS( $m/z$ ) = 451.97 [M+Na]<sup>+</sup>; **Compound 9**: 178mg (45%), off-white crystals, mp 168-170°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.82 (s, 3H), 3.84 (s, 3H), 4.04 (s, 2H), 6.89 (d,  $J$  = 6.9 Hz, 2H), 6.92 (d,  $J$  = 7.2 Hz, 2H), 7.17 (d,  $J$  = 8.5 Hz, 2H), 7.82 (d,  $J$  = 8.8 Hz, 2H), 11.00 (s, 1H). MS( $m/z$ ) = 414.06 [M+Na]<sup>+</sup>; **Compound 6**: 27mg (7%), off-white crystals, mp 170°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.81 (s, 3H), 4.01 (s, 2H), 6.88 (d,  $J$  = 8.6 Hz, 2H), 7.15 (d,  $J$  = 8.6 Hz, 2H), 7.48 (t,  $J$  = 7.8 Hz, 1H), 7.51 - 7.60 (m, 2H), 7.93 – 7.80 (m, 1H), 8.01 (d,  $J$  = 8.2 Hz, 1H), 8.24 (d,  $J$  = 7.3 Hz, 1H), 8.80 – 8.60 (m, 1H), 10.60 (s, 1H). MS( $m/z$ ) = 434.06 [M+Na]<sup>+</sup>; **Compound 3**: 185mg (47%), off-white crystals, mp 134-136°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.83 (s, 3H), 4.09 (s, 2H), 6.91 (d,  $J$  = 8.5 Hz, 2H), 7.19 (d,  $J$  = 8.5 Hz, 2H), 7.39 (t,  $J$  = 7.8 Hz, 1H), 7.51 (d,  $J$  = 7.8 Hz, 1H), 7.78 (d,  $J$  = 7.8 Hz, 1H), 7.91 (s, 1H). MS( $m/z$ ) = 418.01 [M+Na]<sup>+</sup>; **Compound 4**: 113mg (26%), off-white crystals, mp 104-106; <sup>1</sup>H NMR (300 MHz, DMSO) δ 3.74 (s, 3H), 4.13 (s, 2H), 6.92 (d,  $J$  = 8.4 Hz, 2H), 7.24 (d,  $J$  = 8.5 Hz, 2H), 7.60 (dd,  $J$  = 8.3, 2.3 Hz, 1H), 7.82 (d,  $J$  = 2.1 Hz, 1H), 7.99 (d,  $J$  = 8.4 Hz, 1H), 14.27 (s, 1H). MS( $m/z$ ) = 451.97 [M+Na]<sup>+</sup>; **Compound 11**: 214mg (53%), off-white crystals, mp 187-190°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 3.74 (s, 3H), 4.14 (s, 2H), 6.92 (d,  $J$  = 8.7 Hz, 2H), 7.25 (d,  $J$  = 8.7 Hz, 2H), 8.00 (d,  $J$  = 9.0 Hz, 2H), 8.35 (d,  $J$  = 9.0 Hz, 2H), 14.34 (s,

1H). MS( $m/z$ ) = 429.03 [M+Na]<sup>+</sup>; **Compound 7**: 110mg (26%), off-white crystals, mp 178-179°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>) (δ, ppm; J, hertz) 1.27 (s, 9H), 3.75 (s, 3H), 4.12 (s, 2H), 6.93 (d, 2H, 8.7), 7.25 (d, 2H, 8.7), 7.56 (d, 2H, 8.4), 7.67 (d, 2H, 8.4). MS ( $m/z$ ) = 440.15 [M+Na]<sup>+</sup>; **Compound 12**: 254mg (59%), off-white crystals, mp 169-170°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>) (δ, ppm; J, hertz) 3.75 (s, 3H), 4.14 (s, 2H), 6.93 (d, 2H, 8.7), 7.25 (d, 2H, 8.7), 7.93 (d, 2H, 8.4), 7.97 (d, 2H, 8.4). MS ( $m/z$ ) = 452.03 [M+Na]<sup>+</sup>; **Compound 13**: 223mg (59%), off-white crystals, mp 198-200°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.23 (s, 2H), 7.52 – 7.24 (m, 5H), 8.01 (d,  $J$  = 8.7 Hz, 2H), 8.36 (d,  $J$  = 8.7 Hz, 2H), 14.36 (s, 1H). MS( $m/z$ ) = 399.02 [M+Na]<sup>+</sup>; **Compound 20**: 219mg (49%), off-white crystals, mp 199-201°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.26 (s, 2H), 7.35 (dd,  $J$  = 8.1, 1.7 Hz, 1H), 7.63 (d,  $J$  = 8.2 Hz, 1H), 7.66 (d,  $J$  = 1.5 Hz, 1H), 8.03 (d,  $J$  = 8.9 Hz, 2H), 8.36 (d,  $J$  = 8.8 Hz, 2H), 14.41 (s, 1H). MS( $m/z$ ) = 466.94 [M+Na]<sup>+</sup>; **Compound 16**: 201mg (49%), off-white crystals, mp 156-158°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.24 (s, 2H), 7.37 (d,  $J$  = 8.4 Hz, 2H), 7.43 (d,  $J$  = 8.4 Hz, 2H), 8.02 (d,  $J$  = 8.9 Hz, 2H), 8.36 (d,  $J$  = 8.8 Hz, 2H), 13.86 (s, 1H). MS( $m/z$ ) = 432.98 [M+Na]<sup>+</sup>; **Compound 17**: 256mg (66%), off-white crystals, mp 207-209°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 2.29 (s, 3H), 4.16 (s, 2H), 7.17 (d,  $J$  = 8.4 Hz, 2H), 7.22 (d,  $J$  = 8.2 Hz, 2H), 8.00 (d,  $J$  = 8.6 Hz, 2H), 8.35 (d,  $J$  = 8.5 Hz, 2H), 14.34 (s, 1H). MS( $m/z$ ) = 413.04 [M+Na]<sup>+</sup>; **Compound 19**: 215mg (55%), off-white crystals, mp 189-192°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.22 (s, 2H), 7.19 (t,  $J$  = 8.8 Hz, 2H), 7.47 – 7.33 (m, 2H), 8.01 (d,  $J$  = 8.8 Hz, 2H), 8.35 (d,  $J$  = 8.8 Hz, 2H), 14.37 (s, 1H). MS( $m/z$ ) = 417.01 [M+Na]<sup>+</sup>; **Compound 14**: 153mg (36%), off-white crystals, mp 180-183°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.42 (s, 2H), 7.63 (d,  $J$  = 8.7 Hz, 2H), 8.02 (d,  $J$  = 9.0 Hz, 2H), 8.23 (d,  $J$  = 8.8 Hz, 2H), 8.36 (d,  $J$  = 9.0 Hz, 2H), 14.55 (s, 1H). MS( $m/z$ ) = 422.02 [M+H]<sup>+</sup>; **Compound 15**: 194mg (47%), off-white crystals, mp 185-188°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 4.26 (s, 2H), 7.34 – 7.28 (m, 1H), 7.39 (dd,  $J$  = 5.4, 2.9 Hz, 2H), 7.45 (t,  $J$  = 1.9 Hz, 1H), 8.02 (d,  $J$  = 8.8 Hz, 2H), 8.36 (d,  $J$  = 8.8 Hz, 2H), 14.41 (s, 1H). MS( $m/z$ ) = 432.98 [M+Na]<sup>+</sup>; **Compound 18**: 211mg (54%), off-white crystals, mp 178-180°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 2.30 (s, 3H), 4.18 (s, 2H), 7.12 (d,  $J$  = 7.9 Hz, 3H), 7.26 (t,  $J$  = 7.6 Hz, 1H), 8.01 (d,  $J$  = 8.8 Hz, 2H), 8.35 (d,  $J$  = 8.8 Hz, 2H), 14.34 (s, 1H). MS( $m/z$ ) = 413.04 [M+Na]<sup>+</sup>.

