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MATERIALS AND INSTRUMENTATION

All solvents were purchased from Fisher Scientific and were of ACS grade unless otherwise noted. All commercially available compounds were used without further purification unless otherwise noted. All water used was deionized and distilled. Liquid chromatography-electrospray mass spectrometry (LC-MS) was performed on an Agilent 1100 series HPLC in line with an MSD ion trap and a Daly conversion dynode detector. HPLC was performed using a Waters Delta 600 HPLC utilizing a Hychrom Ultrasphere 5 ODS (250 x 4.6mm) analytical C-18 column with the UV-Vis detector set at 225 and 254 nm. ^{1}H NMR and ^{13}C NMR spectra were collected in either $CDCl_3$ or $(CD_3)_2SO$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on a Varian 600 MHz spectrometer. Microplate absorbance and fluorescence readings were performed on a PerkinElmer Victor 1420 plate reader. Working solutions of $CuSO_4$ were diluted from a 100 mM stock solution that was standardized for [Cu] by titration against EDTA with a murexide endpoint.

Synthesis of GGTDTC

Boc-GluPAB(OH)-OtBu (1; GluPAB): To a solution of Boc-Glu(OH)-OtBu (155 mg, 0.51 mmol) dissolved in 5 mL of 4% NMM in DMF was added first a portion of HBTU (291 mg, 0.77 mmol), which was left to stir under N_2 for 15 min, followed by a portion of *p*-aminobenzyl alcohol (63 mg, 0.51 mmol). After stirring for 5 h at rt, the full consumption of Boc-E(OH)-OtBu was confirmed by TLC and the reaction mixture was added to 100 mL of DCM and washed 3 times with 100 mL of 10% LiCl in water. The organic layer was dried over sodium sulphate and evaporated to dryness under vacuum. Flash silica-gel chromatography (3.5 % MeOH in CH_2Cl_2) afforded **1** as a white solid (159 mg, 76%). ^{1} H NMR (400 MHz, $CDCl_3$, 298K) δ (ppm) 8.90 (s, 1H), 7.45 (d, *J*= 8 Hz, 2H), 7.2 (d, *J*= 8 Hz, 1H), 5.44 (d, *J*= 8Hz, 1H), 5.25 (s, 1H), 4.54 (s, 2H), 4.09 – 4.13 (m, 1H), 2.35 (t, *J*= 6 Hz, 2H), 2.13 (br, 1H), 1.86 (br, 1H). ^{13}C NMR (100 MHz, DMSO-d6, 298K) δ (ppm) 171.6, 170.2, 155.5, 137.9, 137.1, 126.9, 118.8, 80.3, 78.1, 62.6, 53.9, 38.9, 38.3, 32.6, 28.2, 27.9, 27.7,26.2. HRMS (m/z): calculated for [MH+] C21H32N2O6 409.2333, observed 409.2332.

Boc-GluPAB(Br)-OH (2): A portion of **1** (60 mg; 0.15 mmol) was dissolved in 10 mL anhydrous THF with stirring and cooled to 4 °C under N_2. A portion of PBr_3 (21 µL, 0.22 mmol) was slowly added under N_2 and the reaction was kept at 4 °C for 2 h. The solvent was

evaporated to yield a yellow solid. The highly reactive brominated intermediate was transferred to a 50-mL centrifuge tube with trifluoroacetic acid. TFA was dried under N_2. To the residue, dry diethylether was added, cooled with liquid N_2, and centrifuged to obtain a yellow solid which was washed with dry ether 3 times. **2** obtained as a precipitate was taken forward for the next step without further purification. HRMS (m/z): calculated for [MH+] C12H15BrN2O3 315.0339 and 317.0320, observed 315.0344 and 317.0324.

GGTDTC: The lot of **2** obtained from above was suspended in dry acetonitrile and sodium diethyl dithiocarbamate trihydrate was added and stirred for 2 h. The precipitate obtained was filtered and washed with ether, then purified by HPLC using a linear gradient running from 80%–2% H_2O:CH_3CN containing 0.1% TFA over 40 min to yield **GGTDTC** (8.5 mg; 15.2 % yield). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.52 (s, 1H), 7.56 (d, *J*= 8.2 Hz, 2H), 7.28 (d, *J*= 8.3 Hz, 2H), 4.41 (s, 2H), 3.96 (q, *J*= 7.0 Hz, 2H), 3.71 (q, *J*= 7.1 Hz, 2H), 3.28 (t, *J*= 6.4 Hz, 1H), 1.95 (q, *J*= 7.5 Hz, 2H), 1.18 (td, *J*= 6.9, 4.9 Hz, 6H).^{13}C NMR (125 MHz, DMSO-d6) δ 193.6, 170.9, 170.0, 138.6, 130.3, 129.5, 119.1, 53.5, 49.0, 46.5, 40.7, 32.9, 27.0, 12.4, 11.4. HRMS (m/z): calculated for [MH+] C21H32N2O6 384.1410, observed 384.1402.

