Structural and Biochemical Analyses Reveal the Mechanism of Glutathione S-Transferase Pi 1 Inhibition by the Anti-cancer Compound Piperlongumine

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Running Title: Hydrolyzed Piperlongumine Inhibits GSTP1

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Abstract

Glutathione S-transferase pi 1 (GSTP1), is frequently overexpressed in cancerous tumors and is a putative target of the plant compound piperlongumine (PL), which contains two reactive olefins and inhibits proliferation in cancer cells but not normal cells. PL exposure of cancer cells results in increased reactive oxygen species and decreased glutathione (GSH). This data in tandem with other information led to the conclusion that PL inhibits GSTP1, which forms covalent bonds between GSH and various electrophilic compounds, through covalent adduct formation at PL’s C7-C8 olefin, while PL’s C2-C3 olefin was postulated to react with GSH. However, direct evidence for this mechanism has been lacking. To investigate, we solved the x-ray crystal structure of GSTP1 bound to PL and GSH at 1.1 Å resolution to rationalize previously reported structure activity relationship studies. Surprisingly, the structure showed a hydrolysis product of PL (hPL) was conjugated to glutathione at the C7-C8 olefin, and this complex was bound to the active site of GSTP1; No covalent bond formation between hPL and GSTP1 was observed. Mass spectrometric (MS) analysis of reactions between PL and GSTP1 confirmed that PL does not label GSTP1. Moreover, MS data also indicated that nucleophilic attack on PL at the C2-C3 olefin led to PL hydrolysis. Although hPL inhibits GSTP1 enzymatic activity in vitro, treatment of cells susceptible to PL with hPL did not have significant anti-proliferative effects, suggesting hPL is not membrane permeable. Altogether, our data suggest a model wherein PL is a prodrug whose intracellular hydrolysis initiates the formation of the hPL:GSH conjugate, which blocks the active site of and inhibits GSTP1 and thereby cancer cell proliferation.

Piperlongumine (PL), a natural product derived from the fruit of Piper longum, selectively causes apoptosis in numerous cancer cell lines as well as cancerous tumors in animal models, but does not demonstrate anti-proliferative behavior in non-transformed cells (1). PL has shown anti-tumor activity in prostate cancer(2,3), breast cancer (4,5), lung cancer(6), primary brain tumors (7), gastric
Hydrolyzed Piperlongumine Inhibits GSTP1

To understand the molecular basis for PL’s effect on GSTP1 and explain the prior structure-activity relationship (SAR) analysis, we determined the high resolution x-ray crystal structure of GSTP1 in complex with PL and GSH. Surprisingly, PL becomes hydrolyzed to a trimethoxycinnamic acid (TMCA) which we term hydrolyzed PL (hPL), eliminating the C2-C3 olefin. Additionally, a covalent complex is formed between PL’s C7-C8 olefin and the thiol from GSH, which is in contrast to the predicted model where GSH undergoes Michael addition at the C2-C3 olefin. Mass spectrometric analysis of reactions between PL and a cysteine-containing peptide confirmed the hydrolysis of PL through nucleophilic attack of PL at the C2-C3 olefin. Further, we demonstrate that in the presence of physiologically relevant concentrations of PL, PL does not covalently modify GSTP1, suggesting that the biological mechanism for GSTP1 inhibition is not through covalent modification by PL. Instead, our x-ray data shows that the hPL:GSH conjugate binds non-covalently in the GSTP1 active site, through the formation of multiple van der Waals and hydrogen bonding interactions. Coupled with in vitro kinetic data which shows a concentration dependent inhibition of GSTP1 by hPL, our data suggest that PL is a prodrug, which becomes activated by hydrolysis to hPL, resulting in the formation of a hPL:GSH conjugate and inhibition of GSTP1.

