Supporting Information:

A structure-based mechanism of cisplatin resistance mediated by Glutathione Transferase P1-1

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This paper is dedicated to the memory of Prof. Mario Lo Bello.

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www.pnas.org/cgi/doi/10.1073/pnas.1903297116

Supporting Materials and Methods

***cis*-DDP preparation**. *cis*-DDP was dissolved in Phosphate Saline Buffer (PBS) to make a stock solution of 3 mM and diluted with complete growth medium to obtain the different working concentrations.

Proteins expression and purification. Human GST P1-1 WT and mutants, C47S and C101S, were expressed in *E. coli* and purified as previously described (1, 2). Enzymatic activity was determined as described above.

Stable transfection. Cells were transfected with pTarget (Promega), WT, C47S, C101S and C47S/C101S GST P1-1 expression vectors using Lipofectamine (Invitrogen) method according to the supplier's instructions. Stable transfectants were isolated by selection in medium containing 500 μg/ml geneticine (G418) for approximately 2 weeks and then diluted in order to select individual clones. Single clones for each transfected cell line were selected for further characterization on the basis of GST activity.

MTS proliferation assay. Proliferation assays (MTS reduction) were carried out in GST P1-1 transfectants expressing high level of GST P1-1 and non-transfected cells, 24 hours after exposure to 5 μM *cis*-DDP, using the MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; Promega] reduction, following the manufacturer's procedure. The values of the untreated samples were regarded as 100%.

Cell death analysis by Fluorescence Activated Cell Sorting (FACS). The effect of GST P1-1 on cell death was investigated by Fluorescence Activated Cell Sorting (FACS) analysis using a FACScan Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and following size

(forward scatter; FCS) and granularity (side scatter; SSC) parameters. Cell clones were seeded in 24-well plates ($2x10^5$ cells/well) and, the day after treated with different *cis*-DDP concentrations. Treatments were performed with 5, 10 and 15 μM *cis*-DDP P for 24 h or for 6 hours of direct exposure to 5, 10 and 15 μM *cis*-DDP, followed by 24 hours of recovery in drug-free medium. For propidium iodide staining, cells were detached from plates with PBS and 2 mM EDTA, washed twice in PBS, gently resuspended with 0.3 ml of solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate and left at 4 °C for 30 min, in the dark, before analysis.

Synthesis of *cis*-DDP-GSH adduct in absence and in presence of GST P1-1. GSH (2 mM) was incubated with *cis*-DDP (1 mM) in PBS (154 mM NaCl and 10 mM potassium phosphate buffer, pH 7.4), at 37°C, final volume 1 ml. The absorption spectrum of the sample was determined every ten min by scanning the differential spectra over a wavelength range of 200-400 nm (Cary 4000 UV-Vis). As a control the same experiment was performed for GSH alone incubated in PBS. The *cis*-DDP-GSH complex was isolated from the reaction mixture by anion-exchange chromatography as previously described (3). The aliquots were further analyzed by electrospray mass spectrometry using a Triple Quadrupole liquid chromatography/mass spectrometry/mass spectrometry mass spectrometer Applied Biosystem Sciex API 365 (Concord, Canada).

To investigate the role of the catalytic activity of GST P1-1 in the formation of the *cis*-DDP-GSH adduct the experiments were performed using 1 cm cuvettes, with 1 ml (final volume) of PBS, pH 7.4, or 10 mM potassium phosphate solution plus 2 mM NaCl, pH 7.4, containing 1 mM GSH, 2 mM *cis*-DDP and increasing amounts of GST P1-1 (from 0 to 50 μg). The enzymatic activities were assayed spectrophotometrically (Cary 4000 UV-Vis), at 260 nm, at 37 °C, for 10 min.

SDS-PAGE analysis under non-reducing condition. WT GST P1-1, C47S and C101S mutants (50 μM in active sites) were incubated at 37°C, in the presence of different *cis*-DDP concentrations (250 μM or 50 μM), in 10 mM potassium phosphate buffer, pH 7.4, for up to 72 hours. At fixed times an aliquot of the sample (5 μg) was withdrawn from the mixture and analyzed by 12% (w/v) SDS-PAGE under non-reducing conditions. The sample corresponding to 0 hour was withdrawn from the mixture immediately after the addition of *cis*-DDP. The samples were mixed in a 1:1 ratio with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% w/v bromophenol blue) to stop the reaction. As a control we used the proteins incubated at 37°C, without *cis*-DPP.

