Blocking Hedgehog Survival Signaling at the Level of the GLI Genes Induces DNA Damage and Extensive Cell Death in Human Colon Carcinoma Cells

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Abstract

Canonical Hedgehog (HH) signaling is characterized by Smoothened (Smo)-dependent activation of the transcription factors Gli1 and Gli2, which regulate HH target genes. In human colon carcinoma cells, treatment with the Gli small-molecule inhibitor GANT61 induces extensive cell death in contrast to the Smo inhibitor cyclopamine. Here we elucidate cellular events upstream of cell death elicited by GANT61, which reveal the basis for its unique cytotoxic activity in colon carcinoma cells. Unlike cyclopamine, GANT61 induced transient cellular accumulation at G1–S (24 hours) and in early S-phase (32 hours), with elevated p21<sup>Cip1</sup>, cyclin E, and cyclin A in HT29 cells. GANT61 induced DNA damage within 24 hours, with the appearance of p-ATM and p-Chk2. Pharmacologic inhibition of Gli1 and Gli2 by GANT61 or genetic inhibition by transient transfection of the Gli3 repressor (Gli3R) downregulated Gli1 and Gli2 expression and induced γH2AX, PARP cleavage, caspase-3 activation, and cell death. GANT61 induced γH2AX nuclear foci, while transient transfection of Gli3R showed expression of Gli3R and γH2AX foci within the same nuclei in HT29, SW480, and HCT116. GANT61 specifically targeted Gli1 and Gli2 substantiated by specific inhibition of (i) direct binding of Gli1 and Gli2 to the promoters of target genes <i>HIP1</i> and <i>BCL-2</i>, (ii) Gli-luciferase activity, and (iii) transcriptional activation of <i>BCL-2</i>. Taken together, these findings establish that inhibition of HH signaling at the level of the GLI genes downstream of Smo is critical in the induction of DNA damage in early S-phase, leading to cell death in human colon carcinoma cells.

Introduction

Binding of the secretory Hedgehog (HH) ligands to their transmembrane receptor Patched (Ptc<sub>1</sub>) initiates the classical HH signaling pathway by releasing Smo from Ptc1-dependent suppression. Smo activates the final arbiters of HH signaling, the Gli transcription factors, that regulate HH target genes (1, 2). Averantly activated HH signaling has been identified in the malignant phenotype of several types of human cancers (reviewed in ref. 3), involving amplification of GLI1 or GLI2 and mutations in <i>PTCH1</i> or <i>SMO</i> (4, 5). There is emerging evidence that the HH pathway progresses during colon carcinogenesis (6, 7) and in metastatic disease (8), whereas in normal colonic tissue, HH signaling is involved in differentiation (9–11). Canonical HH signaling genes are expressed in primary colon cancers, metastatic disease, human carcinoma xenografts (8), and human colon carcinoma cell lines (12, 13). In several studies, HH signaling molecules have been linked to genomic instability, involving inactivation of homologous recombination or nonhomologous end joining, defects in checkpoint activation, and predisposition to development of cancers (14–16). However, little is known functionally about this signaling pathway and how it affects the survival and pathogenesis of colon cancer.

The majority of studies that determine the effects of inhibiting the HH signaling pathway have used the classic Smo inhibitor, cyclopamine, which cross-links Smo (17). Cyclopamine has shown variable activity in several different types of cancer cells (18). Oncogene-driven signaling pathways converge downstream of Smo on the Gli transcription factors providing noncanonical regulation of HH signaling (5, 19–22). Such noncanonical activation of the Gli proteins can therefore circumvent the inhibition of Smo resulting in reduced efficacy of or resistance to Smo inhibitors. The Gli family of transcription factors is composed of Gli1, Gli2, and Gli3 that regulate HH-induced target gene expression (3). Gli2 seems to be the primary activator of HH signaling, with Gli1 as a transcriptional target of Gli2, which may amplify HH-induced target gene expression (4, 23–25). Full-length Gli3 has activator functions, whereas a C-terminus cleaved form mediates repressor activity (3, 5). Expression of the repressor form of Gli3 (Gli3R) inhibited proliferation.

