Hedgehog Signaling Drives Cellular Survival in Human Colon Carcinoma Cells

Tapati Mazumdar, Jennifer DeVecchio, Ting Shi, Janay Jones, Akwasi Agyeman, and Janet A. Houghton

Abstract

Aberrant activation of Hedgehog (HH) signaling is implicated in many human cancers. Classical HH signaling is characterized by Smoothened (Smo)-dependent activation of Gli1 and Gli2, which transcriptionally regulate target genes. A small molecule inhibitor of Gli1 and Gli2, GANT61, was used to block HH signaling in human colon carcinoma cell lines that express HH signaling components. GANT61 administration induced robust cytotoxicity in 5 of 6 cell lines and moderate cytotoxicity in the remaining 1 cell line. In comparison, the classical Smo inhibitor, cyclopamine, induced modest cytotoxicity. Further, GANT61 treatment abolished the clonogenicity of all six human colon carcinoma cell lines. Analysis of the molecular mechanisms of GANT61-induced cytotoxicity in HT29 cells showed increased Fas expression and decreased expression of PDGFRα, which also regulates Fas. Furthermore, DR5 expression was increased whereas Bcl-2 (direct target of Gli2) was down-regulated following GANT61 treatment. Suppression of Gli1 by shRNA mimicked the changes in gene expression observed in GANT61-treated cells. Overexpression of dominant-negative FADD (to abrogate Fas/DR5-mediated death receptor signaling) and/or Bcl-2 (to block mitochondria-mediated apoptosis) partially rescued GANT61-induced cytotoxicity in HT29 cells. Thus, activated Gli genes repress DR5 and Fas expressions while upregulating Bcl-2 and PDGFRα expressions to inhibit Fas and facilitate cell survival. Collectively, these results highlight the importance of Gli activation downstream of Smo as a therapeutic target in models of human colon carcinoma. Cancer Res; 71(3): 1092–102. ©2010 AACR.

Introduction

Activation of the canonical Hedgehog (HH) signaling pathway is initiated by the binding of HH ligands (namely Shh, Ihh, or Dhh) to the transmembrane receptor Patched (Ptc), which becomes internalized leading to the activation of the transmembrane signaling molecule Smo via release from Ptc-dependent suppression. Smo activates the final arbiter of HH signaling, the Gli family of transcription factors that regulate HH target gene expression (1).

HH signaling is important during normal embryonic development and its aberrant activation has been associated with many human cancers (reviewed in ref. 2). HH signaling is also critical in the regulation of cellular proliferation, stemness, cell fate determination, and cellular survival in a variety of organs (3, 4). Gli1 is amplified in glioma (5), osteosarcoma, and rhabdomyosarcoma (6), whereas Gli2 is amplified in oral squamous cell carcinoma (7). Mutations in Ptc or Smo are also prevalent in basal cell carcinomas, medulloblastomas, and cancers of the esophagus and bladder (reviewed in ref. 8), and sustained and activated HH–Gli signaling has led to the development of medulloblastomas in Ptc+/− mice (9). Melanomas and carcinomas of the prostate have further shown a HH–Gli signaling axis, inhibited by cyclopamine at the level of Smo (10, 11). In gastrointestinal cancers, HH signaling activation occurs not by mutation or amplification of signaling molecules, but via transcriptional upregulation of the HH ligands (8). It has recently been suggested that HH signaling progresses during colon carcinogenesis (12, 13) and in metastatic disease (13), whereas in normal colonic tissue, HH signaling is involved in differentiation (14, 15). However, very little is known regarding the specific role of HH signaling in regulating cellular survival and proliferation in colon cancers, and the downstream target genes involved in determination of cell fate.

