Supplementary Data

Supplementary Methods

Hh reporter assay

Human epithelial kidney HEK293 cells were stably transfected with either wild type pRK5-*Smo*^{WT}, or with the Smo-binding site mutant pRK5-*Smo*^{D473H} construct. The cells were then transiently transfected with an 8X-*Gli1* binding site firefly luciferase reporter, and a Renilla luciferase construct as normalization for transfection efficiency, using Fugene6 transfection reagent (Roche). The FBS content of the culture medium was reduced to 0.5% the following morning to induce formation of primary cilia, and the cells were exposed to either HPI-1, or the prototype Hh inhibitor, cyclopamine, for a period of 24 hours. The Hh reporter activity was determined 24 hours later using the Dual-Glo Luciferase Assay System (Promega). The values were expressed as Relative Luciferase Units (RLU), and represent the mean and standard deviation of three independent experiments.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Treated human xenograft or murine allograft samples were homogenized with brief ultrasound sonication (Sonic Dismembrator Model 100, Fisher Scientific, Baltimore, MD) and RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA). RNA was reverse transcribed with oligo-dT primers at 42°C for 50 minutes

using the SuperScript™ First Strand System (Invitrogen), according to manufacturer's recommendations. Thereafter, qRT-PCR for murine *Gli1* (Mm*Gli1*), Mm*Akna*, Mm*Cltb* and Mm*Olig2* transcripts was performed, using Assay-By-Design TaqMan probes on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Each reaction was carried out in triplicate. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with Mm*Actin-β* used as a housekeeping control. In the pancreatic cancer study, we also examined the relative expression of murine *Nestin* (Mm*Nestin*), a marker of stromal endothelial cells, as well as human *HES1* and *CMYC*, two non-canonical Hh readout targets, following NanoHHI administration in the treated xenografts.

Immunohistochemistry for aldehyde dehydrogenase (ALDH)

ALDH immunohistochemistry was performed on formalin-fixed paraffin-embedded xenografts from the control and three treatments arms, as previously described (1, 2).

Statistical analysis

Two-tailed *t*-test and Mann-Whitney-U-test were performed using Prism (GraphPad Software Inc., San Diego, CA, USA) version 5.01. A value of $P<0.05$ was regarded as statistically significant. Pharmacokinetic parameters were summarized using descriptive statistics. All data are presented as mean values with standard error (SE), unless indicated otherwise.

Supplementary Figure Legends

Supplementary Figure 1: Pharmacokinetic analyses of NanoHHI and free HPI-1 following various routes of administration, and absence of demonstrable toxicity with NanoHHI in allografted mice.

(A) Pharmacokinetic disposition of NanoHHI administered intravenously as a single dose (30 mg/kg). The experiments were performed in non-tumor bearing CD1 mice, with four mice per arm. The comparable data for free HPI-1 in 40% ethanolic solution is not shown, as the mice succumbed almost immediately following tail-vein injection, likely due to excipient toxicity. Pharmacokinetic parameters are tabulated in Supplementary Table 1.

(B) Pharmacokinetic disposition of NanoHHI administered orally as a single dose (30 mg/kg) compared to that of free HPI-1 (30 mg/kg) suspended in corn oil. The experiments were performed in non-tumor bearing CD1 mice, with four mice per cohort. Plasma HPI-1 levels were assessed using LC-MS/MS. Pharmacokinetic parameters are tabulated in Supplementary Table 1.

(C) Brain distribution of HPI-1 was obtained at two independent time points following intravenous administration of NanoHHI at 30mg/kg dose in two arms, three mice per arm. HPI-1 levels found to be 3.9±2.1 µg/g after 10 minutes, and 1.4±0.4µg/g at 30 minutes after injection.

(D) Body weight measurements were obtained at three time points in mice bearing subcutaneous Smo^{WT} (left) or Smo^{D477G} (right) allografts that were treated with vehicle, HhAntag or NanoHHI over a two week time period. In both

the study groups no significant body weight losses are encountered in NanoHHI arm, whereas mice administered HhAntag lost ~10% of their baseline body weight.

Supplementary Figure 2: Pharmacodynamic analyses of epithelial and stromal gene expression following NanoHHI treatment in pancreatic cancer

(A) NanoHHI therapy has no significant effects on the expression of Hs*CMYC* (*left*) in orthotopic Pa03C xenografts, while gemcitabine, as single agent or in combination results in significant downregulation (P<0.001). NanoHHI significantly downregulates Hs*HES1* expression in both single agent and combination therapy arms (P<0.01), while single agent gemcitabine has no significant effect. Each qRT-PCR experiment was performed in triplicate, and the relative fold changes are depicted as mean $\pm$ S.E.

(**B**) NanoHHI significantly downregulates Mm*Nestin* (i.e., stromal *Nestin*) expression in both single agent and combination therapy arms (P<0.001). Gemcitabine has a somewhat more modest, albeit significant effect (P<0.01).

Supplementary Figure 3: Absence of demonstrable biochemical and hematological toxicity in mice receiving NanoHHI.

Several hematological and biochemical parameters were examined in terminal blood samples obtained from vehicle versus NanoHHI treated mice, including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), platelet count (PLT), blood urea nitrogen (BUN) and alanine aminotransferase

(ALT); no significant differences were observed in any of the measured parameters.

Supplementary Table 1: Pharmacokinetic analyses of NanoHHI and free HPI-1 following various routes of administration

Pharmacokinetic parameters including C_{max}, T_{max}, $AUC_{0-\infty}$ and $T_{1/2}$, following administration via different routes of administration are tabulated. The values are reported as the mean ± standard deviation except for T_{max} which is reported as median (range). Abbreviations: AUC, area under the concentration-time curve; C_{max}, maximal plasma concentration; T_{max}, time of the maximal plasma concentration; $T_{1/2,}$ terminal half-life.

Acknowledgements: This research was supported by U54CA151838 (AM), and the Flight Attendants Medical Research Institute (AM, SRK, MAR and CMR). This research was also supported by the Analytical Pharmacology Core of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (NIH grants P30 CA006973 and UL1 RR025005) and the Shared Instrument Grant (S10 RR026824). This publication was made possible by Grant Number UL1 RR 025005 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. We thank Ping He and Teresia Wanjiku for technical assistance.

Supplementary References

1. Feldmann G, Fendrich V, McGovern K, Bedja D, Bisht S, Alvarez H, et al. An orally bioavailable small-molecule inhibitor of Hedgehog signaling inhibits tumor initiation and metastasis in pancreatic cancer. Mol Cancer Ther. 2008;7:2725-35.

2. Feldmann G, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, et al. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer Res. 2007;67:2187-96.