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and induced cell death in primary cultures of human colon cancers and metastases. Furthermore, human colon carcinoma cells transduced with Gli3R failed to form xenografts in nude mice (8), indicating the importance of Gli1 and Gli2 and corroborating the role of the HH signaling pathway in colon cancer cell survival.

To identify the mechanisms that regulate HH-driven cellular survival in the context of colon cancer, we employed cyclopamine to target Smo. In addition, to target the Gli proteins downstream of Smo, we employed a small-molecule inhibitor of both Gli1 and Gli2, GANT61, identified in a cell-based small-molecule screen for inhibitors of Gli1-mediated transcription (26). We have previously shown that GANT61 reduced GLI1, GLI2, and PTC1 mRNA expression in human colon carcinoma cell lines and significantly modulated cDNA microarray gene expression profiles downstream of Gli1/Gli2 function (12). Furthermore, inhibition of HH signaling by GANT61 induced greater cytotoxicity in human colon carcinoma cells than targeting Smo with cyclopamine (13). To elucidate the mechanisms regulating this differential response, studies were conducted in the human colon carcinoma cell line HT29, which is mutant for p53. Cells treated with GANT61 (20 µmol/L) accumulated at G1-S and in early S-phase at 24 and 32 hours, respectively, and by 48 hours underwent cell death. In contrast, cyclopamine (20 µmol/L)-treated HT29 cells showed minimal effects on cell-cycle distribution or cell death. GANT61-treated cells accumulated p21<sup>CIP1</sup>, cyclin E, and cyclin A in G1- and S-phase cells at 24 to 40 hours, in contrast to cyclopamine-treated cells, and stable knockdown of p21CIP1 did not influence sensitivity to GANT61. GANT61 but not cyclopamine induced DNA damage by 24 hours with the appearance of γH2AX nuclear foci at the sites of DNA strand breaks. Similar to the cellular effects induced by pharmacologic inhibition of Gli1 and Gli2 by GANT61, transient transfection of the Gli3R mutant, which inhibits the activating functions of Gli1 and Gli2 (3), downregulated expression of Gli1 and Gli2, induced expression of γH2AX, cleavage of PARP, and caspase-3, and cell death. GANT61 activated ATM and Chk2 (but not ATR and Chk1) by 4 hours, which was sustainable. Transient expression of Gli3R in HT29, SW480, and HCT116 cells induced nuclear localization of the Gli3R protein and induced formation of γH2AX nuclear foci within the same cells. Data suggest that exogenously expressed Gli3R is functional and suppression of HH/Gli signaling results in DNA damage in multiple human colon carcinoma cell lines that show active HH signaling. This phenomenon is p53 independent, because HT29 and SW480 cells express a mutant form of p53 whereas HCT116 harbor a wild-type p53 gene. Within 1 hour of exposure, GANT61 (i) reduced the binding of Gli1 and Gli2 to the promoter regions of the Gli target genes HIP1 and BCL-2, but not FAS, which is not a direct Gli target, and (ii) inhibited the transcriptional regulation of BCL-2. Furthermore, GANT61 specifically inhibited Gli-luciferase (luc) activity in contrast to NF-κB-luc or AP1-luc activities, indicating its specificity for Gli1 and Gli2. These findings emphasize the importance of targeting the Gli proteins to functionally inhibit HH signaling and their critical role in the cellular survival of human colon carcinoma cells.