Crystallization

***cis*-DDP soaks**. An artificial mother liquor was made up consisting of 100 mM MES pH 6.0, 20 mM $CaCl_2$ and 30% (w/v) PEG 8000 to which *cis*-DDP solution was added to make a final concentration of 0.1 mM. Crystals, grown in either the absence or presence of GSH, were transferred into 4 μl drops of the *cis*-DDP-containing mother liquor and left to soak for 24 hours. Crystals left to soak for longer periods rapidly lost their diffracting properties. Co-crystallization experiments were not attempted because DTT, a likely *cis*-DDP chelator, was required for crystallization. After soaking in *cis*-DDP solutions, crystals were frozen by soaking for two min in the reservoir solution plus 5% (v/v) methyl-2,4-pentanediol (MPD), then dipped briefly into a reservoir solution plus 10% (v/v) MPD. The crystals were then snap frozen at 100 K in a cryostream. *cis*-DDP and GSH were obtained from Sigma-Aldrich.

Data collection and refinement of the GST P1-1 *cis*-DDP complex in the absence of GSH

Diffraction data from a GST P1-1 frozen crystal, grown in the absence of GSH and soaked for 24 hours in 0.1 mM *cis*-DDP, were collected at the Advanced Photon Source (Chicago, Illinois), on the BioCARS beam line 14-BM-C using an ADSC Quantum-315 CCD detector. The wavelength was

set to 0.90 Å. An X-ray fluorescence scan before data collection indicated the presence of Pt (II). The diffraction data were processed with XDS (4) (Table S1) and scaled with Aimless (5). Molecular replacement was performed with Phaser (6) using a model of GST P1-1 (PDB id:5GSS) (7) after GSH and water molecules were removed, followed immediately by a round of Phenix refine (8), yielding an R_{cryst} of 25.9% (and R_{free} of 28.5%). Water and MES were added using COOT (9), interspersed by rounds of positional and isotropically restrained B-factor refinement using Phenix (8). Three strong peaks (30σ for the central peak and 10 σ for the two satellite peaks) were identified in the difference map at the dimer interface. These were interpreted as three Pt ions: two of the ions binding to a single C101 from each subunit (corresponding to the two satellite peaks) and the third ion being bound by both cysteines (corresponding to the central peak). The interpretation was supported by an anomalous difference Fourier map, calculated with Anode (10), that showed significant peaks at all three positions, from 30σ at the satellite position to greater than 53σ for the central atom. The sites were refined with occupancies of 0.6 and a *B*-factor of 57 $Å^2$ for the satellite Pt and an occupancy of 0.7 with a *B*-factor of 38 $Å^2$ for the central Pt. The C101 side-chains were modeled in a single conformation and had an average B-factor of 42 $Å^2$. The R_{cryst} for the final model is 20.7% (R_{free} of 24.0%) for all data to 1.9 Å resolution. A summary of the refinement statistics is given in Table S1. The model was analyzed with the program PROCHECK (11) which showed that its stereochemical quality was similar or better than expected for structures refined at similar resolutions.

Data collection and refinement of a GST P1-1 *cis*-DDP GSH complex in the presence of GSH. Diffraction data from a GST P1-1 frozen crystal, grown in the presence of GSH and soaked for 24 hours in 0.1 mM *cis*-DDP, were collected using a MAR345 detector with CuK_α X-rays generated by a Rigaku RU200 rotating anode X-ray generator. The diffraction data were processed with Mosflm (12) (Table S1) and scaled with Aimless (5). Molecular replacement was performed with