Experimental Methods

Calcein assay: In a 96-well plate 12.5 μM GGTDTC prochelator or DTC chelator were dissolved in PBS (pH = 7.4) from a 20 mM stock solution in DMSO. To each well Calcein(Cu) was added to reach a final concentration of 1 μM. The fluorescence of calcein was measured using a plate reader with λ_{ex} = 485 nm and λ_{em} = 535 nm. After measuring the initial fluorescence, Gly-Gly and GGT were added in each well to make up to a final concentration of 1 mM and 20 U/L respectively. The plate was scanned at various time points to observe GGTDTC turnover via the development of a fluorescent signal caused by Cu removal from Calcein. The fluorescence emission was plotted as percentage with fluorescence of 1 μM Calcein in PBS as 100% and 1 μM (Cu)Calcein as 0%. The experiment was repeated in triplicate and standard deviation are represented as error bars.

Enzyme cleavage assay: In order to detect DTC liberated from GGTDTC by reactivity with GGT, 100 μM GGTDTC was exposed to 100 U/L GGT in the presence of 1 mM Gly-Gly (PBS; pH 7.4; 37 °C). After 15 min, 500 μM $CuSO_4$ was added to the vial, mixed well, then analyzed by LC/MS using a 20 min H_2O:CH_3CN gradient from 95% to 5% H_2O. A peak eluting at 12 min was observed corresponding to GGTDTC as detected by MS, while a second peak eluting at 22

min corresponded to $Cu(DTC)_2$ as detected by MS. A decrease in GGTDTC peak and formation of $Cu(DTC)_2$ was observed upon treatment with GGT enzyme.

Competition experiments of GSH and GGTDTC vs. γE-pNA for GGT: To perform the inhibition study, a serial dilution of γE-pNA was performed ranging from 1 mM to 3 μM in PBS with 1 mM Gly-Gly (dilution factor = 0.5; pH 7.4; 10% DMSO; 100 μL) using a clear, flat bottom 96 well plate. GGTDTC, DTC, and GSH were added to separate sets of triplicate wells to reach a final concentration of 10 and 100 μM. Before the addition of enzyme, the plate was cooled to 4 °C for 15 min. To initiate the assay, 10 μL of 50 U/L GGT was added to each well and an initial absorbance scan was performed. Subsequent scans were performed every 10 min for ~2 h. In between each scan, the plates were stored at 37 °C in a humidified incubator to reduce solvent evaporation. The experiment was repeated in triplicate.

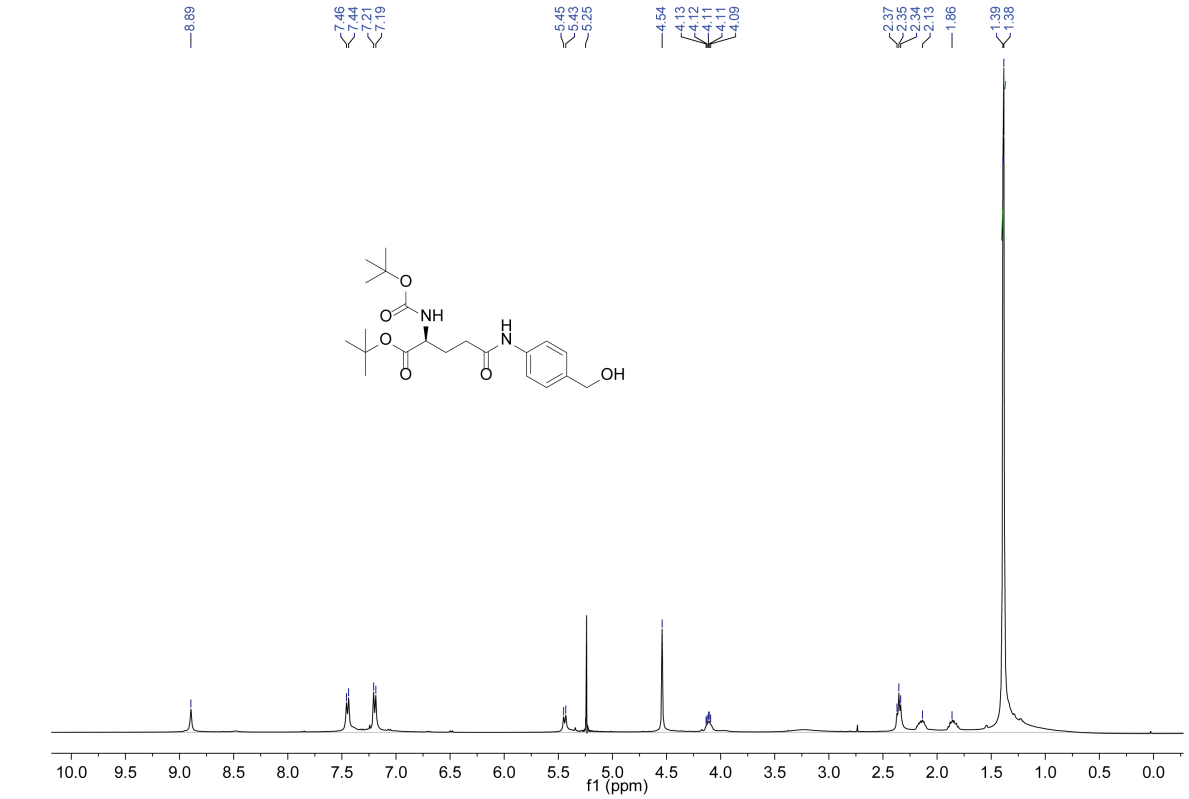
GGTDTC cleavage in cell culture: 22Rv1 and PWR-1E cell lines were plated into a 24 well plate (2×10^5 cells/well) with their respective growth media and allowed to adhere overnight. The following day, the growth media was removed, and each cell line was washed with PBS. 2 mL of 100 μM GGTDTC in serum free media along with 1 mM Gly-Gly was added to the cells. One batch of 22Rv1 was treated with 2 mM Acivicin, a GGT inhibitor, along with GGTDTC and Gly-Gly. 100 μL aliquots were taken at various time points over 72 h. Each aliquot was analyzed via HPLC to generate chromatograms of the progress of GGTDTC digestion. Caffeine was added as reference and the ratio of GGTDTC peak area with respect to caffeine was plotted as percentage considering initial GGTDTC level as 100%.

