

Targeting of the Hedgehog signal transduction pathway suppresses survival of malignant pleural mesothelioma cells in vitro

Min You, PhD,^a Javier Varona-Santos, PhD,^a Samer Singh, PhD,^b David J. Robbins, PhD,^b Niramol Savaraj, MD,^c and Dao M. Nguyen, MD, MSc, FRCSC, FACS^a

Objective: The present study sought to determine whether the Hedgehog (Hh) pathway is active and regulates the cell growth of cultured malignant pleural mesothelioma (MPM) cells and to evaluate the efficacy of pathway blockade using smoothened (SMO) antagonists (SMO inhibitor GDC-0449 or the antifungal drug itraconazole [ITRA]) or Gli inhibitors (GANT61 or the antileukemia drug arsenic trioxide [ATO]) in suppressing MPM viability.

Methods: Selective knockdown of SMO to inhibit Hh signaling was achieved by small interfering RNA in 3 representative MPM cells. The growth inhibitory effect of GDC-0449, ITRA, GANT61, and ATO was evaluated in 8 MPM lines, with cell viability quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell death was determined by annexinV/propidium iodide staining and flow cytometry.

Results: SMO small interfering RNA mediated a two- to more than fivefold reduction of *SMO* and *Gli1* gene expression as determined by real-time quantitative reverse-transcriptase polymerase chain reaction, indicating significant Hh pathway blockade. This was associated with significantly reduced cell viability ($34\% \pm 7\%$ to $61\% \pm 14\%$ of nontarget small interfering RNA controls; $P = .0024$ to $P = .043$). Treating MPM cells with Hh inhibitors resulted in a 1.5- to 4-fold reduction of *Gli1* expression. These 4 Hh antagonists strongly suppressed MPM cell viability. More importantly, ITRA, ATO, GANT61 induced significant apoptosis in the representative MPM cells.

Conclusions: Hh signaling is active in MPM and regulates cell viability. ATO and ITRA were as effective as the prototypic SMO inhibitor GDC-0449 and the Gli inhibitor GANT61 in suppressing Hh signaling in MPM cells. Pharmaceutical agents Food and Drug Administration–approved for other indications but recently found to have anti-Hh activity, such as ATO or ITRA, could be repurposed to treat MPM. (J Thorac Cardiovasc Surg 2014;147:508-16)

Malignant pleural mesothelioma (MPM) is an uncommon, yet deadly, cancer that is etiologically linked to asbestos exposure. No effective standard therapy is available for this disease. Surgically resectable MPM should be treated with a combination of surgery, chemotherapy, and radiotherapy, a treatment strategy with significant morbidity and mortality that results in a median survival of 11

(stage III) to 30 months (stage I).¹ Inoperable MPM should be treated with palliative chemotherapy, which results in a median survival of approximately 9 months.² From the experimental therapeutic perspective, considerable effort has been placed on testing novel anticancer drugs such as histone deacetylase inhibitors, new combinations of known chemotherapy agents, and targeting the usual receptor tyrosine kinases such as epidermal growth factor receptor (EGFR). All without significant improvement in survival, however.³ A new treatment paradigm is urgently needed.

The embryonic/developmental signal transduction pathway mediated by the Hedgehog (Hh) ligands plays a crucial role during embryogenesis by regulating body patterning, cellular proliferation, and differentiation.⁴ It is largely silenced in terminally differentiated cells until reactivated by tissue injury to mediate cellular regeneration and repair.⁴ Activation of the Hh pathway has recently been shown to play a causative or facilitating role in the development and/or maintenance of cancer.⁴ The molecular mechanisms of asbestos-induced carcinogenesis include chronic inflammation and tissue injury and repair, generation of oxygen free radicals, DNA damage, activation of mitogenic signal transduction pathways, and transcription factors.⁵ Significant crosstalk occurs among these activated

From the Thoracic Surgery Section,^a Division of Cardiothoracic Surgery, Department of Surgery, Molecular Oncology Program,^b Department of Surgery, and Division of Oncology,^c Department of Medicine, Miami Veterans Affairs Medical Center, Sylvester Comprehensive Cancer Center, Leonard M. Miller School of Medicine, University of Miami, Miami, Fla.

This study was supported by start-up funds from the Department of Surgery, the Sylvester Comprehensive Cancer Center, and the B. and Donald Carlin Endowed Chair of Thoracic Surgical Oncology (to D. M. Nguyen).

Disclosures: Authors have nothing to disclose with regard to commercial support.

Read at the 93rd Annual Meeting of The American Association for Thoracic Surgery, Minneapolis, Minnesota, May 4-8, 2013.

Received for publication May 5, 2013; revisions received Aug 5, 2013; accepted for publication Aug 9, 2013; available ahead of print Oct 7, 2013.

Address for reprints: Dao M. Nguyen, MD, MSc, FRCSC, FACS, Division of Cardiothoracic Surgery, Department of Surgery, Leonard M. Miller School of Medicine, University of Miami, Jackson Memorial Hospital, 1161 NW 12th Ave, ET 3072, Miami, FL 33136 (E-mail: dnguyen4@med.miami.edu).

0022-5223/\$36.00

Copyright © 2014 by The American Association for Thoracic Surgery

<http://dx.doi.org/10.1016/j.jtcvs.2013.08.035>

Abbreviations and Acronyms

ATO	= arsenic trioxide
GDC	= GDC-0449
EGFR	= epidermal growth factor receptor
FDA	= Food and Drug Administration
Hh	= hedgehog
HPI	= Hedgehog pathway inhibitor
IC ₅₀	= drug concentration that mediates 50% inhibition of cell number or viability
ITRA	= itraconazole
MPM	= malignant pleural mesothelioma
mRNA	= messenger RNA
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCR	= polymerase chain reaction
PI	= propidium iodide
PTCH	= patched
RT	= reverse transcriptase/reverse transcribed
siNT	= nontarget scramble control siRNA
siRNA	= small interfering RNA
siSMO	= siRNA targeting SMO
SMO	= smoothened

biologic processes and Hh signaling.⁵ Whether Hh is reactivated in MPM or even contributes to its tumorigenesis is not completely known.

