

## Fully Human Anti-Hedgehog Antibodies

Novel neutralizing hedgehog antibody MEDI-5304 exhibits antitumor activity by inhibiting  
paracrine hedgehog signaling.

### Authors:

Neil R. Michaud<sup>1\*</sup>, Youzhen Wang<sup>1</sup>, Kristen McEachern<sup>1</sup>, Jerold J. Jordan<sup>1</sup>, Anne Marie  
Mazzola<sup>1</sup>, Axel Hernandez<sup>1</sup>, Sanjoo Jalla<sup>2</sup>, Jon W. Chesebrough<sup>2</sup>, Mark J. Hynes<sup>3</sup>, Matthew  
Belmonte<sup>1</sup>, Lidong Wang<sup>3</sup>, Jaspal S. Kang<sup>4#</sup>, Jelena Jovanović<sup>5</sup>, Naomi Laing<sup>1</sup>, David W.  
Jenkins<sup>1</sup>, Elaine Hurt<sup>2</sup>, Meina Liang<sup>6</sup>, Christopher Frantz<sup>7</sup>, Robert E. Hollingsworth<sup>2</sup>, Diane  
M. Simeone<sup>3</sup>, David C. Blakey<sup>8</sup>, and Vahe Bedian<sup>1\*</sup>

Authors' affiliation: <sup>1</sup>Oncology iMED, AstraZeneca-R&D Boston, Waltham, MA <sup>2</sup>Oncology  
Research, MedImmune LLC, Gaithersburg, MD <sup>3</sup>Translational Oncology Program, University of  
Michigan, Ann Arbor, MI; <sup>4</sup>Amgen British Columbia, Burnaby, BC, Canada <sup>5</sup>Lead Generation-  
Research, Medimmune LLC, Granta Park, Cambridge, UK <sup>6</sup>Clinical Pharmacology and DMPK,  
MedImmune LLC, Hayward, CA <sup>7</sup>Biologics Safety Assessment, MedImmune LLC, Mountain View,  
CA <sup>8</sup>Oncology iMed, AstraZeneca, Alderley Park, Macclesfield. UK

<sup>#</sup>Current address: Paul Kang, CSO, Innovative Targeting Solutions Inc., Burnaby BC, Canada

### \*corresponding authors:

Neil R. Michaud, Ph.D.  
Oncology iMED  
AstraZeneca-R&D Boston  
Waltham, MA 02451  
e-mail: [nrmichaud706@gmail.com](mailto:nrmichaud706@gmail.com)

Vahe Bedian Ph.D.  
Oncology iMED  
AstraZeneca-R&D Boston  
Waltham, MA 02451  
phone: 781-839-4613  
e-mail: [vahe.bedian@astrazeneca.com](mailto:vahe.bedian@astrazeneca.com)

Running title: Fully Human Anti-Hedgehog Antibodies

Key Words: Hedgehog, Antibody, Xenograft, XenoMouse, cancer stem cells

Grant Support: This work was funded by AstraZeneca PLC and MedImmune LLC.

Disclosure of Potential Conflicts of Interest: There are no potential conflicts of interest.

## Fully Human Anti-Hedgehog Antibodies

### Abstract

The Hedgehog pathway has been implicated in the tumorigenesis, tumor progression and metastasis of numerous human cancers. We generated the first fully human hedgehog antibody MEDI-5304 and characterized its antitumor activity and preclinical toxicology. MEDI-5304 bound Sonic and Indian hedgehog with low picomolar affinity and neutralized SHH and IHH activity in cellular mGLI1 reporter assays. The antibody inhibited transcription of hedgehog target genes and osteoblast differentiation of C3H10T1/2 cells. We evaluated the activity of MEDI-5304 *in vivo* in model systems that allowed us to evaluate two primary hypotheses of hedgehog function in human cancer, paracrine signaling between tumor and stromal cells and cancer stem cell (CSC) self-renewal. MEDI-5304 displayed robust pharmacodynamic effects in stromal cells that translated to antitumor efficacy as a single agent in an HT-29/MEF co-implantation model of paracrine hedgehog signaling. MEDI-5304 also improved responses to carboplatin in the HT-29/MEF model. The antibody, however, had no effect as a single agent or in combination with gemcitabine on the cancer stem cell frequency or growth of several primary pancreatic cancer explant models. These findings support the conclusion that hedgehog contributes to tumor biology via paracrine tumor-stromal signaling but not via cancer stem cell maintenance or propagation. Finally, the only safety finding associated with MEDI-5304 was odontodysplasia in rats. Thus, MEDI-5304 represents a potent dual hedgehog inhibitor suitable for continued development to evaluate efficacy and safety in human patients with tumors harboring elevated levels of Sonic or Indian hedgehog.

## Fully Human Anti-Hedgehog Antibodies

### **Introduction**

Hedgehog was identified in *Drosophila* as a mediator of embryonic patterning. There are three members of the hedgehog family in mammals, Sonic (SHH), Indian (IHH), and Desert hedgehog (DHH), which specify tissue patterning and regulate organ homeostasis by affecting cell growth and differentiation (1,2). The hedgehog proteins are ligands for the twelve-pass membrane spanning receptor Patched (PTCH), which normally represses the function of the GPCR-like transmembrane protein Smoothed (SMO) (3). Upon binding by hedgehog ligands, repression of SMO by PTCH is relieved, allowing SMO to signal through members of the Gli family of transcription factors. Activation of transcription leads to the synthesis of pathway components such as GLI1 and PTCH1, which in turn drives proliferation and survival.

Dysregulation of the hedgehog/SMO pathway is implicated in tumorigenesis. Mutations that inactivate PTCH or constitutively activate SMO have been described in basal cell carcinoma, medulloblastoma and rhabdomyosarcoma (4). Over-expression of SHH or IHH has been described in numerous tumor types (5-9), and concomitant upregulation of GLI1 and PTCH1 has been observed (9-11). Ectopic over-expression of SHH in preclinical models can induce tumor formation, including basal cell carcinomas and pancreatic neoplasia (11,12), supporting the view that pathway activation promotes tumorigenesis in a ligand-dependent manner.

Dependence of tumors on HH/SMO signaling for growth and metastasis has been demonstrated with various pathway inhibitors. The natural product cyclopamine and its derivative saridegib (IPI-926) are reported to directly antagonize SMO and inhibit the growth

## Fully Human Anti-Hedgehog Antibodies

of numerous tumor cell lines *in vitro* and *in vivo* either alone or in combination with chemotherapy (13-16). However, a challenge with using cyclopamine-related molecules is the potential for off-target effects in tumor cells when used at elevated concentrations, confounding whether observed biological effects can be attributed to inhibiting HH/SMO function. Yet other more selective inhibitors have implicated hedgehog in tumor growth. The SMO antagonist vismodegib, structurally unrelated to cyclopamine, impaired the growth of a medulloblastoma tumor model driven by mutation of *PTCH1* and human xenograft tumors over-expressing SHH (8,17). Vismodegib has shown clinical activity in patients with basal cell carcinoma and medulloblastoma and was recently approved for the treatment of metastatic and locally advanced basal cell carcinoma (18,19). The mouse monoclonal antibody 5E1 has been used extensively as a tool to probe hedgehog biology by specifically blocking ligand-dependent pathway activation (20). 5E1 neutralized the GLI1 reporter in cells, which correlated with inhibition of primary tumor cell proliferation *in vitro* (6,10), and it attenuated the growth of colorectal tumor xenografts and basal-like mouse mammary tumors *in vivo* (8,9).

Several models have been proposed to explain how over-expression of hedgehog ligands may lead to the development of cancer (4). In the first model, autocrine signaling stimulates tumor cell proliferation. In the second model, paracrine signaling by SHH secreted by tumor cells activates SMO signaling in stromal cells, which in turn results in the production of factors that enhance tumor cell growth. Finally, SHH function has been implicated in self-renewal of cancer stem cells in various tumor types (21). As such, SHH may promote tumorigenesis or relapse following treatment with chemotherapy and/or radiation by driving cancer stem cell self-renewal and propagation.

## Fully Human Anti-Hedgehog Antibodies

We describe here the isolation of two human antibodies, 6D7 and 3H8, with high affinity but differential selectivity for hedgehog proteins. 6D7 neutralizes SHH and IHH signaling and biological activity in osteoblasts, whereas 3H8 neutralizes only SHH activity. 6D7 was renamed MEDI-5304 and used to evaluate the paracrine signaling and the cancer stem cell hypotheses, because it represents a potent and selective agent to probe hedgehog biology in preclinical models. MEDI-5304 demonstrated pharmacodynamic activity and corresponding antitumor activity in an *in vivo* tumor cell-mouse embryonic fibroblast (HT-29/MEF) co-implantation model of paracrine hedgehog signaling and combination efficacy with carboplatin. Yet, MEDI-5304 had no effect on cancer stem cells in primary pancreatic tumor explant models *in vivo* or derived tumorspheres. Finally, there were no drug-related toxicities for MEDI-5304 in exploratory toxicology studies performed in rat and cynomolgus monkeys, except for mild odontodysplasia of rat incisors, a finding that does not translate to humans. These findings suggest targeting the hedgehog pathway with a neutralizing antibody represents a promising therapeutic approach for human cancers in which over-expression of hedgehog ligands play a prominent role.

## Materials and Methods

### Cell lines and Primary Human Samples

Colo205 and HT-29 tumor cell lines and the C3H10T1/2 mouse mesenchymal cell line were obtained from American Type Culture Collection (ATCC). These cell lines were cultured according to ATCC instructions and were tested and authenticated by the AstraZeneca cell bank using DNA fingerprinting short-tandem repeat (STR) assays. All revived cell lines were used within 20 passages for a period of less than 6 months. Mouse embryonic fibroblasts (MEFs) from C57BL/6 mice treated with mitomycin C were obtained from Global

## Fully Human Anti-Hedgehog Antibodies

Stem. Pancreatic adenocarcinoma tissue samples were obtained from patients who underwent pancreatic resection at University of Michigan Medical Center from 2010–2011 using IRB-approved guidelines. The pancreatic tumor tissue was used to develop low passage xenografts in NOD-SCID mice as we have described (22).

