Supplementary Materials for

Small molecule inhibition of GDC-0449 refractory Smoothened mutants and downstream mechanisms of drug resistance

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Running title: Mechanisms of resistance to a Hedgehog pathway inhibitor

Supplemental Materials and Methods

Gli-luciferase reporter assays. C3H10T$^1/_2$ cells (ATCC, CCL-226) were seeded into six-well plates at 1.85 x 10^5 cells/well in DMEM High Glucose with 4 mM glutamine, 10 mM Hepes pH 7.2 and 10% FBS as described (9). Cells were transfected 16 h later with 400 ng of SMO expression construct, 400 ng of the Hh luciferase reporter Gli-BS and 200 ng of pRL-TK per well using GeneJuice Transfection Reagent (Novagen, 70967). After 6 h, cells were trypsinized and replated into four wells of a 12-well plate. The FBS content of the culture medium was reduced to 0.5% the following morning to induce formation of primary cilia and small molecule Hh inhibitors were added at indicated concentrations. Luciferase activity was determined 48 h later with the Dual-Glo Luciferase Assay System (Promega, E2940) using an Envision 2103 Multilabel Reader (Perkin Elmer). Values were divided by *Renilla* luciferase activities to normalize for transfection efficiency and are shown as the mean of four replicates ± standard deviation. Dose response data were fit to a 4-parameter equation [Y=100/(1+10ˆ((X-LogIC_{50})))], where Y=Gli-luciferase signal calculated as a percent of control that did not include inhibitor, and X=inhibitor concentration.

FACS analysis of SMO mutants. 5x10^5 C3H10T$^1/_2$ cells were seeded into 10 cm plates and transfected 6 h later with 3 ug of SMO expression construct and 18 ul of GeneJuice (Novagen). Cells were dislodged 48 h later with 1 mM EDTA in PBS and sequentially incubated for 30 min with anti-SMO antibody (2D11, at 1 μg/ml), followed by 20 min incubations with 1:100 biotin-SP-conjugated Affinipure goat anti-mouse IgG and 1:50 R-Phycoerythrin-conjugated Streptavidin (both Jackson Immunoresearch Labs). Cells were resuspended in propidium iodide (500 ng/ml) and analyzed on a HTS FacsCalibur (BD Biosciences). The percent SMO-positive cells were normalized to SMO-WT controls.

Compounds. GDC-0449 and compounds 1-5 were prepared as described in WO2006028956, WO2006078283, and WO2007059157**.** SAG was prepared according to (15). HhAntag was obtained from Curis, Inc. (Cambridge, MA), cyclopamine and KAAD-cyclopamine were from Toronto Research Chemicals, Inc. (North York, ON) and SANT-1 was from Tocris Bioscience (Ellisville, MO).

Mouse pharmacokinetic studies. Mouse pharmacokinetic studies with compounds 4 and 5 were essentially performed as described for GDC-0449 (41). Briefly, 24 female CD-1 mice weighing 25 to 33g at study initiation were given a single oral 100 mg kg^{-1} dose of either compound 4 or 5 as a suspension in 0.5% methylcellulose with 0.2% Tween-80 (MCT). Blood samples (n=3 mice per time point) were collected in tubes containing EDTA as an anticoagulent by terminal cardiac puncture under isoflurane at the following time points: 5, 15 and 30 min; and 1, 3, 6, 9 and 24 h post-dose. Blood samples were centrifuged to collect plasma, which was stored at -80°C until drug concentrations were quantified by LC/MS/MS.

RNA isolation and qRT-PCR. Total RNA was extracted from tumors using the RNeasy Mini Kit (Qiagen 74106) and qRT-PCR was carried out with 100 ng RNA on an Applied Biosystems 7500 theromocycler. Expression levels were normalized to *Rpl19* and are presented as normalized gene expression values ($2^{-\Delta Ct}$). A TaqMan gene expression assay for *Gli1* was purchased from Applied Biosystems, for which the probe (Assay ID: Mm00494646_g1) spanned the exon 3-4 boundary. The primer and probe sequences for *Rpl19* are F: 5'-AGAAGGTGACCTGGATGAGA-3', R: 5'-TGATACATATGGCGGTCAATCT-3' and P: 5'-CTTCTCAGGAGATACCGGGAATCCAAG-3'.