The canonical signal transduction mediated by members of the secreted Hh proteins (sonic Hh, Indian Hh, and desert Hh) starts with binding of the ligands to their receptor Patched (PTCH), which then relieves its inhibitory activity on the signal transducer smoothened (SMO).⁴ Uninhibited SMO translocates to, and accumulates in, the primary cilium, where it processes and transmits Hh signals to the nucleus by way of the transcription activators Gli2/3, leading to increased expression of many proteins, including the transcription factor Gli1, that are essential for tumor development.⁴ Thus, *Gli1* mRNA levels are physiologically relevant indicators of Hh signaling pathway activity⁴ (Figure 1). Additional advances in Hh signaling research has demonstrated that Hh proteins can also signal through Gli-independent mechanisms, commonly referred to as “noncanonical” Hh signaling cascades (type I, Hh/PTCH/SMO-independent/Gli-independent signaling; or type II, Hh/PTCH/SMO-dependent/Gli-independent signaling).⁴ Moreover, elevated Gli1 expression levels have been observed in many tumors independent of Hh.⁶ The most practical strategy to inhibit Hh activity for therapeutic purposes is to block the function of either SMO or Gli1/2 with pharmacologic antagonists (Hh pathway inhibitors [HPIs]). Many SMO inhibitors are in various stages of clinical development, with 1 (GDC-0449 [GDC] or vismodegib) approved by the US Food and Drug Administration

(FDA) for the treatment of metastatic skin basal cell carcinoma.^{7,8} Itraconazole (ITRA), a commonly used drug to treat invasive mycosis, has recently been identified by drug screening to be a potent SMO inhibitor.⁹ Similarly, many Gli antagonists, such as GANT61, are also in preclinical development.¹⁰⁻¹² Arsenic trioxide (ATO), FDA-approved for the treatment of acute promyelocytic leukemia, has been shown to inhibit Gli transcriptional activity and to be active against Gli-driven tumors in vitro and preclinical in vivo models.^{13,14}

The primary objective of our present study was to determine whether the Hh pathway is active in MPM cells and contributes to cell proliferation. We planned to repurpose and develop ITRA and ATO as HPIs to treat Hh-dependent cancers. Therefore, the second objective of the present study was to comparatively evaluate the therapeutic efficacy of HPIs in cultured MPM cells using SMO antagonists (either ITRA or SMO GDC-0449, specifically designed by the medicinal chemistry inhibitor of SMO) or Gli antagonists (either ATO or GANT61, the selective Gli inhibitor identified by drug screen).

METHODS**Cell Lines and Reagents**

Cultured MPM cells (generously provided by Dr D. S. Schrupp, National Cancer Institute, National Institutes of Health, Bethesda, Md; and Dr H. I. Pass, New York University, New York, NY) were maintained in Roswell Park Memorial Institute-1640 supplemented with 5% fetal calf serum and glutamine (10 mM) and penicillin/streptomycin. The MPM cells used in the present study were H513, Gates, Gardner (epithelioid subtype), H2373, H2052, REN (sarcomatoid subtype), H2452 (biphasic), and H290 (unknown). ITRA and ATO were purchased from Sigma-Aldrich (St Louis, Mo). GANT61 was purchased from Tocris Bioscience (Minneapolis, Minn), and GDC-0449 (GDC) was obtained from Selleck Chemicals (Houston, Tex). ITRA was dissolved in 0.2 N HCl in ethanol. ATO was dissolved in 0.04 N NaOH in Dulbecco's phosphate-buffered saline. GANT61 and GDC were dissolved in dimethyl sulfoxide. All described experiments were performed in Roswell Park Memorial Institute-1640 with 2% fetal calf serum.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Cells seeded in 6-well plates were collected in 0.5 mL of Trizol reagent (Invitrogen, Grand Island, NY) at the indicated times after treatment with HPIs or small interfering RNA (siRNA). Total RNA was isolated and reverse transcribed (RT) using the Complementary DNA Synthesis Kit from Bio-Rad (Hercules, Calif). Real-time polymerase chain reaction (PCR) was conducted using SYBR Green Supermix from Bio-Rad in a MyiQ single-color thermocycler (Bio-Rad). Real-time RT-PCR primers were purchased from Sigma-Genosys (The Woodlands, Tex). The Ct data were generated using the software accompanying the thermocycler, and the messenger RNA (mRNA) levels were calculated using the ΔCt method, with glyceraldehyde-3-phosphate dehydrogenase as the internal control (Ct *Gli* – Ct *GAPDH*) and presented as arbitrary units as $2^{-(\Delta Ct)} \times 10^5$.

siRNA Assays

Representative MPM cells were seeded in 6-well plates and, after an overnight incubation, were transfected with 50 nM of either pooled

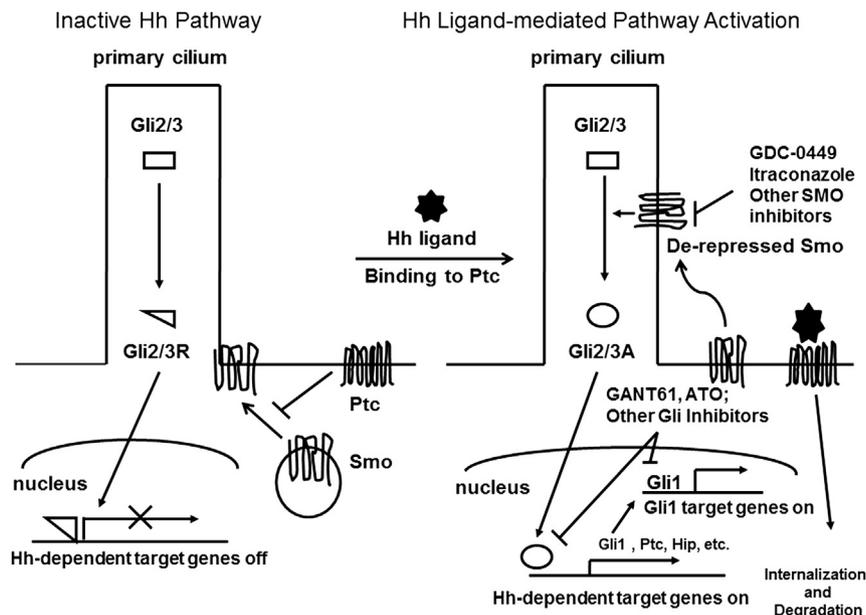


FIGURE 1. Diagrammatic representation of the canonical Hedgehog (*Hh*)-mediated signal transduction pathway in eukaryotes. In the absence of Hh ligands, the cognate Hh receptor patched (*ptc*) suppresses the activity of the signal transducer smoothed (*Smo*) from translocating to the primary cilium, a membrane-encased protrusion located on the apical side of polarized cells that was initially thought to be a vestigial organelle but has now been proved to be essential for Hh-mediated signaling. In the absence of activated SMO in the primary cilium, Gli2/3 transcription factors are processed to become repressors (*Gli2/3R*). Binding of Hh ligands to patched (PTCH) leads to ligand/receptor internalization and degradation, which allows SMO translocation to the primary cilium where it mediates activation of Gli2/3 to form the transcriptional activators Gli2/3A. Gli2/3R move to the nucleus and mediate Hh-dependent gene transcription. The Hh-mediated signal transduction pathway can be attenuated by targeting SMO using pharmacologic inhibitors such as GDC-0449, itraconazole (ITRA) or others currently in development or by targeting the transcription activators Gli1/2 using Gli antagonists such as GANT61, arsenic trioxide (*ATO*), or other selective Gli inhibitors. Interested readers are referred to the study by Robbins and colleagues⁴ for a full description of the Hh signaling network.

