Arsenic Trioxide Inhibits the Hedgehog Pathway Which Is Aberrantly Activated in Acute Promyelocytic Leukemia

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Key Words

Acute promyelocytic leukemia · Arsenic trioxide · Gli1 target transcription factors · Gli2 target transcription factors · Hedgehog signaling pathway · Smoothed receptors

Abstract

Background/Aim: Dysregulated Hedgehog (Hh) signaling has been implicated in several human malignancies. Hh signaling inhibitors are predicted to have a minimal effect when the Smoothed receptor is mutated. Implications that Gli proteins are molecular targets of arsenic trioxide (ATO) action prompted us to investigate the expression of Hh signaling in acute promyelocytic leukemia (APL) and the influence of ATO on the Hh signaling pathway in APL. **Methods:** Quantitative real-time reverse transcription polymerase chain reaction and Western blot were employed to analyze the expression of Hh pathway components and the influence of ATO on the Hh signaling pathway in APL. **Results:** The expression of Hh pathway components was significantly up-regulated in APL. In newly diagnosed APL patients, Gli2 expression was significantly positively correlated with Gli1 ($R = 0.57$, $p < 0.001$) and Smo ($R = 0.56$, $p < 0.001$) and the expression of Hh pathway components was significantly higher in

the high WBC group ($p < 0.05$). ATO can significantly down-regulate the expression of Hh pathway components in vitro and in vivo ($p < 0.05$). **Conclusion:** The Hh pathway is aberrantly activated in APL and associated with a bad prognostic factor. ATO can effectively inhibit the expression of the Hh pathway. The obtained data give the first clinical evidence for the application of ATO in tumors exhibiting an aberrantly activated Hh pathway.

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Introduction

The Hedgehog (Hh) signaling pathway was initially discovered in *Drosophila* with the function of regulating embryonic segment development [1]. The functional homologs of *Drosophila* Hh signaling genes were also isolated later in vertebrates, and Hh signaling has long been recognized as critical in the developmental biology as modulated cell cycle regulators in stem cells [2]. More recently, the Hh signaling pathway was found to be dysregulated in several human malignancies and critical for

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the maintenance and expansion of malignant stem cells [3–5]. The Hh signaling pathway has rapidly become the focus of intense study in oncology; thus, inhibitors of the Hh signaling pathway have emerged in recent years as a promising new class of potential therapeutics for cancer treatment [6, 7]. The Hh signaling pathway comprises Hh ligands, Patched (Ptch) and Smoothened (Smo) receptors, and Gli zinc-finger protein transcription factors [8]. Although the role of mutation is controversial, the mutation of Smo and/or Ptch is present in many malignancies [9, 10].

To date, most efforts to develop Hh signaling pathway antagonists have focused on cyclopamine derivatives or other compounds that mimic cyclopamine action in binding to and antagonizing Smo [11–13]. However, these antagonists are not expected to be effective in diseases with an aberrant activation of Hh signaling pathway-involved Smo mutations [14] or resulted from an increased expression or activity of the Gli proteins [15]. Other Hh signaling pathway antagonists, such as Gli antagonist (GANT) small-molecule compounds, were shown to inhibit Gli at the protein level and to inhibit xenograft tumor growth from the 22Rv1 prostate cancer cell line [16]; they will require significant preclinical testing to assess safety and efficacy before entering clinical trials.

Recently, it has been reported that arsenic trioxide (ATO) can antagonize the Hh signaling pathway through direct binding and inhibition of Gli1 and/or Gli2 transcriptional activity [17, 18]. Data from the two groups suggested that ATO inhibited the growth of human cancer cell lines that depended on upregulated Gli expression in vitro and in vivo in a xenograft model of Ewing sarcoma. They also found that ATO improved the survival of a clinically relevant spontaneous mouse model of medulloblastoma with activated Hh pathway signaling. Even more importantly, the growth of Hh pathway-driven medulloblastoma in mice can be inhibited by ATO within a range of serum levels comparable to those achieved in the treatment of human acute promyelocytic leukemia (APL). Their results are particularly noteworthy. As a drug already approved by the FDA, ATO can be used clinically without the need for clinical trials. However, much work remains to be done to prove the inhibition of the Hh signaling pathway by ATO in cancer patients with an abnormal activation of this pathway.