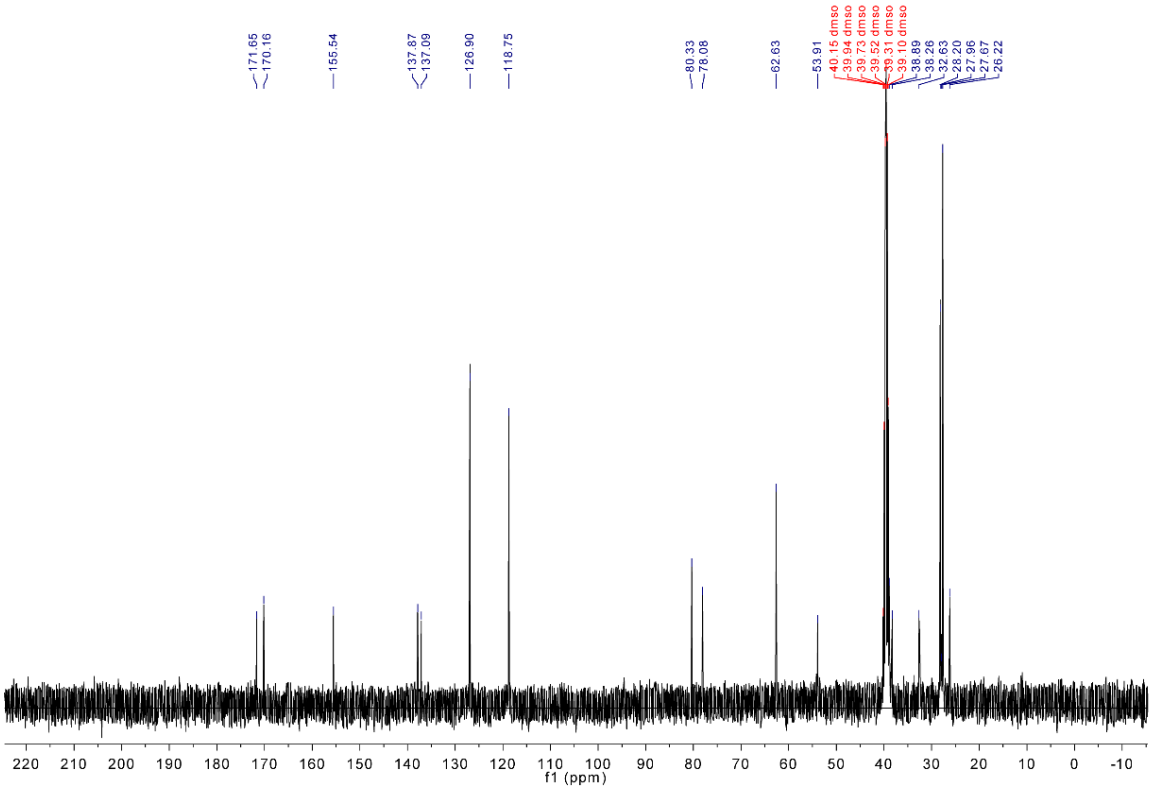
Determination of GGT activity in cells: GGT activity was measured in 22Rv1, LNCaP, MCF-7, PC-3, and PWR-1E cells. Cells (2×10^5 cells/well) were seeded in a 24 well plate and allowed to adhere for 24 h. The cells were dosed with 1 mM γ-Glu(*p*-NA) and 1 mM Gly-Gly in serum free medium (RPMI medium for 22Rv1, LNCaP, and PC-3; DMEM for MCF-7; Keratinocyte-SFM for PWR-1E). The cells were then incubated for 72 h at 37 °C during which 100 μL aliquots were taken at multiple time points and absorbance at 405 nm was measured. Turnover rates (ΔA/min) were acquired via linear regression of the compiled data. A standard curve for GGT activity was obtained by measuring the turnover rates of γ-Glu(*p*-NA) for varying amounts of a standard isolated GGT enzyme. Turnover rates from cellular activity were interpolated to standard curve to obtain the GGT activity in the cells. All experiments were performed in triplicate and the experiments were repeated three times. Standard deviation is reported as