Results

Structure Determination of PL:GSTP1 Complex- We performed x-ray crystallography to understand the molecular interactions between GSTP1 and PL. GSTP1 was expressed and purified from E. coli, and protein crystals were obtained using methods described previously (24). The crystals were grown in the presence of 1 mM final...
concentration of GSH and PL and appeared within 6 days, growing to 200 micrometers in size. After cryoprotection in 25% glycerol diffraction data extended to a resolution of 1.1 Å. Crystals were found to have C2 symmetry with two molecules in the asymmetric unit, forming a functional dimer, as previously reported. Phases were obtained by molecular replacement using a previously solved structure of GSTP1 as a search model, PDBID 3GUS, followed by positional refinement and 2-fold noncrystallographic symmetry averaging. Refinement resulted in final $R_{work}$ and $R_{free}$ values of 16.3 and 18.6 respectively. Final crystallographic and refinement statistics are given in Table 1.

**Overall Architecture and Piperlongumine/Glutathione Binding** - The topology and overall organization of GSTP1 has been reported previously as a biological dimer between chains A and B, with each chain/protomer consisting of eight α-helices and two sets of anti-parallel β-sheets. Consistent with prior reports, each protomer is subdivided into N and C-terminal domains. The GSH binding site (G-site) is formed by the N-terminal domain while the binding site for hydrophobic electrophiles (H-site) is formed with contributions from the C-terminus (Fig 1B). Structural alignment with a previously solved x-ray structure of GSTP1 bound with the GSH:Ethacrynic Acid conjugate (PDB ID 3GSS) showed an almost identical fold (0.22 Å rmsd for Ca atoms).

Inspection of the difference electron density map clearly demonstrated GSH bound to each protomer. The interactions between GSH and GSTP1 have been described in detail previously (27), and are consistent with our findings, as described below for binding in chain A. The carbonyl oxygen from the cysteine portion of GSH makes a 3.0 Å hydrogen bond with the backbone amino nitrogen of Leu52, while the nitrogen from the glycine portion of GSH forms a 3.0 Å hydrogen bond with HOH209, and the C-terminal oxygens are engaged in hydrogen bonding interactions with HOH617, and the side chain nitrogen atoms of Lys44 and Trp38, respectively. The glutamate portion of GSH can be summarized by the following interactions: the backbone nitrogen makes a 2.8 Å hydrogen bond with the carbonyl oxygen of Leu52; the carbonyl oxygen makes a 2.7 Å hydrogen bond with HOH53; the α-amino group makes a 2.8 Å hydrogen bond with the side chain Asp98 O δ2 from chain B; oxygen δ1 of the α-carboxylic acid makes hydrogen bonding interaction of 2.9 Å to HOH3 and 2.9 Å to the backbone amino nitrogen of Ser65, while oxygen δ2 makes a 2.9 Å hydrogen bond with HOH99, and a 2.7 Å hydrogen bond with the side chain oxygen of Ser65.

Electron density for PL was also found in each subunit, occupying the H-site, where other hydrophobic electrophiles such as chlorambucil and ethacrylic acid have been reported to bind. Continuous electron density between the thiol portion of GSH and the C7-C8 olefin of PL indicated that a covalent bond had been formed between the two (Figure 1C). This is in contrast to the proposed adduct formation that would occur between the C2-C3 olefin of PL and the GSH thiol (12). Density for the PL portion of the complex extends from the thiol portion of GSH into a hydrophobic pocket of GSTP1, formed by the side chains of residues Tyr108, Ile104, Phe8, and Val35 (Figure 1D). The trimethoxyphenyl portion of PL fit the electron density very well, and was found to form a parallel-displaced π-stacking interaction with the side chain of Tyr108.

Surprisingly, there was not sufficient density to model the dihydropyridinone portion of PL. Rather, hPL which replaces the dihydropyridinone with a carboxyl group, fit the density well. The hydroxyl oxygen of the carboxyl group makes hydrogen bonding interactions of 2.6 Å with HOH53, and 3.0 Å with the ε nitrogen of Arg13. The carbonyl oxygen makes hydrogen bonding interactions of 2.8 Å with HOH36 and HOH48, respectively. No additional density corresponding to a dihydropyridinone conjugate was observed near the binding pocket.