***In vitro* hedgehog binding ELISA** Microtiter plates coated with 200 ng recombinant hSHH C24II or h/mIHH C28II (R&D Systems #1845-SH-025/CF and 1705-HH/CF) in PBS and subsequently blocked with 1% BSA (Sigma #3156) in PBS for 1 h RT were incubated with serially diluted primary antibodies 6D7 and 3H8 in TBS-T for 16 hours on a shaker at 4°C. After washing in TBS-T, secondary goat anti-human IgG H+L antibody (KPL #074-1006) diluted 1:50,000 in TBS-T was incubated 1 h RT. RLU were determined in a Spectromax 5e luminometer after incubation (10 min) with peroxidase substrate (Sigma #CPS-2-60).

**Binding kinetics determined with surface plasmon resonance (SPR)** SPR experiments were performed on a Biacore T100 biosensor (GE Healthcare, Piscataway, NJ, USA) at 25°C. 150 RU of protein G' (Sigma-Aldrich) was covalently immobilized on the surface of the C1 chip using standard amine coupling chemistry, according to manufacturer's instructions (BIAapplications Handbook, GE Healthcare). The same procedure was used to immobilize 150 RU of 5E1 mouse IgG1 directly on the C1 surface. The first flow cell was left empty and served as a reference surface following EDC/NHS activation and ethanolamine deactivation.

Low density surfaces with  $\leq 100$  RU of each antibody were generated to minimize mass transport effects. Recombinant Hedgehog proteins run in HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.0005% surfactant P20 from GE Healthcare) supplemented with 200  $\mu$ g/ml recombinant human albumin (Sigma) were used as analytes. A titration series of analyte was

## Fully Human Anti-Hedgehog Antibodies

injected for 5 min at 50  $\mu$ l/min followed by 15 min of dissociation. Interaction surfaces were regenerated with 10 mM glycine pH 1.75.

Sensorgrams were analysed using the Biacore T100 evaluation software, version 2.0.1 (GE Healthcare). All sensorgrams were double referenced. The average response of all blank injections was subtracted from all sensorgrams to remove systematic artifacts in the experimental and reference flow cells. The kinetic parameters were derived by fitting the data with a simple 1:1 bimolecular binding model that included a term for mass transport.

**NIH 3T3 mGLI1 Reporter Assay** NIH 3T3 cells engineered with a luciferase reporter driven by eight copies of the GLI1 binding site in the promoter were plated (75,000 cells/well) in media containing 0.5% FBS. Cells were treated with 1  $\mu$ g/ml recombinant hSHH C24II or h/mIHH C28II alone or with serially diluted antibodies for 24 hours in media containing 0.5% FBS. RLU were determined in a luminometer (Tecan Infinite M200) after 10 min incubation RT with firefly luciferase reagent (Promega).

**Osteoblast Differentiation Assay** C3H10T1/2 cells were plated (5000 cells/well in 96-well plates or 3000 cells/well in 384-well plates). Recombinant hedgehog ligands were mixed with serially diluted hedgehog antibodies and incubated with cells for 72 h. Cells were lysed in RIPA cell lysis buffer (Thermo Scientific) and assayed for alkaline phosphatase (AP) activity using the chromogenic substrate p-nitrophenylphosphate (Sigma) at 1 mg/ml, pH 9.8 and absorbance at 405 nm was measured.

**Quantitative RT-PCR** Quantitative RT-PCR was used to determine target modulation in C3H10T1/2 cells, xenograft tumors and skin samples. RNA was isolated from homogenized

## Fully Human Anti-Hedgehog Antibodies

snap-frozen tumor or skin tissue or from cultured cell lines using TRIzol (Invitrogen) or RNeasy (Qiagen), and cDNA was made from RNA using the QuantiTect Reverse Transcription System kit (Qiagen) or Cells-to-Ct kit (Invitrogen) according to the manufacturer's instructions. Quantitative PCR for hedgehog pathway genes and HPRT (Applied Biosystems) was done using TaqMan gene expression assays (Applied Biosystems) employing species-specific primer-probe sets according to the manufacturer's specifications. Modulation of hedgehog target and pathway was determined using the  $2^{-\Delta\Delta Ct}$  method normalizing expression to that of human or mouse HPRT1.

**Pharmacokinetics and pharmacodynamic effects of MEDI-5304** Female NCr nude (athymic) mice were injected subcutaneously in the right flank with  $4 \times 10^6$  Colo205 cells. When the tumors reached an average of  $\sim 400 \text{ mm}^3$ , the mice were randomized into control and treatment groups by stratified sampling (n=5 animals per group). Control groups received a single i.p injection of 0.9% sodium chloride. Treatment groups were given a single i.p. injection of MEDI-5304 diluted in 0.9% sodium chloride. The animals were humanely euthanized and samples were collected at various times post dose administration. Whole blood was collected by cardiac puncture. Serum was prepared and frozen at  $-20^\circ\text{C}$  for pharmacokinetic analysis. The tumor from each animal was resected, cut into small pieces, and stored in RNA stabilization buffer for pharmacodynamic analysis.

**Determination of serum MEDI-504 levels in mouse and rat.** Concentrations of MEDI-5304 in mouse and rat serum were determined using a qualified ELISA method. ELISA plates were coated with 500 ng goat anti-human IgG (Fc specific, Thermo Scientific, cat# 31125) in PBS overnight at  $4^\circ\text{C}$  and blocked with I-Block Buffer (Tropix, Bedford, MA) 1 h



## Fully Human Anti-Hedgehog Antibodies

RT. MEDI-5304 reference-standard, quality-control (QC) and test sample dilutions were prepared in 10% mouse or rat serum (assay matrix) and added to blocked plates for 2 h RT. Washed plates were incubated with diluted (1:20000) secondary HRP-goat anti human IgG (Fc-specific, Thermo Scientific, cat# 31413) 1 h RT. TMB substrate was incubated 5 min and color development was stopped with 2M H<sub>2</sub>SO<sub>4</sub>. Plates were read at 450 nm with a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Concentrations of MEDI-5304 in QC and test-sample dilutions on each plate were interpolated from the reference-standard curve for that plate. The range of the assay for quantification of MEDI5304 in 100% rat serum was 31.25 to 2000 ng/mL.

**Pancreatic tumorsphere assays** Tumorspheres assays were performed using human pancreatic tumor cells from two patient derived xenografts (P479, 947). Briefly, 1000-3000 cells from P479 tumors digested and dissociated into a single cell suspension were cultured with different treatment conditions in tumorsphere medium containing 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), 20 ng/ml bFGF-2 (Invitrogen) and 20 ng/ml EGF (Invitrogen) in 6-well ultralow attachment plates (Corning) for 4 days. All cultures were analyzed for tumorsphere formation by counting using an inverted microscope or by measuring total cell viability using Cell Titer-Glo Luminescent Cell Viability Assay (Promega Biosciences, Inc., San Luis Obispo, CA) according to the manufacturer's recommendations. Luminescence was measured using an Envision plate reader with an integration time of 1 s/well.

***In vivo* efficacy experiments** Experiments performed using the HT-29/mouse embryonic fibroblast (MEF) co-implantation model were conducted at AstraZeneca-R&D Boston according to IACUC guidelines as described in Hwang et al. (23).

## Fully Human Anti-Hedgehog Antibodies

Human Panc479 xenografts (passage 4-5) were maintained as a serially passaged xenograft model using female Rag2 KO mice (Taconic). Donor tumors were excised, cut into fragments, and placed into the right flank of each mouse with 11G trochars. MEDI-5304 diluted in PBS or gemcitabine (Gemzar®, Eli Lilly) diluted in 0.9% saline were administered by i.p. injection twice per week according to body weight (10mL/kg) into mice once mean tumor volume had reached ~130 mm<sup>3</sup>. Tumors were harvested 24 h post last dose, weighed, and preserved in Cryostor (BioLife Solutions) on ice before being stored at -80°C until CSC analysis. Tumor volumes and body weight measurements were recorded twice a week.

Experiments performed by orthotopic implantation of primary pancreatic explant models 890 and 947 were done using eight-week-old male NOD-SCID mice that were anesthetized using an i.p. injection of 100 mg/kg ketamine and 5 mg/kg xylazine. Single cell suspensions of patient derived pancreatic cancer cells were made with serum-free RPMI/Matrigel (BD Bioscience, San Jose, CA) mixture (1:1 volume), infected with luciferase-expressing retrovirus and  $5 \times 10^5$  cells injected into the pancreatic tail as previously described (22). Animals were either untreated, or treated with MEDI-5304 and gemcitabine (Gemzar®, Eli Lilly), either alone or in some cases in combination, administered by intraperitoneal (i.p.) injection at the indicated dose levels twice per week according to body weight (10 mL/kg). Tumor measurements of orthotopically implanted tumors were performed weekly using bioluminescent imaging. After 4 weeks of treatment, some tumors were harvested 24 hours post last dose and digested for CSC analysis as described (22). General health of mice was monitored daily and all studies done at MedImmune and the University of Michigan were performed in accordance with IACUC guidelines.

## Fully Human Anti-Hedgehog Antibodies

Additional experimental protocols describing the exploratory rat and cynomolgus monkey toxicology studies including pharmacokinetic and pharmacodynamic analyses are provided in the Supplementary Materials and Methods.

## Results

### Isolation of Hedgehog antibodies and characterization of their binding properties

XenoMice™ (24) were immunized with recombinant protein corresponding to the N-terminal signaling domain of Sonic hedgehog (R&D Systems) or Chinese Hamster Ovary cells transiently transfected with full length human SHH to generate specific neutralizing monoclonal antibodies. Hybridomas producing antibodies capable of binding SHH were identified using an ELISA measuring antibody binding to immobilized SHH and binding to native human SHH on the surface of 293T cells transfected with full length human SHH cDNA. 808 hits were identified and advanced into NIH3T3-Gli reporter assays. Forty hybridomas were chosen from this group for further scale-up and analysis, of which sixteen were successfully cloned and sequenced, representing twelve independent lineages.