error. PWR-1E cells displayed < 0.1 U/L activity which was the least measurable activity at the given conditions and duration of the experiment.

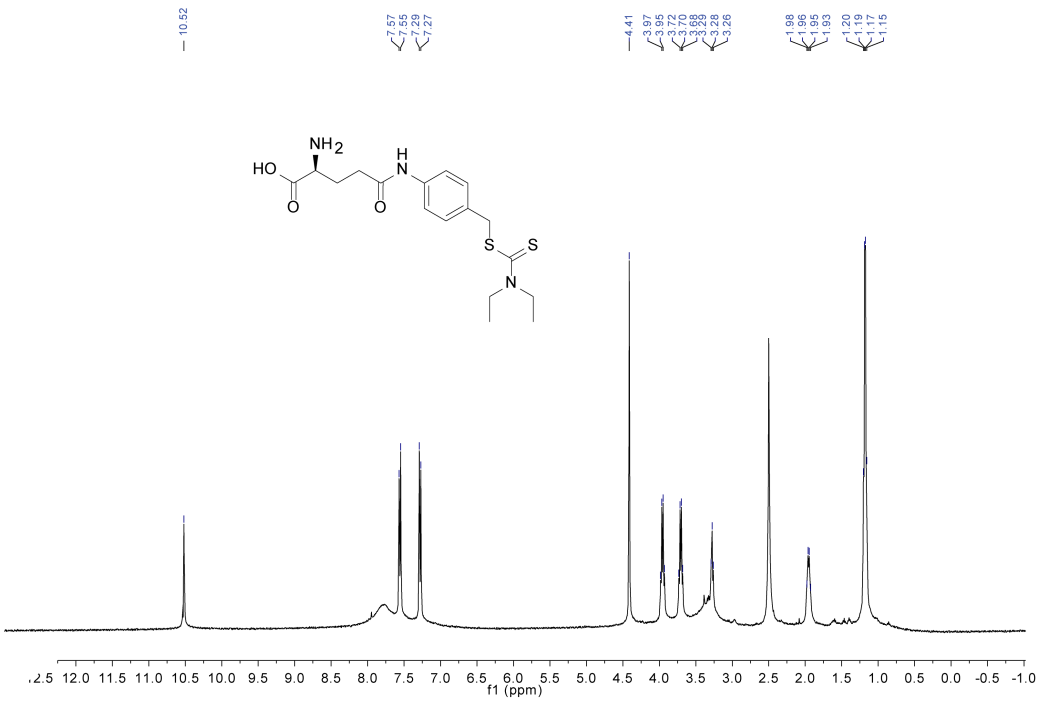
Antiproliferation assay: GGTDTC and DSF show a Cu dependent activity. To determine the amount of Cu required for cell studies a checkerboard assay was performed by varying the prodrug or drug concentration and $CuSO_4$ concentration. 22RV1, LNCaP, PC3, MCF-7, and PWR-1E (5,000 cells/well) were seeded in 96-well plates with regular media (22Rv1, LNCaP, and PC-3 in RPMI with 10% FBS; MCF-7 in DMEM with 10% FBS; PWR-1E in Keratinocyte-SFM) and let adhere for 24 h. The following day, the media was replaced with serum free media and cells were treated as indicated for an additional 24 h or 72 h. Cell proliferation was measured using a fluorometric resazurin reduction method. 20 µL of 700 µM resazurin (Sigma R7017) was added in each well and incubated at 37 ºC for 2 h. Fluorescence at 580 nm was measured by exciting at 555 nm with a plate reader. Each experiment was performed in triplicate, and the experiments were repeated three times. Plots of viability vs drug concentration from representative experiments are shown with standard deviation from the experiment as error bars. The error bars in reported IC_{50} values represent the standard deviation from three different experiments.