**Comparison with other GSTP1 Inhibitor Structures** - GSTP1 has been shown to conjugate several chemotherapeutics including chlorambucil (PDB 4HJ2) and ethacrylic acid (PDB 3GSS). It has also been shown to be inhibited by NBDHEX (PDB 3GUS), though no conjugation occurs in the reported GSTP1 structure. Structural alignment of our PL-GSH crystal structure with each, shows nearly identical overall folds for GSTP1 (rmsd Ca ≤ 0.25 Å), and the binding of GSH to the G site is identical, with the exception of the thiol, which can rotate approximately 45 degrees relative to the inhibitor in the H site. Despite the conservation in
the overall fold of GSTP1 and of GSH binding, the H site binders show subtle yet significant differences in positioning, hydrogen bonding, and side chain orientation, supporting the assignment of GSTP1 as a significant cellular target of PL.

Most notably, hPL extends deeper into the H pocket by 2.8 Å which allows it to form a direct hydrogen bond with Arg13 described above, making 4 hydrogen bonding interactions altogether (Figures 2A-D). The positioning also causes the side chain of Ile104 to flip away from the H site relative to the other three structures, and make van der Waals interactions with the aliphatic backbone of hPL. If Ile104 did not flip, then presumably there would be a steric clash with the carboxyl group of hPL. The entropy lost due to the rotation of Ile104 may be offset by the enthalpy gained through the multiple hydrogen bonding interactions of the carboxylate oxygens. Of note, I104V is a common polymorphism found in malignant tumors (28). NBDHEX shows approximately 4-fold greater affinity for the I104V mutant GSTP1 (Kd = 0.21 ± 0.06 µmol/min) (24), owing in part to a shift in position resulting in a direct hydrogen bond from the carbonyl oxygen to the ε nitrogen of Arg13, which in the wild type only hydrogen bonds through a bridging water molecule. This suggests the importance of this particular direct hydrogen bond towards the binding affinity, which is also found in the hPL-GSH structure even without an I104V mutation.

No Covalent Labeling of PL to GSTP1-
Previous hypotheses about PL binding to GSTP1 suggested a model whereby a covalent adduct forms between the C7-C8 olefin and a side chain cysteine of the protein. Despite no indication in our crystal structure of a covalent adduct between PL and GSTP1 at either olefin, we sought to determine if PL could efficiently covalently modify GSTP1 in solution. Recombinant GSTP1 at a concentration of 10 µM was incubated with 10 µM PL and 1 mM L-glutathione. These conditions were chosen to imitate those in which cellular effects of PL are seen. Electrospray ionization mass spectrometry revealed that none of the GSTP1 protein became labeled with PL at these concentrations (Figures 3A, B). Notably, the conjugate of GSH and PL was also detected (M+H⁺ at m/z 625; Figure 3B).

Mass Spectrometry Analysis of PL Hydrolysis- To address the possibility that PL may react non-specifically in the cell with free cysteines in solution we conducted experiments using the model peptide FGLCSGPADTG (FPEP; containing a single cysteine residue) with PL and GSH using the same buffer conditions employed for the crystallography experiment. Mass spectrometry analysis of the resulting reaction products identified FPEP-PL, FPEP-PL-GSH, and PL-GSH conjugates (Figures 3C,D). MALDI MS/MS analysis of FPEP-PL (Figure 3E) confirmed PL labeling of the peptide cysteine residue. Interestingly, peaks at m/z 405 (Figure 3D, “5”) and 1277.8 (Figure 3C, “1”) did not correspond to starting materials or expected products. Consideration of the possible products of the reaction led us to the realization that PL likely reacts with a thiol at the C2-C3 olefin, but ultimately becomes unstable, leading to amide hydrolysis and the formation of hPL. The proposed reaction scheme is shown in Figure 3F. Of note, we did not detect the hPL adduct of GSH, suggesting that GSTP1 is necessary for the reaction at the C7-C8 olefin to occur, which is also consistent with the kinetic observations reported for chlorambucil (29).

hPL Does Not Inhibit Cell Proliferation- With a predicted pKa of 3.7 we considered that in physiologic pH conditions hPL likely exists in a deprotonated form and therefore does not efficiently pass through cell membranes owing to its negative charge. To confirm this we studied cancer cell lines which had previously shown sensitivity to PL (Raj et al., 2011; Adams et al., 2012). We assayed for cell viability using cellular ATP content as a surrogate following a 72 hour treatment with PL or hPL. IC₅₀ values for PL were of 5.8, 7.9, and 17 µM for HeLa, SW620, and PANC-1 cells respectively, consistent with prior observations. However, under identical conditions hPL had little effect on cell viability (Figure 4A) suggesting that hydrolysis of PL occurs only after passage into cells.