We identified two lead antibodies, 6D7 and 3H8, that differed in their binding specificity to mammalian hedgehog proteins. 6D7 bound both Sonic and Indian Hedgehog in ELISAs, whereas 3H8 bound only SHH (Fig 1). Neither antibody bound Desert hedgehog. Binding kinetics for the antibodies was determined using surface plasmon resonance (SPR). 6D7 bound human and mouse SHH, which differ by only one amino acid, with low picomolar affinities (Table 1 and Supplementary Fig S1), but it displayed reduced affinity for IHH. The affinities of 3H8 for human and mouse SHH were 6-7 fold lower than the affinities observed for 6D7. The differences in the binding specificity of 6D7 and 3H8 suggest they could have

## Fully Human Anti-Hedgehog Antibodies

differential biological activity depending on the expression pattern of hedgehog ligands in a given biological system. The profile and affinity of 6D7 and 3H8 binding to hedgehog proteins also differ from those seen with 5E1. Both novel antibodies bind SHH with higher affinity than 5E1, and 6D7 displays higher affinity binding to IHH than 5E1 (Table 1). 5E1 is reported to bind DHH, albeit with lower affinity (25), whereas neither 6D7 nor 3H8 interact with DHH.

### **Anti-Hedgehog antibodies block mGLI1 reporter activation and inhibit osteogenesis of mouse C3H10T1/2 cells**

We examined the effect of the antibodies on hedgehog signaling and function. Hedgehog ligands activate a programmed transcriptional response controlled by the transcription factor GLI1 (26). Stimulation of mouse mesenchymal cells expressing a functional Smoothed signaling cascade with hedgehog ligands results in the activation of a GLI1 reporter. Recombinant SHH or IHH stimulated the mGLI1 luciferase reporter in stable NIH 3T3 cells in a dose dependent manner (Supplementary Fig S2A), though SHH showed greater maximal activity than IHH. 6D7 effectively inhibited reporter activation by either SHH or IHH, as did 5E1, whereas 3H8 interfered with only SHH-dependent reporter activation (Fig 2A,B).

Although numerous tumor cell lines express SHH and IHH, they typically do not respond to autocrine or paracrine ligand stimulation in culture because of inactivation of functional Smoothed signaling (8,27), though the mechanism by which this occurs is unknown. Some cell lines do retain the ability to respond to exogenous hedgehog ligands, including mesenchymal C3H10T1/2 cells which differentiate into osteoblasts and express the osteogenesis marker alkaline phosphatase (AP) when treated with SHH or IHH (28,29). SHH consistently displayed greater osteogenic activity than IHH (Supplementary Fig S2B). 6D7

## Fully Human Anti-Hedgehog Antibodies

inhibited induction of osteoblast differentiation of C3H10T1/2 cells by either SHH or IHH in a dose-dependent manner, yet 3H8 blocked differentiation due to SHH but not IHH (Fig 2C,D). Transcriptional targets of hedgehog signaling are elevated coincident with the phenotypic effects in target cells stimulated with exogenous hedgehog ligands (28,29). SHH and IHH induce dramatic increases in GLI1 and PTC1 RNA levels in C3H10T1/2 cells that occur in a time-dependent manner over 72 hours (not shown), though the cells were more responsive to SHH treatment and the effects on mGLI1 levels by either ligand were larger than for PTC1. The neutralizing effects of 6D7 and 3H8 on ligand-dependent induction of mGLI1 and mPTC1 RNA mirrored their phenotypic effects (Fig 2E,F and Supplementary Fig S3A,B). 5E1 exhibited similar neutralizing activity to 6D7 in C3H10T1/2 cells (Fig 2C,D and Supplementary Fig S3C). The potency of each of the antibodies observed in the reporter assay and C3H10T1/2 cell systems was low ( $IC_{50} \sim 10\text{-}15$  nM) given their picomolar affinities for the target. However, at the high ligand concentrations ( $\geq 30$  nM) required to see biological responses in these systems, the binding of the antibody is stoichiometrically rather than  $K_D$ -controlled which would explain why the potencies do not reflect affinity values determined with SPR.

We chose to focus on characterizing the preclinical antitumor efficacy of 6D7 *in vivo* in models of paracrine hedgehog signaling and CSC self-renewal because of its higher affinity and broader selectivity profile. 6D7 was renamed MEDI-5304 and is referenced as such in the remainder of the paper.

### **Pharmacokinetics and pharmacodynamics of MEDI-5304**

It was important to determine whether MEDI-534 demonstrates biological activity *in vivo* and what doses and levels of circulating antibody are required for activity. Secreted human

## Fully Human Anti-Hedgehog Antibodies

hedgehog ligands stimulate expression of hedgehog target genes in mouse stromal cells but not in the tumor cells of human tumor xenografts (8,23,30). We employed the Colo205 colon tumor model, which expresses SHH when grown as a xenograft, to ascertain whether MEDI-5304 inhibits paracrine hedgehog signaling *in vivo*. Administration of MEDI-5304 effectively inhibited hedgehog-dependent transcription in the stroma but had no effect on GLI1 RNA levels in Colo205 tumor cells, as determined with species-specific quantitative RT-PCR (TaqMan) primers (Supplementary Fig S4A). The differential effect of antibody treatment on tumor and stromal GLI1 RNA levels indicates the antibody's effects are specific. We determined the effect of varying doses of MEDI-5304 on mouse GLI1 and PTC1 RNA levels in Colo205 xenografts 24 h post-dosing. Doses of 1 mg/kg and greater decreased mGLI1 levels by ~90% and mPTC1 levels by about 75% (Fig 3A), but lower doses had no effect. MEDI-5304 and 5E1 exhibited similar pharmacodynamic effects at doses of 1 mg/kg and above (Supplementary Fig S4B).

The pharmacokinetic-pharmacodynamic (PK/PD) relationship for MEDI-5304 was examined over a 14 day period following an acute 1 mg/kg bolus i.p. dose. Circulating levels of MEDI-5304 reached a maximum of nearly 8 µg/ml at 4 hours and decayed over the next several days to undetectable levels by 7 days post dose administration (Fig 3B). Pharmacodynamic effects on mGLI1 and mPTC1 RNA emerged within 4-6 hours after dosing and reached a maximum effect level at 24 hours, which was maintained through at least 72 hours. By 7 days when circulating antibody was no longer detected, mGLI1 and mPTC1 RNA levels were returning to normal levels. This PK/PD experiment showed that MEDI-5304 exerts extended inhibitory effects on hedgehog signaling in xenograft tumors, and that twice weekly dosing at 1 mg/kg should be sufficient to maintain target coverage.

## Fully Human Anti-Hedgehog Antibodies

### **MEDI-5304 efficacy in an *in vivo* model of paracrine hedgehog signaling**

MEDI-5304 is a candidate therapeutic agent well suited to assess the importance of hedgehog function in *in vivo* efficacy models representing distinct mechanistic hypotheses because of its potency and selectivity. Yauch *et al.* (8) have argued that paracrine hedgehog signaling is the primary mechanism by which hedgehog proteins influence tumor growth. We evaluated the antitumor activity of MEDI-5304 in their *in vivo* co-implantation model using HT-29 colon carcinoma cells and mouse embryonic fibroblasts (MEFs). Establishment and growth of subcutaneous HT-29 xenograft tumors are dependent on MEF co-implantation when suboptimal numbers of HT-29 cells are implanted. Based on PK/PD findings, we expected MEDI-5304 to have biological activity at 1 mg/kg and above. The growth of tumors treated with doses of MEDI-5304  $\geq 1$  mg/kg was inhibited in a statistically significant manner ( $P < 0.05$ ) (Fig 4A), whereas the 0.1 mg/kg dose was not efficacious. 5E1 administered at 10 mg/kg displayed virtually identical antitumor activity as 10 mg/kg MEDI-5304 (Fig 4A). All doses of MEDI-5304 and 5E1 were well tolerated. The effects of MEDI-5304 on RNA levels of several stromal hedgehog target genes were evaluated in samples collected 24 hours after the last dose. Dramatic inhibition of each target gene transcript was observed at each dose level of MEDI-5304 and 5E1 that resulted in tumor growth inhibition (Fig 4B). The lack of effects on mGLI3 and mSMO indicated that the pharmacodynamic effects were specific for transcriptionally regulated hedgehog pathway targets.

We extended the PD analysis to the same set of hedgehog target genes in mouse skin samples collected during the efficacy study to understand whether MEDI-5304 activity could be detected in other tissues as a potential biomarker. Hedgehog signaling occurs in normal epidermis and modulation of hedgehog target genes by Smoothed antagonists in mice and human patients demonstrates skin can be used as a surrogate tissue to assess PD effects of

## Fully Human Anti-Hedgehog Antibodies

hedgehog inhibitors (31-33). Significant inhibitory effects of MEDI-5304 were limited to mGLI1 at doses of 1 mg/kg and above (Fig 4C), which corresponds to the doses at which MEDI-5304 showed activity in xenograft tumors (Fig 4B). Trends of dose-dependent activity were apparent for mGLI2, mPTC1, mPTC2, mHHIP, but they did not reach statistical significance. 5E1 displayed similar PD effects. The explanation for the lower activity of MEDI-5304 in mouse skin than in xenograft tumors is unclear, but one possibility is that MEDI-5304 did not distribute as effectively into skin as tumors, which can be more highly vascularized. Nonetheless, significant decreases in mGLI1 suggested the pharmacodynamics of MEDI-5304 could be assessed in skin from animals used in safety studies.