nontarget scramble control siRNA (siNT) or pooled siRNA targeting SMO (siSMO; Dharmacon, Lafayette, Colo) using INTERFERin (Polyplus-transfection, New York, NY) according to the transfection protocol of the manufacturer. The cells were collected 72 hours after siRNA transfection and subjected to real-time RT-PCR for *SMO* and *Gli1* expression. For growth proliferation assays, the cells transfected with either siNT or siSMO and were cultured for 96 hours. Cell viability was assayed using the MTT and were expressed as percentages of siNT-treated cells.

Assay for AntiHh Activity of HPIs

The Hh pathway reporter sHh-Light2 cells (NIH-3T3 cells stably transfected with the reporter cassette 8XGli1-luciferase that respond to SMO stimulation) were activated with either conditioned media of iHH cells¹⁵ containing the sHhN ligand or the SMO agonist SAG with or without the presence of HPIs, which were added to the cells 2 hours before SMO activation. Unactivated (negative controls) and activated (positive controls) and activated sHHhLight2 cells treated with HPIs were assayed for luciferase activity and expressed as the percentage of luciferase activity of SMO-stimulated sHh-Light2 cells.

Cell Proliferation and Cell Death Assays

For the dose-dependent cell viability assay, MPM cells were seeded in 96-well plates at predetermined densities to avoid overconfluence of the control cells at the end of the experiments. After an overnight incubation, the cells were treated at the indicated concentrations of HPIs or drug carriers in 200 μ L for 96 hours with replacement of fresh media with drugs or carriers 48 hours after the onset of drug treatment. The cell number or viability was measured using the MTT assay and expressed as the

percentage of untreated controls. For the cell proliferation assay, 20,000 cells were seeded in 24-well plates and treated with HPIs at the indicated concentrations (with a change of media at 48 and 96 hours after the onset of the experiments), and the cell number or viability was enumerated daily for 5 days using the MTT assay and expressed as the x-fold increase of the MTT optical density at wavelengths of 570 nm/650 nm. For the cell death assay, MPM cells were seeded in 12-well plates at 40,000 cells and treated with the indicated concentrations of HPIs for 60 hours and subjected to annexinV/PI staining and flow cytometry for enumeration of cell death: apoptosis (early, annexinV+/PI-; late, annexinV+/PI+) and necrotic death (annexinV-/PI+). For cell cycle analysis, MPM cells were treated with the indicated concentrations of HPIs for 48 hours, harvested by trypsinization, fixed with paraformaldehyde, and stained with PI/RNase. The cell cycle distribution was determined using flow cytometry.

Data Presentation and Statistical Analysis

The experiments were performed at least in triplicate, and the data are expressed as the mean \pm standard deviation. The 2-tail Student *t* test and analysis of variance with Bonferroni's post test for pairwise comparison was used as indicated for the statistical analysis (GraphPad software, San Diego, Calif).

RESULTS

Hh Pathway Is Active and Controls Cell Growth in MPM

Real-time quantitative RT-PCR analysis for the expression of key components of the Hh pathway (PTCH, SMO,

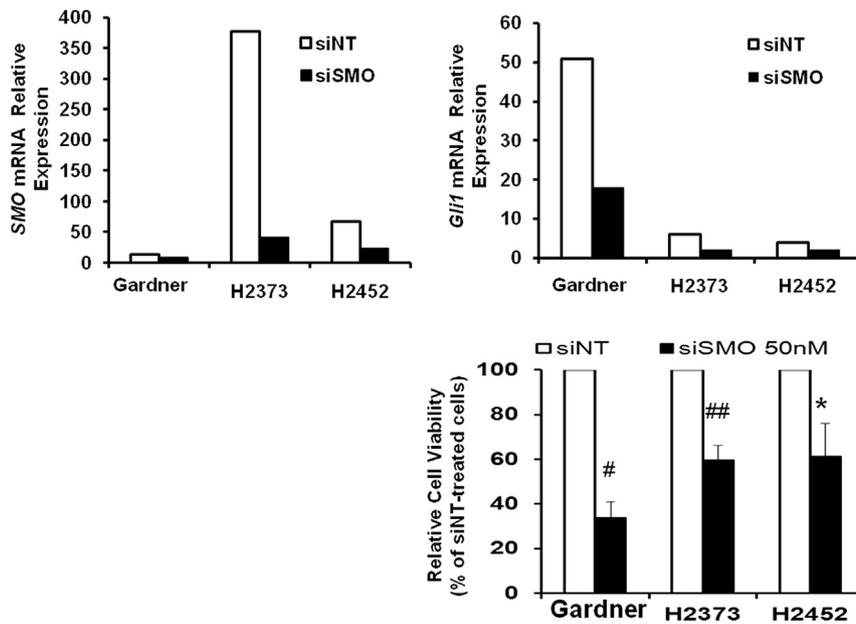


FIGURE 2. The Hedgehog pathway is active and contributes to cell proliferation in representative cultured malignant pleural mesothelioma cells. The cells were transfected with pooled scramble nontarget small interfering RNA (*siNT*) or small interfering RNA targeting smoothened (*siSMO*) and assayed for *SMO* messenger RNA (*mRNA*) (on-target effect) and *Gli1* mRNA (Hedgehog pathway activity) and cell viability (n = 3, mean ± standard deviation, #*P* = .002, ##*P* = .009, **P* = .04) and representative quantitative reverse-transcriptase polymerase chain reaction data of 3 independent experiments.

