Hedgehog signaling drives cellular survival in human colon carcinoma cells

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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 5/6 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 6 human colon carcinoma cell lines. Analysis of the molecular mechanisms of GANT61-induced cytotoxicity in HT29 cells, demonstrated increased Fas expression and decreased expression of PDGFRα, which also regulates Fas. Furthermore, DR5 expression was increased while 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 expression while up-regulating Bcl-2 and PDGFRα expression 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.

INTRODUCTION

Activation of the canonical 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 (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), while 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 (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 demonstrated 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 up-regulation 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), while 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 Gli1 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 G1 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 expressed and functional 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 demonstrates a high degree of selectivity for HH/Gli signaling (31). In a panel of six well-characterized human colon carcinoma cell lines, we demonstrated that inhibition of the HH signaling pathway by targeting the Gli genes using GANT61, induced significant cell death in all of the cell lines while the conventional Smo inhibitor, cyclopamine, demonstrated 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 expression, and decreased Gli-luciferase reporter activity. In addition, partial knockdown of both Gli1 and Gli2 expression using shRNA conferred equivalent and partial resistance to GANT61-induced cytotoxicity confirming that cytotoxicity is dependent upon 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 (32), and decreased expression of the anti-apoptotic factor Bcl-2, were demonstrated, 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; (33) to inhibit the function of both Fas and DR5, partial protection from GANT61-induced cell death was observed. When Bcl-2 was over-expressed prior to GANT61 treatment, partial protection from GANT61-induced cytotoxicity was also observed. Similar partial protection was obtained when DNFADD and Bcl-2 were over-expressed 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/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 nM [6RS]5-methyltetrahydrofolate. Antibodies against SHH, Ptc, Smo, PARP, PDGFR\(\alpha\), Fas, \(\beta\)-actin and HSP90\(\alpha/\beta\) were purchased from Santa Cruz Biotechnology (CA). Antibodies against Gli2, DR5 and caspase-3 were obtained from Cell Signaling Technology (MA), and against Gli1 from Novus Biologicals (CO). GANT61 was purchased from Alexis Biochemicals (CA), and cyclopamine from Toronto Research Chemicals, Canada.

Gli1\(\text{shRNA}\), Gli2\(\text{shRNA}\), DNFADD and Bcl-2 transduction: Gli1\(\text{shRNA}\) (5'-CGCCTTGAGAACCTCAGGCTGGACCAGC-3’) and Gli2\(\text{shRNA}\) (5’-CCACGGAAAGCACTGGCTTCTCTGACAAC-3’) were obtained from OriGene Technologies, Inc, (MD). HT29 cells stably expressing Gli1\(\text{shRNA}\) or Gli2\(\text{shRNA}\) were generated by transducing HT29 cells with either scrambled shRNA (control), Gli1\(\text{shRNA}\) or Gli2\(\text{shRNA}\) expressing retroviruses for 48 hr (50% retroviral supernatant and 50% normal growth media). Following transduction, the cells were washed three times with 1X 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 Gli1\(\text{shRNA}\) and Gli2\(\text{shRNA}\) was ensured by culturing cells in the presence of a selection antibiotic, puromycin (1 \(\mu\)g/ml). The HT29 cell line simultaneously expressing both Gli1\(\text{shRNA}\) and Gli2\(\text{shRNA}\) was generated by transducing HT29 cells stably expressing Gli2\(\text{shRNA}\) with Gli1\(\text{shRNA}\) expressing retroviruses, for 48 hr (50% retroviral supernatant and 50% normal growth media). The transduced cells were washed three times with 1X PBS and used for experiments. HT29Bcl-2 (30) and HT29DNFADD (30), stably overexpressing Bcl-2 or DNFADD, respectively, were generated by retroviral gene transduction, as described. The HT29 cell line simultaneously expressing both DNFADD and Bcl-2 was generated by transducing HT29 cells stably expressing Bcl-2, with DNFADD expressing retroviruses, for 48 hr (50% retroviral supernatant and 50% normal growth media). The transduced cells were washed three times with 1X PBS and used for experiments.