The mechanism of ATO action involves inducing apoptosis and differentiation, altering cellular redox and inhibiting angiogenesis [19]. In APL, the proposed mechanism of action of ATO is via degradation of the promyelocytic leukemia-retinoic acid receptor- α (PML-RAR α)

Table 1. Patient characteristics

Patients	45
Males/females	22/23
Age, years	42 (14–79)
Hemoglobin, g/dl	83 (40–165)
WBC count, $\times 10^9/l$	3.7 (0.4–142)
Platelets, $\times 10^9/l$	31.2 (4–260)
PML-RAR α , %	52.12 (10.66–86.6)

Values represent patient number or median (range).

fusion protein [20], and this mechanism is still under evaluation.

In the present study, it is proposed that Hh signaling is another dysregulated pathway in APL, and ATO inhibits the growth of APL cells partly through inhibiting the Hh/Gli pathway. Our data provide clinical evidence for the hypothesis that ATO could be clinically useful in the treatment of tumors exhibiting activated Hh signaling.

Materials and Methods

Samples and ATO Induction Therapy Protocol

There are 84 APL samples from 45 APL patients altogether in this study, including 45 samples from newly diagnosed APL patients and 39 samples after complete ATO induction. This study also included 44 healthy donors as normal controls. The APL patients included in this study were inpatients of the First Affiliated Hospital of Harbin Medical University from January 2009 to January 2011. The diagnosis of APL was established on the basis of the WHO guidelines, and the characteristic French-American-British M3 bone marrow morphology, the positive results of both cytogenetic evaluation of t(15;17) and a molecular study of PML-RAR α were required. Patients' characteristics are shown in table 1.

ATO solution (10 mg/10 ml) was supplied by the Harbin Yida Pharmaceutical Company. Intravenous ATO infusion, prepared in 5% dextrose, was administered daily at a dose of 7 mg/m² body surface area, with a maximum daily dose of 10 mg. The total daily dose was infused intravenously over the course of 2–4 h. ATO infusion was given daily until achievement of hematological complete remission or up to a maximum of 35–42 doses. Mononuclear cells were obtained after informed consent had been given. The study was approved by the Harbin Medical University Institutional Review Board.

Cell Culture and Cell Treatment

Human APL NB4 cells were maintained in RPMI 1640 (Gibco) medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin (both from Invitrogen). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. NB4 cells were treated with ATO at a concentration of 0, 0.5, 1 and 2 μ M, respectively, for 48 h.

Table 2. Primers for Hh pathway genes

Gene name	GenBank accession No.	Primer sequences
Gli2	NM_005270	F: 5'-AGGACAAGGCCAACATCGTCATCT-3' R: 5'-TCCCTGGCTTTCATTTGCAGTTGG-3'
Smo	NM_005631.4	F: 5'-ACCTATGCCTGGCACACTTC-3' R: 5'-GTGAGGACAAAGGGGAGTGA-3'
Ptch	NM_000264	F: 5'-AAACCTCCTTTGCGGTGGACAAAC-3' R: 5'-TGTAACCATGACCAACCTCAGCCT-3'
Gli1	NM_001160045.1	F: 5'-CCCAATCACAAGTCAGGTTCT-3' R: 5'-CCTATGTGAAGCCCTATTTGCC-3'
β-Actin	NM_001101.3	F: 5'-GGCACCCAGCACAAATGAAG-3' R: 5'-CGTCATACTCCTGCTTGCTG-3'

Table 3. Sequences and positions of the PML-RARα and c-ABL primers and probe

EAC code	Primers/probe localization 5'-3' position (size)	Sequence 5'-3'
ENF903	PML, 1690–1708 (19)	TCTTCCTGCCCAACAGCAA
ENF906	PML, 1642–1660 (19)	ACCTGGATGGACCGCCTAG
ENF905	PML, 1198–1216 (19)	CCGATGGCTTCGACGAGTT
ENP942	RARA, 439–458 (20)	AGTGCCAGCCCTCCCTCGC
ENR962	RARA, 485–465 (21)	GCTTGTAGATGCGGGGTAGAG
ENF1003	Abelson, 372–402 (31)	TGGAGATAACACTCTAAGCATAACTAAAGGT
ENP1043	Abelson, 467–494 (28)	CCATTTTGGTTTGGGCTTCACACCATT
ENR1063	Abelson, 495–515 (21)	GATGTAGTTGCTTGGGACCCA