The Gli family of transcription factors has activator and repressor functions that are defined only partially and can respond to combinatorial and cooperative Gli activity (3). Although dispensable for normal development (16, 17), Gli1 plays a key role in HH-driven cancers (4, 16), whereas less is known about the role of Gli2 in HH signaling in tumors (17). Gli2 appears to be the primary activator of HH signaling, with Gli2 as a transcriptional target of Gli2, which may amplify HH-induced, Gli2-mediated transcription of Gli1 target genes (8, 18–20); Gli2 and Gli1 also induce transcription of overlapping and distinct sets of target genes (17). The roles of Gli1 and Gli2 in HH-driven cellular survival and cell death responses remain
ill-defined, and specifically, their role in cellular survival of colon cancer is unknown.

We have recently profiled genes that are regulated downstream of Gli1 and GlI2 that are either direct or indirect targets and are involved in cellular proliferation including genes that regulate the cell cycle, such as Cyclin D, CYCLIN B, FOXM1, CDC25 family members, and CDC2 (4, 8, 21–23). Other genes that are involved in cell signaling or the regulation of cell survival including PDGFRα (24, 25) and BCL-2 (26) also function downstream of the Gli proteins. PDGFRα is a gene that encodes a cell surface tyrosine kinase receptor, expressed in human cancers, and whose expression is regulated by Gli1 (25). Thus, decreased expression of Gli1 reduces the expression of PDGFRα, which via Erk, removes the repression imposed on Fas expression (24, 27), a molecule that is well known to be an important regulator of cell death in colon cancer cells (28, 29). Further, overexpression of Gli1 or PDGFRα has rendered basal cell carcinoma cells resistant to cyclopamine (27). The BCL-2 gene encodes an integral outer membrane protein that blocks apoptotic cell death and is known to be an important regulator of cell death in colon carcinoma cells (30). Bcl-2 is transcriptionally regulated by Gli2 (26), in contrast to its family member Bcl-xL.

To identify downstream targets of the Gli genes that regulate cellular survival in the context of colon cancer, we employed a small molecule inhibitor of Gli1 and Gli2, GANT61, identified in a cell-based small molecule screen for inhibitors of Gli1-mediated transcription (31). GANT61 acts in the nucleus to block Gli function, inhibits both Gli1- and Gli2-mediated transcription, and shows a high degree of selectivity for HH/Gli signaling (31). In a panel of 6 well-characterized human colon carcinoma cell lines, we showed that inhibition of the HH signaling pathway by targeting the Gli genes using GANT61 induced significant cell death in all the cell lines whereas the conventional Smo inhibitor, cyclopamine, showed only modest cytotoxic activity. The components of the canonical HH signaling pathway were present in all cell lines. In further detailed analyses of HT29 cells, GANT61 decreased both Gli1 and Gli2 expressions, and decreased Gli-luciferase reporter activity. In addition, partial knockdown of both Gli1 and Gli2 expressions using shRNA conferred equivalent and partial resistance to GANT61-induced cytotoxicity confirming that cytotoxicity is dependent on Gli inhibition in response to GANT61 treatment. Further, decreased expression of PDGFRα concomitant with elevation in Fas, elevated expression of the death receptor DR5 (known to be functional in colon carcinoma cells; ref. 32), and decreased expression of the antiapoptotic factor Bcl-2 were shown, and PARP cleavage and activation of caspase-3 were also induced. Similar changes in gene expression were obtained by Gli1 knockdown using Gli1shRNA. Using HT29 cells transfected with a dominant-negative mutant form of FADD (DNFADD; ref. 33) to inhibit the function of both Fas and DR5, partial protection from GANT61-induced cell death was observed. When Bcl-2 was overexpressed prior to GANT61 treatment, partial protection from GANT61-induced cytotoxicity was also observed. Similar partial protection was obtained when DNFADD and Bcl-2 were overexpressed simultaneously in HT29 cells, suggesting that the extrinsic pathway via death receptors and the intrinsic pathway via the mitochondria are not mutually exclusive for the induction of cell death, and contribute to the regulation of HH-dependent cell survival in colon cancer cells.