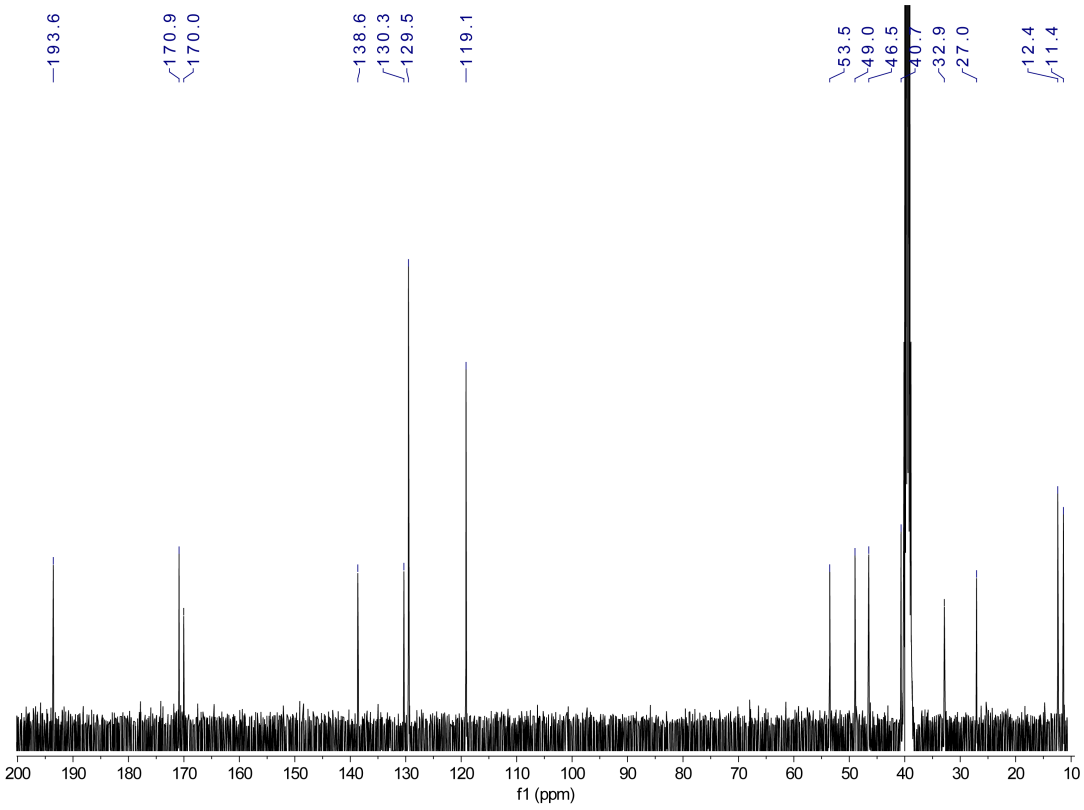
^{1}H and ^{13}C NMR of Compound **1**





^{1}H and ^{13}C NMR of GGTDTC





LC trace for GGTDTC

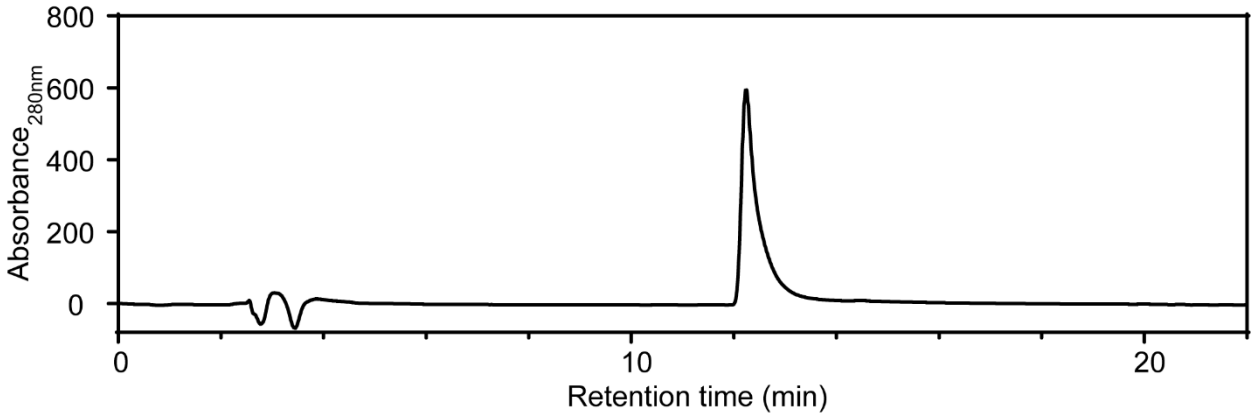


Figure S1. LC trace of GGTDTC observed at 280 nm absorbance confirming sample purity. A gradient of 10% Solvent A (90:10 H_2O: MeOH with 0.1% formic acid) to Solvent B (90:5:5 CH_3CN: H_2O:MeOH with 0.1% formic acid) was run for 20 min through a C18 column followed by 100% solvent B.