RNA Isolation

Mononuclear cells were isolated by Ficoll gradient centrifugation (MD Pacific, Tianjin, China, density: 1.077 g/ml) from APL marrow samples and normal controls, snap-frozen, and stored at –80°C. Total RNA was isolated from the bone marrow sample using Trizol (Invitrogen, Paisley, UK) according to the manufacturer's instructions. mRNA was reverse-transcribed into cDNA using random hexamers and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Promega, Madison, Wisc., USA).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

For quantified Hh pathway gene expression, a total 50-μl reaction with FastStart Universal SYBR Green PCR Master (Roche, Basel, Switzerland) was used to perform quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) in STARTGENE Mx3005P (Agilent, Santa Clara, Calif., USA) according to the manufacturer's instructions. PCR for β-actin was used as a control. The primers for each gene are shown in table 2. Patient-specific PML-RARα gene expression was used as PCR targets for quantitative assessment of minimal residual disease, and it

was performed as described in the literature [21]. The primer and TaqMan probe for each gene is shown in table 3. The values of genes of interest were normalized to those of β-actin.

Western Blot Analysis

Treated and untreated NB4 leukemic cells were washed twice in ice-cold PBS and protein extracts were prepared by lysis in RIPA buffer [150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8), 10 mM EDTA and 1 mM PMSF (Beyotime, China)] for 30 min at 4°C. Samples were then centrifuged for 15 min at 15,000 g. Protein concentrations of supernatants were determined. For each sample, 60 μg of protein was loaded onto a 6.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and transferred to a PVDF membrane (Beyotime). Each membrane was blocked for 1 h at room temperature with blocking buffer [TBS containing 0.1% (v/v) Tween 20 (Sigma) and 5% (w/v) milk powder]. Primary antibodies (applied for 1 h at room temperature and overnight at 4°C) were anti-Gli-2 antibody (C-10; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-Gli1 antibody (Abcam, Inc., Cambridge, Mass., USA), anti-Smoothed antibody (Abcam), Anti-Patched/PTCH antibody (Abcam) and anti-actin (mouse

monoclonal C-2, Santa Cruz Biotechnology). Antibodies were diluted 1:1,000 except for the anti-Gli2 antibody (1:500). Thereafter, membranes were incubated for 1 h with AP-labeled secondary antibodies (Amersham Pharmacia Biotech, Uppsala, Sweden), either sheep anti-mouse (diluted 1:2,500) or donkey anti-rabbit (1:5,000), and the protein bands were detected by FluorChem Imaging Systems (Alpha Innotech, Corp., San Leandro, Calif., USA).

Statistical Analysis

Experiments were performed 3 times and data are expressed as mean $\pm$ SD. The qRT-PCR results were converted into fold change based on a doubling of PCR product in each cycle according to the manufacturer's guidelines. The $\Delta\Delta C_t$ was used for statistical analysis. Wilcoxon rank sum two-sample tests were used to compare the groups. The correlation of Hh pathway gene expression and clinical data was analyzed with Pearson correlation. R software (version 2.14.1) was used to perform this analysis. *p* values were obtained between variables, and *p* < 0.05 was considered to be significant.

Results

Expression of Hh Pathway Genes in Newly Diagnosed APL Patients

The expression levels of components of the Hh signaling pathway were determined in newly diagnosed APL patients and normal controls using qRT-PCR. The expression of transcripts revealed that the target transcription factors Gli1 and Gli2, and the Hh pathway receptors Ptch and Smo were expressed in newly diagnosed APL patients (*n* = 45) and normal controls (*n* = 44). Compared with normal controls, there was a significantly upregulated expression of Gli2 (*p* < 0.05), Smo (*p* < 0.001), Ptch (*p* < 0.01) and Gli1 (*p* < 0.01) (fig. 1). The relative expression of Gli1, Gli2, Ptch and Smo was a 3.6-, 2.1-, 7.8- and 2.0-fold change compared with normal controls, respectively.