Materials and Methods

Cell culture and reagents
HT29, SW480 and HCT116 cells were obtained from American Type Culture Collection and routinely verified by morphology, growth characteristics, response to cytotoxic agents [Annexin V/propidium iodide (PI) staining], cDNA microarray gene profiles were also characteristic. Cells were verified biannually to be mycoplasma-free. Cells were maintained in folate-free RPMI 1640 medium containing 10% dFBS and 80 nmol/L [6R5]-methyltetrahydrofolate. The cells were trypsinized and counted using a Z2 Coulter particle count and size analyzer (Beckman Coulter). For Western analysis, antibodies against p21<sup>Cip1</sup>, β-actin, and HSP90α/β were purchased from Santa Cruz Biotechnology, anti-Gli1 antibody was from Novus Biologicals, and anti-Gli2 antibody was from Cell Signaling Technology. Anti-c-myc antibody (9E10) was obtained from the Hybridoma Core, Lerner Research Institute. Anti-p21<sup>Cip1</sup>, anti-cyclin E, and anti-cyclin A antibodies used for bivariate flow cytometry were purchased from BD Biosciences. For Western analysis and confocal microscopy, antibodies against γH2AX, p-Chk1, Chk-1, p-ATR, ATR, p-Chk2, Chk-2, and ATM were purchased from Cell Signaling Technology; the p-ATM antibody was from Rockland Immunochemicals Inc. AlexaFluor 488 goat anti-rabbit and AlexaFluor 633 goat anti-mouse secondary antibodies were obtained from Invitrogen. GANT61 was purchased from Alexis Biochemicals and cyclopamine from Toronto Research Chemicals.

Annexin V-fluorescein isothiocyanate/PI staining and flow cytometric analysis
These were as described previously (27).

Cell-cycle distribution, bivariate flow cytometric analysis, and bromodeoxyuridine incorporation
For cell-cycle distribution and bivariate flow cytometry, cells were analyzed as previously described (28). For analysis of bromodeoxyuridine (BrdU) incorporation, cells were plated (50,000 cells/well) in a 6-well format and treated with GANT61 (20 µmol/L) or cyclopamine (20 µmol/L) for up to 48 hours. Cells were pulsed with BrdU (10 µmol/L; BD Biosciences) for 30 to 45 minutes and analyzed by flow cytometry for distribution within the cell cycle as per the manufacturer protocol.

Western analysis
This was carried out as previously described (27).

RNA interference studies
HT29 cells stably expressing p21<sup>Cip1</sup> shRNA were generated by transducing HT29 cells with scrambled-shRNA or the gene-specific short hairpin RNA (shRNA)-expressing retroviruses. Details are provided in Supplementary Materials and Methods.

COMET assay
Cells were processed, and electrophoresed in agarose gels as described (29). Tail moment (TM) and tail length (TL) were used to characterize the DNA damage within individual cells.
Image analysis and quantification were conducted using the NIH ImageJ software. TM = %DNA in the tail × TL where %DNA in the tail = tail area (TA) × tail area intensity (TAI) × 100/(TA × TAI) + [head area (HA) × head area intensity (HAI)].

Confocal microscopy

Cells were plated (50,000/well) on coverslips in 6-well plates. The cells were treated with GANT61 (20 μM) or cyclopa-mine (20 μM) for up to 48 hours and processed for microscopy. Details of microscopy are described in the Supplementary Materials and Methods.

Gli3R and transient transfections

The myc-tagged C-terminus deleted construct Gli3R (gift of Dr. Ariel Ruiz i Altaba, University of Geneva Medical School, Geneva, Switzerland) has been previously described (3). HT29 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with Gli3R or the empty vector pCS2-MT (gifted by Dr. David Turner, University of Michigan’s The Molecular & Behavioral Neuroscience Institute, Ann Arbor, MI). Cells were used for experiments 24, 48, or 72 hours posttransfection.

Chromatin immunoprecipitation analysis

HT29 cells were treated with GANT61 (20 μM) for 1 or 24 hours and chromatin immunoprecipitation (ChIP) analysis was conducted using Gli1 or Gli2 antibodies and Abcam ChIP kit, according to the manufacturer’s protocol. Details are provided in Supplementary Materials and Methods.