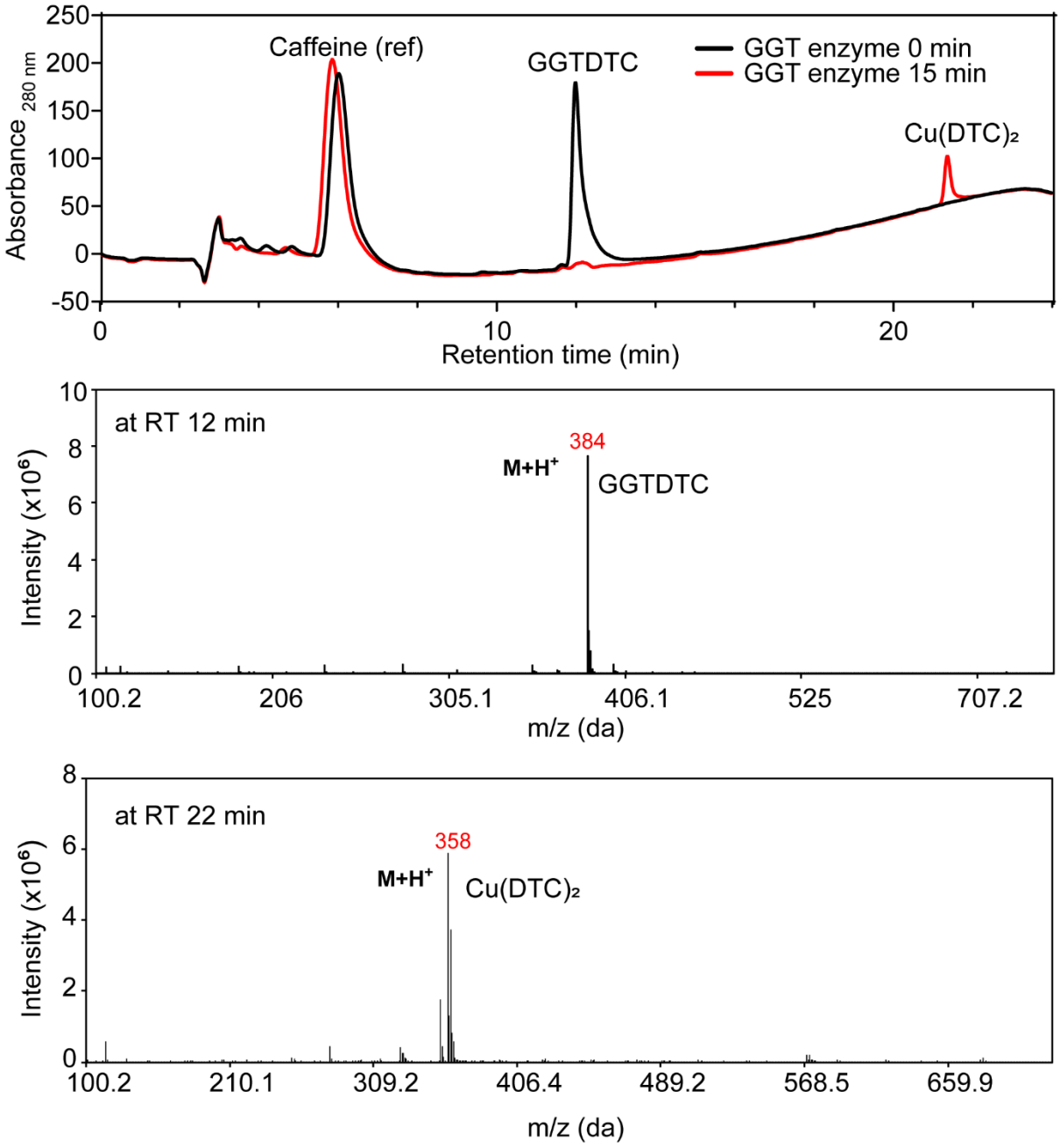


Figure S2. a) LC trace of GGTDTC observed at 280 nm absorbance before (black) and after (red) exposure to 100 U/L of GGT enzyme for 15 min. A gradient of 10% Solvent A (90:10 H2O: MeOH with 0.1% formic acid) to 90% Solvent B (90:5:5 ACN:H2O:MeOH with 0.1% formic acid) was run for 25 min through a C18 column followed by 100% solvent B. **b)** Extracted mass spectrum at retention time 12 min matches that expected for GGTDTC. **c)** Extracted mass spectrum at retention time 22 min matches that expected for the $Cu(DTC)_2$ complex.