**Synthesis of Compound 5 (Fig. 2C)**—Compound 11 (130 mg, 0.32 mmol) and 10%Pd/C (15.4 mg) were dissolved in EtOAc (7 mL). The reaction was degassed 5 times and stirred at RT for 24 hr under H<sub>2</sub>. The reaction was filtered through celite and concentrated *in vacuo*. Product was recrystallized from EtOAc and Hexanes. The characteristics of **Compound 5** are: 112 mg (89%), off-white crystals, mp 167-168°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 3.74 (s, 3H), 4.10 (s, 2H), 6.83 (d,  $J$  = 8.8 Hz, 2H), 6.92 (d,  $J$  = 8.6 Hz, 2H), 7.24 (d,  $J$  = 8.6 Hz, 2H), 7.52 (d,  $J$  = 8.7 Hz, 2H), 8.65 (s, 1H), 8.93 (s, 1H), 13.87 (s, 1H). MS( $m/z$ ) = 415.05 [M+Na]<sup>+</sup>.

## Enzyme Isolation

Human GGT1 (P19440), lacking the transmembrane domain, was expressed in *Pichia pastoris* and isolated as previously described (14). The specific activity of the purified enzyme was 406.5 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.

## Hydrolysis Reaction

The assay buffer contained: 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.2 mM KCl, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, and 27.5 mM NaCl pH 7.4. The concentration of the D-GpNA (Bachem, Torrance, CA) substrate was varied from 0.25 mM to 3 mM D-GpNA. The reaction was initiated with the addition of 19 mU GGT. The reaction was incubated at 37°C and monitored continuously at 405 nm by a

Bio-Rad model 680 microplate reader with Microplate Manager 5.2 software (Bio-Rad, Hercules, CA).

### Transpeptidation Reaction

The same assay buffer was used for both the hydrolysis reaction and the transpeptidation reaction. The transpeptidation reaction included 40 mM glycylglycine (glygly, Sigma, St. Louis, MO) as the acceptor. The concentration of the substrate for the transpeptidation reaction, LGpNA (Sigma) was varied from 0.25 mM to 3 mM. The concentration of L-GpNA was 3mM for experiments in which the concentration of glygly was varied. To initiate the transpeptidation reaction, 4 mU GGT were added. The reaction was incubated at 37°C and monitored continuously at 405 nm.

### Data Analysis

Samples in each experiment 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 (BIOSOFT, Cambridge, UK). Kinetic parameters with standard errors were estimated using a simple weighting method.

Data adhering to Michaelis-Menten Kinetics were fitted to eq. 1. Data for initial rate patterns with D-, L-GpNA varied at different fixed levels of glygly were fitted to eq. 2, which describes a ping pong kinetic mechanism. Data for competitive and uncompetitive inhibition were fitted to eqs. 3 and 4. Data for the dependence of  $V_{max}$  and  $V_{max}/K_{D-GpNA}$  on the concentration of activator were fitted using eq. 5 (25).

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

$$v = \frac{V_{max}AB}{K_aB + K_bA + AB} \quad (2)$$

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

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

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

In eqs 1-5,  $v$  and  $V_{max}$  are the initial and maximum rates, respectively,  $K_a$  and  $K_b$  are Michaelis constants for substrates A and B, respectively, 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 (25).

For irreversible inhibition studies, time courses were fit to eq. 6.

$$P=A(1-e^{-kt}) \quad (6)$$

In eq. 6,  $A$  is the burst amplitude,  $t$  is time, and  $k$  is the first order rate constant for formation of the inactivated enzyme. Graphs, averages, and standard error between experiments were calculated using Prism GraphPad Software (San Diego, CA).

## Results

### Initial Rate Studies with D- and L-GpNA

In this study, we examined the inhibition of both the GGT hydrolysis and transpeptidation reactions by OU749 and a new series of structural analogs. The hydrolysis and transpeptidation reactions were analyzed separately by using different stereo-isomers of the  $\gamma$ -glutamyl-*p*-nitroanilide (GpNA) substrate. We first characterized the kinetics of both reactions in the absence of inhibitors. Cleavage of the  $\gamma$ -glutamyl bond of either D- or L-GpNA releases *p*-nitroaniline, which has an  $\epsilon_{405}$  of  $7,680 \text{ M}^{-1}\text{cm}^{-1}$ , allowing for the reactions to be monitored continuously with high sensitivity. The D-glutamyl isomer of GpNA was used as the substrate for the hydrolysis reaction, in which water serves as the acceptor. The L-glutamyl isomer of GpNA, with glycylglycine (glygly) as an acceptor, was used for the transpeptidation reaction. LGpNA can serve as both a substrate and a low affinity acceptor, while D-GpNA is unable to serve as an acceptor (18, 21, 26). Therefore, in the absence of an added acceptor GGT exclusively catalyzes a hydrolysis reaction with D-GpNA as the substrate. When L-GpNA is used as a substrate, the GGT reaction is a mixture of both the hydrolysis and transpeptidation reactions. Under these conditions, the rate of the reaction is the sum of hydrolysis and transpeptidation reactions, with the hydrolysis reaction contributing more at low L-GpNA concentrations and transpeptidation dominating at saturating substrate concentrations. For the hydrolysis reaction, the second order rate constants,  $V/K_{GpNA}$ , were similar for D-GpNA and LGpNA ( $13.2 \pm 0.1$  vs  $7.8 \pm 0.2 \text{ min}^{-1}\text{nM}^{-1}$ ), indicating that, at limiting substrate concentrations, the rate of the hydrolysis reaction is similar with D-GpNA and L-GpNA (Table 1). However, the maximum rate ( $V_{max}$ ) obtained with L-GpNA was approximately 3-times faster than that measured with D-GpNA, and the  $K_{GpNA}$  for L-GpNA was 5-times higher than that measured for D-GpNA. This is at least partially due to the ability of L-GpNA to serve as an acceptor, resulting in a transpeptidation reaction, which is faster than the hydrolysis reaction. Therefore, to study the hydrolysis reaction in isolation, we used D-GpNA as the substrate without any added acceptor.

Characterization of the transpeptidation reaction with both D- and L-GpNA showed that addition of the acceptor, glygly, accelerates the reaction with both substrates (Table 1). In the presence of glygly, the kinetic mechanism is ping-pong with D-GpNA or L-GpNA as the donor and glygly acting as an acceptor (21, 27). In both cases, data fit well to the rate equation for a ping-pong mechanism, eq. 2, as demonstrated by the parallel lines in the double reciprocal plots (Fig. 3A and B). The measured  $V_{max}$  for D- and L-GpNA reactions increased in the presence of glygly by 25- and 22-fold, respectively (Table 1). However, some notable differences were observed in the kinetics of the transpeptidation reaction with D- versus L-GpNA. The ratio of the maximum rates of the two reactions was almost 3:1 in favor of L-GpNA over D-GpNA in the presence of glygly ( $141 \pm 11$  vs  $53 \pm 11 \text{ mM}/\text{min}\cdot\text{nM}$ ). While there was no change in the  $V/K$  for D-GpNA in the presence of an acceptor, the  $V/K$  for L-GpNA increased by about 15-fold in the presence of glygly. Insights into the mechanism of the reaction with L-GpNA provided by these data are included in the

discussion. To evaluate the affect of the inhibitors on the transpeptidation reaction, we used the physiological L-isomer as a substrate with glygly as the acceptor.