Materials and Methods

Cell culture and reagents

HT29, HCT116, SW480, and HCT8 cells were obtained from ATCC. GC3/c1 and VRC5/c1 cells were established in our laboratories from a human colon adenocarcinoma xenograft model (34). Cell lines were routinely verified by morphology, growth characteristics, response to cytotoxic agents (Annexin V/propidium iodide (PI) staining), cDNA microarray gene profiles were also characteristic. Cell lines were verified biannually to be mycoplasma-free. Cells were maintained in the presence of folate-free RPMI 1640 medium containing 10% dFBS and 80 nmol/L [6RS]-methyltetrahydrofolate. Antibodies against SHH, Ptc, Smo, PARP, PDGFRα, Fas, β-actin, and HSP90/β were purchased from Santa Cruz Biotechnology. Antibodies against Gli2, DR5, and caspase-3 were obtained from Cell Signaling Technology, and against Gli1 from Novus Biologicals. GANT61 was purchased from Alexis Biochemicals, and cyclopamine from Toronto Research Chemicals.

Gli1shRNA, Gli2shRNA, DNFADD, and Bcl-2 transduction

Gli1shRNA (5′-CGCTTGGACAACCTCAGCTGACCAGCT-3′) and Gli2shRNA (5′-CCACGGAAAGCCTGGCTCTG-GACAAC-3′) were obtained from OriGene Technologies, Inc. HT29 cells stably expressing Gli1shRNA or Gli2shRNA were generated by transducing HT29 cells with scrambled shRNA (control), Gli1shRNA, or Gli2shRNA expressing retroviruses for 48 hours (30% retroviral supernatant and 50% normal growth media). Following transduction, the cells were washed 3 times with 1× PBS and allowed to grow for 3 passages before screening for gene expression. Once decreased expression of the targeted gene was confirmed, the cells were used for experiments. Stable expression of Gli1shRNA and Gli2shRNA was ensured by culturing cells in the presence of a selection antibiotic, puromycin (1 μg/mL). The HT29 cell line simultaneously expressing both Gli1shRNA and Gli2shRNA was generated by transducing HT29 cells stably expressing Gli1shRNA with Gli1shRNA expressing retroviruses, for 48 hours (50% retroviral supernatant and 50% normal growth media). The transduced cells were washed 3 times with 1× PBS and used for experiments. GANT61 was stably expressed by transducing HT29 cells expressing DNFADD, for 48 hours (50% retroviral supernatant and 50% normal growth media). The transduced cells were washed 3 times with 1× PBS and used for experiments.
Clonogenic assays

The cells were plated at densities of 1,500 (HT29, HCT8, HCT116) and 3,000 (SW480, GC3/c1, VRC5/c1) cells/well in 6-well plates. Following overnight attachment, cells were treated, in triplicate, with varied concentrations of GANT61 (0–20 μmol/L) for 72 hours. Drug was removed and replaced with fresh media containing dThd (20 μmol/L) for a period equivalent to 7 cell doublings (7 days for HT29, SW480, GC3/c1, and VRC5/c1; 5 days for HCT8 and HCT116). Cells were washed with 1× Dulbecco’s PBS (without Ca²⁺ or Mg²⁺) and allowed to dry overnight. The following day, cells were stained with crystal violet, and colonies analyzed using an Alpha Innotech imager.

Annexin V/PI staining and flow cytometric analysis

Annexin V/PI staining and flow cytometric analysis was performed as described previously (35). Briefly, cells were treated, in duplicate, as described in the figure legends, after which they were collected by trypsinization and incubated treated, in duplicate, as described in the figure legends, after which they were collected by trypsinization and incubated with Annexin V FITC (BD Biosciences) and PI (Sigma) prior to analysis using a FACSCalibur flow cytometer. Raw data were analyzed by CellQuest software.