hPL inhibits GSTP1 in a concentration dependent manner in vitro- To confirm inhibition of GSTP1 by hPL we developed an enzymatic in vitro assay that utilizes purified GSTP1 in solution. Catalysis of the conjugation of glutathione and 1-chloro-2,4-binitrobenzene by GSTP1 was found to be inhibited by hPL in a concentration dependent manner (IC₅₀ = 384 µM) (Figure 4B). It should be noted that in this case the IC₅₀ is not interchangeable with a binding constant because hPL is competitive with the substrate needed for the...
Hydrolyzed Piperlongumine Inhibits GSTP1

assay (400 µM 1-chloro-2,4- dinitrobenzene, see Methods). Using the Cheng-Prusoff equation, the K_i for hPL was calculated to be 199 µM.

Discussion

The structure of GSTP1 in complex with a hydrolysis product of PL gives novel structural insights into how PL interacts with GSTP1, and demonstrates that PL is able to sequester GSH through GSTP1-assisted conjugation at the C7-C8 olefin, with no covalent bond formation between PL and GSTP1. The extensive van der Waals and hydrogen bonding contacts between GSTP1 and the hPL:GSH conjugate provides a possible mechanism for the anti-cancer activity of PL, through inhibition of GSTP1. Additionally, the hydrolyzed form of PL may hold biological significance.

Identification of hPL as the inhibitory form of PL is consistent with prior SAR of PL. In the absence of C2-C3 olefin, PL derivatives exerted no effect on H1703 or HeLa breast cancer cell lines (12). Similarly an analog lacking the more reactive C7-C8 olefin produced no increase in ROS or reduction of cell viability in all cancer cells tested. In conjunction with our results this suggests a model wherein PL requires activation to its hydrolyzed form in order to effectively interact with GSH in the GSTP1 binding site (Figure 4C). Indeed, hydrolysis of the C2-C3 olefin is predicted to enhance the reactivity of the C7-C8 olefin due to electron withdrawing effects of the carboxyl group, as well as a decrease in steric hindrance.

We note that the high K_i of hPL relative to the active concentration of PL in cells suggests additional mechanisms must contribute to the activity of hPL. A leading hypothesis is that entrapment of hPL within cells leads to elevated intracellular concentrations of hPL that are required for inhibition of GSTP1. Of note, this mechanism of entrapment where compound exit from the cell is impeded has been reported for other compounds including chemotherapeutics such as Vinblastine (30).

It should be noted that despite initial reports that the primary target of PL is GSTP1, additional potential targets for the molecular mechanism of PL’s action have been put forward, including; Keap1 (31), PI3K/AKT/mTOR (6), the nuclear transporter CRM (32), NF-κB pathway (33-35), peroxiredoxin 4 (PRDX4)(36), C/EBP homologous protein (CHOP) activation (37), Signal transducer and activator of transcription (STAT) 3(38), p38(7,39) and the ubiquitin-proteasome system (UPS) (40). This long list of targets and the fact that relatively high concentrations of PL are required for anti-cancer effects, raises the possibility that PL also acts on these targets through relatively non-specific means, perhaps through direct interactions with biologically important small molecules such as GSH. The observation that treatment of leukemia cells with PL depletes reduced glutathione stores in a dose dependent fashion (11) is consistent with this model. While our data provides supporting evidence for this idea it does not exclude other possibilities such as direct inhibition of GSTP1, nor does it rule out the possibility of effects from a different byproduct of PL hydrolysis or conjugation. Additional work will be required to clarify this question.

In conclusion, we have provided the first structural model for interactions between PL, GSH and GSTP1 as summarized in Figure 4C. In this model, PL is a prodrug which enters the cell and conjugates to GSH through Michael addition at the C7-C8 olefin, but only after PL is activated by hydrolysis of the C2-C3 olefin. GSTP1 is inhibited by the hPL:GSH conjugate, and rotation of Ile104 allows the formation of several important hydrogen bonding interactions, most notably with Arg13, which resides deeper in the H-site and does not form a direct hydrogen bond with any other known inhibitors. This suggests that PL has thermodynamic advantages which enable the cellular effects seen with PL, but not with other compounds previously demonstrated to interact with GSTP1. Finally, our results give an atomic level understanding of the mechanism of action for PL, and form the basis for a structure-guided design approach for novel PL or PL:GSH conjugates, which may be used as chemotherapeutic agents.

