Supplementary information

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Shp2 protein tyrosine phosphatase inhibitor activity of estramustine phosphate and its triterpenoid analogs

Materials and methods.

Chemical library screening. NCI Approved Oncology Drug set and NIH Clinical Collection chemical libraries were obtained from the NCI Developmental Therapeutics Program and screened with Shp2 PTP at 20 μM. Compounds were classified as hits upon inhibition of Shp2-mediated DiFMUP hydrolysis greater than 50%. Estramustine phosphate used for characterization was purchased from Sigma-Aldrich (St. Louis, MO); 17-β-Estradiol was purchased from Tocris Bioscience (Ellisville, MO); celastrol from Cayman Chemical (Ann Arbor, MI); enoxolone and estramustine from AK Scientific (San Diego, CA); estradiol phosphate (NSC18312), NSC233064, and maitenin (NSC608781) were obtained from the Development Therapeutics Program of the NCI/NIH.

***In vitro* phosphatase activity assay.** Recombinant Shp2, Shp1, PTP1B, and HePTP proteins were prepared as previously described.[1, 2] PTP activity was assayed using the fluorogenic 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP; Invitrogen, Carlsbad, CA) as the substrate similar to that described previously.[1-3] Briefly, each reaction contained 50 mM Bis-Tris, pH 7.0, 50 mM NaCl, 0.01% Triton X-100, 1 mM dithiothreitol, 1mM EDTA, 40 μM DiFMUP, 100 nM PTP, and 10 μl of test compound prepared in 20% dimethyl sulfoxide (DMSO) in a total reaction volume of 75 μl in black 96-well plates (Fisher Scientific, Pittsburgh, PA). The reactions were initiated by addition of PTP, and the incubation time was 15 min at room temperature.

For IC_{50} determination, eight concentrations of each inhibitor at one-third dilution (~0.5 log) were tested (300-0 μM). Unless noted, each experiment was performed in triplicate and IC_{50} data were derived from at least three independent experiments. IC_{50} was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity normalized to the control PTP activity as determined by the equation: $Y=Ymax/(1+(X/IC_{50})^{Hillslope}$ using the GraphPad Prism program.

For kinetics analysis, the Shp2 PTP activity was measured for 30 minutes at 2-minute intervals over varying combinations of five estramustine phosphate and DiFMUP concentrations. Enzyme velocity was calculated as the slope of linear regression fit to DiFMU product formation by Shp2 over time. Enzyme velocity versus substrate concentration at each inhibitor concentration examined was plotted and competitive, uncompetitive and mixed model inhibition fits using the Levenberg-Marquart algorithm with constant weighting were determined using the VisualEnzymics program (Softzymics, Princeton, NJ). The statistically best model fit was calculated using ANOVA and F-test statistics in VisualEnzymics. Data presented were from two experiments performed in duplicate.

Surface Plasmon Resonance (SPR) Assay of Direct Binding. SPR analysis of direct binding of estramustine phosphate to the Shp2 PTP was carried out as previously reported[2] with the following modifications: the His-Shp2 protein used contained amino acids 237-529 of the Shp2 PTP domain, 50 mM NaCl was used in the running buffer, and no DMSO was included in the running buffer as estramustine phosphate was soluble in aqueous buffer at the concentrations examined.

For K_D determination, six concentrations of estramustine phosphate at one-half dilution were tested (25-0 μM). Two separate experiments were performed on independently immobilized

CM5 chip surfaces in duplicate and k_d/k_a data reported were derived from these independent experiments.

Computer docking. Schrödinger's Maestro 9.1 was used as the primary graphical user interface (GUI). Schrödinger's LigPrep 2.41 was used for molecule preparation for docking. During that process, LigPrep was instructed to generate tautomers, ionization states, and stereoisomers. Schrödinger's Protein Preparation workflow was used in the preparation of the protein structure. Schrödinger's GLIDE 5.6 was used for the generation of grid files and docking. PyMol 0.99rc6 (DeLano Scientific L.L.C.), was used for graphical presentation of the results in the figures.

Structure 3B7O from the protein databank (www.rcsb.org) was used for the protein structure of the catalytic region of Shp2 and 1GWZ was used for the catalytic site of Shp1. In order to prepare the protein for use in virtual screening, the pdb file was analyzed with Schrödinger's Prime application to correct any missing residues due to incomplete electron density. The corrected structure was then imported into Maestro. Water molecules were removed, hydrogens were added, and the protein was then prepared for use in docking studies by processing it with Schrödinger's protein preparation workflow. This preparation process minimizes the protein's potential energy gradient to an RMSD of 0.30 using the OPLS-2005 force field. For all docking studies we employed Schrödinger's GLIDE 5.6 SP using the default settings with the exception of expanded sampling. The GLIDE 5.6 docking scores (GScores) provide estimates of relative binding free energies for each docked structure. Further analysis was based on hydrogen bond formation potential and interaction with the electrostatic potential at the protein surface. Also considered were the relative depths of the binding pockets and exposure of the small molecules to solvent.

References

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