Western blot analysis

Total cellular lysates were prepared using RIPA lysis buffer (Cell Signaling Technology). Protein (54 μg) was loaded and separated on a 10% or 5% Tris-HCl gel. Proteins from the gel were transferred on polyvinylidene difluoride membranes and subsequently blocked in blocking buffer [5% nonfat dry milk in 1× Tris Buffer Saline with 0.1% Tween 20 (TBS-T)] for 1 hour. Membranes were washed in 1× TBS-T and incubated with primary antibody overnight at 4°C; they were subsequently washed and incubated with the secondary antibody for 1 hour after which they were developed using Super Signal Pico substrate from Pierce Biotechnology.

RNA isolation and mRNA analysis

Total RNA was isolated using the Qiagen RNeasy mini kit according to the manufacturer’s protocol. Total RNA was converted to cDNA using random primers (Script Select cDNA synthesis kit; BIO-RAD), and used for real-time mRNA expression analysis using 40 cycles of Applied Biosystems 7500 Real-Time PCR instrumentation and software. Basal levels of canonical HH signaling components were analyzed by 35 cycles of conventional PCR using the cDNA prepared as described earlier in the text. Primers specific for each of the signaling molecules were designed using NCBI/Primera-BLAST and used to generate the PCR products, subsequently resolved and visualized on a 1% agarose gel. The following primers were used:

\[
\text{PTC-Forward: } 5'-\text{CCAGAGAGGAGCTTCTTAC-3'}
\]
\[
\text{PTC-Reverse: } 5'-\text{CTGGATATTTGCGCTTCC-3'}
\]
\[
\text{GLI1-Forward: } 5'-\text{GCCAGACAGAGCCACTC-3'}
\]
\[
\text{GLI1-Reverse: } 5'-\text{CTGAGCATCCATTACAAGGCA-3'}
\]
\[
\text{GLI2-Forward: } 5'-\text{CCCGCTGTCAGAAAGAGAA-3'}
\]
\[
\text{GLI2-Reverse: } 5'-\text{TCCACCGGCCTTGCTATT-3'}
\]
\[
\text{GAPDH-Forward: } 5'-\text{CAGGCTCAAGATCTACGCA-3'}
\]
\[
\text{SHH-Forward: } 5'-\text{CGGGAGGAGGACCCCTCC-3'}
\]
\[
\text{SHH-Reverse: } 5'-\text{GTACCTCTGTGGGCTGGT-3'}
\]
\[
\text{SMO-Forward: } 5'-\text{CAGGGAGAGCCAGCAGC-3'}
\]
\[
\text{SMO-Reverse: } 5'-\text{TCCGGAGGCTATGACG-3'}
\]
\[
\text{DR5-Forward: } 5'-\text{GTTCGCTCTGATCCCAAC-3'}
\]
\[
\text{DR5-Reverse: } 5'-\text{CTGCAAACTGACTCTATG-3'}
\]

Gli-luciferase assay

The Gli-luciferase reporter construct (kindly provided by Dr. Rune Toftgard, Karolinska Institutet) contains 12 consensus Gli binding sites (36). HT29 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with 4 μg Gli-luc and 0.4 μg pRLTK (renilla luciferase driven by TK promoter). Twenty-four hours posttransfection, cells were treated with GANT61 (20 μmol/L) for 36 hours, and harvested using the dual luciferase kit (Promega Corporation) according to the manufacturer’s protocol. Luciferase activity was detected by a Victor2 multilabel counter, and normalized to renilla luciferase activity as a control for transfection efficiency.

Results

Canonical HH pathway signaling components are expressed in human colon carcinoma cell lines

We have shown that canonical HH signaling pathway components, including the ligand, Shh, and the signaling molecules Ptc, Smo, Gli1, and Gli2, are expressed in human colon carcinoma cell lines, determined by RT-PCR (Fig. 1A) or by Western analysis (Fig. 1B). Shh, Smo, and Gli1 were consistently expressed among the cell lines, whereas the levels of both Ptc and Gli2 were more variable. Of interest, all the 6 human colon carcinoma cell lines expressed the secretory HH ligand, Shh, shown by both RT-PCR and by Western analysis, which supports the existence of an autocrine HH signaling pathway in these cells, and is consistent with the known transcriptional upregulation of HH ligands in gastrointestinal malignancies (8).