EXPERIMENTAL PROCEDURES

Expression and purification of GSTP1-A construct encoding human GSTP1 cDNA was synthesized and cloned into the pJExpress 411 vector (DNA2.0), then transformed into the BL21 (DE3) bacterial strain. Cells were grown in Luria Broth to an OD_600 of 0.8 and induced with 250 µM isopropyl b-D-1-thiogalactopyranoside. Cells were grown at 16°C for 18 hours, then harvested by
Hydrolyzed Piperlongumine Inhibits GSTP1

centrifugation for 15 minutes at 6000 x g, and re-suspended in lysis buffer [100 mM sodium phosphate (pH 8.0), 750 mM NaCl, 10 mM imidazole, 10 mM EDTA (pH 8.0), 25% (vol/wol) glycerol, 1 mM PMSF, 1 mM benzamidine, and 1 mg/ml lysozyme]. This preparation was flash frozen and stored at -80°C. Thawed cells were pelleted my centrifugation to remove debris, and soluble protein was loaded onto a glutathione agarose column (Bio-Rad) and eluted with glutathione-containing buffer (40mM glutathione, 200 mM Tris pH 7.0 and 10 mM EDTA (pH 8.0). The purified protein was then buffer exchanged to 10 mM HEPES pH 7.0, 50 mM ammonium sulfate and 5 mM glutathione using Amicon 10kD cutoff filters (EMD Millipore) and concentrated to 10 mg/ml for crystallization experiments.

**Crystallization**- The crystallization was carried out as previously described, however, 2mM final concentration of PL and reduced L-Glutathione were added to the protein prior to crystallization (24). Hanging drop vapor diffusion plates were set up at a ratio of 1:1 (protein to well solution containing 1.6 M Ammonium Sulfate, and 100 mM MES pH 6.5). Crystals were grown at 4°C and appeared after 5 days. Crystals were cryoprotected in a mother liquor solution supplemented with 25% glycerol and flash froze in liquid nitrogen prior to data collection.

**X-Ray Diffraction, Structure Determination and Refinement**- X-ray diffraction data were collected at the Advanced Photon Source beamline 19-ID. Images were processed, integrated, and scaled with HKL-2000/3000 packages (HKL Research Inc)(41). Molecular replacement and model refinement were performed using Phenix (42) and CCP4 software (43) with PDB ID 3GUS as the initial search model (24). The final structure was submitted to the Protein Data Bank under ID code 5J41. Images were prepared using PyMOL, version 1.5.0.4 (Schrodinger, LLC).

**Mass spectrometry of Intact GSTP1**-
Labeling and liquid chromatography-electrospray ionization-MS analysis of GSTP1 by PL for crystallography was performed similarly to the previously described procedure (44). Briefly a solution of 10 μM GSTP1 was mixed with a 10 μM PL and Glutathione and incubated at 27°C for 2 h. Mass spectra were de-convoluted using MagTran software (version 1.03b2, (45)).

**Proteolytic digestion, nano-LC/MS and data analysis** were performed essentially as described (46),(47).

**Mass spectrometry of PL and Peptide FGLCSGPADTGR**- The peptide FGLCSGPADTGR was synthesized using Fmoc chemistry and purified by reversed phase HPLC. The peptide was reconstituted with 10 mM HEPES pH 7.0, 50 mM ammonium sulfate, incubated with a 10-fold molar excess of PL and a 5-fold molar excess of GSH, and incubated for 6 days at 4°C. An aliquot of the reaction was acidified with 10% TFA, further diluted with 0.1% TFA, and peptides were extracted using μC18 zip tips (Millipore, Billerica, MA). After desalting, peptides were eluted to a MALDI plate (384 well opti-tof), mixed with matrix (1 µL 10 mg/mL α-cyano-4-hydroxycinnamic acid in 80% acetonitrile, 0.1% TFA), allowed to air dry, and analyzed by MALDI-MS and -MS/MS as described (48).