### Inhibition of the GGT hydrolysis and transpeptidation reactions by OU749

OU749 (Fig. 4A) is an uncompetitive inhibitor of GGT transpeptidation (14). To determine the mechanism by which OU749 inhibits the hydrolysis reaction, the rate of the hydrolysis reaction was determined as a function of D-GpNA concentration and different concentrations of OU749, including zero. Double reciprocal plots of the data gave rise to a series of parallel lines (Fig. 4B). This pattern is indicative of uncompetitive inhibition and binding of OU749 to the covalent E- $\gamma$ -glutamyl complex (the F-form of the enzyme). This is the same mode of inhibition observed previously for OU749 versus L-GpNA with glygly as an acceptor (14). A  $K_{ij}$  of 73  $\mu$ M was obtained for OU749 in the hydrolysis reaction, which is similar to the  $K_{ij}$  of 68  $\mu$ M that was obtained in the transpeptidation reaction with the L-GpNA substrate plus acceptor glygly (Table 2). These values are comparable to the  $K_{ij}$  value of 73  $\mu$ M for OU749 measured previously in the transpeptidation reaction (14). Maintaining the L-GpNA substrate concentration at 3 mM while varying the concentration of the acceptor, glygly, gave rise to a competitive inhibition profile with a  $K_{is}$  of 54  $\mu$ M, confirming previously reported data with glygly as an acceptor (14). Thus, OU749 inhibits GGT by binding to the acceptor site.

### OU749 substructure inhibition of GGT

Sodium benzosulfonamide (SBS, Fig. 4C) is a substructural component of OU749. The effect of SBS on both the hydrolysis and transpeptidation reactions was evaluated. The data show that SBS is an uncompetitive inhibitor vs. D-GpNA in the hydrolysis reaction (Fig. 4D). This is the same mechanism observed for OU749 (Fig. 4B). However, in the hydrolysis reaction, SBS was more than 2,000-fold less effective than OU749 with a  $K_{ij}$  of  $160 \pm 81$  mM, (Table 2). These data suggest that, although the SBS moiety provides some of the binding energy of OU749, it is a minor contributor. SBS also qualitatively mimics OU749 as an inhibitor of transpeptidation. It is uncompetitive vs L-GpNA (Fig. 4E) and competitive vs glygly (Fig. 4F). SBS was several orders of magnitude less potent than OU749 at inhibiting the transpeptidation reaction. For SBS, the  $K_{ij}$  and  $K_{is}$  values were,  $28.8 \pm 1.6$  mM and  $2.28 \pm 0.07$  mM, respectively, compared with 68  $\mu$ M and 54  $\mu$ M for OU749. The difference in the potency of SBS versus OU749 as an inhibitor of both the hydrolysis and transpeptidation reactions implies that the benzosulfonamide portion of OU749 contributes to the inhibition of GGT by blocking access of the acceptor to the acyl bond, although SBS is much less effective than OU749 in this regard. To determine the impact of the benzosulfonamide ring on GGT inhibition, we modified this substructure in the context of OU749 and assessed the effect of these modifications in the hydrolysis and transpeptidation reactions.

### Modification of the benzosulfonamide ring of OU749

A series of structural analogs of OU749 with substitutions at the *para* (X), *meta* (W), or *ortho* (V) positions on the sulfoxibenzene ring were evaluated as inhibitors of both the hydrolysis and transpeptidation reactions of GGT (Table 2). The compounds fall into a number of classes of inhibitory potency compared to OU749. Compounds 2-4, with *p*-methyl, *m*-chloro, or *p*, *o*-dichloro substitutions had no effect on inhibition of the hydrolysis reaction, but were 3-5 times more effective inhibitors of the transpeptidation reaction. These data suggest that the aforementioned substitutions do not alter the stability of the ES complex. These compounds are equivalent to OU749 in their ability to block access of water to the acyl bond, but are more effective than OU749 in blocking access of the dipeptide acceptors to the acyl bond in the transpeptidation reaction. Compounds 5-7 with the hydrophilic *p*-hydroxylamino, or the bulky *m*, *o*-benzyl, or *p*-tBu substitutions are 2-5 times less effective than OU749 in inhibiting the hydrolysis reaction but have smaller affects as

inhibitors of transpeptidation. The latter is also true of compounds 8-10 with *p*, *m*-dichloro, *p*-methoxy or *p*-chloro substituents, which are 40-75 times poorer inhibitors of the hydrolysis reaction but, with the exception of the bulkier *p*-methoxy, are better inhibitors of the transpeptidation reaction. Comparison of the data obtained with compounds 3 and 4, to the data for compounds 8 and 10, reveals that the combination of Cl substituents alters the ability of the inhibitor to block hydrolysis of the acyl bond but has little effect on the transfer of the acyl bond to an acceptor. For the hydrolysis reaction, the detrimental effect of having a Cl substituent in the *para* position can be ameliorated by *ortho*, but not *meta* substitution in the same molecule, while *meta* substitution alone generates no effect.

Introduction of a highly electron-withdrawing group, -NO<sub>2</sub> or -CF<sub>3</sub>, in the *para* (X) position (Compound 11 and 12, Table 2) resulted in activation of the hydrolysis reaction. However, addition of these groups maintained inhibition of the transpeptidation reaction, with less than a 2-fold affect on the  $K_{ij}$  relative to OU749. Data are shown for Compound 11 (Fig. 5). A detailed analysis of the kinetics of the hydrolysis reaction is shown in Figs. 5A and B. At each of the concentrations of Compound 11, including zero, data were first fitted to eq. 1 to obtain estimates of  $V_{max}$  and  $V_{max}/K_{D-GpNA}$ . The limiting rate constants were fitted to eq. 5, which allows for an estimate of the values of the rate constant at zero and infinite concentration of the compound and a determination of its activation constant ( $K_{act}$ ). Note that activation is observed on  $V_{max}$ , while inhibition is observed for  $V_{max}/K_{D-GpNA}$  (Fig. 5B). The compound is thus a V-type activator in the hydrolysis reaction. GGT inhibition by Compound 11 was also analyzed in the transpeptidation reaction. Compound 11 is an uncompetitive inhibitor vs. L-GpNA in the transpeptidation reaction, indicating that it binds to the L- $\gamma$ -glutamyl intermediate, presumably at the acceptor site (Fig. 5C). The  $K_{ij}$  was 93  $\mu$ M. The  $K_{ij}$  of Compound 11 while varying glygly in the transpeptidation reaction was 96  $\mu$ M (Fig. 5D). The affinity of Compound 11 for the D- $\gamma$ -glutamyl enzyme intermediate and the L- $\gamma$ -glutamyl enzyme intermediate differ by only 2.5-fold, suggesting that there may be a minor difference in the acceptor site or a slight difference in the orientation of the acyl bond depending on the isomer of glutamate that is bound.

### Modification of the benzyl ring, structure activity studies

In order to determine whether modification of the phenyl ring remote to the benzenesulfonamide ring has an effect on the behavior of compound 11, a series of structural analogs of compound 11 were synthesized with modifications to this portion of the inhibitor. Kinetic parameters for modifications of the benzyl ring of nitro compounds are shown in Table 3. A 3- to 6-fold activation of the  $V_{max}$  for hydrolysis (the ratio of  $V_{\infty}$  to  $V_0$  in Table 3) was observed with an activation constant,  $K_{act}$  of 22-54  $\mu$ M for compounds 13-19. These activators may alter the conformation of the enzyme or be oriented differently than OU749, allowing greater access of water to the acyl bond.

In the transpeptidation reaction, the nitro-containing derivatives of compound 11 behave in a manner that is both qualitatively and quantitatively similar to the parent compound, OU749 (Table 3). Addition of a chlorine group at the Y position resulted in an inhibitor (Table 3, Compound 16) with a  $K_{ij}$  of 61.9  $\mu$ M in the transpeptidation reaction. An OCH<sub>3</sub> at the Y position increased the L-GpNA  $K_{ij}$  in the transpeptidation reaction less than 2-fold and increased the glygly  $K_{is}$  more than 2-fold (Table 3, Compound 11) when compared with Compound 16. No substitution on the benzyl ring (Table 3, Compound 13) increased the L-GpNA  $K_{ij}$  by almost 2-fold compared to Compound 16 while only slightly increasing the glygly  $K_{is}$ . Addition of a methyl group at the Y position yielded results similar to having no substitution (Table 3, Compound 17). Addition of any of a series of chemical groups at the Z position reduced the strength of this family of inhibitors in the transpeptidation reaction by 2-fold while slightly increasing the strength of the compound as a competitive inhibitor of the acceptor, glygly (Table 3, Compound 15 and 18). Addition of an NO<sub>2</sub> at the Y position



(Table 3, Compound 14) more than doubled the  $K_{ij}$  and  $K_{is}$  relative to Compound 16 in the transpeptidation. There is very little difference in the behavior of any of these compounds in the transpeptidation reaction with the exception of Compound 19 and Compound 20, which exhibited time dependent inhibition and, together with acivicin, will be discussed below.