Luciferase reporter assays

The Gli-luc reporter construct (kindly provided by Dr. Rune Tofgård, Karolinska Institutet, Stockholm, Sweden) has been previously described (30). The NF-kB-luc plasmid p5XP10KB was previously reported (27). The AP1-luc (kindly provided by Dr. Philip H. Howe, Cleveland Clinic, Cleveland, OH) contains a basic promoter element (TATA box) joined to tandem repeats of the AP1 binding element (Stratagene). Transient transfection for 24 hours with luciferase reporters was carried out 24 hours after GANT61 (20 μM) treatment, as described (13).

RT-PCR

HT29 cells were treated with GANT61 (20 μM) for 1 hour, followed by RNA isolation for up to a further 4 hours. Following conversion into cDNA, samples were used for quantitative PCR (qPCR) as described previously (13). Primers used were BCL-2 forward 5'-GGAGGATTGTGGGCCTTTT-3' and BCL-2 reverse 5'-GCCGTACAGTCCACAAAGG-3'.

Results

GANT61 and cycloamine exhibit differences in cell-cycle regulation and induction of cell death

We have shown previously in a panel of 6 human colon carcinoma cell lines that at equimolar concentrations (10–30 μM/L), GANT61 induced more than 80% cell death by 72 hours of treatment in contrast to cycloamine (13). These concentrations and time frames for the induction of cellular effects are similar to those determined in other model systems for inhibitors of HH signaling (8, 26, 31, 32). A more detailed study of the mechanisms regulating the differential effects between GANT61 and cycloamine was conducted in HT29 cells, which express mutant p53. Cells were treated with GANT61 (20 μM/L) or cycloamine (20 μM/L), followed by PI staining and flow cytometric analysis of cell-cycle distribution. GANT61-treated cells accumulated at G1–S by 24 hours, moving into early S-phase by 32 hours, and subsequently becoming sub-G1 by 48 hours (Fig. 1A). In contrast, treatment with cycloamine resulted in a modest increase in G1 to S-phase cells by 48 hours; by 72 hours cells had not progressed either into S-phase or into sub-G1 (Fig. 1A).

BrdU incorporation analysis

In GANT61-treated cells, cellular accumulation at the G1–S boundary was evident by 24 hours, as shown by a 37% increase in BrdU incorporation, which increased to 52% by 32 hours, and an 8% increase in S-phase cells at this time. By 40 hours, there was a decrease in cells in G1–S (40% decrease in BrdU incorporation), in S-phase (17%), and an increase (60%) in cells within the sub-G1 compartment (Fig. 1B and Supplementary Fig. S1). These effects were consistent with decreased BrdU-labeled cells in G2–M. In contrast, following cycloamine treatment, the appearance of a stronger G1–S peak by 48 hours observed in cell-cycle analysis (Fig. 1A) was paralleled by an 11% to 14% increase in BrdU-labeled cells in the S-phase (Fig. 1B), clearly showing differences in cell-cycle regulation between GANT61- and cycloamine-treated HT29 cells.

Bivariate flow cytometric analysis of the distribution of p21Cip1, cyclin E, and cyclin A within the cell cycle