Current therapy for colon cancer includes the chemotherapeutic regimen FOLFOX (Folinic acid [leucovorin], Fluorouracil [5-FU], and Oxaliplatin). We evaluated whether treatment of HT-29/MEF tumors with MEDI-5304 exhibited greater efficacy in combination with carboplatin, which we used as a surrogate for the oxaliplatin component of FOLFOX. The combination of MEDI-5304 with carboplatin significantly inhibited tumor growth compared to control antibody, and the effect of combination treatment was significantly different ( $P < 0.05$ ) than inhibition seen with MEDI-5304 or carboplatin alone (Fig 4D). These results suggest that blockade of paracrine hedgehog signaling could improve the efficacy of chemotherapy regimen that include platin agents in patients with colon tumors.

### **MEDI-5304 does not alter the prevalence of cancer stem cells in primary pancreatic tumor explants *in vivo***

In addition to paracrine signaling, hedgehog proteins are implicated in the biology of cancer stem cells (CSCs) in pancreatic adenocarcinoma. We used MEDI-5304 to investigate the hedgehog dependence of pancreatic CSCs by assessing its effects *in vivo* on tumor growth



## Fully Human Anti-Hedgehog Antibodies

and CSC numbers of three primary pancreatic explant models implanted either subcutaneously (P479) or orthotopically (890 and 947). The CSCs from each model expressed elevated SHH RNA (Supplementary Fig S5A), as seen previously (22). Each model exhibited sensitivity to gemcitabine (Fig 5A), a chemotherapeutic agent commonly used to treat patients with pancreatic cancer, but MEDI-5304 did not display single agent activity or enhance the activity of gemcitabine in any of the models (Fig 5A). Gemcitabine increased the CSC population in each model (Fig 5B), as has been reported previously (34,35), yet MEDI-5304 had no effect on CSC frequency and did not enhance the effect of gemcitabine on CSC numbers for any of the tumors studied (Fig 5B). Biological activity of MEDI-5304 in each model was confirmed, as it decreased stromal mGLI1 levels by >90% (data not shown).

We evaluated whether use of MEDI-5304 in a maintenance setting would improve responses to gemcitabine by impairing CSCs. We treated P479 tumors with gemcitabine to enrich for CSCs and subsequently administered MEDI-5304 alone or in combination with gemcitabine. Administration of MEDI-5304 following gemcitabine treatment resulted in rapid tumor regrowth, and addition of MEDI-5304 to gemcitabine did not improve responses (Fig 5C). Gemcitabine-containing treatment arms were discontinued on day 53 to allow tumor outgrowth, but prior MEDI-5304 treatment had no inhibitory effect on regrowth. Finally, MEDI-5304 lacked activity in tumorsphere assays using dissociated cells from two different patient tumors, whereas the anti-CSC compound salinomycin (36) blocked tumorsphere formation (Figs 5D and Supplementary Fig S5B). These results indicate that MEDI-5304 lacked activity against pancreatic CSCs *in vitro* and *in vivo* and was unable to affect corresponding tumorsphere or tumor growth of these models, supporting the conclusion that hedgehog function is not essential for pancreatic CSC maintenance or self-renewal.

## Fully Human Anti-Hedgehog Antibodies

### **Exploratory toxicology in rats and cynomolgus monkeys**

We performed a two week pilot toxicity study with MEDI-5304 in rats during which animals received vehicle, 10 or 50 mg/kg MEDI-5304 on day 0 and day 7, and the subjects were subsequently necropsied on day 14. Doses were chosen based on a preliminary single dose rat PK study, which indicated a trend of an antigen sink that is saturated at MEDI-5304 doses of greater than 10 mg/kg (Supplementary Fig S6). MEDI-5304 did not affect appearance, behavior, body weights, hematology, coagulation, or clinical chemistry parameters.

Microscopic evaluation of tissues showed the only drug-related finding was odontodysplasia noted in the incisor teeth in the 50 mg/kg group. The change was characterized by loss of odontoblast cell layer polarity accompanied by deposits of dentin-like material (Supplementary Fig S7), a finding consistent with disruption of SHH function in the tooth germ (37).

In addition, a non-terminal safety study was conducted in cynomolgus monkeys to evaluate PK/PD and toxicity of MEDI-5304. Animals received either PBS or MEDI-5304 i.v. at an initial single low dose of either 0.3, 1, or 3 mg/kg followed two weeks later by escalated doses of 3, 10, or 75 mg/kg antibody qwk x 3wks. MEDI-5304 levels in serum were within the expected range for a human IgG in cynomolgus monkey, with a trend toward an antigen sink saturated at 1 mg/kg (Supplementary Fig S8). There were no changes in clinical signs, body weights, or clinical pathology parameters, including Troponin I, Troponin T, or creatine kinase isozymes. However, PD effects were observed in skin punch biopsies taken on day four. GLI1, PTCH1, and PTCH2 RNA levels were decreased (Supplementary Fig S9) at all doses, similar to observations in mouse skin samples (Fig 4C). The results of the pre-clinical rat and cynomolgus toxicology studies support the preliminary conclusion that MEDI-5304

## Fully Human Anti-Hedgehog Antibodies

likely has a sufficient safety margin to allow testing the clinical hypothesis that neutralizing hedgehog function with MEDI-5304 in tumors with elevated Sonic or Indian hedgehog will be efficacious.

### **Discussion**

We describe here the isolation of two novel neutralizing hedgehog monoclonal antibodies with differential selectivity for the three mammalian isoforms of hedgehog. 6D7 (MEDI-5304) binds Sonic and Indian hedgehog with high affinity and inhibits their activity in cellular reporter assays and functional assays of osteoblast differentiation. 3H8 is selective for SHH, inhibiting its activity in reporter and osteogenesis assays, but it exhibits slightly lower binding affinity than 6D7. MEDI-5304 (6D7) is biologically active *in vivo*, demonstrating pharmacodynamic effects on hedgehog signaling in stromal cells at doses as low as 1 mg/kg. MEDI-5304 displayed antitumor activity in the HT-29/MEF co-implantation model when administered as a single agent and in combination with carboplatin. MEDI-5304 exhibited higher affinity binding to SHH and IHH than mouse mAb 5E1, but MEDI-5304 and 5E1 displayed similar activity in cellular mechanistic and phenotypic assays and in *in vivo* PD and efficacy studies. These findings may be explained by preliminary competition binding studies indicating MEDI-5304 and 5E1 do not cross-compete for binding to SHH and as such bind different epitopes. (data not shown). Finally, the only signal attributed to MEDI-5304 in preclinical rat and cynomolgus primate safety studies was mechanism-based odontodysplasia of rat incisors at the 50 mg/kg dose. These findings indicate MEDI-5304 represents an attractive candidate to continue clinical development in patients with tumors resulting from elevated Sonic or Indian hedgehog.

## Fully Human Anti-Hedgehog Antibodies

Initial observations linking the hedgehog pathway to tumorigenesis and tumor growth suggested autocrine signaling (5-7,38). However, it has been difficult to model autocrine effects on tumor cells because the pathway appears to be inactivated shortly after initial model propagation *in vitro* and *in vivo* (27). Additionally, paracrine hedgehog signaling has been described in primary patient samples. Coincident expression of SHH in tumor cells and GLI1 in the stroma has been reported in invasive breast, pancreatic, and metastatic liver tumors (8,9,39,40). Recent experimental evidence supports a paracrine signaling model whereby hedgehog ligands produced by tumor cells exert biological effects on stromal cells which then in turn promote tumor growth and metastasis (8,40). Potential stromal target cells include myofibroblasts and perivascular stromal cells (23,30).

We employed the potent and selective hedgehog pathway inhibitor MEDI-5304 to assess the paracrine hedgehog signaling hypothesis. Co-implantation of MEFs, myofibroblasts or human pancreatic stellate cells (HPSCs) has been used to support tumor establishment and growth of colon and pancreatic xenograft tumor models (8,23). MEDI-5304 inhibited the growth of the HT-29/MEF coimplant model, which corresponded to pharmacodynamic effects on hedgehog target genes in the stroma but not in tumor cells. Similar results were seen with the SMO inhibitor AZD8542 (23). MEDI-5304 had no effect on the growth of HT-29 xenografts implanted without MEFs (data not shown). Similarly, BxPC3 pancreatic xenograft tumor growth was sensitive to disruption of HH/SMO signaling due to AZD8542 treatment, but only when co-implanted with HPSCs (23). Thus, the antitumor activity of MEDI-5304 in the HT-29/MEF co-implant model supports the hypothesis that paracrine hedgehog signaling can promote tumor formation and growth.

## Fully Human Anti-Hedgehog Antibodies

Hedgehog function is implicated in cancer stem cell biology (21). SHH and IHH RNA expression is elevated in CSCs of multiple primary pancreatic tumor explant models [(22) and Supplementary Fig S5A]. Pancreatic tumorspheres, which are enriched in cells with stem-cell like properties, have increased levels of hedgehog pathways components (41). Treatment of cell lines and tumorspheres *in vitro* with SMO antagonists cyclopamine and IPI-926 reduced the number of CSCs defined as ALDH+ or CD133+, and cyclopamine inhibited Panc-1 tumorsphere growth (16,41-43). However, the anti-proliferative and apoptotic effects of cyclopamine and related compounds may be off-target as they are observed broadly in tumor cells in which active ligand-dependent hedgehog signaling is not evident. These effects may be the result of neutral sphingomyelin phosphodiesterase 3 (nSMase2) activity and ceramide production rather than inhibition of hedgehog signaling (44). These findings led us to evaluate whether hedgehog signaling is required for CSC self-renewal or maintenance in primary pancreatic cancer explant models using the highly selective and potent antibody MEDI-5304. MEDI-5304 had no effect on tumorspheres from two independent primary models, and it did not alter CSC frequency or inhibit tumor growth of three primary pancreatic cancer xenograft models when used as a single agent, indicating ligand-dependent hedgehog pathway activity does not regulate CSCs numbers in primary models of pancreatic cancer.