Phaser (6) using a model of GST P1-1 (PDB id:5GSS) (7) after GSH and water molecules were removed, followed immediately by a round of Phenix refine (8), yielding an R_{cryst} of 24.5% (and R_{free} of 29.2%). Water, MES, GSH, calcium, sulfate and carbonate ions were added using COOT (9), interspersed by rounds of positional and isotropically restrained B-factor refinement using Phenix (8). One major peak (19.5σ) was observed at the dimer interface and interpreted as a Pt ion bound by C101 of both subunits with Pt-S distances of ~ 2.4 Å (Fig. 4(a)). In addition two minor peaks (6σ), located on either side of the major peak, were interpreted at Pt ions bound by a single C101 of either subunit with Pt-S distances of 2.3 Å (Fig. 4(a)). An anomalous difference Fourier map, calculated with ANODE (10) showed peaks in the same positions for the major and minor sites (14σ and 5σ respectively) (Fig. 4(a)). The central Pt ion refined to an occupancy of 0.3 with a B-factor of 26.4 $Å^2$ and the satellite Pt ions refined to an occupancy of 0.2 with B-factors of ~58 $Å^2$. The Cys residues were modeled in two alternate conformations. The R_{cryst} for the final model is 14.9% (R_{free} of 17.7%) for all data to 1.8 Å resolution. A summary of the refinement statistics is given in Table S1. The model was analyzed with the program PROCHECK (11) which showed that its stereochemical quality was similar or better than expected for structures refined at similar resolutions.

Table S1. Summary of crystallographic data collection and structure refinement.

Data Collection	***cis*-DDP**	***cis*-DDP GSH**
Temperature (K)	100	100
Space group	C2	C2
Cell dimensions		
a (Å)	77.5	77.5
b (Å)	90.1	89.9
c (Å)	68.9	68.8
β (°)	97.9	98.0
Maximum resolution (Å)	1.9	1.8
No. of crystals	1	1
No. of observations	268519 (17634)	175679 (9899)
No. of unique reflections	36278 (3620)	43040 (2477)
Data completeness (%)	98.2 (98.0)[a]	99.6 (98.4)
R_{merge}	6.3 (77.5)	7.3 (50.6)
R_{pim}	3.8 (45.8)	4.1 (28.8)
I/σ	18.2 (2.9)	9.6 (2.9)
Multiplicity	7.4 (7.6)	4.1 (4.0)

Refinement	*cis*-DDP	*cis*-DDP-GSH
Non-hydrogen atoms		
Protein	3110	3293
cis-DDP	3	3
GSH	0	40
MES	24	24
SO_4^{2-}	-	5
CO_3^{2-}	-	4

Ca^{2+}	-	2
Solvent (H_2O)	105	528
Resolution (Å)	1.9	1.8
R_{cryst}[c] (%)	20.7	14.9 (21.0)
R_{free}[d] (%)	24.0	17.7 (25.9)
Unique reflections used in R_{conv} calculations:		
Number	36278 (3620)	43039 (4216)
Completeness (%)	98.0 (97.89)	99.6 (98.7)
rmsd's from ideal geometry:		
Bonds (Å)	0.013	0.004
Angles (°)	1.42	0.96
Mean *B* (protein) ($Å^2$)	32.8	19.2
Mean *B* (solvent) ($Å^2$)	29.2	29.0
Mean *B* (ligands) ($Å^2$)	40.3	26.8
Residues in most favored regions of Ramachandran plot (%)	96.0	97.0