### Acivicin and Acivicin-like Inhibition

Acivicin is an analog of  $\gamma$ -glutamyl donor substrates [GpNA, GSH, etc.] and exhibits reversible competitive inhibition against these substrates with a very slow rate of release (27). The time course for production of pNA in the presence of increasing concentrations of acivicin is shown in Fig. 6. The time course for hydrolysis of 0.287 mM D-GpNA in the absence of acivicin is linear for at least 30 minutes (Fig. 6A). In the presence of increasing concentrations of acivicin, the initial rate is unchanged, but the rate decreases with increasing time, with the most pronounced decrease seen at the highest acivicin concentration (Fig. 6A). A fit of the time courses to the equation for a first-order process gives an observed rate constant,  $k_{obs}$ , that is a linear function of acivicin concentration. This linear dependence is expected for an irreversible inhibitor, where  $k_{obs}$  is the pseudo-first order net on-rate constant for modification of enzyme by acivicin times the concentration of acivicin. The slope of the line depicted in the inset of Fig. 6 thus describes the second order rate constant,  $k_{on}$ , as  $0.171 \text{ mM}^{-1}\text{min}^{-1}$ . Since acivicin is competitive vs. D-GpNA,  $k_{obs}$  is equal to the pseudo-first order rate constant divided by  $(1 + [\text{DGpNA}]/K_{D-\text{GpNA}})$ . Given a  $K_{D-\text{GpNA}}$  of 0.16 mM (Table 1)  $k_{on}$  corrected for the presence of D-GpNA is  $0.48 \text{ mM}^{-1}\text{min}^{-1}$ .

Kinetic analysis of acivicin was also carried out for the transpeptidation reaction using LGpNA or L-GpNA with glygly, and the results were qualitatively the same (data not shown). In the transpeptidation reaction with L-GpNA, the time courses were curvilinear even in the absence of acivicin. Unlike D-GpNA, which can only act as a donor to form the  $\gamma$ -glutamyl enzyme intermediate (F-form), L-GpNA can also act as an acceptor (18, 21, 26). As a result, a complete analysis was only carried out for the hydrolysis reaction using D-GpNA.

Increasing the D-GpNA concentration to saturation eliminated the development of inhibition by acivicin over the same time course as would be expected for competitive inhibition against D-GpNA (Fig. 6B). Time dependent inhibition of the transpeptidation reaction was observed for the monofluoro- (Compound 19, Table 3) and dichloro- (Compound 20, Table 3) substituted compounds shown in Table 3, suggesting that they also exhibit either irreversible or slow onset inhibition. A complete analysis of this phenomenon is outside the scope of the present studies.

All of the nitro-containing OU749 analogs accelerated the hydrolysis reaction except Compound 20 (Table 3). Compound 20 has chlorines both *para* (Y) and *meta* (Z) on the benzyl ring and a  $\text{NO}_2$  at the *para* position on the benzosulfonamide ring. The inhibition mechanism of Compound 20 is unique among the OU749 analogs, because it is competitive with the  $\gamma$ -glutamyl substrate in the hydrolysis reaction, yielding a  $K_{ij}$  with D-GpNA of  $78 \mu\text{M}$  (Fig. 7A). In addition to being competitive with the  $\gamma$ -glutamyl substrate, Compound 20 is also competitive with the acceptor glygly in the transpeptidation reaction (Fig. 7B), with a  $K_{is}$  of  $10 \mu\text{M}$  (Table 3). However, we were unable to establish a  $K_{ij}$  with L-GpNA in the transpeptidation reaction, because Compound 20 exhibits slow onset binding similar to acivicin. Thus, the transpeptidation reaction is too complex for accurate analysis and establishment of inhibition constants for this compound.

## Discussion

In this analysis, new structural analogs of OU749 were evaluated for their inhibitory activity in both the hydrolysis and transpeptidation reactions. All of the new analogs were weaker inhibitors of the hydrolysis reaction relative to OU749, but some were more potent inhibitors than OU749 in the transpeptidation reaction. In addition to structure-activity information, the divergent results provide insights into the reaction catalyzed by GGT. In both the hydrolysis and transpeptidation reactions, OU749 is an uncompetitive inhibitor of the donor  $\gamma$ -glutamyl substrate. The data provided herein are consistent with OU749 binding to the F-form of the enzyme. Despite the fact that L-GpNA has the capacity to act as an acceptor in the transpeptidation reaction, its maximum turnover rate was only about 3-times higher than that measured for D-GpNA in the absence of glygly (Table 1). Thus, although L-GpNA can act as an acceptor, it is not very effective. The second order rate constant measured with L-GpNA is similar to the rate constant with D-GpNA ( $8 \text{ min}^{-1}\text{nM}^{-1}$  and  $13 \text{ min}^{-1}\text{nM}^{-1}$ , respectively), indicating that formation of the  $\gamma$ -glutamyl-enzyme intermediate (F-form) is independent of the stereochemistry of the  $\gamma$ -glutamyl moiety on the nitroanilide substrate. To develop effective inhibitors for therapeutic use, each compound must be evaluated in the physiologic hydrolysis reaction.

We began our comparative analyses by characterizing the substructural elements of OU749 that contribute to its efficacy as an inhibitor. The terminal benzosulfonamide moiety of OU749, SBS, was evaluated for activity as an inhibitor (Fig. 4A and 4C). SBS is an uncompetitive inhibitor versus both D- and L-GpNA and a competitive inhibitor versus glygly in the L-GpNA reaction. We expanded our analysis to a series of OU749 analogs with modifications on the benzosulfonamide ring. As with SBS, compounds 2-10 were more potent inhibitors of the transpeptidation reaction than the hydrolysis reaction (Table 2). However, compared to OU749, all of these structural analogs bound with either equal (compounds 2-4) or lower (compounds 5-10) affinity to the D- $\gamma$ -glutamyl enzyme. As observed for glygly, analogs of OU749 with a strong electron-withdrawing group ( $-\text{NO}_2$  or  $-\text{CF}_3$ ) *para* to the sulfonamide group activate the hydrolysis reaction with D-GpNA (Compounds 11 and 12, Table 2). Both analogs inhibit the reaction with L-GpNA and exhibit an affinity very similar to that of compounds 2-10 in Table 2. Thus, as was observed for glygly in the absence of inhibitors, compounds 11 and 12 activate the hydrolysis reaction. This suggests that like glygly, they bind to the acceptor site of the E- $\gamma$ -D-glutamyl intermediate (F-form), and in doing so, they may alter the conformation of the intermediate in a manner that allows a more facile attack by water on the ester carbonyl. The  $\text{SO}_2$  of the benzosulfonamide group may interact with residues in or near the active site.

All of the OU749 analogs containing a nitro group (compounds 11, 13-20), except Compound 20, accelerated the hydrolysis reaction. However, these same analogs inhibited the transpeptidation reaction with an affinity ( $K_{ij}$ ) very similar to that of compounds 2-10 (Table 2). A series of analogs in which the methoxyphenyl ring of compound 11 was modified (Table 3) elicited no major effect on the inhibition of the transpeptidation reaction with the exception of *p*-F or *p,m*-dichloro analogs (compounds 19 and 20), which exhibited a distinct mode of inhibition, similar to that observed for acivicin.