Cell-cycle progression is regulated by different cyclin–Cdk complexes. Cyclin E, which regulates Cdk2, is expressed in late G1 and early S-phase (33). Cyclin A, expressed in late G1, begins to accumulate in S-phase and is rapidly destroyed at the onset of mitosis (34). Furthermore, p21Cip1 could have a potential role at the G1–S boundary. Expression of these proteins was analyzed by bivariate flow cytometric analysis, simultaneously with DNA content. In GANT61-treated cells, p21Cip1 was induced and continued to be elevated in G1 phase cells over a period of 24 to 40 hours (Fig. 1C). Similarly, cyclin E appeared at 24 hours in G1 and S-phase cells at 32 to 40 hours; the largest accumulation of cyclin E occurred in G1 phase cells where most remained accumulated at 40 hours. Cyclin A accumulated significantly in the G1 phase following GANT61 treatment, whereas the percentage of cells expressing cyclin A in S-phase as well as G2–M phase cells declined. In cycloamine-treated cells, p21Cip1 and cyclin E remained at low levels in all cell-cycle phases for up to 40 hours. Cyclin A was expressed in untreated cells in G1, S, and G2–M but decreased in all phases by 24 hours following cycloamine treatment (Fig. 1C and Supplementary Fig. S2). Data are consistent with cellular accumulation at the G1–S boundary and in early S-phase in GANT61-treated HT29 cells with accumulation of p21Cip1, cyclin E, and cyclin A mostly in G1 and partially in S-phase cells. In contrast, no effects on p21Cip1 or cyclin E distribution,
Figure 1. GANT61 induces accumulation of HT29 cells at G1–S and in early S-phase in contrast to cyclopamine. A, HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for up to 72 hours. DNA was extracted, stained, and cell-cycle distribution was analyzed using flow cytometry. Data are representative of 3 independent experiments. B, BrdU incorporation shows accumulation of GANT61-treated cells at the G1–S boundary and in early S-phase. HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for up to 48 hours. % BrdU incorporation was determined using flow cytometry. Data are representative of duplicate determinations. C, bivariate flow cytometric analysis of p21Cip1, cyclin E, and cyclin A expression in different phases of the cell cycle. HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for up to 40 hours, followed by staining and cell-cycle analysis. Data are representative of duplicate determinations. D, influence of stable p21Cip1 shRNA knockdown on sensitivity of HT29 cells to GANT61. HT29 cells stably expressing p21Cip1- or scrambled-shRNA (Scr-shRNA) were treated for 72 hours with GANT61 (20 μmol/L). Cell death was analyzed by Annexin V/PI staining and flow cytometry. Data represent the mean ± SD of duplicate determinations. Reduced expression of p21Cip1 was confirmed by Western analysis. WT, wild type.
or sustained accumulation of cyclin A, were evident in cyclopamine-treated cells, consistent with lack of significant cell-cycle perturbation or induction of cell death.

**GANT61-induced cell death is independent of p21Cip1**

HT29 cells stably transduced with p21Cip1 shRNA or scrambled-shRNA (control) were treated with GANT61 (20 μmol/L) for 72 hours, followed by Annexin V/PI staining and flow cytometric analysis (Fig. 1D). GANT61 induced similar levels of cell death (75%–80%) in scrambled-shRNA- or p21Cip1 shRNA-transduced cells, indicating the lack of a functional role for p21Cip1, as well as p53, in the mechanism of GANT61-induced cell death.

**GANT61 induces DNA damage**

To determine whether GANT61 induces DNA damage following cellular accumulation at G1–S and in early S-phase, HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for 24 or 48 hours. Single cells were analyzed by the COMET assay, which detects DNA damage by alteration in the pattern of cellular elution through agarose gels (Fig. 2A). Significant changes in elution profiles were detected in GANT61-treated cells by fluorescence microscopy, tail moment, and tail length (Fig. 2B). In contrast, cyclopamine-treated cells showed an increase in tail moment but not in tail length at 48 hours. HT29 cells were also exposed to GANT61 (20 μmol/L) or dimethyl sulfoxide (DMSO; control) in the absence or presence of nucleosides (thymidine, adenosine, cytidine, and guanosine; 20 μmol/L each). Supplementation with nucleosides conferred partial protection (~50% cell death) from GANT61-induced cytotoxicity (~80% cell death; Fig. 2C), indicating a role of DNA damage signaling in GANT61-induced cytotoxicity.

Figure 2. GANT61 induces DNA damage in HT29 cells. A, COMET assay for determination of DNA damage in single cells following treatment with GANT61 or cyclopamine. HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for up to 48 hours, harvested, and analyzed by COMET assay. B, graphical representation of DNA damage by tail length and tail moment, calculated as described in Materials and Methods. C, cell death analysis of HT29 cells treated with either DMSO or GANT61 in the presence and absence of nucleosides. HT29 cells were simultaneously exposed to nucleosides (thymidine, adenosine, cytidine, and guanosine; each 20 μmol/L) and/or GANT61 (20 μmol/L) for 72 hours. Cell death was determined by flow cytometry following Annexin V-fluorescein isothiocyanate/PI staining.