Targeting Gli1 and Gli2 (GANT61) induced greater cell death than targeting Smo (cyclopamine)

Previous studies have targeted Smo with cyclopamine, a natural inhibitor, and have reported modest cytotoxicity in human colon cancer cell models (13, 37). GANT61 has been recently identified as a small molecule inhibitor of Gli1 transcriptional activity, which also abrogates Gli2-mediated transcription (31). We compared the efficacy of both cyclopamine and GANT61 in the panel of 6 human colon cancer cell lines. Cells were treated, in duplicate, with either cyclopamine (20 μmol/L) or GANT61 (20 μmol/L) for up to 72 hours prior to flow cytometric analysis to determine the extent of cell death by Annexin V/PI staining, as described in Materials and Methods. Cell death was initiated within 24 hours following exposure to GANT61, but was maximally observed between 48 hours and 72 hours for all cell lines except for HCT8, cell death was more than 80% at 72 hours, and for HCT8, this was approximately 60% (Fig. 2A). In contrast, cyclopamine induced
Figure 1. Components of the canonical HH signaling pathway are expressed in human colon carcinoma cell lines. A, mRNA expression, with GAPDH employed as the endogenous control; B, Western blot analysis, with β-actin for loading control.

Figure 2. Inhibition of HH signaling induced cell death in human colon carcinoma cell lines. A, GANT61 induced greater cell death than cyclopamine at equimolar concentrations (20 μmol/L) for up to 72 hours. B, dose response to GANT61 (0–30 μmol/L) after 72 hours exposure. Cell death was determined by Annexin V FITC/PI staining and FACS analysis. Data represent mean ± SD, n = 2.
modest cytotoxicity in all cell lines examined, except for SW480 (approximately 55% cell death; Fig. 2A) when administered at equimolar concentrations and for the same period of time as GANT61. All cell lines responded to GANT61 treatment for 72 hours in a dose-dependent manner for a range of concentrations (0–30 μmol/L; Fig. 2B), with 20 μmol/L achieving almost maximal cytotoxicity. Thus, GANT61 was further employed in mechanistic studies.

GANT61 inhibited colony forming ability

Cells were treated, in triplicate, with increasing concentrations of GANT61 (0–20 μmol/L) for 72 hours, and clonogenic survival determined (Fig. 3). HCT116, VRC5/c1, and GC3/c1 cells were sensitive at 10 μmol/L GANT61 (<10% clonogenic survival), and almost no cell survival in all 6 colon carcinoma cell lines following exposure to 20 μmol/L GANT61 (IC50: 5–15 μmol/L; Fig. 3).

GANT61 downregulates Gli1 and Gli2 expressions in HT29 cells

The influence of GANT61 (20 μmol/L) on the expressions of both Gli1 and Gli2 was subsequently determined by real-time PCR in HT29 cells for up to 72 hours after treatment (Fig. 4A). Gli2 expression was more rapidly decreased in comparison to Gli1, with 50% decreased expression by 48 hours; Gli1 downregulation was approaching these levels by 72 hours. In addition, Western analysis confirmed reduced expressions of both Gli1 and Gli2 in HT29 cells by 72 hours after GANT61 treatment (Fig. 4B). In a third experiment, transient transfection of HT29 with a Gli-dependent luciferase reporter construct followed by exposure to GANT61 (0–20 μmol/L) for 36 hours showed a 50% decrease (at 20 μmol/L concentration) in Gli-dependent luciferase reporter activity and hence reduced transcriptional activity of the Gli genes (Fig. 4C). To confirm the Gli-dependent cytotoxic effects of GANT61, we generated an HT29-derived stable cell line simultaneously expressing Gli1shRNA and Gli2shRNA. Partial knockdown of both Gli1 and Gli2 expressions resulted in partial (50% cell death in Gli1shRNA + Gli2shRNA compared with 80% in cell death in scrambled shRNA) but significant (p < 0.0005) protection from GANT61-induced cytotoxicity at 72 hours posttreatment (Fig. 4D), supporting Gli-mediated cytotoxic effects of GANT61 in human colon cancer cells.