Acivicin is an analog of the  $\gamma$ -glutamyl donor substrate (27-30). It exhibits time-dependent inhibition at low but not at high concentrations of L-GpNA (Fig. 6). The second order rate constant for binding acivicin,  $k_{on}$ , is  $0.48 \text{ mM}^{-1}\text{min}^{-1}$ . This is the same type of inhibition of the transpeptidation reaction as was observed for compounds 19 and 20, indicating that they also exhibit either irreversible or time-dependent inhibition. Of further interest is the fact that the *p,m*-dichloro analog (compound 20) is the only compound in this series of analogs that does not accelerate the hydrolysis reaction but rather serves as a competitive inhibitor of

the D-GpNA substrate. This is the only OU749 analog that inhibits a GGT reaction by competing with the  $\gamma$ -glutamyl substrate.

To fully understand the affect of the OU749 analogs in the hydrolysis and transpeptidation reactions, the reactions had to be independently analyzed with both donor substrates, D- and L-GpNA. In the absence of the acceptor glygly, the second order rate kinetics are similar for both D- and L-GpNA in the GGT hydrolysis reaction, indicating the stereochemistry of the  $\gamma$ -glutamyl group of the substrate does not affect the binding and cleavage of the substrate in the hydrolysis reaction. However, when the acceptor glygly is present in the transpeptidation reaction with L-GpNA serving as the donor substrate, the second order rate kinetics increase by 15-fold. This dramatic increase suggests three possibilities 1) the binding of glygly increases the rate of hydrolysis, 2) glygly could be binding to another site, perhaps an affector site, instead of to the acceptor site that increases the turnover rate, or 3) glygly could be binding free enzyme forming an E-glygly complex prior to L-GpNA binding. The third possibility would imply a sequential mechanism instead of a ping-pong mechanism. In a ping-pong mechanism, the donor substrate's second order rate constant ( $V/K$ ) will not change in the absence or presence of an acceptor molecule. A sequential mechanism can also yield the classic ping-pong plots if the  $K_m$  for glygly is greater than the  $K_i$  of glygly for the E-glygly complex. This is only possible when the glygly concentration is varied around the  $K_m$  of glygly, as was carried out in these studies and previous studies of the GGT transpeptidation reaction (14, 31-32).

It is difficult at best to model the results obtained in this study on a molecular level. The crystal structure for human GGT has not yet been determined. Homology models based on the crystal structures of bacterial GGT lack the resolution necessary for molecular modeling (33-35). Mutational analysis have indicated that the active nucleophile Thr-381, Arg-107 and Asp-423 interact with the  $\alpha$ -amino and the  $\alpha$ -carboxylate of glutamate (29-30, 36). Two adjacent serines (Ser-451 and Ser-452) have also been proposed to stabilize the rate-limiting transition state of the catalytic reaction (29). Identification of amino acids involved in the binding of the acceptor has been more elusive, and there is little solid structural work available to delineate the "acceptor" site of GGT from any organism. All of the inhibitors tested, with the exception of acivicin, target the acceptor site of human GGT. Although one could speculate as to the residues or sites with which different inhibitors interact, no definitive statements can be made until a reasonable structure is obtained with the acceptor site occupied.

A schematic representation of the hydrolysis and transpeptidation reaction in the presence of a high affinity competitive inhibitor (acivicin) and an uncompetitive inhibitor (i.e. OU749 and analogs, I) is presented in Fig. 8. In the hydrolysis reaction (Fig. 8A), D-GpNA binds to GGT, and the  $\gamma$ -glutamyl moiety is cleaved, releasing pNA. The D- $\gamma$ -glutamyl group is retained by GGT establishing the F-form. Water can then hydrolyze the acyl bond of the F-form, releasing D-glutamate and free enzyme. Acivicin can inhibit GGT by binding the free enzyme, competing with the  $\gamma$ -glutamyl substrate, thus inhibiting both the hydrolysis and transpeptidation reactions equally. After establishment of the F-form of GGT, in the transpeptidation reaction with LGpNA (Fig. 8B), the acyl bond can be hydrolyzed by water, or attacked by an acceptor (i.e. glygly or L-GpNA). L-glutamate, L- $\gamma$ -glutamyl-glygly, or L- $\gamma$ -glutamyl-L-GpNA are then released, and the enzyme returns to the free enzyme state. OU749 and its analogs, are uncompetitive, in that they bind the F-form of the enzyme competing with the acceptor. Therefore, OU749 and its analogs only inhibit the second half of the reaction of GGT; the attack of the acyl bond by either water or an acceptor.

Expression of GGT on the surface of the cell initiates the cleavage of extracellular GSH thereby releasing cysteine and providing an additional source of cysteine for increased

intracellular GSH synthesis (5, 37). GSH and free cysteine within the cell are potent reducing agents that protect the cell against oxidative stress and detoxify electrophilic metabolites. Conjugation of many chemotherapy drugs to GSH result in their inactivation, and the conjugates are exported from the cell (38). GSH has been shown to be increased in various cancers including breast (39), lung (40), bone marrow (41), ovarian (42), and head and neck laryngeal (43). Increased intracellular GSH has also been shown to contribute to the inhibition of apoptosis by inducing Bcl-2 and inducing resistance to antihormonal therapy (44-45). Studies have shown that, in mice, GGT-positive tumors are more resistant to treatment with cisplatin than GGT-negative tumors (6). In Phase I clinical trials acivicin was found to be neurotoxic and cannot be used clinically as an inhibitor of GGT (10-13). Therefore, less toxic inhibitors need to be developed. A complete understanding of the mechanism of inhibition of GGT is crucial to optimize GGT inhibitors for clinical use. In the current study, the stereochemistry of the donor substrates provided insight into the heterogeneity of the acceptor site and the potential interaction of the OU749 analogs within the acceptor site. These studies also emphasize the need to study the mechanism of inhibition of GGT in both the hydrolysis and transpeptidation reactions. The hydrolysis reaction is the physiologic reaction and inhibitors of GGT need to be thoroughly analyzed and optimized in this reaction.

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## The abbreviations used are

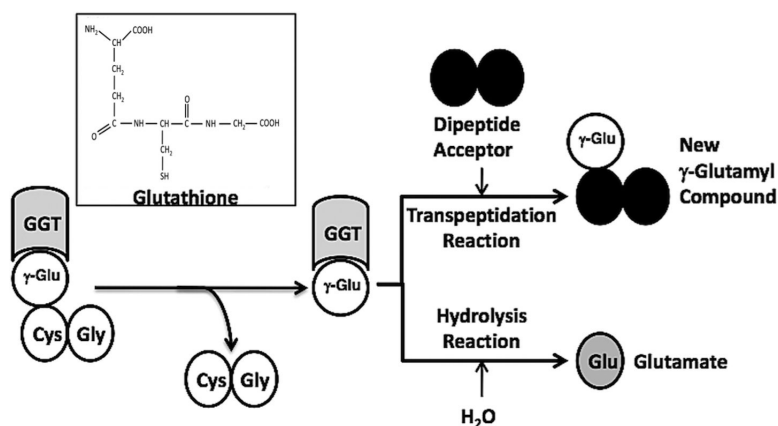
|               |  |
|---------------|--|
| <b>GGT</b>    | gamma-glutamyl transpeptidase                      |
| <b>GpNA</b>   | D- or L-glutamic acid $\gamma$ -4-nitroanalide HCl |
| <b>glygly</b> | glycyl-glycine                                     |
| <b>DMSO</b>   | dimethyl sulfoxide                                 |