SMO immunostaining. S12 cells (42) were plated to confluency and serum-starved for 16 h ± 200 ng/ml octyl-Shh in the presence of saturating compound levels (5μM for cyclopamine, 1μM for the others). Cells were then fixed in 100% methanol, stained with anti-SMO (5928B, a rabbit pAb raised against the C-terminal tail of mouse SMO (43) and detected with Cy3-anti-rabbit [Jackson Immunoresearch]) as well as anti-acetylated and anti-gamma tubulins (mAbs 6-11B-1 and GTU88 respectively [both SIGMA], co-detected with FITC-anti-mouse [Jackson Immunoresearch]) and imaged as described (43). At least 200 cilia from three or more independent experiments were analyzed for robust SMO staining all along the cilium shaft (excluding those with weak signals or staining confined to the cilium base).

Immunoblotting. Frozen tumor samples were lysed in either M-PER Mammalian Protein Extraction Reagent (Pierce) or Cell Extraction Buffer (Invitrogen), both supplemented with protease and phosphatase inhibitors. Lysates were separated on 4-

12% Bis-Tris gels and proteins were transferred onto PVDF membranes with an iBlot (Invitrogen). Blots were blocked and incubated overnight at 4°C with 5% milk containing one of the following primary antibodies; anti-cyclin D1 (Cell Signaling, #2922), anti-phospho(Ser473)-AKT (Cell Signaling, #9271), anti-total AKT (Cell Signaling #9272), anti-phospo(Ser235/236)-S6 (Cell Signaling, #2211), anti-total S6 (Cell Signaling #2317), anti-GLI2 (44), or anti-Actin (Santa Cruz Biotechnology, sc-47778), followed by a 1 h incubation at RT with appropriate HRP-conjugated secondary antibodies. Antigen-antibody interactions were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce) on an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Supplemental References

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Supplemental Figure legends

Supplemental Figure 1. GDC-0449 inhibition and cell surface expression of various SMO-D473 mutants. *A*, as in Fig. 1A, but with various other amino acid substitutions at position 473. *B*, *Gli*-luciferase reporter activity of CH310T$^{1}/_{2}$ cells transfected with SMO-WT (black squares) or SMO-D473V (green triangles) following a dose response of GDC-0449. SMO-D473V is partially resistant to this HPI with an approximately 20-fold higher IC_{50}. *C*, relative cell surface expression of several SMO-D473 mutants. *D*, FACS profiles used to calculate the relative cell surface expression of the SMO-D473 mutants in *C*.

Supplemental Figure 2. Tumor volume traces and group fits of the HPI study presented in Fig. 4A. Tumor volume traces on the natural scale (mm^3) from individual mice with data present up to the last day of measurement are colored in grey, while traces from mice with missing data on the last day are colored in red. Group fits are generated from the linear mixed effects analysis of log2-transformed volume data over time (solid black lines), with the group fit of vehicle treated mice (dashed blue lines) being superimposed for comparison purposes.

Supplemental Figure 3. SMO localization in S12 cells treated with either KAAD-Cyclopamine or HhAntag in the absence or presence of Shh. As in Fig. 4C, but with other compounds.

Supplemental Figure 4. Summary of copy number variations across (A) chromosome 7 in model SG102 and (B) chromosome 1 in model SG152. Log2 ratio is plotted on the y-axis and chromosomal location is plotted on the x-axis, in relationship to the ideogram. Outer green and red lines indicate pre-defined thresholds as described in Materials and Methods. The yellow line represents the moving average of neighboring probes on the chromosome.

Supplemental Figure 5. Tumor volume traces and group fits of the PI3K inhibition study presented in Fig. 6B. As in Supplemental Figure 2, but with the PI3K inhibitor GDC-0941.

Supplemental Figure 6. PI3K inhibition moderately reduces cyclin D1 levels in SG152 and SG274 allografts**.** Immunoblots showing cyclin D1 and Actin (loading control) protein levels in expanded tumors of the four models (*n*=3/group) following a 6-hour treatment with either vehicle (Veh) or GDC-0941 (941).