Figure S1

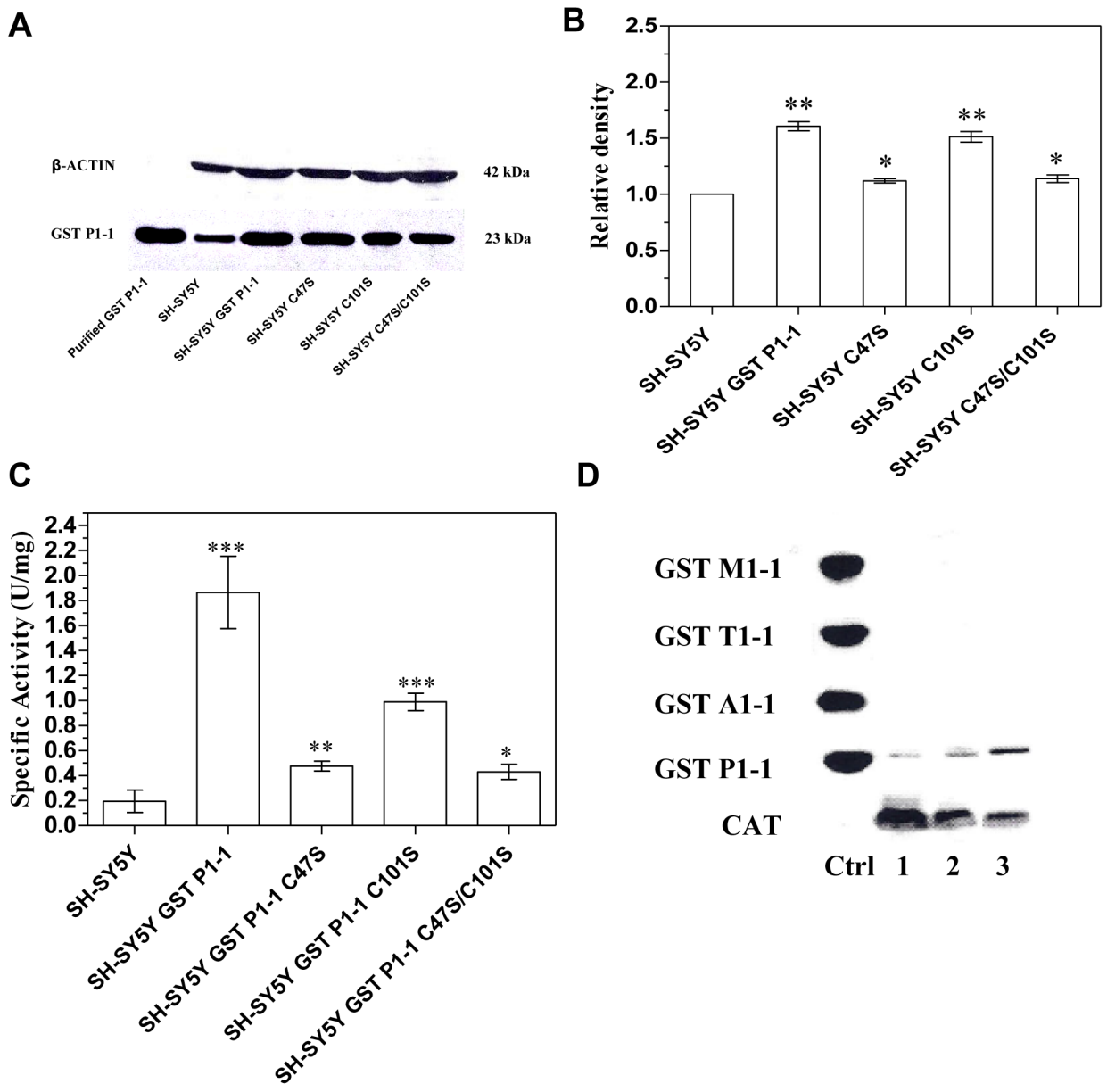

Figure S1. Expression of WT and Cys-mutant GST P1-1 enzymes in SH-SY5Y human neuroblastoma cell line. (**A**) Western blot analysis of GST P1-1 expression in stably transfected SH-SY5Y clones. Each lane was loaded with 40 μg of total proteins except lane 1, which was loaded with 0.5 μg of purified enzyme. β-actin was used as loading control. (**B**) Densitometric analysis of the protein bands obtained by Western blotting. A densitometric analysis using β-actin as control was performed to assess the entity of the signals related to GST P1-1 enzyme. The protein pattern was analyzed through ImageJ software. Values are expressed as means ± standard deviation from 3 independent experiments. Statistical significance: *p<0.05, **p<0.01 as compared

with non-transfected SH-SY5Y cells (Student's t test). (**C**) Enzymatic activity of GST P1-1 in cells overexpressing GST P1-1 WT and C47S, C101S and C47S/C101S mutant enzymes. The enzymatic activity was determined as reported in the Methods section. Values are expressed as means ± standard deviation from 3 independent experiments. Statistical significance: *$p<0.05$, **$p<0.01$, *** $p<0.001$, as compared with non-transfected SH-SY5Y cells (Student's t test). (D) Expression of GSTs A1-1, M1-1, P1-1, T1-1, and catalase (CAT) in human neuroblastoma SH-SY5Y. CTRL: the purified protein of the different classes as positive control; (1) SH-SY5Y pTARGET cells; (2) SH-SY5Y pTARGET GST P1-1 cells.