Gli1/2, and the Hh ligands) indicated that all cultured MPM cells had detectable mRNA levels of these genes (data not shown). To determine whether the Hh pathway is active in MPM cells, we selectively suppressed *SMO* gene expression using siRNA in representative MPM cells H2373, Gardner, and H242 and assayed for *Gli1* mRNA using quantitative RT-PCR. Compared with the siNT, siSMO significantly reduced not only the *SMO* transcripts, but also *Gli1* gene expression, indicative of attenuation of pathway activity. More importantly, suppression of Hh signaling was associated with a 40% to 60% reduction of cell proliferation (Figure 2), implicating the role of the Hh pathway in cell proliferation.

Suppression of Hh Pathway Activity by HPIs

Using the prototypic *SMO* inhibitor GDC as the positive control, we sought to determine the Hh pathway inhibitory activity of the Gli inhibitor GANT61 and also of the two repurposed Hh antagonists ITRA and ATO, first in the reporter cells sHH-Light2 stimulated with either the Hh ligand or the pharmacologic *SMO* agonist SAG and, subsequently, in representative MPM cells. Complete attenuation of Hh signaling was observed in the activated sHH-Light2 cells treated with GDC at concentrations as low as 50 nM or ITRA at concentrations of ≥2.5 μM. Both *SMO* inhibitors were much more effective in blocking pathway activation mediated by Hh ligand acting on PTCH upstream of *SMO* than that mediated by SAG acting on *SMO* itself. The two Gli inhibitors, GANT61 and ATO, exhibited a

similar profile of inhibiting pathway activation mediated by either Hh ligand or SAG (Figure 3, A). More importantly, treating representative MPM cells with these HPIs for 24 hours resulted in attenuation of Hh signaling, indicated by a 1.5- to >5-fold reduction of *Gli1* mRNA (Figure 3, B).

Suppression of MPM Cell Growth by HPIs

We next evaluated the growth inhibitory effects of these 4 HPIs on a panel of 8 MPM cells by performing dose-response cell viability and time-course cell proliferation assays. Data from the representative experiments are shown in Figure 4. Significant dose-dependent suppression of cell number or viability was observed in the cells treated with these HPIs, with ITRA exerting the strongest effect. In contrast, the selective *SMO* inhibitor GDC demonstrated only mild to moderate reduction of cell viability. The cell proliferation assay showed that although GDC resulted in a mild reduction of cell proliferation, ITRA, ATO, or GANT61, not only suppressed cell growth, but also caused a net loss of cell numbers, indicative of a strong induction of cell death. The susceptibility of the 8 MPM cells to the antiproliferation effects of these 4 HPIs was quantified by estimating the drug concentration that mediated 50% inhibition of cell number or viability (IC₅₀) from respective dose-response curves similar to those shown on Figure 4, A. All MPM cells evaluated in our study were very sensitive to ATO and ITRA with IC₅₀s <5 μM. However, the IC₅₀s of GDC ranged from 15 to 20 μM in 5 of 8 and was not reached in 3 of 8 other MPM cells (Figure 5, A). In the following set