Clonogenic assays: The cells were plated at a density 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 \(\mu\)M) for 72 hr. Drug was removed and replaced with fresh media containing dThd (20 \(\mu\)M) 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 1X Dulbecco’s PBS (without Ca\(^{++}\) or Mg\(^{++}\)) 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 with Annexin V FITC (BD Biosciences, CA) and propidium iodide (Sigma, MO) 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 PVDF membranes and subsequently blocked in blocking buffer (5% non fat dry milk in 1X Tris Buffer Saline with 0.1% Tween 20 [TBS-T]) for 1 hr. Membranes were washed in 1X TBS-T and incubated with primary antibody overnight at 4°C; they were subsequently washed and incubated with the secondary antibody for 1 hr 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 (iScript 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 above. Primers specific for each of the signaling molecules were designed using NCBI/Primer-BLAST and used to generate the PCR products, subsequently resolved and visualized on a 1% agarose gel. The following primers were used:

- PTC-Forward: 5’-CCACAGAAGCGCTCTTACA-3’
- PTC-Reverse: 5’-CTGTAATTTCGCCCTTCC-3’
- GL1-Forward: 5’- GCCCAGACAGAGGCCCACCT-3’
- GL1-Reverse: 5’- CTGCAGCCATCCCAACGCA-3’
- GL2-Forward: 5’-CACCGCTGCTCAAGAGAA-3’
- GL2-Reverse: 5’-TCTCCAGCCACTGCTT-3’ (10)
- GAPDH-Forward: 5’-CAGCCTCAAGATCATCAGCA-3’
- GAPDH-Reverse: 5’-GTCTTCTGGGTGGCAATGTAT-3’
- SHH Forward: 5’- CCCCCAGAACAGAGGACCA-3’
- SHH Reverse: 5’- GTACTTGTGCTGGGCAATGTAT-3’
- SMO Forward: 5’-CAGGAGAACGCACCGCAA-3’
- SMO Reverse: 5’-TGACGAGCGAGTAATCGGCA-3’
- DR5 Forward: 5’-GTCTGCTGCTGATACCATGCAC-3’
- DR5 Reverse: 5’-CTGCAAACTGTGACTCTATG-3’

**Gli-Luciferase assay:** The Gli-luciferase reporter construct (kindly provided by Dr. Rune Toftgård, 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 hr post-transfection, cells were treated with GANT61 (20 µM) for 36 hr, and harvested using the dual luciferase kit (Promega Corporation, WI) 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 demonstrated 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 (Figure 1A) or by Western analysis (Figure
1B). Shh, Smo and Gli1 were consistently expressed among the cell lines, while the levels of both Ptc and Gli2 were more variable. Of interest, 6/6 human colon carcinoma cell lines expressed the secretory HH ligand, Shh, demonstrated 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 μM) or GANT61 (20 μM) for up to 72 hr 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 hr following exposure to GANT61, but was maximally observed between 48 hr and 72 hr. In 6/6 cell lines examined, except for HCT8, cell death was >80% by 72 hr, and for HCT8, this was ≈ 60% (Figure 2A). In contrast, cyclopamine induced modest cytotoxicity in all cell lines examined, except for SW480 (~55% cell death; Figure 2A) when administered at equimolar concentrations and for the same period of time as GANT61. All cell lines responded to GANT61 treatment for 72 hr in a dose-dependent manner for a range of concentrations (0-30 μM; Figure 2B), with 20 μM 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 μM) for 72 hr, and clonogenic survival determined (Figure 3). HCT116, VRC5/c1 and GC3/c1 cells were sensitive at 10 μM GANT61 (<10% clonogenic survival), and almost no cell survival in 6/6 colon carcinoma cell lines following exposure to 20 μM GANT61 (IC50: 5-15 μM; Figure 3).