**Cell Culture**- Cells were maintained in a 37°C incubator (5% CO2). HeLa (cervical cancer) cells were obtained from the ATCC and cultured in EMEM medium containing 10% FBS and 1% penicillin/streptomycin/ampicillin B (antibiotics). PANC-1 (pancreatic carcinoma) cells were a gift of Paul Chiao, M. D. Anderson Cancer Center, Houston and cultured in DMEM medium containing 10% FBS and antibiotics. SW620 (colorectal adenocarcinoma) cells were a gift of Nathanael Gray, Dana Farber Cancer Institute, Boston and cultured in L-15 medium containing 10% FBS and antibiotics.

**Cell Viability Assay**- Cells were plated at 1000 viable cells per well in 50 μl of medium in white 96-well plates and allowed to attach for 4 hr. At that time, another 50 μl of medium containing PL or hPL was added to create nine sets of triplicate wells of serial 3-fold dilutions spanning a final concentration range of 0.015 – 100 μM compound and 1% DMSO. A triplicate set of wells with medium containing 1% DMSO alone served as untreated controls. After addition of compounds, plates were incubated for 72 hr. At that time, 100 μl of a 1:1 solution of PBS containing 1% Triton X-100:CellTiter-Glo (Promega) was added to each well. Following 10 minutes of gentle shaking, luminescence was read using a BioTek Synergy NEO plate reader. Cell viability (% of DMSO alone) was calculated from the data and plotted using GraphPad Prism 6. IC10’s were extrapolated.
as best-fit values of log(inhibitor) vs. response (three parameters) curves.

GSTP1 Kinetic Assay - Inhibition of GSTP1 activity by hPL was analyzed using the Glutathione-S-Transferase Assay Kit (Sigma-Aldrich), which measures the conjugation of L-glutathione to 1-chloro-2,4-dinitrobenzene (CDNB). Reactions in Dulbecco’s phosphate buffered saline containing 10 nM GSTP1, 2000 mM glutathione, and various concentrations of CDNB and hPL were performed at 25°C in 96-well UV Flat Bottom Microtiter Plates (Fisher Scientific). The resultant time-dependent change of A340 was determined with a Biotech Synergy NEO plate reader. Data was graphed and processed using GraphPad Prism 7 software. A K_m value was determined by nonlinear regression of initial rates of GSTP1-dependent catalysis of various concentrations of CDNB. Nonlinear regression of first-order rate constants from assays containing fixed CDNB and various hPL concentrations was used to obtain an IC_{50} value for the latter compound.

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Conflict of Interest: The authors declare that they have no conflict of interest with the content of this article.

Author Contribution: KDW conceived and coordinated the study. WH, SG, and KDW wrote the paper with contributions from all authors. SG and DU purified the protein. JH and DG collected the x-ray data. WH and JH solved the structure and performed refinement. SF, JM, and SG conducted mass spec experiments. WH, JH and KDW analyzed and interpreted structural data. WS and YL performed cell based assays. WS and LL performed kinetic assays. All authors reviewed the results and approved the final version of the manuscript.
Hydrolyzed Piperlongumine Inhibits GSTP1

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Hydrolyzed Piperlongumine Inhibits GSTP1

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Hydrolyzed Piperlongumine Inhibits GSTP1

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FIGURE LEGENDS

Figure 1. Hydrolyzed piperlongumine inhibits GSTP1 (A) Structure of piperlongumine (PL) and hydrolyzed piperlongumine (hPL). (B) Architecture of GSTP1 dimer. Protomer A is colored brown and protomer B is grey. The hPL-GSH complex is shown as green sticks, and the glutathione (G-site) and hydrophobic (H-site) binding sites are each labeled. (C) 2Fo-Fc map for hPL-GSH complex is shown in green and scaled to 1σ. (D) Binding of the hPL-GSH complex (green sticks) to GSTP1. Residues that make notable hydrogen bonding or van der Waals interactions are shown as grey sticks.

Figure 2. Surface representation of GSTP1 bound by inhibitors: (A) hPL-GSH; (B) Chlorambucil-GSH; (C) NBDHEX-GSH; and (D) Ethacrynic acid-GSH. Inhibitor structures are shown as green sticks. Red arrow indicates the portion of the hPL-GSH structure that extends deeper into the binding cleft compared to the other inhibitor bound structures shown.