Cancer stem cells are often insensitive to chemotherapeutic agents, resulting in accumulation of CSCs following treatment with such agents (21). Gemcitabine treatment of primary pancreatic models results in CSC enrichment (34,35), and gemcitabine-resistant pancreatic cells express elevated levels of pancreatic CSC markers (45,46). This evidence suggests simultaneous or sequential combination treatment with chemotherapy and agents that kill cancer stem cells will lead to more effective antitumor responses and prevent development of

## Fully Human Anti-Hedgehog Antibodies

resistance, as has been reported for combination treatment of primary colon xenografts with DLL4 antibodies and irinotecan (47). It has been argued that blocking hedgehog signaling with cyclopamine sensitizes pancreatic CSCs to chemotherapy (41,46). For example, treatment of primary pancreatic model Panc185 with gemcitabine alone resulted in tumor stasis, whereas administration of cyclopamine and gemcitabine caused tumor regression (48). Because of concerns that cyclopamine can act on pathways other than the hedgehog pathway, we asked whether treating primary pancreatic tumor models with gemcitabine to kill differentiated cells but spare CSCs in combination with the specific neutralizing antibody MEDI-5304 would lead to synergistic responses *in vivo*.

We observed that gemcitabine treatment caused accumulation of CSCs in the three pancreatic models we evaluated, which coincided with tumor growth inhibition (Fig 5A,B). However, addition of MEDI-5304 to gemcitabine therapy did not modulate its effects on CSC prevalence or tumor growth *in vivo*. Further, exploration of sequential and combination dosing of gemcitabine and MEDI-5304 in the P479 model indicated that MEDI-5304 did not improve responses to gemcitabine under conditions where gemcitabine treatment would have enriched for CSCs and presumably rendered tumors more dependent on this cell population for growth (Fig 5C). These results indicate the hedgehog pathway is not essential for CSC function in pancreatic cancer. However, pancreatic CSC populations defined by markers other than ESA/CD44/CD24 may differentially depend on hedgehog function. Our results also do not preclude the possibility that MEDI-5304 may inhibit tumor growth, metastasis or development of resistance by impairing CSCs in other tumor types. Nonetheless, our findings support the paracrine hedgehog signaling hypothesis, suggesting tumors that depend on paracrine hedgehog signaling between tumor cells expressing hedgehog ligands and responsive stromal cells are most likely to respond to MEDI-5304.

## Fully Human Anti-Hedgehog Antibodies

Several Smoothed antagonists have advanced to clinical trials and the first of those agents, vismodegib, has been approved for the treatment of locally advanced and metastatic BCC (18,49). Vismodegib has also displayed clinical activity in medulloblastoma (19). The clinical activity of vismodegib in these indications is observed in patients with tumors harboring mutations in *Patched* or *Smoothed* that constitutively activate the pathway. However, results from Phase 2 clinical trials with vismodegib or sarigedib in disease indications where over-expression of hedgehog ligands rather than mutational activation of the pathway is observed have been less encouraging (49). These outcomes suggest alternative approaches to inhibiting hedgehog signaling, such as neutralizing hedgehog antibodies, may be required for improved responses in these settings.

In summary, we describe high affinity fully human antibodies to Sonic and Indian hedgehog that demonstrated neutralizing activity *in vitro* and *in vivo*. The dual hedgehog inhibitor MEDI-5304 exhibited robust PD effects and antitumor activity in a xenograft model of paracrine hedgehog signaling. As such, MEDI-5304 is an attractive clinical candidate for the treatment of hedgehog pathway-dependent tumors resulting from elevated hedgehog proteins.

**Acknowledgments** We would like to thank Christine Pien, Shenghua Wen, Hong Lu, Ling Du, Ling Fu, Patrick Strout, Zach Brohawn, and Ying Li for technical assistance. We also thank Jin Gao, Yong Chang, and Alex Cao for productive discussions during the course of this work, and Stephen Scott, Lily Cheng, and Mark Mense for contributions to the toxicology studies.

## Fully Human Anti-Hedgehog Antibodies

### References

1. Borzillo GV, Lippa B. The hedgehog signaling pathway as a target for anticancer drug discovery. *Curr Top Med Chem* 2005;5:147-57.
2. Varjosalo M, Taipale J. Hedgehog: Functions and mechanisms. *Genes Dev* 2008;22:2454-72.
3. Evangelista M, Tian H, de Sauvage FJ. The hedgehog signaling pathway in cancer. *Clin Cancer Res* 2006;12:5924-8.
4. Teglund S, Toftgard R. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta* 2010;1805:181-208.
5. Kar S, Deb M, Sengupta D, Shilpi A, Bhutia SK, Patra SK. Intricacies of hedgehog signaling pathways: A perspective in tumorigenesis. *Exp Cell Res* 2012;318:1959-72.
6. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, et al. Widespread requirement for hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 2003;425:846-51.
7. Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* 2004;431:707-12.
8. Yauch RL, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, et al. A paracrine requirement for hedgehog signalling in cancer. *Nature* 2008;455:406-10.
9. O'Toole SA, Machalek DA, Shearer RF, Millar EK, Nair R, Schofield P, et al. Hedgehog overexpression is associated with stromal interactions and predicts for poor outcome in breast cancer. *Cancer Res* 2011;71:4002-14.



## Fully Human Anti-Hedgehog Antibodies

10. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 2003;422:313-7.
11. Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003;425:851-6.
12. Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH, Jr, Scott MP. Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science* 1997;276:817-21.
13. Lee MJ, Hatton BA, Villavicencio EH, Khanna PC, Friedman SD, Ditzler S, et al. Hedgehog pathway inhibitor saridegib (IPI-926) increases lifespan in a mouse medulloblastoma model. *Proc Natl Acad Sci U S A* 2012;109:7859-64.
14. Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009;324:1457-61.
15. McCann CK, Growdon WB, Kulkarni-Datar K, Curley MD, Friel AM, Proctor JL, et al. Inhibition of hedgehog signaling antagonizes serous ovarian cancer growth in a primary xenograft model. *PLoS One* 2011;6:e28077.
16. 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.

## Fully Human Anti-Hedgehog Antibodies

17. Romer JT, Kimura H, Magdaleno S, Sasai K, Fuller C, Baines H, et al. Suppression of the shh pathway using a small molecule inhibitor eliminates medulloblastoma in Ptc1(+/-)p53(-/-) mice. *Cancer Cell* 2004;6:229-40.
18. Sekulic A, Migden MR, Oro AE, Dirix L, Lewis KD, Hainsworth JD, et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *N Engl J Med* 2012;366:2171-9.
19. Rudin CM, Hann CL, Lattera J, Yauch RL, Callahan CA, Fu L, et al. Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. *N Engl J Med* 2009;361:1173-8.
20. Beachy PA, Hymowitz SG, Lazarus RA, Leahy DJ, Siebold C. Interactions between Hedgehog proteins and their binding partners come into view. *Genes Dev* 2010;24:2001-12.
21. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: Challenges and opportunities for anticancer drug discovery. *Nat Rev Drug Discov* 2009;8:806-23.
22. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. *Cancer Res* 2007;67:1030-7.
23. Hwang RF, Moore TT, Hattersley MM, Scarpitti M, Yang B, Devereaux E, et al. Inhibition of the hedgehog pathway targets the tumor-associated stroma in pancreatic cancer. *Mol Cancer Res* 2012;10:1147-57.
24. Green LL. Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. *J Immunol Methods* 1999;231:11-23.

## Fully Human Anti-Hedgehog Antibodies

25. Maun HR, Wen X, Lingel A, de Sauvage FJ, Lazarus RA, Scales SJ, et al. Hedgehog pathway antagonist 5E1 binds hedgehog at the pseudo-active site. *J Biol Chem* 2010;285:26570-80.
26. Ruiz i Altaba A, Mas C, Stecca B. The gli code: An information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol* 2007;17:438-47.
27. Sasai K, Romer JT, Lee Y, Finkelstein D, Fuller C, McKinnon PJ, et al. Shh pathway activity is down-regulated in cultured medulloblastoma cells: Implications for preclinical studies. *Cancer Res* 2006;66:4215-22.
28. Nakamura T, Aikawa T, Iwamoto-Enomoto M, Iwamoto M, Higuchi Y, Pacifici M, et al. Induction of osteogenic differentiation by hedgehog proteins. *Biochem Biophys Res Commun* 1997;237:465-9.
29. Williams KP, Rayhorn P, Chi-Rosso G, Garber EA, Strauch KL, Horan GS, et al. Functional antagonists of sonic hedgehog reveal the importance of the N terminus for activity. *J Cell Sci* 1999;112:4405-14.
30. Chen W, Tang T, Eastham-Anderson J, Dunlap D, Aliche B, Nannini M, et al. Canonical hedgehog signaling augments tumor angiogenesis by induction of VEGF-A in stromal perivascular cells. *Proc Natl Acad Sci U S A* 2011;108:9589-94.
31. Ikram MS, Neill GW, Regl G, Eichberger T, Frischauf AM, Aberger F, et al. GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *J Invest Dermatol* 2004;122:1503-9.
32. Rohner A, Spilker ME, Lam JL, Pascual B, Bartkowski D, Li QJ, et al. Effective targeting of hedgehog signaling in a medulloblastoma model with PF-5274857, a potent and selective

## Fully Human Anti-Hedgehog Antibodies

smoothened antagonist that penetrates the blood-brain barrier. *Mol Cancer Ther* 2012;11:57-65.

33. LoRusso PM, Rudin CM, Reddy JC, Tibes R, Weiss GJ, Borad MJ, et al. Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. *Clin Cancer Res* 2011;17:2502-11.

34. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007;1:313-23.

35. Bednar F, Simeone DM. Pancreatic cancer stem cell biology and its therapeutic implications. *J Gastroenterol* 2011;46:1345-52.

36. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 2009;138:645-59.

37. Cobourne MT, Sharpe PT. Sonic hedgehog signaling and the developing tooth. *Curr Top Dev Biol* 2005;65:255-87.

38. Varnat F, Duquet A, Malerba M, Zbinden M, Mas C, Gervaz P, et al. Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Mol Med* 2009;1:338-51.