Relationship of Hh Pathway Gene Expression and Clinical Feature Variables

The correlation of Hh pathway genes and clinical feature variables was estimated with correlation analysis. As was shown in correlation analysis, in newly diagnosed APL patients, the Gli2 expression was significantly positively correlated with Gli1 (*R* = 0.57, *p* < 0.001) and Smo (*R* = 0.56, *p* < 0.001). The percent of bone marrow blasts was significantly positively correlated with the expression of Hh pathway genes, i.e., Smo (*R* = 0.56, *p* < 0.001), Ptch (*R* = 0.44, *p* < 0.01), Gli1 (*R* = 0.78, *p* < 0.001) and Gli2 (*R* = 0.67, *p* < 0.001) in newly diagnosed APL patients (table 4). We then divided the newly diagnosed APL patients into a high WBC group and a not high WBC group according to pretreatment WBC counts of $10 \times 10^9/l$, and

Fig. 1. Relative expression calculated by $2^{-\Delta\Delta C_t}$ of Gli1, Gli2, Ptch and Smo in newly diagnosed APL patients as determined by the qRT-PCR. Samples were obtained from newly diagnosed APL patients (ND) or normal controls (NC). There was a differential gene expression of Gli2 (*p* < 0.05), Smo (*p* < 0.001), Ptch (*p* < 0.01) and Gli1 (*p* < 0.01) in newly diagnosed APL patients compared with normal controls.

Table 4. Correlation analysis table

Gene	Type	Blast	ND-Smo	ND-Ptch	ND-Gli1
Blast	R	1	0.5565	0.4411	0.7792
	p	0	0.0003	0.0056	0
ND-Gli2	R	0.6719	0.5597	0.2439	0.5747
	p	0	0.0003	0.14	0.0002

R = Correlation value; *p* = *p* value; ND = newly diagnosed APL patients.

compared the expression levels of components of the Hh pathway in the two groups. Our data showed that the expression of Hh pathway components was significantly higher in the high WBC group (*p* < 0.05).

Influence of ATO on the Expression of Hh Pathway Molecules in APL Patients and Cell Line

Forty-five newly diagnosed APL patients were included in this study and were given a single ATO induction therapy. At a median follow-up of 28 months (range: 10–46 months), 39 patients (86.67%) achieved hematological

Fig. 2. Relative expression calculated by $2^{-\Delta\Delta CT}$ of Gli1, Gli2, Ptch and Smo in newly diagnosed APL patients and APL patients after ATO induction therapy by qRT-PCR. Samples were obtained from newly diagnosed APL patients (ND) or APL patients after ATO induction therapy (AT). There was a significantly downregulated expression of Gli2 ($p < 0.01$) and Smo ($p < 0.001$) and a trend to decreased Ptch (0.66-fold change, $p = 0.053$) in APL patients after ATO induction therapy.

complete remission, the remaining 6 patients either died early or died prior to achieving a complete remission secondary to disseminated intravascular coagulation or uncontrolled sepsis. For analyzing whether ATO can influence Hh pathway in patients, we compared the expression of Hh signaling components in 39 APL patients who achieved complete remission before and after single ATO induction therapy at the mRNA level. As was shown, there was a significantly downregulated expression of Gli2 ($p < 0.01$) and Smo ($p < 0.001$) after ATO induction therapy. There was a trend to decreased Ptch (0.66-fold change, $p = 0.053$) in APL patients after ATO induction therapy, but the decrease was not statistically significant. Gli1 mRNA expression was only marginally inhibited by ATO, and the reduction was not statistically significant ($p > 0.05$) (fig. 2). We cultured the APL cell line with 0, 0.5, 1 and 2 μM of ATO for 48 h and subjected it to qRT-PCR. At the mRNA level, none of the Smo and Ptch was reduced when the ATO concentration was below 1 μM ($p > 0.05$). However, when the concentration of ATO increased to 2 μM , it could be clearly seen that the expression of Smo and Ptch was significantly downregulated ($p < 0.05$). Furthermore, the mRNA of Gli2 was reduced with the increase in the ATO concentration (fig. 3). There was no significant change of

Fig. 3. The expression of Hh signaling pathway components at the mRNA in NB4 cells cultured under different ATO concentrations for 48 h. Neither Smo nor Ptch was reduced when the ATO concentration was below 1 μM ($p > 0.05$). However, when treated with 2 μM ATO, it could be clearly seen that the expression of Smo and Ptch was significantly downregulated ($p < 0.05$) and the mRNA of Gli2 was reduced with the increase in the ATO concentration.