**GANT61 down-regulates Gli1 and Gli2 expression in HT29 cells:** The influence of GANT61 (20 μM) on the expression of both Gli1 and Gli2 was subsequently determined by Real-Time PCR in HT29 cells for up to 72 hr after treatment (Figure 4A). Gli2 expression was more rapidly decreased in comparison to Gli1, with 50% decreased expression by 48 hr; Gli1 down-regulation was approaching these levels by 72 hr. In addition, western analysis confirmed reduced expression of both Gli1 and Gli2 in HT29 cells by 72 hr after GANT61 treatment (Figure 4B). In a third experiment, transient transfection of HT29 with a Gli-dependent luciferase reporter construct followed by exposure to GANT61 (0-20 μM) for 36 hr, demonstrated a 50% decrease (at 20 μM concentration) in Gli-dependent luciferase reporter activity and hence reduced transcriptional activity of the Gli genes (Figure 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 expression resulted in partial (50% cell death in GLI1shRNA+Gli2shRNA compared to 80% in cell death in scrambled shRNA) but significant protection from GANT61-induced cytotoxicity at 72 hr post-treatment (Figure 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 μM, 20 μM) for 72 hr 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 (Figure 5A). No increase in expression of the TRAIL receptor DR4 was observed, in contrast to DR5 expression, where 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 (Figure 5A). Maximal effects on gene expression were obtained with 20 μM 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 (Figure 5B). Following Gli1 knockdown, PDGFRα and Bcl-2 expression were both decreased, while Fas and DR5 expression were simultaneously increased, similar to the effects of GANT61 (Figure 5B). Further, up-regulation of DR5 by GANT61 (20 μM) at the level of transcription was also demonstrated at 48 hr after treatment (Figure 5C). The clonogenic cell survival of HT29 cells, stably expressing Gli1shRNA, was reduced by ~ 60% compared to scrambled shRNA expressing HT29 cells in the presence of GANT61 (20 μM; Figure 5D).

**Overexpression of DNFADD and Bcl-2 inhibit GANT61-induced cell death:** Since GANT61 induced the expression of both Fas, and the short isoform of DR5, DR5S, signaling via both receptors at the level of the adaptor molecule, FADD, was inhibited by transduction of DNFADD (33). HT29 cells stably transected with DNFADD were less sensitive to GANT61-induced cytotoxicity (20 μM, 72 hr; ~ 45% cell death). Further, upon over-expression of Bcl-2, HT29 cells were also partially protected (~ 50% cell death) from GANT61-induced cytotoxicity (20 μM, 72 hr; Figure 6A). When DNFADD and Bcl-2 were overexpressed simultaneously in HT29 cells, confirmed by western analysis (Figure 6B), GANT61-induced cell death also remained partially inhibited (Figure 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 demonstrated 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 the current 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 while 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 (Figure 2). Recent studies in pancreatic ductal adenocarcinoma demonstrate 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, since both aberrant activation of HH signaling and RAS mutations are found in colon cancers (reviewed in (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 > 90%, thereby demonstrating 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). Since the HH signaling pathway is already activated in human colon carcinoma cell lines, studies using shRNA knockdown of both Gli1 and Gli2 were conducted. Since HH signaling is critical to cellular survival, complete knockdown of the Gli genes could not be obtained. However, stable expression of Gli1shRNA and 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 demonstrate 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 upon the known regulation of PDGFRA 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 demonstrated that GANT61 treatment reduces Bcl-2 expression and over-expression of Bcl-2 partially rescues from GANT61-induced cytotoxicity in HT29 cells (Figure 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 tumor necrosis factor (TNF) family (reviewed in (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, since no Gli binding sites can be identified in the promoter region of DR5. However, GANT61 induced up-regulation 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, that would inhibit death receptor signaling downstream of the receptor complex. Data demonstrate 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.

The present 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 Figure 7), this study demonstrates 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. In the presence of GANT61 (targeting Gli) the functions of Gli activators are inhibited; PDGFRα and Bcl-2 are down-regulated, in contrast Fas and DR5 are up-regulated. GANT61 induces significant cell death, while targeting Smo with cyclopamine is less effective at inducing cytotoxicity. These findings underscore the critical role of HH signaling in human colon cancer cells and the possibility of targeting Gli1 and Gli2 activator functions using GANT61 in this disease.

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REFERENCES


FIGURE LEGENDS:

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 μM) for up to 72 hr. B: Dose response to GANT61 (0 - 30 μM) after 72 hr exposure. Cell death was determined by Annexin-V-FITC/PI staining and FACS analysis. Data represent mean ± SD, n=2.