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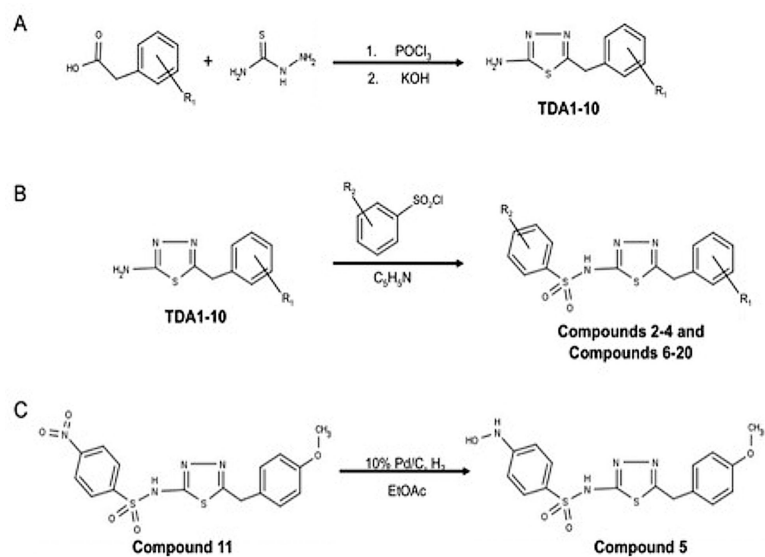
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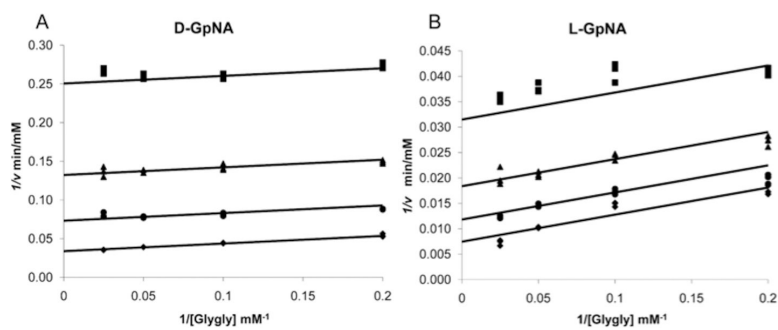


**Fig. 1.** GGT reactions with  $\gamma$ -Glutamyl Substrates. Cleavage of glutathione, a physiologic substrate of GGT, is shown. The  $\gamma$ -glutamyl bond between the  $\gamma$ -carbon of glutamate and the amine of cysteine is cleaved by GGT. The  $\gamma$ -carbon of glutamate forms an acyl covalent bond with the hydroxyl group on the side chain of Thr-381 of human GGT. The acyl bond can either be hydrolyzed releasing glutamate (Glu), or an acceptor nucleophile can attack forming a new  $\gamma$ -glutamyl compound (transpeptidation reaction). The reactions proceed via a Ping-Pong mechanism.

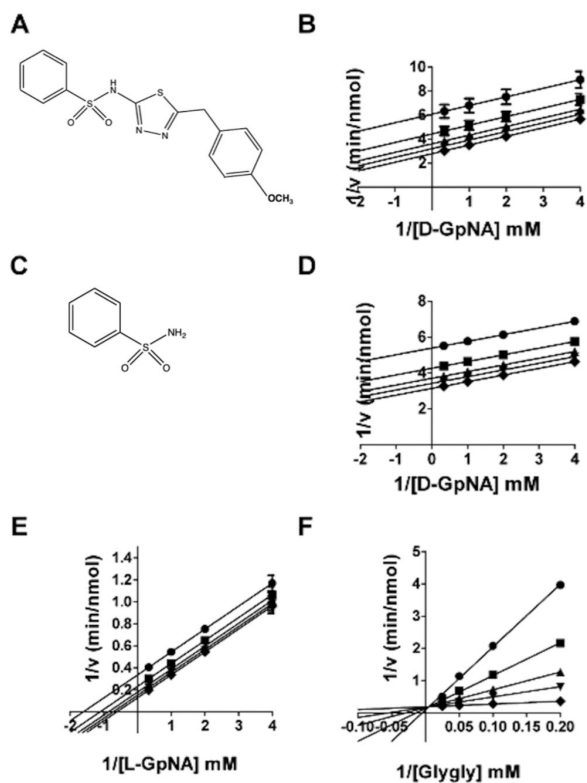


**Fig. 2.** Synthesis scheme for the OU749 analogs. Synthesis and purification of the synthetic intermediates TDA1-10 (A) was required before synthesis of the OU749 analogs Compounds 2-4 and Compounds 6-20 (B). Synthesis of Compound 5 is derived from Compound 11 (C).

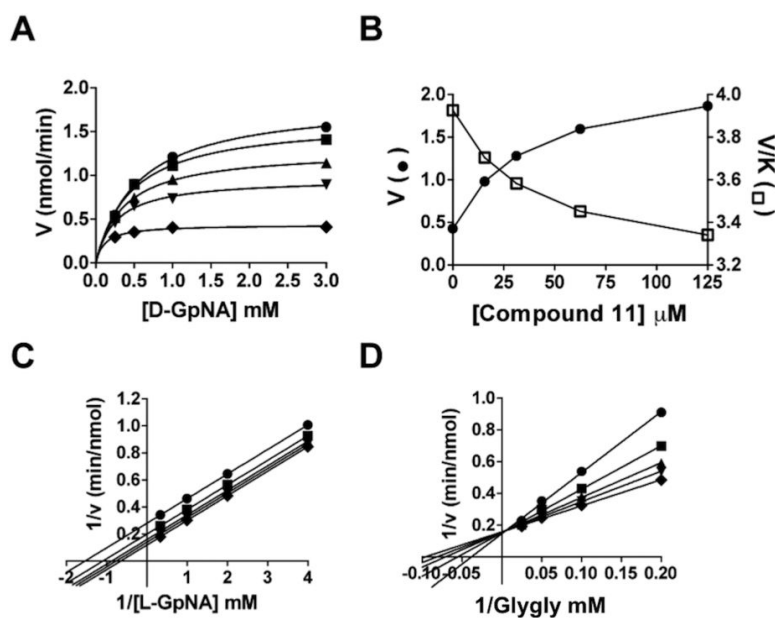




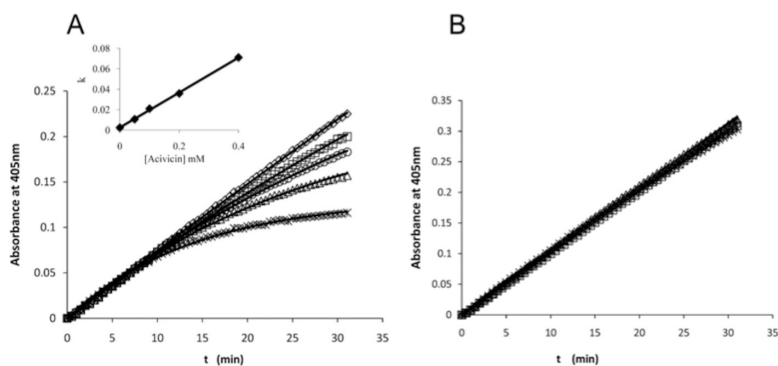
**Fig. 3.** Initial velocity pattern with D-GpNA or L-GpNA as the substrate and glygly as an acceptor. Initial rates were measured at pH 7.4 and 37 °C. Double reciprocal plots of the initial velocity versus the glygly concentration with 0.25 mM (◆), 0.5 mM (●), 1 mM (▲), or 3 mM (■) D-GpNA (A) or L-GpNA (B).



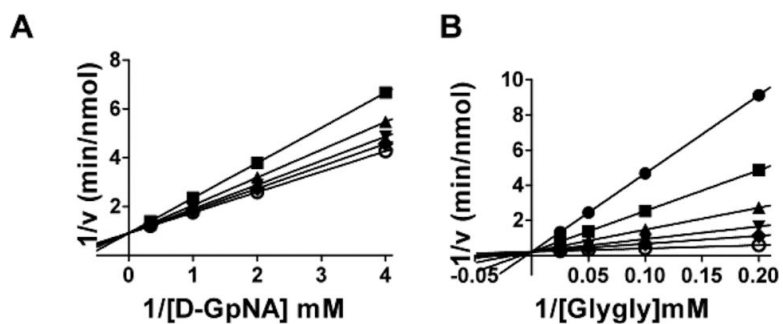
**Fig. 4.** Kinetic analysis of GGT inhibition by OU749 and SBS. The structure of OU749 (A). Double-reciprocal plot of the initial velocity of the hydrolysis of D-GpNA in the presence of 0 (◆), 15.2  $\mu\text{M}$  (▼), 31.25  $\mu\text{M}$  (▲), 62.5  $\mu\text{M}$  (■), 125  $\mu\text{M}$  (●) OU749 (B). The structure of SBS (C). Double reciprocal plot of the initial velocity of the hydrolysis of D-GpNA in the presence of 0 (◆), 6.25 mM (▼), 12.5 mM (▲), 25 mM (■), 50 mM (●) SBS (D). Double reciprocal plot of the initial velocity of the transpeptidation reaction with varying concentrations of L-GpNA and 40 mM glygly (E) or varying concentrations of glygly with 3 mM L-GpNA (F) in the presence of 0 (◆), 6.25 mM (▼), 12.5 mM (▲), 25 mM (■), 50 mM (●) SBS. Data shown are average triplicate values  $\pm$  S.D. For many data points the S.D. is smaller than the symbol.



