Supplemental Material

A New Nitrobenzoxadiazole-Based GSTP1-1 Inhibitor with a Previously Unheard of Mechanism of Action and High Stability

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MATERIALS AND METHODS

Expression and Purification of GSTP1-1 and TRAF2

GSTP1-1 and TRAF2 were expressed and purified as previously described (1, 2). The protein concentration was determined by measuring the absorbance at 280 nm, and using an extinction coefficient of 17,780 and 25,460 M\(^{-1}\) cm\(^{-1}\) for TRAF2 and GSTP1-1 monomers, respectively.

HPLC evaluation of stability of compounds 1 and 2 in the presence of GSH

General incubation procedure and sample preparation. Compound 1 or 2 (final concentration, 10 μM) was incubated in a medium (final volume, 0.2 mL) containing 0.1 M potassium phosphate (pH 7.4) and 1 mM GSH. Preliminary control incubations were performed in the absence of GSH. Incubations were conducted at 37°C for different time periods, and terminated by adding 10 µL of 20% (p/v) perchloric acid, and 100 µL of ice-cold acetonitrile. Samples were then centrifuged (4°C) at 20,000g for 10 min, and aliquots of the supernatants were analyzed by HPLC with visible absorbance detection, as described below.

HPLC analysis. Analysis were conducted using a Hewlett-Packard series 1100 HPLC system (Agilent Technologies Inc., formerly Hewlett-Packard) equipped with a degasser, a quaternary pump, an autosampler, a column oven, and a UV-visible detector; data were collected and integrated using the Agilent ChemStation software (Rev. A.10.02). Chromatographic conditions were as follows: column, Agilent Zorbax Eclipse XDB-C18 (3.0 x 150 mm, 5 μm; Agilent Technologies Inc.); mobile phase, 10 mM ammonium bicarbonate, pH 6.8/acetonitrile (90:10 v/v; solvent A) and acetonitrile (solvent B); elution program, isocratic elution with 100% solvent A for 2 min, linear gradient from 0 to 70% solvent B in 8 min, followed by an isocratic elution with 70% solvent B for 8 or 14 min (incubations containing compound 1 or 2, respectively); post-run time, 7 min; flow rate, 0.4 mL/min.; injection volume, 30 μL; column temperature, 28°C; detection, absorbance at 433 nm. Under the above conditions, retention times of compounds 1, 2, and GS-NBD were 13.1, 20.5 and 6.3 min, respectively. Stability of compounds 1 and 2, expressed as percent of compound remaining, was calculated by comparing the corresponding chromatographic peak area at each time point relative to that at time 0 min.

Protein-ligand docking

The protein-ligand docking technique was applied as previously reported (3), with some variations here described, by using the AutoDock program, via AutoDockTool (4). The structure of 2 was initially built using the Chimera program (5) and then processed in AutoDockTools assigning partial
charges using the Gasteiger method. All suitable ligand dihedrals were allowed to rotate. The GSTP1-1 receptor structure was built starting from the PDB file 3GUS, removing water and the co-crystallized inhibitor (6), while preserving the bound GSH. A large grid volume (18.50 × 18.50 × 26.25 Å), centered in the GSTP1-1 active site, was used to allow the ligand to sample the most favorable conformations. Each Lamarckian Genetic algorithm job consisted of 250 runs and the initial population was 150 structures. To enhance sampling ten runs were executed independently, each starting from a different random seed, and the results were merged. Grid spacing (0.375 Å) and other parameters were set to default values. The final 2500 structures were clustered and ranked according to the most favorable docking energy. The distribution of these clusters spans from -7.8 to -3.8 kcal/mole in binding energy. Most of these clusters represent poses that are similar to those found for 1, thus lining the H-binding site of GST. However a family of clusters reports a different binding mode for compound 2 (see figure 6); binding energy of this cluster spans from -7.0 to -5.0 kcal/mole. It should be noted that energetically, although this is not the most favorite pose, we cannot exclude that the docking experiments are in some way biased by the crystal conformation of the receptor, i.e. the complex with 1, that may tend to over-represent ligand conformations close to the crystallized one. Figure 6 has been generated using the PyMol (7) graphic program

**Normal mode analysis**

The protein starting structure was used as input in the WebNMA server that computes low frequency normal modes from a static structure in order to predict large amplitude movements connected with the protein function (8). Figure S2 has been generated using the VMD (9) graphic program
Figure S1. HPLC evaluation of the stability of compounds 1 and 2 in the presence of GSH.

Compound 1 or 2 (final concentration, 10 μM) was incubated with 1 mM GSH in 0.1 M potassium phosphate (pH 7.4) at 37°C, for 0, 5, 10, 15, or 30 min, after which each sample was analyzed by HPLC as described under the Methods Section. (A) Time-disappearance curves of compounds 1 and 2. Data are expressed as percent of compound remaining at each time compared with time 0, and represent the mean ± SD of 3 independent determinations; the SD bars are smaller than the circles denoting the mean. Representative stacked HPLC traces of supernatants from mixtures containing GSH and compound 2 (B) or 1 (C).
Figure S2. Normal mode analysis. Trace representation of the protein backbone is colored from red to blue showing the movements predicted by the normal mode analysis server WebNMA. The position of Tyr49 is shown as a labeled bead. For reference the position of GSH in the starting structure is shown in spacefill representation colored by atom type.
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