GANT61 differentially regulates genes involved in the balance between cell death and cell survival

HT29 cells were exposed to GANT61 (10 μmol/L, 20 μmol/L) for 72 hours followed by Western analysis to determine the expression of genes involved in the regulation of cell death [PDGFRα, Fas, DR4, DR5 (DR5L, DR5S)] and cell survival (Bcl-2), of which PDGFRα can be regulated by Gli1 (25), and Bcl-2 is a direct transcriptional target of Gli2 (26). Expression of PDGFRα was decreased following GANT61 treatment, with concomitant increase in Fas (Fig. 5A). No increase in expression of the TRAIL receptor DR4 was observed, in contrast to DR5 expression, in which the short isoform of DR5, DR5S, was elevated. In contrast, Bcl-2 expression was decreased by GANT61 treatment. Cleavage of PARP and activation of caspase-3, both markers of apoptosis, were also increased after GANT61 exposure, correlating with the change in expression of genes that regulate cell death (Fig. 5A). Maximal effects on gene expression were obtained with 20 μmol/L GANT61 exposure, correlating with the extent of cell death determined in Annexin V/PI staining and clonogenic survival assays. To genetically determine the effects of Gli1 knockdown, Gli1 expression was inhibited in HT29 cells.
using Gli1shRNA (Fig. 5B). Following Gli1 knockdown, PDGFRα and Bcl-2 expressions were both decreased, whereas Fas and DR5 expressions were simultaneously increased, similar to the effects of GANT61 (Fig. 5B). Further, upregulation of DR5 by GANT61 (20 μmol/L) at the level of transcription was also shown at 48 hours after treatment (Fig. 5C). The clonogenic cell survival of HT29 cells, stably expressing Gli1shRNA, was reduced by approximately 60% compared with scrambled shRNA expressing HT29 cells in the presence of GANT61 (20 μmol/L; Fig. 5D).

Overexpression of DNFADD and Bcl-2 inhibit GANT61-induced cell death
Since GANT61 induced the expressions of both Fas and the short isoform of DR5, DR5 S, signaling via both receptors at the level of the adaptor molecule, FADD, was inhibited by transduction of DNFADD (33). HT29 cells stably transfected with DNFADD partially protected (approximately 50% cell death) from GANT61-induced cytotoxicity (20 μmol/L, 72 hours; approximately 45% cell death). Further, on overexpression of Bcl-2, HT29 cells were also partially protected (approximately 50% cell death) from
GANT61-induced cytotoxicity (20 μmol/L, 72 hours; p < 0.005; Fig. 6A). When DNFADD and Bcl-2 were overexpressed simultaneously in HT29 cells, confirmed by Western analysis (Fig. 6B), GANT61-induced cell death also remained partially inhibited (Fig. 6A).

**Discussion**

HH signaling events have been implicated in the tumor cell survival and growth of many human cancers that include basal cell carcinoma (27), subsets of medulloblastoma (9, 38), prostate cancer (18), pancreatic cancer (39), rhabdomyosarcoma (40), and breast cancer (41). Recent reports also suggest that HH signaling is required for colon cancer cell survival, such that blocking active HH signaling with the pharmacologic inhibitor cyclopamine (37) or shRNA, both targeting Smo, induced apoptosis (13). We have shown that human colon carcinoma cell lines consistently express HH signaling components including Ptc, Smo, Gli1, Gli2, and, most importantly,
the ligand, Shh, suggesting an autocrine mode of HH signaling in these cells. Activation of the HH signaling cascade consistently induces Gli1 transcription (42), hence Gli1 mRNA and protein expression, expressed in all cell lines in this study, is indicative of active HH signaling.