39. Bailey JM, Swanson BJ, Hamada T, Eggers JP, Singh PK, Caffery T, et al. Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin Cancer Res* 2008;14:5995-6004.

## Fully Human Anti-Hedgehog Antibodies

40. Tian H, Callahan CA, DuPree KJ, Darbonne WC, Ahn CP, Scales SJ, et al. Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proc Natl Acad Sci U S A* 2009;106:4254-9.
41. Huang FT, Zhuan-Sun YX, Zhuang YY, Wei SL, Tang J, Chen WB, et al. Inhibition of hedgehog signaling depresses self-renewal of pancreatic cancer stem cells and reverses chemoresistance. *Int J Oncol* 2012;41:1707-14.
42. 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.
43. Mueller MT, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, et al. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. *Gastroenterology* 2009;137:1102-13.
44. Meyers-Needham M, Lewis JA, Gencer S, Sentelle RD, Saddoughi SA, et al. Off-target function of the Sonic hedgehog inhibitor cyclopamine in mediating apoptosis via nitric oxide-dependent neutral sphingomyelinase 2/ceramide induction. *Mol Cancer Ther* 2012;11:1092-1102.
45. Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. *Int J Cancer* 2009;125:2323-31.
46. Yao J, An Y, Wie JS, Ji ZL, Lu ZP, Wu JL, et al. Cyclopamine reverts acquired chemoresistance and down-regulates cancer stem cell markers in pancreatic cancer cell lines. *Swiss Med Wkly* 2011;141:w13208.

## Fully Human Anti-Hedgehog Antibodies

47. Hoey T, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, Fitch-Bruhns M, et al. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 2009;5:168-77.

48. Jimeno A, Feldmann G, Suarez-Gauthier A, Rasheed Z, Solomon A, Zou GM, et al. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. *Mol Cancer Ther* 2009;8:310-4.

49. McMillan R, Matsui W. Molecular pathways: The hedgehog signaling pathway in cancer. *Clin Cancer Res* 2012;18:4883-8.

Fully Human Anti-Hedgehog Antibodies

**Table 1. Binding properties of anti-hedgehog antibodies**

	Interaction	$k_{on}$ ( $M^{-1} s^{-1}$ ) ( $\times 10^7$ )	$k_{off}$ ( $s^{-1}$ ) ( $\times 10^{-4}$ )	$K_D$ ( $k_{off}/k_{on}$ ) ( $\mu M$ )
6D7 IgG1	human Shh	$2.21 \pm 0.85$	$1.11 \pm 0.36$	$5.13 \pm 1.04$
	mouse Shh	$2.39 \pm 0.46$	$2.92 \pm 0.45$	$12.6 \pm 4.31$
	human/mouse Ihh	$3.15 \pm 0.90$	$10.9 \pm 2.93$	$34.7 \pm 0.07$
3H8 IgG1	human Shh	$0.24 \pm 0.08$	$0.83 \pm 0.09$	$35.90 \pm 7.59$
	mouse Shh	$0.20 \pm 0.07$	$1.45 \pm 0.03$	$78.2 \pm 30.6$
	human/mouse Ihh	no binding	no binding	no binding
5E1 mIgG1	human Shh	$14.2 \pm 3.0^*$	$182 \pm 16$	$130.8 \pm 17.3$
	human/mouse Ihh	$6.21 \pm 0.76^*$	$103 \pm 13$	$159 \pm 10$

The binding kinetics of hedgehog antibodies to human and mouse hedgehog SHH and IHH proteins were determined using SPR. SPR-derived kinetic ( $k_{on}$ ,  $k_{off}$ ) and equilibrium dissociation constants ( $K_D$ ) for the binding of recombinant human and mouse SHH and IHH to 6D7 and 3H8 human IgG1 and 5E1 mouse IgG1 antibodies are shown. Values represent mean  $\pm$  SD from two or three independent experiments and were uniquely determined by the Biaevaluation software. Asterisk (\*) indicates that the rates of SHH and IHH association with 5E1 are at the limit of detection for the Biacore instrument and are likely mass transport limited.

## Fully Human Anti-Hedgehog Antibodies

### Figure Legends

#### **Figure 1. Differential binding of human anti-hedgehog antibodies to recombinant**

**human hedgehog proteins** Monoclonal antibodies 6D7 (A) and 3H8 (B) were serially diluted and binding to recombinant human SHH C24II, IHH C28II, and DHH C23II was detected after 16 h incubation.

#### **Figure 2. Neutralizing activity of anti-hedgehog mAbs in cellular assays**

(A, B) Hedgehog antibodies inhibit the mGLI1 luciferase reporter expressed in NIH3T3 cells. Varying concentrations of 6D7, 3H8, 5E1 or negative control mAb R347 were mixed with 1  $\mu\text{g/ml}$  (A) human SHH C24II or (B) human/mouse C28II IHH and incubated with cells for 24 h. Values plotted are per cent ligand stimulation in the absence of antibody and they represent means  $\pm$  SEM. (C, D) Hedgehog antibodies inhibit osteoblast differentiation of C3H10T1/2 cells induced by hedgehog ligands. C3H10T1/2 cells were treated with 600 ng/ml SHH C24II (C) or 3  $\mu\text{g/ml}$  IHH C28II (D) for 3 days in the absence or presence of serially diluted antibody. Osteoblast differentiation was measured by alkaline phosphatase activity. Values are means  $\pm$  SEM. (E, F) Hedgehog antibodies inhibit induction of endogenous mGLI1 by hedgehog proteins. C3H10T1/2 cells were treated with 600 ng/ml SHH C24II (E) or 2  $\mu\text{g/ml}$  IHH C28II (F) for 24 h in the absence or presence of serially diluted antibody. Modulation of endogenous levels of mGLI1 RNA by hedgehog stimulation and anti-hedgehog antibody treatment was determined by qRT-PCR. mGLI1 fold stimulation was calculated relative to unstimulated cells in the absence of antibody. Values are means  $\pm$  SEM.

#### **Figure 3. Pharmacokinetics and pharmacodynamics of MEDI-5304** (A) MEDI-5304

displays biological activity *in vivo*. Ncr nude mice bearing Colo205 xenograft tumors were



## Fully Human Anti-Hedgehog Antibodies

treated i.p. with vehicle or varying concentrations of antibody for 24 h. RNA expression of mGLI1 and mPTC1 in excised tumors was determined by qRT-PCR. Values represent means  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparison tests, and significance (\*\*\*)  $P < 0.001$ ) is indicated when comparing antibody treatment to vehicle controls. (B) PK/PD relationship of MEDI-5304 in athymic mice bearing Colo205 xenograft tumors. Athymic mice with  $\sim 200 \text{ mm}^3$  Colo205 tumors were treated with vehicle ( $t=0$ ) or a single 1 mg/kg i.p. dose of MEDI-5304. Tumors were excised at various times and relative mGLI1 and mPTC1 RNA levels were determined. Serum levels of MEDI-5304 were determined with a human IgG-specific ELISA.

### **Figure 4. MEDI-5304 exhibits single agent and combination efficacy with carboplatin in a coimplant model of paracrine hedgehog signaling**

(A) MEDI-5304 inhibits the growth of HT-29/MEF coimplant tumors. HT-29 and MEFs were mixed (1:5 cell ratio) and implanted subcutaneously into Ncr nude mice, which were dosed (10 animals/group) i.p. with varying amounts of MEDI-5304, 10 mg/kg 5E1 or 10 mg/kg R347 twice weekly starting on day 11. Tumor volumes are geometric means  $\pm$  SEM. Student's t test was used to determine significance of MEDI-5304 or 5E1 treatment relative to R347 treatment on day 35 ( $*P < 0.05$ ). (B,C) End of efficacy study PD effects of MEDI-5304 and 5E1 on hedgehog target gene expression in tumor stroma (B) and mouse skin (C). Tumors were harvested and skin samples were obtained 24 h post last dose and RNA expression of stromal hedgehog target and pathway genes (mGLI1, mGLI2, mGLI3, PTC1, PTC2, mHHIP, mIGFBP-5, and mSMO) was measured using qRT-PCR. Values represent means  $\pm$  SEM. One-way ANOVA and Dunnett's multiple comparison tests were used to determine statistical significance ( $*P < 0.05$ ,  $*P < 0.001$ ) of antibody treatment compared to the R347 control group. (D) MEDI-5304 enhances tumor growth inhibition of carboplatin treatment of the HT-29/MEF

## Fully Human Anti-Hedgehog Antibodies

coimplant model. Mice with HT-29/MEF coimplant tumors received MEDI-5304 (10 mg/kg twice per week i.p.), carboplatin (30 mg/kg once per week i.p.) or both agents in combination for four weeks starting on day 12. Tumor volumes are geometric means  $\pm$  SEM. Student's t test was used to determine statistical significance of combination treatment compared to R347 ( $***P < 0.001$ ) and significance of combination treatment compared to each agent alone ( $*P < 0.05$ ).