To further characterize the DNA damage response, expression of γH2AX, a marker of double-strand breaks (DSB; ref. 35), was determined by Western analysis in HT29 cells treated for up to 72 hours with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L; Fig. 3A). Appearance of γH2AX was detected at 24 hours after GANT61 treatment upstream of cell death and was strongly expressed at 48 hours when cells were undergoing apoptosis. In contrast, γH2AX was barely detectable in cyclopamine-treated cells at 24 hours by Western analysis and only slightly increased at 48 hours. Further evaluation of γH2AX expression by confocal microscopy is shown in Figure 3B. Changes in cellular morphology by confocal microscopy and γH2AX foci were not detectable within 48 hours of cyclopamine exposure (Fig. 3B).

**GANT61 activates ATM and Chk2 in HT29 cells**

To determine the molecular mechanism underlying GANT61-induced DNA damage signaling, HT29 cells were treated with GANT61 (20 μmol/L) or cyclopamine (20 μmol/L) for up to 24 hours, and expression of the
phosphorylated (active) forms of ATM, ATR, Chk1, and Chk2 were examined by Western analysis (Fig. 3C) and p-Chk1 and p-Chk2 by confocal microscopy (Fig. 3D). In GANT61-treated cells, p-ATM and p-Chk2 were detected as early as 4 hours and their expression was sustained for 24 hours. In contrast, p-ATR and p-Chk1 expression remained undetectable. Furthermore, p-Chk2 but not p-Chk1 nuclear foci were detected by confocal microscopy in GANT61-treated cells, indicating an active ATM–Chk2 axis in the GANT61-induced DNA damage response.

**Genetic downregulation of Gli1 and Gli2 by Gli3R induces DNA damage and cell death**

The critical role of Gli1 and Gli2 function in cellular survival in colon carcinoma cells was further investigated by genetic downregulation of Gli1 and Gli2. A C-terminus deleted mutant form of Gli3 [Gli3R, Gli3CΔC1α with a myc tag; refs. 3, 8] was employed, which contains the N-terminus region that determines nuclear localization and repressor activity. Transient transfection of HT29 cells with Gli3R-pCS2-MT reduced cell growth by 60% over a period of 72 hours (microscopy, cell number), induced cell death (microscopy, Annexin V/PI staining), and decreased Gli1 and Gli2 protein expression (Western). By 72 hours posttransfection, Gli2 protein was reexpressed whereas decreased Gli1 protein was sustained (Fig. 4A). Gli3R was determined by expression of the myc tag, which was detected by 24 hours and was highest at 48 hours posttransfection (Fig. 4A). Similar effects on cell growth, cell death, and Gli1 and Gli2 protein expression were induced by GANT61 (20 μmol/L; Fig. 4B). Furthermore, induction of DNA damage was detected following transient transfection with Gli3R, marked by elevated expression of γH2AX, detected within 24 hours. This was associated with cleavage of full-length PARP and caspase-3, also determined in GANT61-treated cells (Fig. 4C). To ensure that the effects of Gli3R were not specific to HT29 only, Gli3R was transiently expressed in SW480 and HCT116 cells and γH2AX expression was determined by confocal microscopy. The expression of Gli3R was visualized using an anti-myc antibody. Within 24 hours of transient transfection, myc-tagged Gli3R was detected in nuclei of cells that also expressed γH2AX nuclear foci in all 3 cell lines, HT29, SW480, and HCT116 (Fig. 5). Collectively, data show that both pharmacologic inhibition and genetic inhibition of HH signaling result in reduced cell growth, increased cell death, and induction of DNA damage that is associated with reduced Gli1 and Gli2 protein levels. These findings emphasize the significance of the HH signaling pathway in human colon cancer cell growth and survival, regulated at the level of the Gli genes.