Figure S2

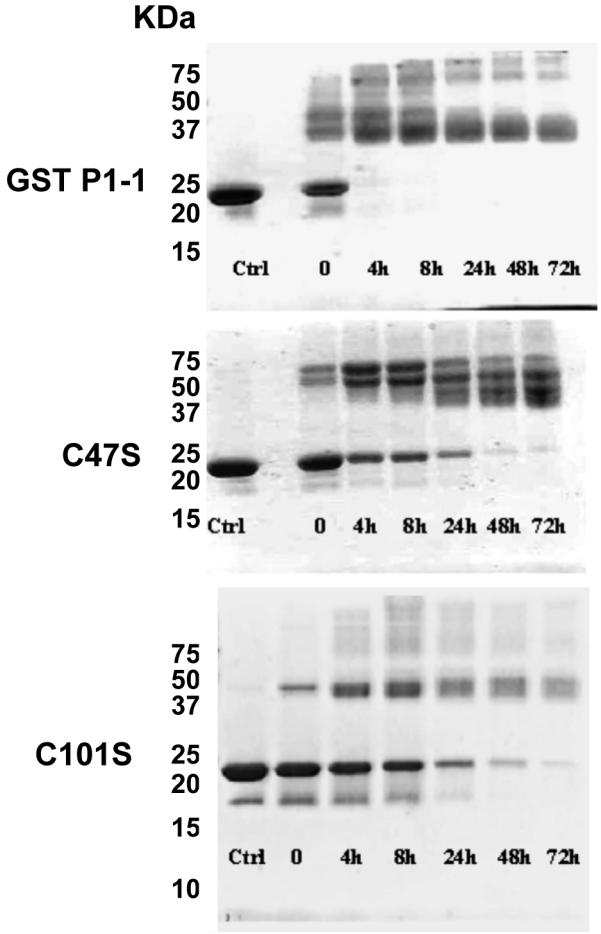

Figure S2. *cis*-DDP induces the complete GST P1-1 crosslinking in absence of GSH. SDS-PAGE analysis, under non-reducing condition, of the GST P1-1, C47S and C101S (50 μM) crosslinking process induced by *cis*-DDP (250 μM) during up to 72 hours incubation at 37°C. As control we used the proteins incubated alone for up to 72 hours.

References

1. Battistoni A*, et al.* (1995) Cytoplasmic and periplasmic production of human placental glutathione transferase in Escherichia coli. *Protein expression and purification* 6(5):579-587.
2. Lo Bello M*, et al.* (1995) Site-directed mutagenesis of human glutathione transferase P1-1. Spectral, kinetic, and structural properties of Cys-47 and Lys-54 mutants. *The Journal of biological chemistry* 270(3):1249-1253.
3. Ishikawa T & Ali-Osman F (1993) Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *The Journal of biological chemistry* 268(27):20116-20125.
4. Kabsch W (2010) Xds. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125-132.
5. Collaborative Computational Project N (1994) The CCP4 Suite: Programs for Protein Crystallography. *Acta Crystallogr D* 50:760-763.
6. McCoy AJ*, et al.* (2007) Phaser crystallographic software. *Journal of applied crystallography* 40(Pt 4):658-674.
7. Oakley AJ*, et al.* (1997) The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. *Journal of molecular biology* 274(1):84-100.
8. Adams PD*, et al.* (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):213-221.
9. Emsley P & Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography* 60(Pt 12 Pt 1):2126-2132.
10. Thorn A & Sheldrick GM (2011) ANODE: anomalous and heavy-atom density calculation. *Journal of applied crystallography* 44(Pt 6):1285-1287.
11. Laskowski RAM, V; Moss, D. S. and Thornton J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Cryst.* 26:283-291.
12. Battye TG, Kontogiannis L, Johnson O, Powell HR, & Leslie AG (2011) iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr D Biol Crystallogr* 67(Pt 4):271-281.