Gli1 mRNA expression at this time point, as assessed by qRT-PCR ($p > 0.05$), in line with the results from clinical samples. Next we estimated the influence of ATO on Gli2, Smo, Ptch and Gli1 at the protein level. It was found that ATO below 1 μM did not decrease the expression of Gli2, Smo and Ptch. However, when the concentration of ATO increased to 2 μM , it could be clearly seen that the expression of Gli2, Smo and Ptch decreased significantly. There was no significant change of Gli1 protein expression at this time point, in line with the results from qRT-PCR (fig. 4).

Discussion

The results of this study presented for the first time clinical evidence for ATO as an inhibitor of tumors exhibiting an aberrantly activated Hh pathway. The potential of ATO can be used since an Hh signaling antagonist prompted us to investigate the expression of this pathway in APL, a highly curable disease since the introduction of ATO [22], and whether ATO can inhibit the aberrant activation of this pathway in APL.

The cancer stem cell hypothesis suggests that the capacity for tumor initiation and propagation is limited to

Fig. 4. The expression of Gli2, Ptch, Smo and Gli1 at plus level between protein and in NB4 cells cultured with 0, 0.5, 1 and 2 μ M ATO for 48 h. Gli2, Ptch, Smo and Gli1 proteins in NB4 cells cultured with 0, 0.5, 1 and 2 μ M ATO for 48 h were transferred onto the PVDF

membrane (a) and the relative expression of these proteins (b) are shown. ATO below 1 μ M did not decrease the expression of Gli2, Smo and Ptch. The expression of Gli2, Smo and Ptch decreased significantly ($p < 0.05$) when the concentration of ATO increased to 2 μ M.

a small number of very primitive cells within the tumor bulk. Although exhibiting similar characteristics to normal stem cells, cancer stem cells are predominantly quiescent and therefore resistant to conventional therapeutic approaches [23]. The existence of such a population has been conclusively demonstrated in chronic myeloid leukemia and acute myeloid leukemia, and there is accumulating evidence for their existence in other hematological and solid tumors such as myeloma or lymphoma. Evidence has suggested that the Hh signaling pathway, a pathway prominent in cancer stem cells [24], has a key role in chronic myeloid leukemia, lymphoma and multiple myeloma [4, 25, 26].

However, less is known about the Hh signaling pathway and its influence on acute myeloid leukemia. Not much has been reported in the literature regarding the status of Hh or Gli activity in APL patients. One report of a small cohort of 3 patients with APL showed evidence of Gli1 expression in all 3 patients [27]. To study the role of the Hh pathway in APL, we first profiled the expression of Hh pathway members. We found a higher expression of Hh pathway components in APL patients compared with normal controls. The expression of Hh pathway genes was positively correlated to bone marrow blast. Our data confirmed earlier findings in a larger sample size and supported our hypothesis that the Hh pathway may be another pathway essential in the development of APL. In addition, our data supported that the expression of Hh pathway components increased in newly diagnosed APL

patients as a whole, as the correlation analysis showed a strongly positive correlation between Hh pathway components. Since the Hh pathway is frequently activated in APL, the markers for the activation, including Smo, Ptch, Gli1 and Gli2, may be useful for the diagnosis of APL. APL with pretreatment WBC counts $>10 \times 10^9/l$ is considered to carry a poorer prognosis than APL with WBC $<10 \times 10^9/l$ [28]. We divided the newly diagnosed APL patients into two groups according to pretreatment WBC counts $>10 \times 10^9/l$ or $<10 \times 10^9/l$. We found a significantly higher expression of Hh pathway components in the high WBC group. Thus, the expression of Hh pathway components may be prognostic in APL biology.