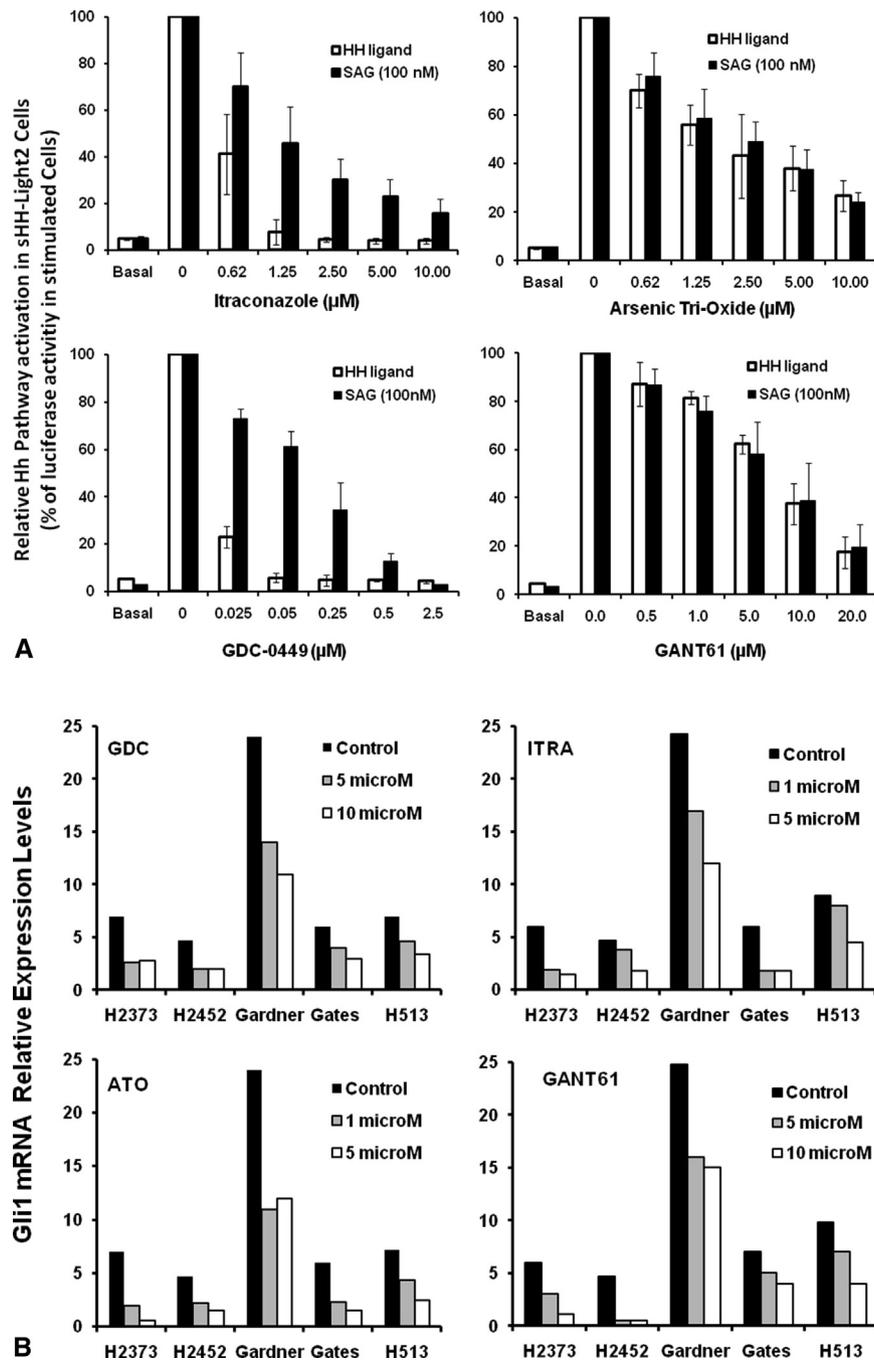


FIGURE 3. A, Attenuation of Hedgehog (*HH*) pathway activation by the selective smoothened inhibitor GDC-0449, the selective Gli inhibitor GANT61, and the repurposed *HH* antagonists itraconazole and arsenic trioxide. Pathway activity was bioassayed using the sHH-Light2 cells stimulated with either sHH ligand or the pharmacologic smoothened agonist SAG, with or without Hedgehog pathway inhibitors (HPIs). Luciferase activity of unstimulated (basal), stimulated without (0) and with HPI treatment (at indicated drug concentrations) was measured and is expressed as the percentages of luciferase activity in the stimulated reporter cells $n = 4$, mean \pm standard deviation. B, Suppression of *HH* activity in representative malignant pleural mesothelioma cells by the HPIs. The cells were treated with 1 of the 4 HPIs at indicated concentrations for 24 hours and assayed for *Gli1* messenger RNA using quantitative reverse transcriptase polymerase chain reaction. Representative data of 3 independent experiments with similar results shown. *GDC*, GDC-0449; *ITRA*, itraconazole; *ATO*, arsenic trioxide; *mRNA*, messenger RNA.

of experiments, we attempted to define the cellular mechanism of the HPI-mediated growth inhibitory effect. Representative MPM cells were treated with 5 or 20 μM

of each of these HPIs for 60 hours and were subjected to cell cycle analysis or assayed for cell death (early, late apoptotic, and necrotic death). Exposure to GDC did not

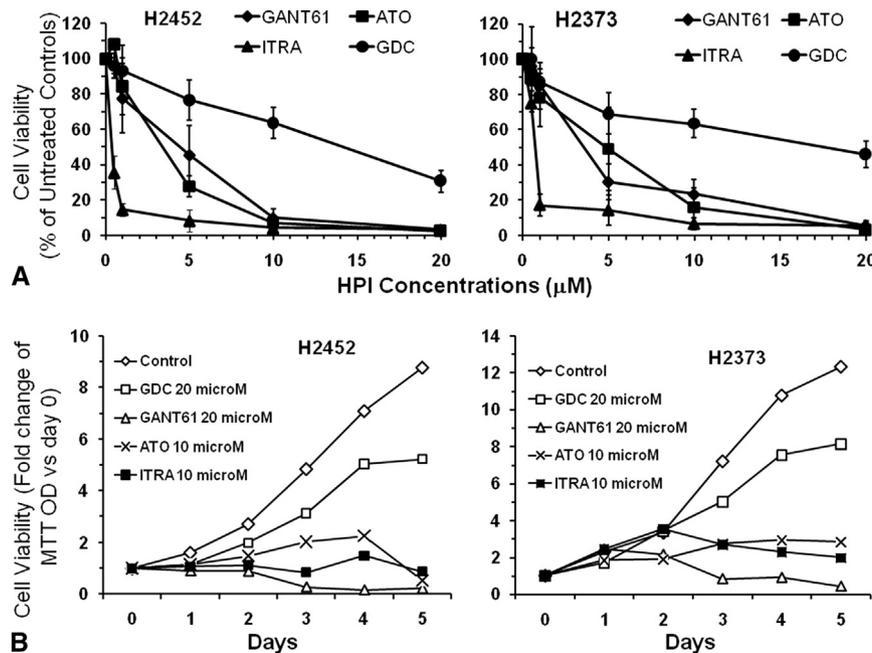


FIGURE 4. A, Dose-dependent cytotoxicity of Hedgehog pathway inhibitors (HPIs) on 2 representative cultured malignant pleural mesothelioma cells (n = 4-6, mean ± standard deviation). Cells seeded in 96-well plates were continuously exposed to HPIs at the indicated concentrations for 96 hours, and the cell number/viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B, Suppression of cell proliferation by HPIs, in particular, with GANT61, itraconazole (ITRA), or arsenic trioxide (ATO), mediated a net reduction in cell viability, indicative of a significant induction of cell death (representative data from 3 independent experiments). Cells seeded in 24-well plates were continuously treated with HPI at the indicated concentrations for 5 days, and the cell number or viability was quantified daily using MTT under constant experimental conditions and expressed as the x-fold changes from the baseline MTT optical density of the control cells on day 0 when HPI treatment started. GDC, GDC-0449.

induce any discernible loss of cell viability; however, significant cell death was observed after ITRA, ATO, or GANT61 treatment (Figure 5, B). In addition to causing cell death, ATO, ITRA, and GANT61 also inhibited cell progression through the cell cycle at G₁/S and/or G₂/M checkpoints with accumulation of cells in G₀/G₁ or G₂/M subpopulations. Delayed progression through the S phase was also observed in the H2373 cells treated with the Gli inhibitors ATO and GANT61. Delayed transition at the G/S checkpoint was observed in the GDC-treated cells, with the accumulation of cells in G₀/G₁ and concomitant reduction of cells in S and G₂/M phases (Figure 5, C).

DISCUSSION

Molecularly targeted therapy, either alone or combined with standard cytotoxic chemotherapeutic agents, is now a well-established treatment strategy for hematologic and solid cancer. Targeted therapy is most effective when applied to tumors that are addicted to, or at least dependent on, the molecular pathway or process being targeted. The prime example is the efficacy of the EGFR-tyrosine kinase erlotinib or gefitinib in treating advanced adenocarcinoma harboring activating EGFR mutations.¹⁶ In addition to many well-described membrane-associated receptor tyrosine kinase or the intracellular serine/threonine kinase

signal transduction pathways that can be oncogenic when dysregulated, other signaling cascades, such as Notch, Wnt, or Hh, well recognized as being crucial for embryonic development and stem cell function, have recently been implicated in cancer initiation and development.^{17,18} Hh-mediated signaling is the oncogenic driver for skin basal cell carcinomas, with constitutively active Hh pathway due to loss of PTCH expression.^{19,20} Consequently, selective targeting of SMO with GDC-0449 results in significant clinical responses in advanced skin basal cell carcinoma refractory to standard therapy.⁷ Our laboratory and others have demonstrated the role of Hh signaling on the tumorigenesis of many malignant tumors, including primary cancer of the lung, prostate, bladder, pancreas, and brain.^{17,18,21-23} A number of novel Hh inhibitors, identified by screening of the chemical library or medicinal chemistry design for SMO or Gli inhibitory activity, have now entered clinical trials,^{19,20} where they will be extensively tested for many years for efficacy and toxicity, with only a small percentage of them eventually receiving FDA approval. Alternatively, screening of FDA-approved pharmaceutical agents currently used to treat various medical conditions has also identified certain agents that can effectively inhibit Hh signaling, such as ITRA or ATO.^{9,13,14} These 2 drugs have been used for years, and