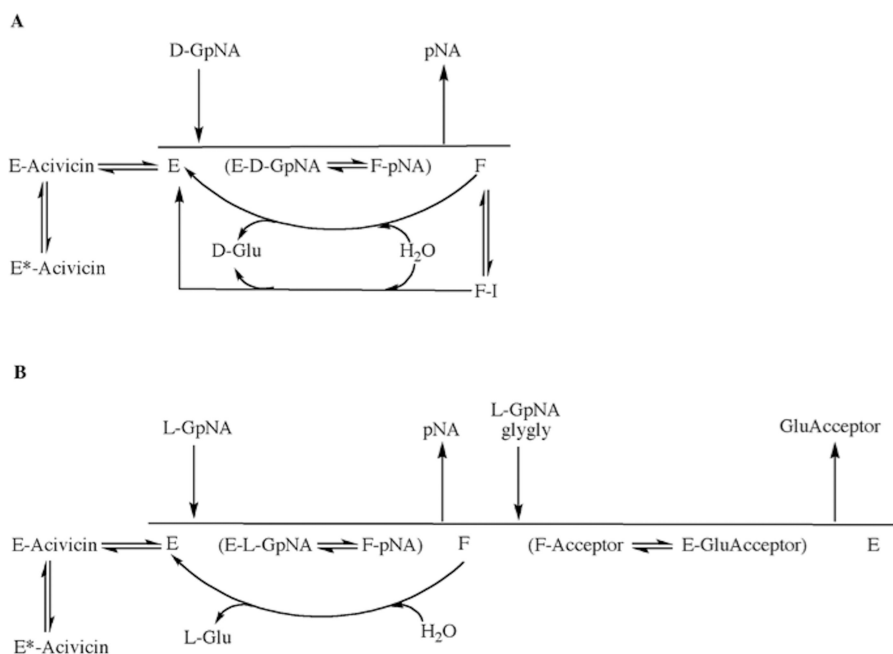
**Fig. 5.** Kinetic analysis of GGT inhibition by Compound 11. Substrate velocity curve of the hydrolysis reaction versus D-GpNA concentration in the presence of 0 (◆), 15.2  $\mu\text{M}$  (▼), 31.25  $\mu\text{M}$  (▲), 62.5  $\mu\text{M}$  (■), 125  $\mu\text{M}$  (●) Compound 11 (A). Velocity vs Compound 11 concentration (closed symbols) and  $V/K$  vs Compound 11 concentration (open symbols) plots illustrate the activation of the hydrolysis reaction (B). Double-reciprocal plots of the initial velocities of the transpeptidation reaction varying L-GpNA with 40 mM glygly (C) or varying glygly with 3 mM L-GpNA (D) in the presence of 0 (◆), 15.2  $\mu\text{M}$  (▼), 31.25  $\mu\text{M}$  (▲), 62.5  $\mu\text{M}$  (■), 125  $\mu\text{M}$  (●) Compound 11. Data shown are average triplicate values  $\pm$  S.D.



**Fig. 6.** Time courses for the hydrolysis of D-GpNA in the presence of acivicin. The release of pNA from 0.287mM D-GpNA (A) or 2 mM D-GpNA (B) was monitored over time in the presence of acivicin (concentrations of acivicin noted in A). The reactions were conducted at pH 7.4. The increase in the  $K_m$  of D-GpNA with increasing concentrations of acivicin is shown (insert 6A).



**Fig. 7.** Kinetic analysis of GGT inhibition by Compound 20. Double-reciprocal plot of the initial velocity of the hydrolysis of D-GpNA in the presence of 0 ( $\blacklozenge$ ), 15.2  $\mu\text{M}$  ( $\blacktriangledown$ ), 31.25  $\mu\text{M}$  ( $\blacktriangle$ ), 62.5  $\mu\text{M}$  ( $\blacksquare$ ), 125  $\mu\text{M}$  ( $\bullet$ ) Compound 20 (A). Double reciprocal plot of the initial velocity of the transpeptidation reaction with varying concentrations of glygly with 3 mM L-GpNA (B) in the presence of 0 ( $\circ$ ), 15.2  $\mu\text{M}$  ( $\blacklozenge$ ), 31.25  $\mu\text{M}$  ( $\blacktriangledown$ ), 62.5  $\mu\text{M}$  ( $\blacktriangle$ ), 125  $\mu\text{M}$  ( $\blacktriangledown$ ), 250  $\mu\text{M}$  ( $\bullet$ ) Compound 20. Data shown are average triplicate values  $\pm$  S.D. For many data points the S.D. is smaller than the symbol.

**Fig. 8.**

Proposed overall kinetic mechanism of inhibition and activation of GGT. Mechanism of hydrolysis of D-GpNA (A). Enzyme (E) is g-glutamylated by D-GpNA with release of pNA to give a form of the enzyme (F) that can transfer the g-glutamyl moiety to water. D-GpNA is not capable of acting as an acceptor. The ester bond can be hydrolyzed with rate constant  $k_{hyd}$  and this rate is decreased in the presence of an inhibitor such as OU749 (F-I), or increased in the presence of an activator such as Compound 11 giving rate  $k'_{hyd}$ . Acivicin can bind to the free enzyme at the acceptor site, and is slowly trapped (tightly bound) on enzyme. Mechanism of transpeptidation of GpNA (B). Enzyme (E) is g-glutamylated by GpNA with release of pNA to give a form of the enzyme (F) that can transfer the g-glutamyl moiety to an acceptor (L-GpNA and/or glygly). The ester bond can be hydrolyzed with rate constant  $k_{hyd}$  but competition between water, L-GpNA and/or glygly decreases the rate of hydrolysis. As in panel A, acivicin can bind to the free enzyme at the acceptor site, and is slowly trapped on enzyme.

**Table 1**

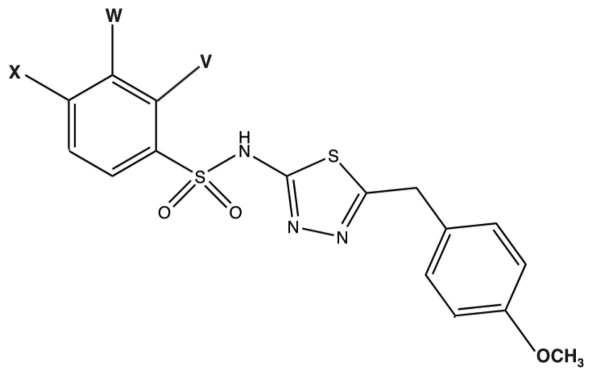
Kinetic Parameters for Reaction With D- and L-GpNA.

| Kinetic parameter                                  | D-GpNA Minus acceptor | D-GpNA Plus acceptor | L-GpNA Minus acceptor | L-GpNA Plus acceptor |
|--|-----------------------|----------------------|-----------------------|----------------------|
| $V$ (mM/min/nM)                                    | $2.1 \pm 0.2$         | $53 \pm 11$          | $6.5 \pm 0.2$         | $141 \pm 11$         |
| $V/K_{glygly}$ ( $\text{min}^{-1}\text{nM}^{-1}$ ) |                       | $7.6 \pm 1.5$        |                       | $14 \pm 1$           |
| $V/K_{GpNA}$ ( $\text{min}^{-1}\text{nM}^{-1}$ )   | $13.2 \pm 0.1$        | $12.7 \pm 2.5$       | $7.8 \pm 0.2$         | $117 \pm 9$          |
| $K_{glygly}$ (mM)                                  |                       | $7.0 \pm 0.3$        |                       | $9.9 \pm 0.9$        |
| $K_{GpNA}$ (mM)                                    | $0.16 \pm 0.02$       | $4.2 \pm 0.1$        | $0.83 \pm 0.06$       | $1.2 \pm 0.1$        |

<sup>a</sup>The acceptor is glygly.