**Figure 5. MEDI-5304 lacks effects on tumor growth and cancer stem cells in primary pancreatic explants models** (A) MEDI-5304 lacks antitumor activity against three primary pancreatic explant models. RAG2 KO mice (10 mice/group) with subcutaneous P479 tumors were treated with R347 (10 mg/kg), MEDI-504 (10 or 30 mg/kg) or gemcitabine (60 or 120 mg/kg) given i.p. twice a week starting on day 16. Animals with orthotopic tumors from models 890 and 947 (8 mice/group) were untreated or received MEDI-5304 (10 mg/kg), gemcitabine (120 mg/kg), or both treatments in combination. Student's t test was used to determine significance of each treatment compared to the untreated control ( $*P < 0.05$ ,  $***P < 0.001$ ). (B) MEDI-5304 has no effect on cancer stem cell frequency in primary pancreatic tumor models. End of study samples were collected 24 h post last dose on a minimum of three samples per treatment arm and flow cytometry analysis of the CSC population (ESA+, CD44+, CD24+) was performed. Data is presented as % CSC, the percentage of cells staining positive for all three markers. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparison tests ( $** P < 0.01$ ,  $*** P < 0.001$ ). (C) MEDI-5304 does not improve responsiveness to gemcitabine pretreatment or co-administration. Mice with subcutaneous P479 tumors were either untreated (closed black squares) or treated i.p. with 120 mg/kg gemcitabine every four days for four doses (closed green squares). Animals receiving gemcitabine were then re-randomized on day 29 into groups of 10

## Fully Human Anti-Hedgehog Antibodies

mice/group, which then received one of the following treatment regimen every four days: 10 mg/kg R347 (closed black triangles), 120 mg/kg gemcitabine (closed green squares), 1 mg/kg MEDI-5304 (closed blue circles), 10 mg/kg MEDI-5304 (open blue circles), combination of 120 mg/kg gemcitabine and 1 mg/kg MEDI-5304 (closed red diamonds), or combination of 120 mg/kg gemcitabine and 10 mg/kg MEDI-5304 (closed purple diamonds). Tumors in mice that did not receive gemcitabine during the second dosing phase grew to  $\sim 1000 \text{ mm}^3$  and these mice were sacrificed on day 50. The second phase of dosing for animals that received gemcitabine as part of the dosing regimen was discontinued on day 53, and outgrowth of those tumors was monitored until day 71. No differences among these three groups were detected. (D) MEDI-5304 does not affect P479 tumorsphere growth.

Dissociated cells from P479 tumors were plated in media that promotes tumorsphere formation. Cells were plated in the absence or presence of 10 or 30  $\mu\text{g/ml}$  negative control antibody R347, 10 or 30  $\mu\text{g/ml}$  MEDI-5304, 5 or 10  $\mu\text{M}$  cyclopamine, or salinomycin (2  $\mu\text{M}$ ). Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparison tests. Statistical significance ( $***P < 0.001$ ) is indicated for salinomycin in comparison with the DMSO control.

Fig 1

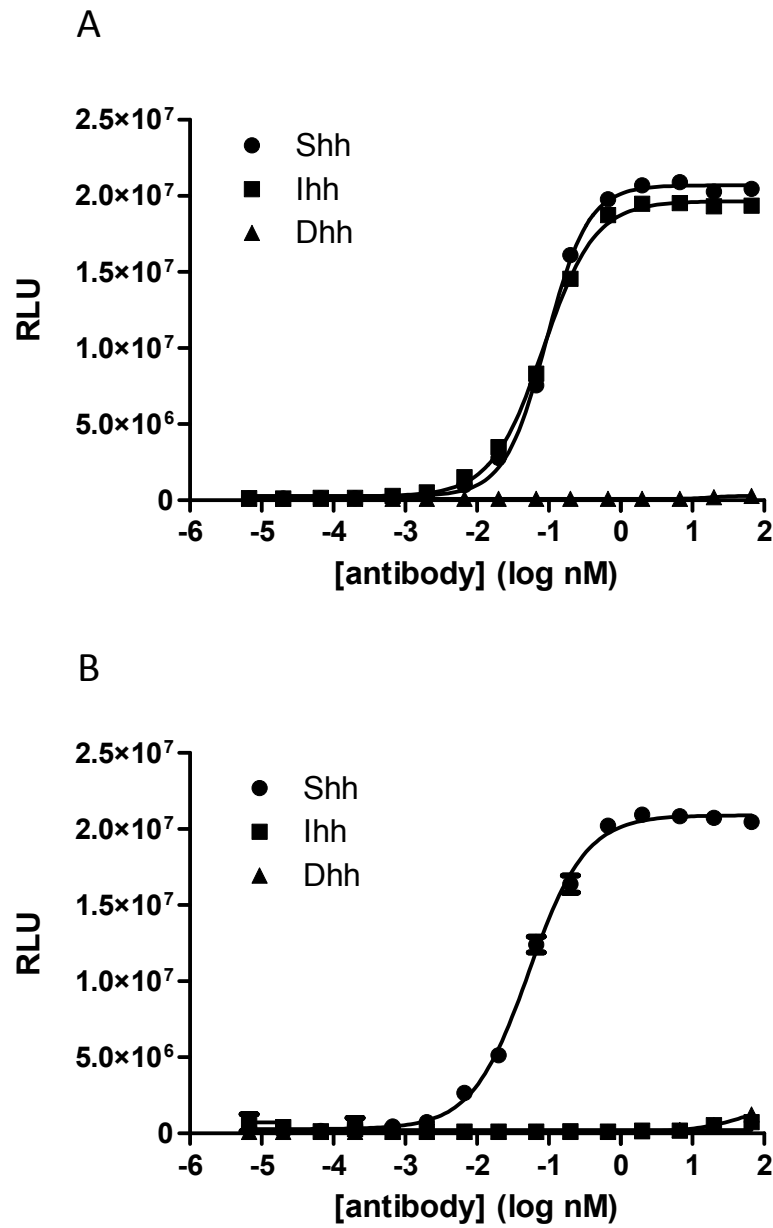


Fig 2

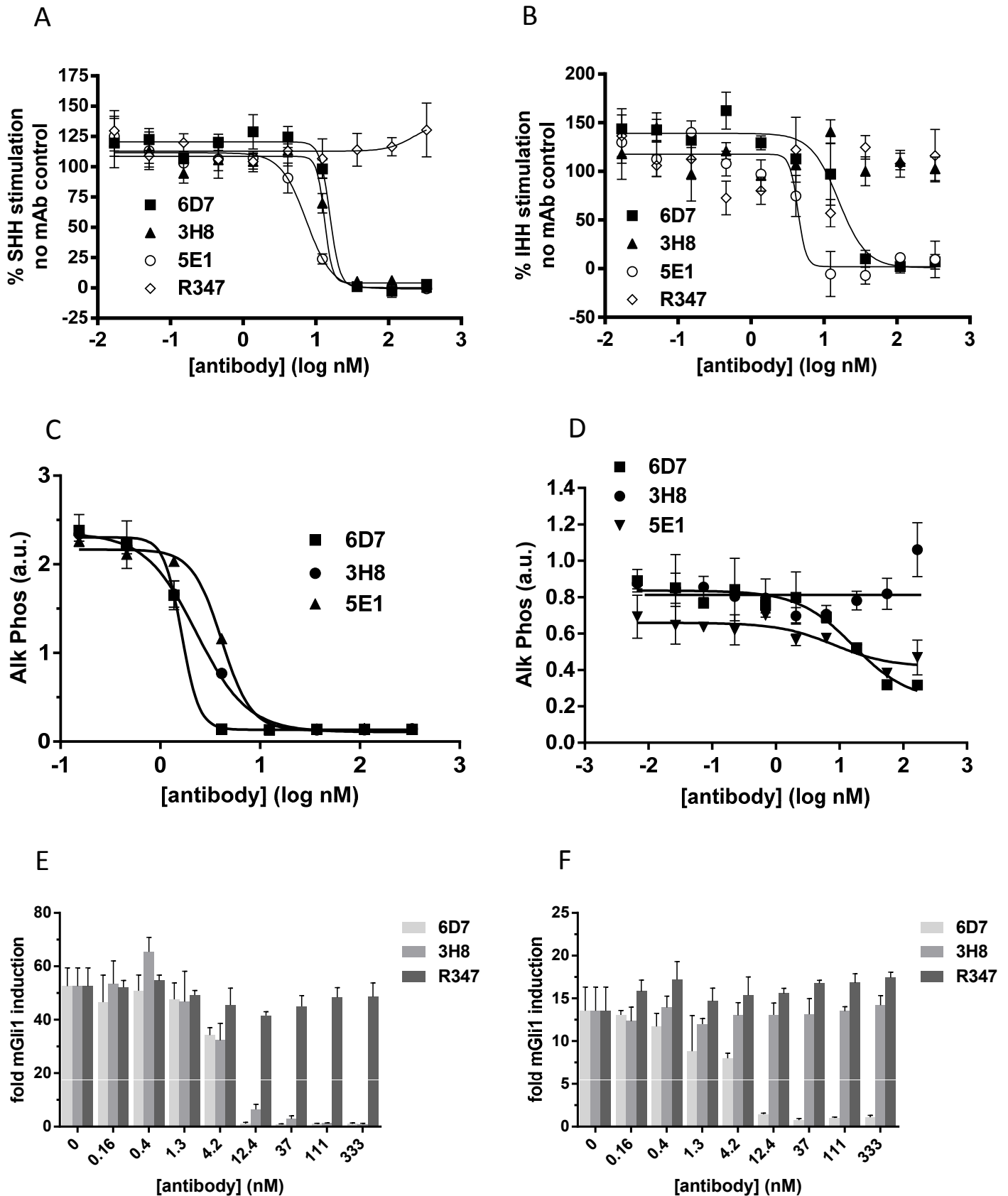
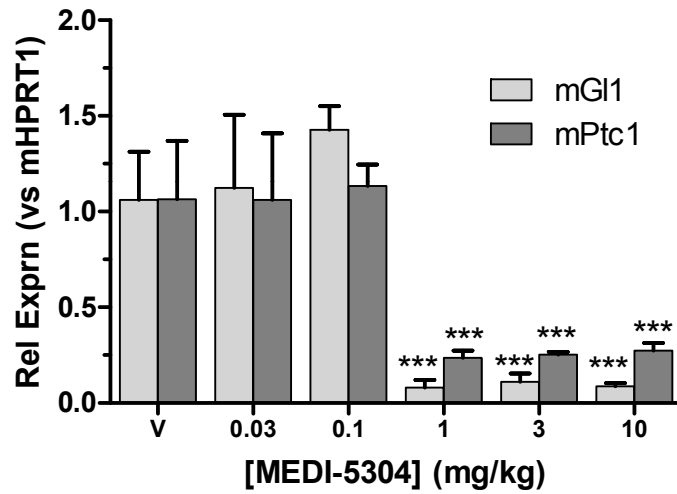