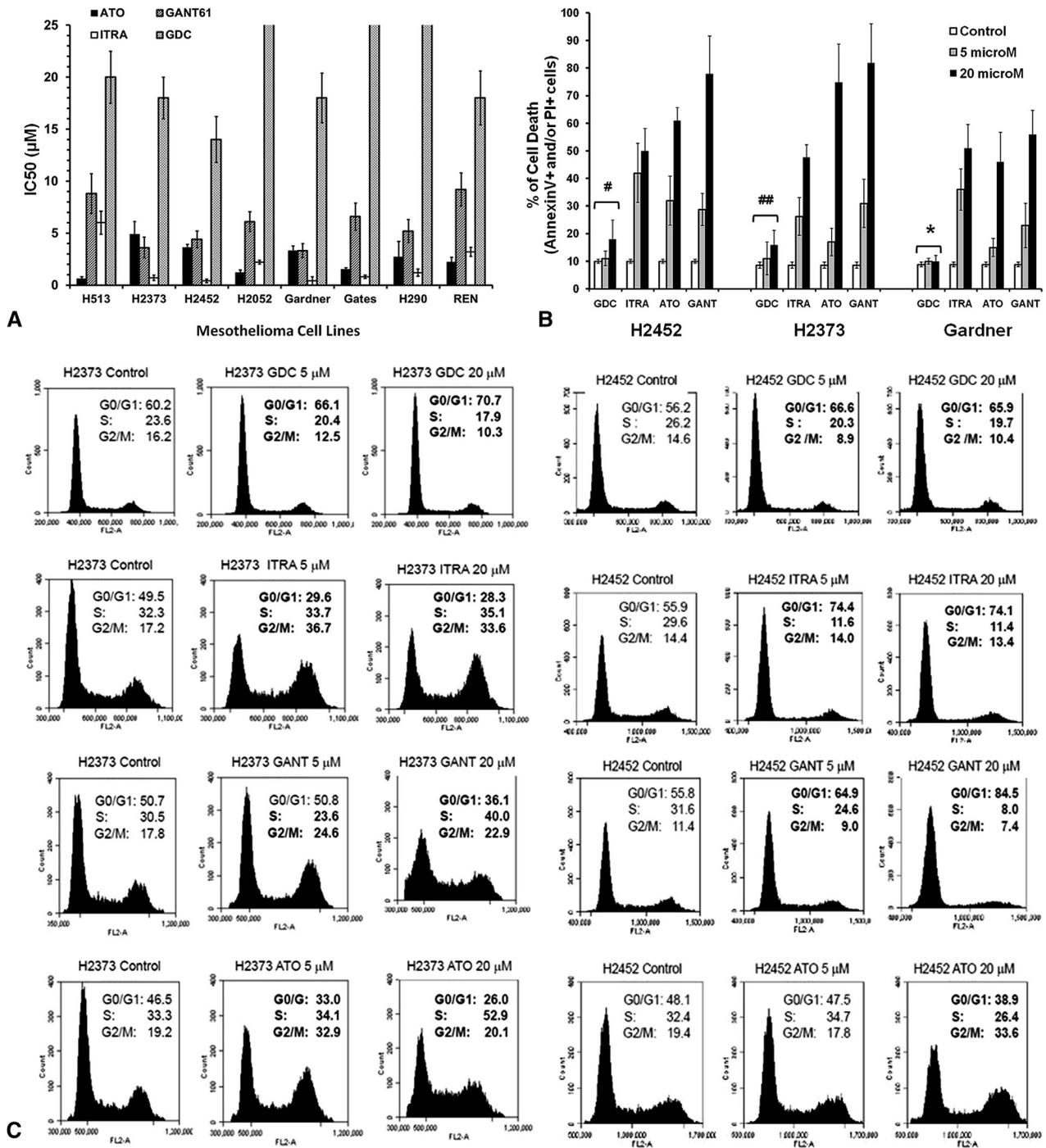


FIGURE 5. A, Comparative analysis of growth inhibitory effect of Hedgehog pathway inhibitors (HPIs) on the panel of 8 cultured malignant pleural mesothelioma cells as demonstrated by the drug concentration that mediated 50% inhibition of cell number or viability. These values were estimated from the respective dose–response curves shown in Figure 3, A. B, Induction of cell death by itraconazole (*ITRA*), arsenic trioxide (*ATO*), and GANT61, but not GDC-0449 (*GDC*) on 3 representative malignant pleural mesothelioma cells (n = 4-6, mean ± standard deviation; #analysis of variance, P = .027, with P < .05 for GDC 20 μM vs control only using Bonferroni’s post test analysis; ##analysis of variance, P = .026, with P < .05 for GDC 20 μM vs control using Bonferroni’s post test analysis; *no significant difference between GDC-treated and control cells). C, Suppression of cell cycle progression by HPIs. Cell cycle arrest at G₁/S, S/G₂, or G₂/M checkpoints were observed in malignant pleural mesothelioma cells treated with HPIs with concomitant accumulation of cells at the G₀/G₁ or S or G₂/M compartments (percentages shown in bold) of the cell cycle. Representative data of 3 independent experiments are shown.

ET/BS

their toxicity profiles and pharmacologic properties have been well described. Repurposing of FDA-approved drugs with known toxicity profiles for cancer therapy circumvents the lengthy process of phase I drug development. Multiple clinical trials (NCT00769600, NCT00887458, NCT00798135, and NCT01108094)²⁴ have been conducted to evaluate the efficacy of ITRA in treating non-small-cell lung cancer (combined with pemetrexate),²⁵ prostate cancer,²⁶ and breast cancer.