To identify new therapeutic targets, inhibition of HH signaling has been attempted in various human cancer models including basal cell carcinoma (27), prostate cancer (11), rhabdomyosarcoma (40), and pancreatic cancer (43). Historically, Smo antagonists have been used to abrogate HH signaling in human cancers with moderate success (40). Natural and synthetic pharmacologic agents including cyclopamine (11) and GDC-0449, respectively, have inhibited survival and anti-tumor functions in preclinical models of human cancers whereas GDC-0449 has recently obtained responses in one medulloblastoma (44) and certain basal cell carcinomas in patients (45). However, various levels of resistance have been observed with each of the clinical trials that have been performed. These observations highlight the need for identifying better therapeutic targets that will effectively block HH signaling. One potential druggable target lies in the family of Gli transcription factors, Gli1 and Gli2, which are the final arbiters of transcriptional regulation in the HH signaling pathway.

A recent study identified a small molecule inhibitor, GANT61, which effectively blocked Gli1 DNA-binding and transcriptional activity, and also interrupted Gli2 activator functions (31). In the context of colon cancer, previous attempts to block HH signaling at the level of Smo, induced only moderate cytotoxicity in these cells (37). In contrast, exposure to GANT61 induces significant cytotoxicity in human colon cancer cells, (60%–90% cell death), which is greater than that induced by exposure to cyclopamine at equimolar concentrations and equivalent durations. This finding suggests that direct targeting of the Gli transcription factors, rather than Smo, upstream of Gli1 and Gli2, is more efficient in inducing cytotoxicity in colon cancer cells (Fig. 2). Recent studies in pancreatic ductal adenocarcinoma show Smo-independent activation of Gli proteins that are dependent on TGF-β and KRAS signaling (46). Although comprehensive data are lacking, it has been suggested that oncogenic signals such as KRAS may affect HH signaling, because both aberrant activation of HH signaling and RAS mutations are found in colon cancers (reviewed in ref. 47).

GANT61 treatment decreased constitutive Gli1 and Gli2 mRNA and protein expression and Gli-luciferase reporter activity in the human colon carcinoma cell line, HT29, consistent with previous observations in HEK293 cells engineered to express Gli1 (31). Of particular interest, GANT61 treatment of human colon carcinoma cell lines markedly reduced their colony forming ability by more than 90%, thereby showing potential for therapeutic application. It was previously reported that GANT61 was identified as an inhibitor of Gli1 transcriptional activity, and also abrogated Gli2-mediated transcription (31). Subsequently, we have observed that reduction in Gli2 mRNA and protein expression preceded that of Gli1 in human colon carcinoma cells. Further, Gli2 is known to transcriptionally regulate Gli1 expression (8, 18–20). Because the HH signaling pathway is already activated in human colon carcinoma cell lines, studies using shRNA knockdown of both Gli1 and Gli2 were conducted. Because HH signaling is critical to cellular survival, complete knockdown of the Gli genes could not be obtained. However, stable expressions of Gli1shRNA and

Figure 6. Overexpression of DNFADD and Bcl-2 inhibit GANT61-induced cell death. A, expression of DNFADD or overexpression of Bcl-2, or simultaneous expression of both DNFADD and Bcl-2, in HT29 cells, impart partial protection against GANT61-induced cell death. HT29 cells were stably transduced with either DNFADD or Bcl-2, as described in Materials and Methods. HT29 cells expressing Bcl-2 were further transduced with DNFADD to generate dual expression of both Bcl-2 and DNFADD. Cells were treated with GANT61 (20 μmol/L) for 72 hours and cell death determined by Annexin V FITC/PI staining and FACS analysis. Data represent the mean ± SD of 3 determinations. B, expression of Bcl-2 and DNFADD was confirmed by Western analysis. DNFADD is a truncated form of FADD. GFP was the control for transgene expression, and β-actin for the loading control.
Gli2shRNA, and partial knockdown of Gli1 and Gli2, in HT29 cells, conferred partial but significant protection from GANT61-induced cytotoxicity. These data further support the Gli-specific mode of action of GANT61, and further show the importance of functional Gli genes in maintaining cellular survival in human colon carcinoma cells.