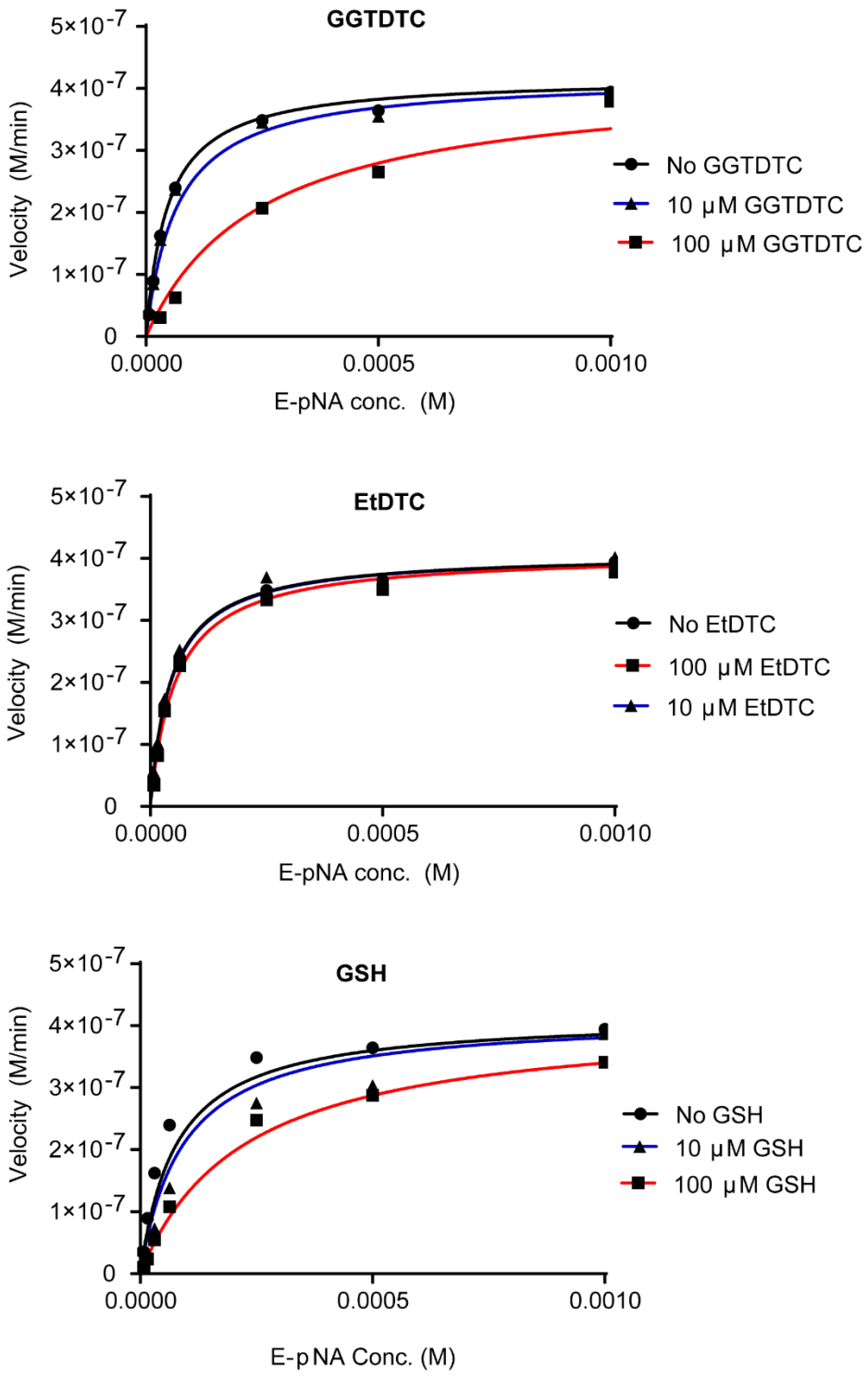


Figure S3: Determination of GGT K_i values for GGTDTC, DTC, and GSH vs. E-pNA (top, middle and bottom, respectively).

a)

DSF [μM]

$CuSO_4$ [μM]	0.00	0.02	0.04	0.08	0.16	0.31	0.63	1.25	2.50
0.0	100.0	87.2	94.2	91.1	92.8	89.6	86.7	70.7	84.6
0.1	100.0	88.2	86.7	81.9	52.7	47.3	52.7	71.1	74.6
0.3	100.0	83.9	90.0	81.1	24.0	25.9	37.4	58.1	66.9
0.5	100.0	93.7	92.7	77.9	15.4	5.6	9.0	27.1	38.5
1.0	100.0	92.7	95.4	86.8	8.9	-0.3	-0.6	-0.5	-0.6
2.0	100.0	100.6	100.3	91.1	9.2	-0.2	-0.4	-4.5	-5.6

b)

GGTDTC [μM]

$CuSO_4$ [μM]	0.00	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00
0.0	100.0	93.8	92.7	88.4	90.7	86.2	90.5	47.5	84.8
0.1	100.0	88.9	93.6	89.8	89.0	58.5	62.3	67.4	67.7
0.3	100.0	101.2	98.1	97.4	80.7	57.5	25.7	21.9	22.0
0.5	100.0	90.4	78.0	89.7	71.5	17.5	0.0	0.2	-0.5
1.0	100.0	94.3	92.2	88.7	58.1	-1.8	-1.2	-1.1	-4.1
2.0	100.0	101.1	96.9	98.5	62.8	-1.1	-0.4	-1.0	-0.7

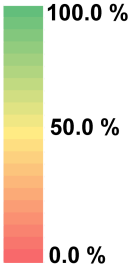


Figure S4. Checker board assays with varying copper concentrations (0 – 2 μM) and **a)** DSF (0 – 2.5 μM) and **b)** GGTDTC (0 – 10 μM) concentrations. %viability is shown. Green indicates more viability while red indicates less viability.