Our data have demonstrated that Hh signaling is active in cultured MPM cells and contributes to cell growth in vitro, in keeping with only 1 previous report on the mitogenic role of Hh signaling in primary MPM tumors.²⁷ Although mediating a reduction of *Gli1* expression in MPM cells (Figure 3, B) comparable to that of other HPIs, GDC had the lowest antiproliferative activity (high IC₅₀ values; Figure 5, A). ITRA and ATO likely have other Hh-independent (off-target) growth inhibitory effects. The molecular mechanism of ATO-mediated anticancer activity is diverse, including generation of reactive oxygen free radicals, inhibition of mitogenic and survival signal transduction pathways, and induction of apoptosis.^{28,29} ITRA has been shown to inhibit mammalian target of rapamycin signaling and membrane growth factor trafficking, causing death of endothelial cells²⁴; however, its effect on cancer cell growth and survival has not been well elucidated. GANT61, being a Gli inhibitor, targets both SMO-dependent and SMO-independent Gli transcriptional activity and has been shown to exert profound anticancer property in preclinical models of solid tumors.^{11,12} The cell culture condition used in our study could influence the magnitude of Hh activation and, thus, the efficacy of the selective SMO inhibitor GDC. Significant activation of Hh signaling and sensitization to the growth inhibitory effect of pathway attenuation was observed in bladder cancer cells with low intrinsic Hh activity (which were thus resistant to the antiproliferative effect of pathway blockade) when these cells were cultured in anchorage-independent growth conditions, instead of grown as a monolayer.²¹ Even if the Hh-independent drug effect might be largely responsible for the profound cytotoxicity of ITRA or ATO on MPM cells, drug-induced reduction of *Gli1* expression could still serve as a pharmacodynamic biomarker.

The results of our study raise many questions and thus provide guidance for the direction of our future works. The frequency and magnitude of Hh pathway activation needs to be determined in MPM tissues using immunohistochemical staining for *Gli1* expression in tissue microarrays. A more comprehensive analysis of Hh pathway activation and the growth inhibitory effect of pathway attenuation (either by siSMO-mediated gene knockdown or by selective SMO inhibitors) will be done in a larger panel of MPM cells cultured using either anchorage-dependent or anchorage-

independent conditions. Biomarkers predictive of the cellular sensitivity to HPIs need to be developed and validated early in the course of drug development. Such biomarkers will be used to properly select tumors most likely to respond to Hh-targeted therapy. The molecular mechanism of ITRA-induced cytotoxicity will also be the focus of our future investigation. Finally, we plan to translate our in vitro findings to preclinical in vivo study using animal models of human MPM xenografts.

In conclusion, our data have indicated that Hh signaling is active and regulates cell proliferation of cultured MPM lines. Repurposing pharmaceutical agents approved by the FDA for other clinical indications but also shown to be HPIs, such as ITRA or ATO, to treat Hh-dependent cancers such as MPM might shorten the lengthy process of drug development and allow rapid entrance into clinical application.

References

- Flores RM, Pass HI, Seshan VE, Dycoco J, Zakowski M, Carbone M, et al. Extrapleural pneumonectomy versus pleurectomy/decortication in the surgical management of malignant pleural mesothelioma: results in 663 patients. *J Thorac Cardiovasc Surg*. 2008;135:620-6. 6.e1-3.
- Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol*. 2003;21:2636-44.
- Jakobsen JN, Sorensen JB. Review on clinical trials of targeted treatments in malignant mesothelioma. *Cancer Chemother Pharmacol*. 2011;68:1-15.
- Robbins DJ, Fei DL, Riobo NA. The Hedgehog signal transduction network. *Sci Signal*. 2012;5:re6.
- Heintz NH, Janssen-Heininger YM, Mossman BT. Asbestos, lung cancers, and mesotheliomas: from molecular approaches to targeting tumor survival pathways. *Am J Respir Cell Mol Biol*. 2010;42:133-9.
- Hui CC, Angers S. Gli proteins in development and disease. *Annu Rev Cell Dev Biol*. 2011;27:513-37.
- Von Hoff DD, LoRusso PM, Rudin CM, Reddy JC, Yauch RL, Tibes R, et al. Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med*. 2009;361:1164-72.
- Axelson M, Liu K, Jiang X, He K, Wang J, Zhao H, et al. U.S. Food and Drug Administration approval: vismodegib for recurrent, locally advanced, or metastatic basal cell carcinoma. *Clin Cancer Res*. 2013;19:2289-93.
- Kim J, Tang JY, Gong R, Kim J, Lee JJ, Clemons KV, et al. Itraconazole, a commonly used antifungal that inhibits Hedgehog pathway activity and cancer growth. *Cancer Cell*. 2010;17:388-99.
- Scales SJ, de Sauvage FJ. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends Pharmacol Sci*. 2009;30:303-12.
- Wickstrom M, Dyberg C, Shimokawa T, Milosevic J, Baryawno N, Fuskevag OM, et al. Targeting the hedgehog signal transduction pathway at the level of GLI inhibits neuroblastoma cell growth in vitro and in vivo. *Int J Cancer*. 2013;132:1516-24.
- Mazumdar T, Devecchio J, Agyeman A, Shi T, Houghton JA. Blocking Hedgehog survival signaling at the level of the GLI genes induces DNA damage and extensive cell death in human colon carcinoma cells. *Cancer Res*. 2011;71:5904-14.
- Beauchamp EM, Ringer L, Bulut G, Sajwan KP, Hall MD, Lee YC, et al. Arsenic trioxide inhibits human cancer cell growth and tumor development in mice by blocking Hedgehog/GLI pathway. *J Clin Invest*. 2011;121:148-60.
- Kim J, Lee JJ, Kim J, Gardner D, Beachy PA. Arsenic antagonizes the Hedgehog pathway by preventing ciliary accumulation and reducing stability of the Gli2 transcriptional effector. *Proc Natl Acad Sci U S A*. 2010;107:13432-7.
- Tokhunts R, Singh S, Chu T, D'Angelo G, Baubert V, Goetz JA, et al. The full-length unprocessed hedgehog protein is an active signaling molecule. *J Biol Chem*. 2010;285:2562-8.