We characterized the molecular mechanisms of GANT61-induced cell death in HT29 cells. Exposure to GANT61 resulted in caspase-3 activation and PARP cleavage, characteristic of apoptotic cell death (48). We also determined the contributions of both the mitochondria-mediated intrinsic and death receptor signaling-mediated extrinsic apoptotic cell death pathways, based on the known regulation of PDGFRα upstream of Fas (24), and of Bcl-2, which may be a direct transcriptional target of both Gli1 and Gli2, in HH-dependent cell survival (26). We have shown that GANT61 treatment reduces Bcl-2 expression and overexpression of Bcl-2 partially rescues from GANT61-induced cytotoxicity in HT29 cells (Fig. 7). These observations underscore the important role of Bcl-2 in contributing to HH-dependent colon cancer cell survival.

A second important cell death signaling mechanism is mediated by the death receptor signaling pathway. Death receptors are cell surface proteins that belong to the TNF family (reviewed in ref. 49). These receptors contain an 80 amino acid long cytoplasmic region, the death domain, which interacts with the death domains of adaptor molecules that in turn transduce cell death signals (49). The specific death receptors, Fas (29) and DR5 (50), are expressed in colon cancers. Exposure to GANT61 induced a marked increase in the expression levels of both Fas and the short isoform of DR5, DR5S, but not TRAIL receptor DR4, suggesting the potential involvement of Fas and DR5 in GANT61-induced cytotoxicity. The regulation of DR5 expression by the Gli genes is currently unknown and may be via an indirect mechanism, because no Gli binding sites can be identified in the promoter region of DR5. However, GANT61 induced upregulation of DR5 mRNA in HT29 cells, suggesting transcriptional regulation of DR5 by a currently unknown mechanism. The adaptor molecule FADD is a critical component of death receptor signaling, and functions downstream of both the death receptors Fas and DR5. Thus, we inhibited the function of FADD with DNFADD, which would inhibit death receptor signaling downstream of the receptor complex. Data show that DNFADD had a protective effect from GANT61-induced cell death in HT29 cells, confirming the importance of suppression of the death receptor signaling pathway in HH-dependent colon cancer cell survival. GANT61-elicited cytotoxicity in human colon cancer cells may be mediated by multiple mechanisms such as cell cycle checkpoints, DNA damage response and autophagy and are currently under investigation.

This work has defined two molecular determinants of cell death following the inhibition of HH signaling by GANT61 in human colon cancer cells that include a functional death receptor signaling pathway, and suppression of Bcl-2 expression. Inhibition of Gli1 expression and function in HT29 cells using RNA interference technology mimicked the effects of GANT61 administration, supporting the specificity of GANT61. Collectively (see schema in Fig. 7), this study shows the significance of HH signaling to cellular survival via activation of Gli1 and Gli2 in human colon carcinoma cells. Activated Gli proteins regulate downstream targets of HH signaling, including Bcl-2, PDGFRα, Fas, and DR5. GANT61 (targeting Gli) blocks the direct functions of Gli activators, resulting in greater cell death than that induced by cyclopamine, which targets Smo.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Grant Support**

This work was financially supported by NCI awards R01 CA 32613 (J.A. Houghton) and NCI RO1 108929 (J.A. Houghton), and by the Cleveland Clinic. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 28, 2010; revised November 2, 2010; accepted December 1, 2010; published OnlineFirst December 6, 2010.
References


www.aacrjournals.org

Cancer Res; 71(3) February 1, 2011

1101

Published OnlineFirst December 6, 2010; DOI: 10.1158/0008-5472.CAN-10-2315

Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 2011 American Association for Cancer Research.
Hedgehog Signaling Drives Cellular Survival in Human Colon Carcinoma Cells

Tapati Mazumdar, Jennifer DeVecchio, Ting Shi, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-2315

Cited articles
This article cites 47 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/3/1092.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/71/3/1092.full.html#related-urls

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.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.