Next, we concentrated on whether ATO can inhibit the abnormal activation of the Hh pathway in APL patients. We found that in the 39 patients who achieved hematological complete remission, the expression of Gli2 and Smo was significantly downregulated, whereas the expression of Ptch only showed a declining trend. We repeated the amplification procedure several times, but still no difference could be found. The reason might be that the primary CD34+ leukemic cells had not been separated. To rule out the influence of various factors on the expression of Hh signaling pathway components in vivo, we replicated the action of ATO on APL in the NB4 cell line. We found that the expression of Gli1 was still not significantly inhibited by ATO at concentrations of up to 2 μ M. The expression of Gli2 was inhibited by ATO in a dose-dependent way, and the expression of Smo and Ptch was

significantly inhibited by ATO at concentrations of up to 2 μM . Considering the important role of Gli proteins in the Hh signaling pathway, we searched the literature for the mechanism of ATO acting on Gli proteins. Gli proteins are believed to be the targets of ATO acting on the Hh pathway [17, 18]. Gli is a cysteine-rich protein and has zinc finger binding domains that contain cysteine residues. Data showed that arsenic interacts readily with sulfhydryls of peptides derived from the zinc fingers of estrogen receptor, and the basis for arsenic action on the Gli transcriptional effectors could be a direct interaction with the Gli zinc fingers [29]. Data from other researchers [18] have shown that Gli1 mRNA and protein were not significantly decreased by the treatment with 3 μM ATO for 12 h. However, ATO altered the expression of the endogenous Gli1 target gene Ptch. Therefore, they believed that the decrease in target gene expression Ptch was not due to a decrease in Gli1 protein levels, but a decrease in Gli activity. Other scientists [18] reported that ATO antagonized the Hh pathway by targeting Gli transcriptional effectors, in the short term, by blocking Hh-induced ciliary accumulation of Gli2 and with prolonged incubation by reducing steady-state levels of Gli2. We did not find any changes of Gli1 in APL at mRNA and protein level after ATO treatment in our experiment. Therefore, we presumed that through inhibiting the activity of Gli1 and reducing the expression of Gli2, ATO effectively antagonized the Hh signaling pathway. The results at the protein level confirmed our hypothesis, and the expression of Gli2 and the target gene of Gli1, i.e., Ptch and Smo, was significantly inhibited at the protein level when treated with ATO at the concentration of 2 μM . It has been shown that ATO presented dose-dependent dual effects in vitro and in vivo, i.e., low-dose ATO (0.1–0.5 μM) induces partial differentiation, while high-dose ATO (1–2 μM) triggers apoptosis [30]. ATO exerts apoptotic effects through a variety of other signal pathways not only on APL cells but also on other cancer cells. In this research, when a concentration of 1 μM ATO was used, no change could be seen in Hh pathway components at the protein level. Therefore, the influence of ATO on the Hh pathway components may mean a unique change in APL independent of the apoptosis pathway. In addition, it is still unclear whether Gli1 or Gli2, as important transcription factors of Hh signaling pathway, plays a more important part in the action of ATO on APL, and this needs to be discussed further.

Finally, we would like to discuss the feasibility of the application of ATO as an Hh pathway antagonist. Although several other Hh signaling pathway antagonists,

such as Gli antagonist (GANT) small-molecule compounds, have been reported to function at the level of the Gli proteins, significant preclinical testing is required to assess safety and efficacy before entering clinical trials. Unlike ATO, an already FDA-approved treatment for APL is safe enough to enter clinical practice. For Hh-driven malignancies and malignancies with elevated levels of Gli protein, the ATO dosing regimen used in APL can serve as a baseline for determining the appropriate dose.

In conclusions, the present study indicates that Hh signaling is another dysregulated pathway associated with a bad prognostic factor in APL patients and the action of ATO on APL may be partly through inhibiting the Hh/Gli pathway. Our data provide the first clinical evidence for the hypothesis that ATO could be clinically useful against tumors exhibiting activated Hh signaling.

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Disclosure Statement

None of the authors has any potential conflicts of interest to declare.

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