Table 2

Inhibition of GGT by structural analogs of OU749.



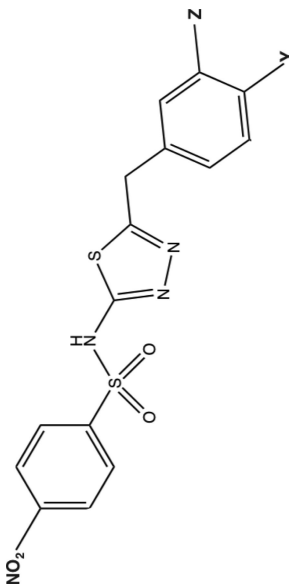
| Compound # | Substitution                         | Hydrolysis D-GpNA $K_{ii}$ ( $\mu\text{M}$ ) | Transpeptidation L-GpNA $K_{ii}$ ( $\mu\text{M}$ ) | Transpeptidation glygly $K_{ii}$ ( $\mu\text{M}$ ) |
|------------|--------------------------------------|--|--|--|
| OU749      | None                                 | 73.2 $\pm$ 8.9                               | 67.6 $\pm$ 6.3                                     | 54.1 $\pm$ 3.9                                     |
| 2          | X = CH <sub>3</sub>                  | 73.3 $\pm$ 4.0                               | 22.7 $\pm$ 0.5                                     | 10.0 $\pm$ 0.4                                     |
| 3          | W = Cl                               | 74.9 $\pm$ 2.2                               | 22.0 $\pm$ 0.7                                     | 11.9 $\pm$ 1.1                                     |
| 4          | X = Cl;<br>V = Cl;                   | 75.6 $\pm$ 7.2                               | 15.7 $\pm$ 0.7                                     | 3.7 $\pm$ 1.0                                      |
| 5          | X = NHOH                             | 149.4 $\pm$ 13.6                             | 64.4 $\pm$ 5.1                                     | 25.7 $\pm$ 1.3                                     |
| 6          | WV = Benzene                         | 161.0 $\pm$ 4.5                              | 74.9 $\pm$ 3.6                                     | 45.7 $\pm$ 3.4                                     |
| 7          | X = C(CH <sub>3</sub> ) <sub>3</sub> | 341.5 $\pm$ 6.4                              | 149.9 $\pm$ 13.0                                   | 143.2 $\pm$ 8.7                                    |
| 8          | X = Cl;<br>W = Cl                    | 3160 $\pm$ 90                                | 18.2 $\pm$ 0.7                                     | 10.8 $\pm$ 0.8                                     |
| 9          | X = OCH <sub>3</sub>                 | 3800 $\pm$ 720                               | 149 $\pm$ 23                                       | 133.3 $\pm$ 10.3                                   |
| 10         | X = Cl                               | 5530 $\pm$ 1460                              | 32.5 $\pm$ 0.9                                     | 13.4 $\pm$ 0.8                                     |
| 11         | X = NO <sub>2</sub>                  | Activator                                    | 92.8 $\pm$ 4.3                                     | 95.7 $\pm$ 7.6                                     |
| 12         | X = CF <sub>3</sub>                  | Activator                                    | 116.7 $\pm$ 7.4                                    | 46.3 $\pm$ 3.5                                     |



Table 3

Kinetic Parameters for Activators of the D-GpNA Hydrolysis Reaction and Inhibition of the L-GpNA Transpeptidation Reaction.

| Compound #      | Substitution         | Hydrolysis D-GpNA   |                          |                      |                 | Inhibition L-GpNA                           |   |   |
|-----------------|----------------------|---------------------|--------------------------|----------------------|-----------------|---|---|---|
|                 |                      | $V_o^a$ (mM/min/nM) | $V_{\infty}$ (mM/min/nM) | $K_{Act}$ ( $\mu$ M) | Fold Activation | Transpeptidation L-GpNA $K_{ij}$ ( $\mu$ M) | Transpeptidation glygly $K_{ij}$ ( $\mu$ M) | Transpeptidation glygly $K_{ij}$ ( $\mu$ M) |
| 13              | None                 | 1.59 $\pm$ 0.06     | 9.09 $\pm$ 0.55          | 54.4 $\pm$ 3.0       | 5.72 $\pm$ 0.32 | 111.5 $\pm$ 5.5                             | 59.6 $\pm$ 3.7                              |   |
| 14              | Y = NO <sub>2</sub>  | 1.27 $\pm$ 0.01     | 5.08 $\pm$ 0.41          | 51.9 $\pm$ 0.8       | 4.00 $\pm$ 0.11 | 132.5 $\pm$ 5.5                             | 81.2 $\pm$ 8.8                              |   |
| 15              | Z = Cl               | 1.30 $\pm$ 0.01     | 5.86 $\pm$ 0.23          | 38.6 $\pm$ 0.3       | 4.51 $\pm$ 0.05 | 119.1 $\pm$ 7.1                             | 43.8 $\pm$ 7.7                              |   |
| 11 <sup>b</sup> | Y = OCH <sub>3</sub> | 1.59 $\pm$ 0.14     | 8.63 $\pm$ 1.21          | 37.1 $\pm$ 5.6       | 5.43 $\pm$ 2.04 | 92.8 $\pm$ 4.3                              | 95.7 $\pm$ 7.6                              |   |
| 16              | Y = Cl               | 1.71 $\pm$ 0.09     | 7.70 $\pm$ 0.46          | 31.4 $\pm$ 2.2       | 4.50 $\pm$ 0.63 | 61.9 $\pm$ 2.4                              | 44.4 $\pm$ 3.4                              |   |
| 17              | Y = CH <sub>3</sub>  | 2.62 $\pm$ 0.79     | 11.25 $\pm$ 4.27         | 25.3 $\pm$ 7.6       | 4.29 $\pm$ 2.20 | 112.5 $\pm$ 5.2                             | 56.9 $\pm$ 8.3                              |   |
| 18              | Z = CH <sub>3</sub>  | 1.15 $\pm$ 0.01     | 5.08 $\pm$ 0.20          | 25.2 $\pm$ 0.2       | 4.42 $\pm$ 0.64 | 121.5 $\pm$ 8.8                             | 34.3 $\pm$ 0.9                              |   |
| 19              | Y = F                | 1.53 $\pm$ 0.09     | 5.39 $\pm$ 0.28          | 22.2 $\pm$ 2.0       | 3.52 $\pm$ 0.60 | Time Dependent                              | 31.8 $\pm$ 0.7                              |   |
| 20 <sup>c</sup> | Y = Cl<br>Z = Cl     | --                  | --                       | --                   | --              | Time Dependent                              | 10.3 $\pm$ 0.5                              |   |



<sup>a</sup>  $V_o$  = maximum velocity at zero activator;  $V_{\infty}$  =  $V_o(K_{ij}/K_{iN})$  in eq. 2, maximum rate at infinite activator;  $K_{act}$  activation constant or concentration that gives  $1/2(V_{\infty} + V_o)$ ;  $K_{ij}$  in eq. 2; fold activation =  $V_{\infty}/V_o$ .

<sup>b</sup> Normalization of all values to Compound 11 yielded values similar to Compound 11

<sup>c</sup> Compound 20 competitively inhibited the hydrolysis reaction with a  $K_{ij}$  with D-GpNA of 78.2  $\pm$  1.9  $\mu$ M.