Fig 3

A



B

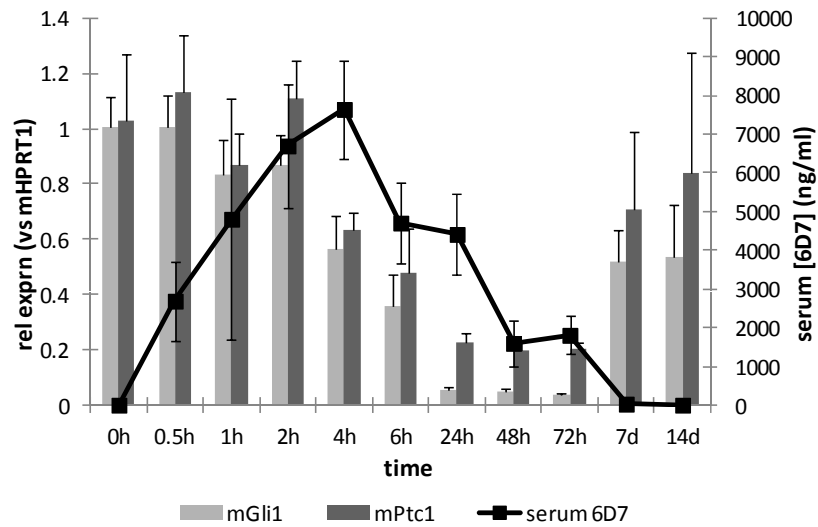


Fig 4

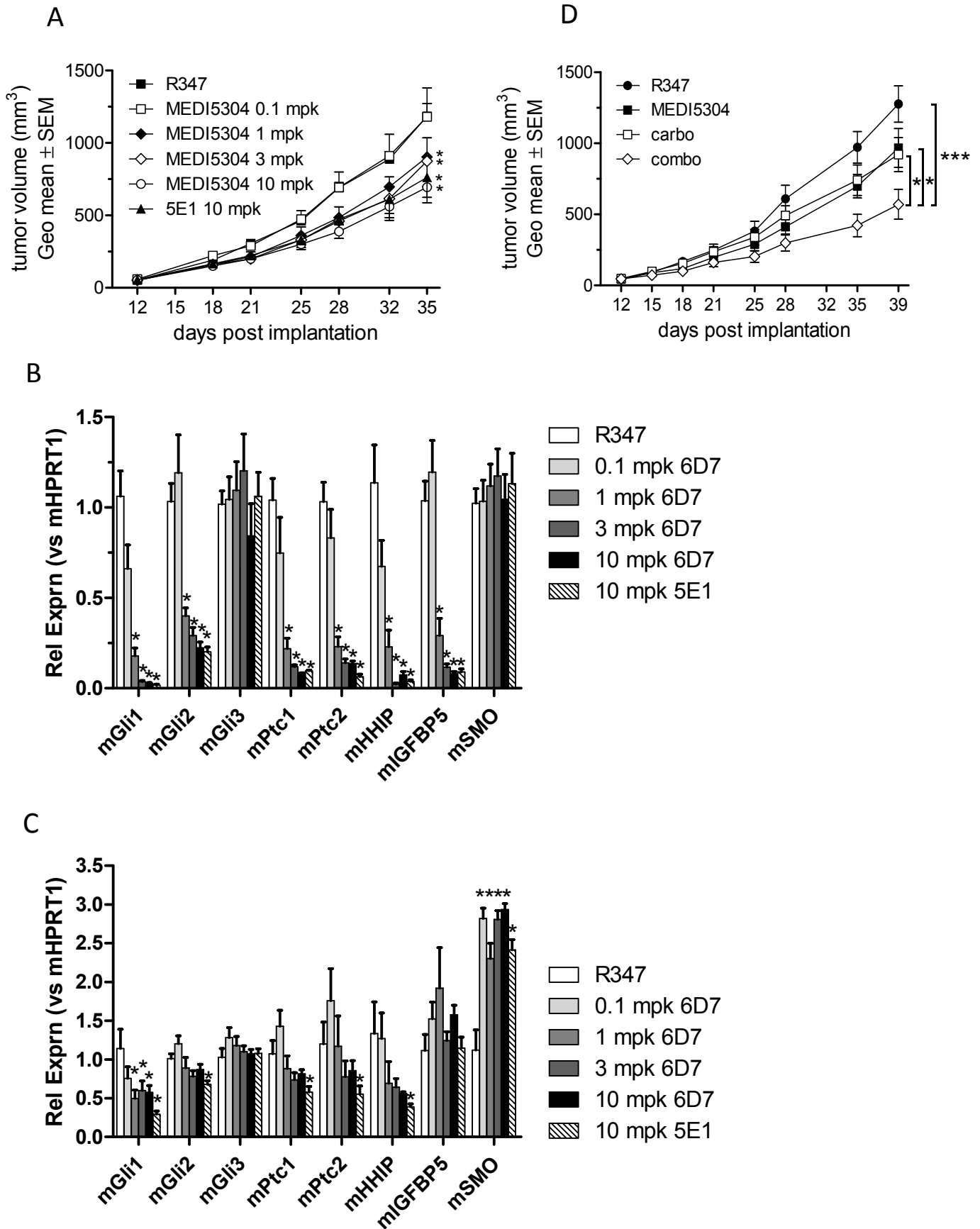
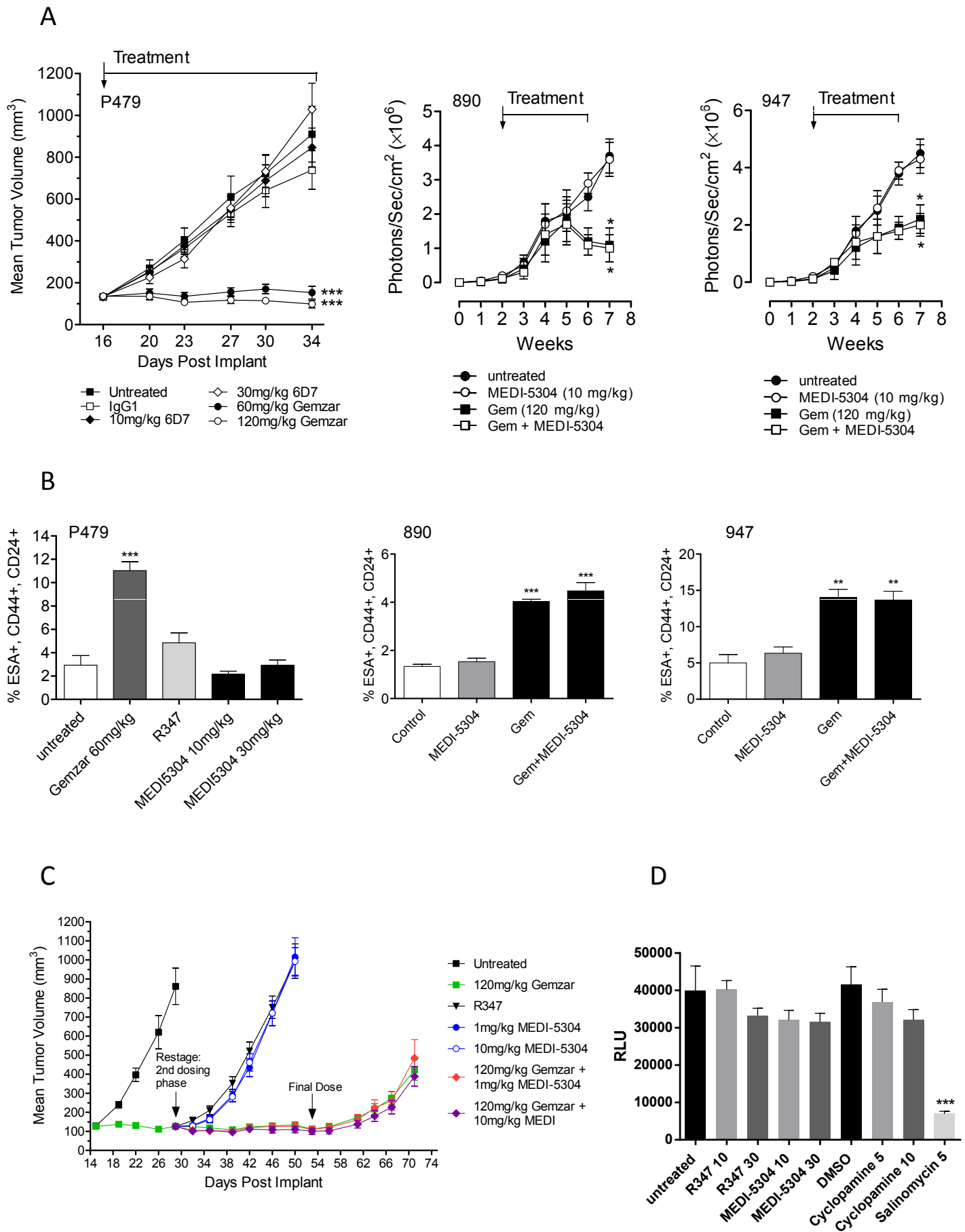


Fig 5





# Molecular Cancer Therapeutics

## Novel neutralizing hedgehog antibody MEDI-5304 exhibits antitumor activity by inhibiting paracrine hedgehog signaling

Neil R Michaud, Youzhen Wang, Kristen McEachern, et al.

*Mol Cancer Ther* Published OnlineFirst December 16, 2013.

<b>Updated version</b>	Access the most recent version of this article at: doi: <a href="https://doi.org/10.1158/1535-7163.MCT-13-0420">10.1158/1535-7163.MCT-13-0420</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://mct.aacrjournals.org/content/suppl/2013/12/16/1535-7163.MCT-13-0420.DC1">http://mct.aacrjournals.org/content/suppl/2013/12/16/1535-7163.MCT-13-0420.DC1</a>
<b>Author Manuscript</b>	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link <http://mct.aacrjournals.org/content/early/2013/12/14/1535-7163.MCT-13-0420>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.