Figure 3. PL does not label GSTP1. Purified GSTP1 was incubated at 10 μM (A) without or (B) with PL and GSH. Deconvoluted electrospray mass spectra (A, B upper panels) detected GSTP1 (23,349 Da; A and B; green circles correspond to full length protein while yellow circles indicate loss of N-terminal methionine) but not GSTP1+PL (expected MW 23,666 Da). PL reacts with cysteine thiols and readily forms hydrolysis products (C-E). A model peptide, FGLCSGPADTGR, was incubated with PL and GSH and the reaction mixture analyzed by mass spectrometry. Prominent peaks corresponding to [M+H]+ of FPEP-PL hydrolysis product (1), FPEP-PL (2), FPEP-PL-GSH (3), GSH-PL (4), GSH-PL hydrolysis product (5), PL (6) and GSH (7) were observed (D,E). MALDI-MS/MS analysis confirmed labeling of the cysteine residue (E). Ions of type y are marked by red circles, water loss is denoted by *, and C(*) corresponds to the cysteine-PL adduct. An ion at m/z 1277. colored green, corresponds to the indicated PL hydrolysis product (F, “A”). (F) Proposed PL, GSH and FPEP reaction scheme.

Figure 4. PL requires cell entry prior to being hydrolyzed to active form. (A) Cell proliferation in the presence of PL vs. hPL. Exposure of cancer cell lines previously shown to be sensitive to PL (PANC1, HEla, SW620) show impaired growth in the micromolar range when exposed to PL (blue hue curves) consistent with prior studies. Cells are unaffected at similar concentrations by hPL (red hue curves). (B) Dose response curve shows the inhibition of GSTP1 activity by hPL. (C) Schematic of PL’s entry, activation by free thiols, and inhibition of GSTP1, leading to reduced cellular glutathione, increased ROS, and cell death.
Table 1. Crystallography statistics

| Data Collection              |       |
|-----------------------------|-------|
| X-ray source                | APS 19-1D |
| Wavelength (Å)              | 0.97924 |
| Space Group                 | C2    |
| Unit Cell                   |       |
| a, b, c (Å)                 | 78.5, 89.5, 69.0 |
| α, β, γ (°)                 | 90.0, 98.2, 90.0 |
| Resolution (Å)              | 50.00-1.18 |
| Unique Reflections          | 152,714 |
| Redundancy                  | 3.6 (2.5) |
| Completeness (%)            | 99.3 (87.6) |
| Wilson B-factor             | 9.2 |
| R_merge (%)                 | 4.6 (33.6) |
| <I/σ>                       | 24.16 (2.65) |

| Refinement                  |       |
| Resolution                  | 50-1.18 |
| Reflections Used            | 145,077 |
| R_free reflections          | 7,637 |
| R_wat/R_free (%)            | 16.3/18.6 |
| Non-Hydrogen Atoms          | 4117 |
| Protein                     | 3,291 |
| Water                       | 728 |
| Ligand                      | 98 |
| RMS deviations              |       |
| Bond lengths (Å)            | 0.009 |
| Bond Angles (°)             | 1.1 |
| Average B-factor (Å²)       | 14.0 |
| Ramachandran plot (%)       | favored/allowed/disallowed 97.3/1.2/1.5 |

* Statistics for the last shell are given in parentheses.
Figure 1
Figure 2

(A) hPL:GSH

(B) Chlorambucil:GSH

(C) NBDHEX-GSH

(D) Ethacrynic acid:GSH
Figure 3
Figure 4

A. 

B. 

C. 

Figure 4

A. 

B. 

C. 

Figure 4
Structural and Biochemical Analyses Reveal the Mechanism of Glutathione S-Transferase Pi 1 Inhibition by the Anti-cancer Compound Piperlongumine
Wayne Harshbarger, Sudershan Gondi, Scott B. Ficarro, John Hunter, Durga Udayakumar, Deepak Gurbani, William Singer, Yan Liu, Lianbo Li, Jarrod A. Marto and Kenneth D. Westover

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