Figure 3. GANT61 inhibited clonogenic survival of human colon carcinoma cell lines. Cells were plated overnight and exposed to GANT61 (0, 5, 10, 20 μM) for 72 hr followed by growth in fresh culture media for the equivalent of 7 cell doublings, as described in Materials and Methods; surviving colonies were stained and counted. Data represent the mean ± SD of 3 determinations.

Figure 4. GANT61 down-regulates Gli1 and Gli2 expression in HT29 cells. A: Exposure to GANT61 (20 μM; 0 - 72 hr) reduced expression of both Gli1 and Gli2 mRNA, determined by Real-Time PCR. Data represent the mean ± SD of 3 determinations. B: Expression of Gli1 and Gli2 proteins were decreased following treatment with GANT61 (0-20 μM; 72 hr). HSP90α/β was used as the loading control. A representative blot from 3 independent experiments is shown. C: Gli-dependent luciferase activity is reduced by GANT61. HT29 cells were transfected with a Gli-dependent luciferase reporter construct, and after 24 hr were treated with GANT61 (10 μM or 20 μM) for a further 36 hr. Lysates were prepared, and luciferase activity measured as described in Materials and Methods. Normalized luciferase activity is presented as mean ± SD, n=3. D: Stable expression of Gli1shRNA and Gli2shRNA partially inhibits GANT61-induced cell death in HT29 cells. HT29 cells stably expressing Gli2shRNA were transduced with Gli1shRNA to generate a cell line with reduced, partial expression of both Gli1 and Gli2 (Gli1sh+Gli2sh). ScrambledshRNA (Scrsh) expressing cells were used as control. Cells were treated with GANT61 (20 μM) for 72 hr and cell death determined by Annexin-V-FITC/PI staining and FACS analysis. Data represent the mean ± SD of 3 determinations. Inset shows decreased expression of Gli1 and Gli2 by Western analysis. HSP90α/β was used for loading control.

Figure 5. GANT61 differentially regulates genes involved in the balance between cell death and cell survival. A: HT29 cells were treated with GANT61 (0, 10 μM or 20 μM) for 72 hr, and lysates extracted for western analysis. β-Actin was used as the loading control. B: Gli1shRNA expression in HT29 cells mimics the effects of GANT61 administration. HT29 cells were stably transduced with Gli1shRNA. Lysates were prepared and subjected to western analysis. HSP90α/β was employed as the loading control. Wt = wild type HT29 cells; ScrshRNA = HT29 cells expressing scrambled shRNA; Gli1shRNA = HT29 cells expressing Gli1shRNA. C: GANT61-induced up-regulation of DR5 mRNA. Exposure to GANT61 (20 μM; 0 - 48 hr) increased expression of DR5 mRNA, determined by Real-Time PCR. Data represent the mean ± SD of 3 determinations. D: Gli1shRNA expression reduced the GANT61-induced loss in clonogenic survival of HT29 cells. Cells were grown in growth media (also containing the selection antibiotic, Puromycin (1 μg/ml)) for 7 cell doublings, followed by staining and scoring of the remaining colonies. ScrshRNA-expressing cells were used as a control. Data represent mean ± SD, n=3.
Figure 6. Over-expression of DNFADD and Bcl-2 inhibit GANT61-induced cell death. A: Expression of DNFADD, or over-expression 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 µM) for 72 hr 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.

Figure 7. Schematic representation of the inhibition of HH signaling and genes involved in the balance between cell survival and cell death. Activated Gli1 and Gli2, downstream of Shh-Ptc-Smo, regulate 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.
### Human Colon Carcinoma Cell Lines

#### RT-PCR

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#### Western

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Figure A: Graph showing cell death percentage with treatment. The graph compares control and GANT61 (20 μM) treated HT29 cells. Significance is indicated as * * * * with p<0.005.

Figure B: Western blot analysis showing expression levels of FADD, DNFADD, Bcl-2, and GFP in HT29 cells treated with different conditions.
Hedgehog signaling drives cellular survival in human colon carcinoma cells

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

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