16. Lee CK, Brown C, Gralla RJ, Hirsh V, Thongprasert S, Tsai CM, et al. Impact of EGFR inhibitor in non-small cell lung cancer on progression-free and overall survival: a meta-analysis. *J Natl Cancer Inst.* 2013;105:595-605.
17. Dodge ME, Lum L. Drugging the cancer stem cell compartment: lessons learned from the hedgehog and Wnt signal transduction pathways. *Annu Rev Pharmacol Toxicol.* 2011;51:289-310.
18. Garcia Campelo MR, Alonso Curbera G, Aparicio Gallego G, Grande Pulido E, Anton Aparicio LM. Stem cell and lung cancer development: blaming the Wnt, Hh and Notch signalling pathway. *Clin Transl Oncol.* 2011;13:77-83.
19. Pasca di Magliano M, Hebrok M. Hedgehog signalling in cancer formation and maintenance. *Nat Rev Cancer.* 2003;3:903-11.
20. Ng JM, Curran T. The Hedgehog's tale: developing strategies for targeting cancer. *Nat Rev Cancer.* 2011;11:493-501.
21. Fei DL, Sanchez-Mejias A, Wang Z, Flaveny C, Long J, Singh S, et al. Hedgehog signaling regulates bladder cancer growth and tumorigenicity. *Cancer Res.* 2012;72:4449-58.
22. Singh S, Wang Z, Liang Fei D, Black KE, Goetz JA, Tokhunts R, et al. Hedgehog-producing cancer cells respond to and require autocrine Hedgehog activity. *Cancer Res.* 2011;71:4454-63.
23. Rodriguez-Blanco J, Schilling NS, Tokhunts R, Giambelli C, Long J, Liang Fei D, et al. The Hedgehog processing pathway is required for NSCLC growth and survival. *Oncogene.* 2013;32:2335-45.
24. Nacev BA, Grassi P, Dell A, Haslam SM, Liu JO. The antifungal drug itraconazole inhibits vascular endothelial growth factor receptor 2 (VEGFR2) glycosylation, trafficking, and signaling in endothelial cells. *J Biol Chem.* 2011;286:44045-56.
25. Rudin CM, Brahmer JR, Juergens RA, Hann CL, Ettinger DS, Sebree R, et al. Phase 2 study of pemetrexed and itraconazole as second-line therapy for metastatic nonsquamous non-small-cell lung cancer. *J Thorac Oncol.* 2013;8:619-23.
26. Antonarakis ES, Heath EI, Smith DC, Rathkopf D, Blackford AL, Danila DC, et al. Repurposing itraconazole as a treatment for advanced prostate cancer: a noncomparative randomized phase II trial in men with metastatic castration-resistant prostate cancer. *Oncologist.* 2013;18:163-73.
27. Shi Y, Moura U, Opitz I, Soltermann A, Rehrauer H, Thies S, et al. Role of Hedgehog signaling in malignant pleural mesothelioma. *Clin Cancer Res.* 2012;18:4646-56.
28. Eguchi R, Fujimori Y, Takeda H, Tabata C, Ohta T, Kuribayashi K, et al. Arsenic trioxide induces apoptosis through JNK and ERK in human mesothelioma cells. *J Cell Physiol.* 2011;226:762-8.
29. Pettersson HM, Pietras A, Munksgaard Persson M, Karlsson J, Johansson L, Shoshan MC, et al. Arsenic trioxide is highly cytotoxic to small cell lung carcinoma cells. *Mol Cancer Ther.* 2009;8:160-70.

Discussion

Dr Jules Lin (*Ann Arbor, Mich*). Dr Nguyen, congratulations on your work and an excellent presentation.

You have identified potential therapeutic targets for MPM in the Hh pathway, SMO and Gli, and identifying a novel target is a good first step. We are often frustrated by the lack of inhibitors, and this approach you have taken is exciting, to find a commonly used drug that has been extensively tested in humans that happens to inhibit the targets you have identified to treat MPM.

I have 3 questions for you. ITRA has many different effects, and it affects other pathways, including the mammalian target of rapamycin pathway. Did you confirm that the effects that you see here are from the inhibition of the Hh pathway? For example, did you inhibit a downstream mediator, such as Gli, with a small hairpin RNA to see whether that eliminates the effect of ITRA?

Dr Nguyen. That is a very good point. ITRA has been shown to target the mammalian target of rapamycin in signaling of the vascular endothelial growth factor receptors in endothelial cells. I did a lot of searching. I could not really find good studies on the effect of ITRA in cancer cells. It will be the next step for us to determine what ITRA does in cancer cells. Your comment about

selectively targeting an Hh pathway component such as Gli to determine the relative contribution of Hh inhibition in mediating the growth inhibitory effect of itraconazole is valid. In this case, I would knockdown SMO because it is the target of ITRA. I agree with you that ITRA has other effects that inhibit cancer cell growth. The antiproliferation effect we observed might not all be coming from SMO inhibition. Also, even if the anticancer effect is SMO independent, we can always use Gli knockdown as a marker of the drug effect on the cells. That is a very good point.

Dr Lin. My second question is, in basal cell carcinoma, as you mentioned, PTCH was decreased and that was the mechanism for Hh overexpression in the basal cells. In MPM, what is the exact alteration? In your cell lines, did you see certain genes that were overexpressed and that correlated with the treatment response? Which would be the best biomarker for the treatment response?

Dr Nguyen. I appreciate those comments a lot. A recently published study in the *International Journal of Oncology* (2012;41:1751-61) examined the antiproliferation effect of cyclopamine—a prototypic SMO inhibitor—in a large collection of cancer cell lines of epithelial origin. The investigators screened for activating mutations of PTCH or SMO, and they observed none. It only seems to occur in basal cell carcinoma or medulloblastoma. However, the Hh pathway can be activated by other mechanisms, including overexpression of the ligands. More importantly, evidence has shown that the Hh pathway can be activated by a paracrine effect with ligands coming from the tumor microenvironment (low Hh activity in in vitro cultures but high levels of activation in tumor xenografts). We do not see that in the in vitro system. The other report has shown that the method we use to grow cells can affect Hh signaling. We grow cells as a monolayer on plastic ware. So, my next step would be to study cells grown as spheroids. Other investigators have shown that in the spheroid-grown condition, pathways such as Notch, Wnt, or Hh are activated. Thus, that would be another way of looking at it. What you referred to in your last question is the identification of a biomarker or gene signature predictive of the treatment response. That is the next step of our research project. I appreciate that insightful comment.

Dr Lin. Then, my final question, Dr Jablons' group at the University of California, San Francisco, found that Gli2 was overexpressed, and they thought that resulted from a SMO-independent pathway, perhaps transforming growth factor- β . Did you consider that at all, and did you find any synergistic effects if you inhibited both SMO and Gli at the same time?

Dr Nguyen. Can you repeat that question?

Dr Lin. Did you find any synergistic effects if you inhibited both SMO and Gli simultaneously?

Dr Nguyen. Yes. A report was published by Beachy's group from Stanford studying combining ITRA and ATO (*Cancer Cell.* 2013;23:23-34). Yes, that would be something that we can do, is to combine, targeting different levels of the pathway. As you know, when you treat cells with GDC, SMO mutants emerge that are resistant to the drug. We must find a method to overcome that. One way of doing it would be to target downstream of SMO, such as at the Gli level.

Dr Lin. Congratulations on your work.