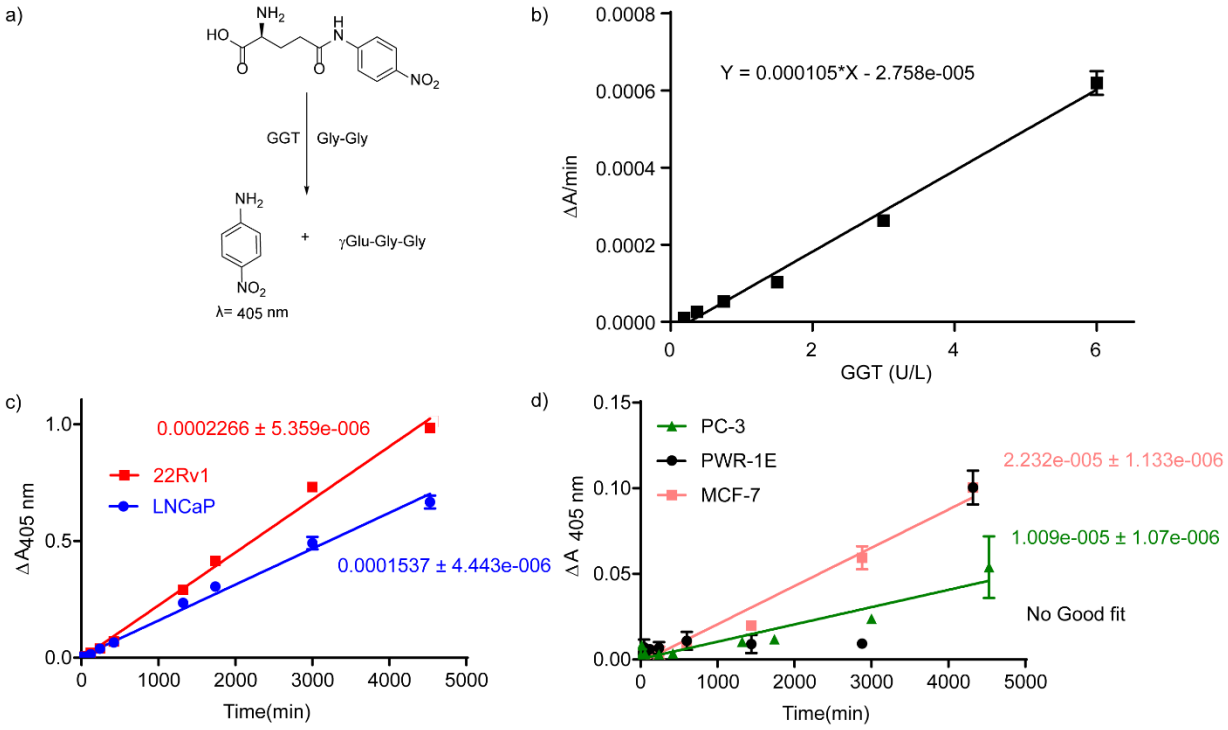


Figure S5: a) Scheme for *p*-nitroaniline release from *γ*-glutamate *p*-nitroanilide by GGT enzyme. **b)** Standard curve for the determination of GGT activity. **c, d)** Representative plots of change in absorbance vs time for (**c**) 22Rv1 (red) and LNCaP (blue), and **(d)** MCF-7 (pink), PC-3 (green), and PWR-1E (black). Slope obtained through linear fit mentioned in the plots. The obtained slope is interpolated in standard curve in fig S5b to obtain GGT activity. Note: PWR-1E data points did not fit a straight line but demonstrated change in absorbance around 72 h.

Table S1. IC50 (μM) with 1 μM $CuSO_4$ in different cell lines arranged in the order of decreasing GGT activity.

Cell line	Aggressive prostate cancer, 22Rv1	Prostate Cancer, LNCaP	Breast Cancer, MCF-7	Prostate Cancer, PC-3	Prostate normal, PWR-1E
Disulfiram at 24 h	0.072 ± 0.01	0.13 ± 0.02	0.18 ± 0.01	0.07 ± 0.01	0.08 ± 0.02
GGTDTC at 24 h	0.76 ± 0.09	0.62 ± 0.04	1.7 ± 0.1	5.5 ± 0.1	16.8 ± 4.4
Disulfiram at 72 h	0.092 ± 0.005	0.14 ± 0.01	0.047 ± 0.002	0.057 ± 0.01	0.07 ± 0.01
GGTDTC at 72 h	0.29 ± 0.11	0.5 ± 0.09	0.48 ± 0.04	1.32 ± 0.25	1.55 ± 0.02