**GANT61 inhibits specific binding of Gli1 and Gli2 to Gli target genes and blocks transcriptional activity**

To further determine the specificity of GANT61 for targeting the function of Gli1 and Gli2, ChIP analysis was conducted using GANT61-treated HT29 cells (20 μmol/L; 24 hours). Following isolation of chromatin, immunoprecipitation with Gli1- or Gli2-specific antibodies, or antibodies against IgG (negative control) or histone H3 (positive control), was carried out. Subsequent qPCR using primers that flanked the promoter regions of the Gli target genes HIP1 and BCL-2, or FAS (negative control), revealed inhibition of binding by GANT61 of both Gli1 and Gli2 to their target gene promoters of HIP1 and BCL-2 but not to FAS, where no Gli binding sites were detected (Fig. 6A). GANT61 (20 μmol/L; 24 hours) specifically inhibited Gli-luc reporter activity in transiently transfected HT29 cells. In contrast, neither p65-luc nor AP1-luc activities were affected (Fig. 6B). In addition, using short, 1-hour GANT61 (20 μmol/L) exposure, (i) inhibition of Gli1 and...
Gli2 binding to the promoter regions of HIP1 and BCL-2 was determined at this early time point, similar to data derived after 24-hour drug exposure (Fig. 6C), and (ii) inhibition of the transcriptional regulation of BCL-2 for up to a further 4 hours was examined (Fig. 6D). Collectively, these data substantiate the specificity of GANT61 in targeting Gli transcriptional activity.

Discussion

Regulated function of the HH signaling pathway is critical during embryonic development, whereas deregulated HH signaling is documented in a variety of human cancers (1, 3–5). Although recent literature suggests the involvement of HH signaling in colon carcinogenesis, progression (6, 7), and metastatic disease (8), there is limited understanding about its mechanistic role in colon cancer. Primary human colon carcinomas, liver metastases, xenografts (8), and human colon carcinoma cell lines (12) express HH signaling pathway components. siRNA-mediated downregulation of Gli1 or Gli2 has reduced proliferation and induced apoptosis in primary cultures of human colon cancers and liver metastases, with the Gli3R acting with greater potency. Furthermore, human colon carcinoma cells transduced with Gli3R have failed to grow as xenografts in nude mice (8).

In the context of colon cancer, previous attempts to block HH signaling at the level of Smo has induced only moderate cytotoxicity in these cells (36). A recent study reported
GANT61, a small-molecule inhibitor of both Gli1 and Gli2, which effectively blocks Gli function (26). We have shown that GANT61 induced considerably greater cytotoxicity in 6 human colon carcinoma cell lines (60%–90% cell death) than that induced by cyclopamine (≈30% cell death; ref. 13). These findings indicate that direct targeting of the Gli transcription factors downstream of Smo is more efficient in interrupting HH signaling, likely due to noncanonical activation of Gli proteins independent of Smo (5, 19–22).

Limited information exists about the genes or mechanisms involved in the inhibition of the HH signaling response in cancer cells. In an oral squamous carcinoma cell line, cyclopamine induced a modest (10%) increase in cells at the G1–S boundary (37). Knockdown of Smo using siRNA reduced proliferation of 2 human colon cancer cell lines, with decreased expression of cyclin E and increased expression of p21(Cip1), consistent with accumulation at G1–S (38). However, no cytotoxic effects were described in either study. These results are consistent with only modest cytotoxicity shown by cyclopamine in human colon carcinoma cell lines. In detailed studies conducted in HT29 cells, inhibition of Smo by cyclopamine induced a modest increase in cells at the G1–S boundary and modest perturbations in cell-cycle distribution, with minimal entry of cells into the sub-G1 compartment, even after 72 hours of exposure. In contrast, inhibition of Gli1/Gli2 downstream of Smo by GANT61 induced transient accumulation of cells at the G1–S boundary and in early S-phase, followed by entry into the sub-G1 compartment. Previously, cDNA microarray gene profiling showed upregulated expression of p21(Cip1) mRNA and downregulated expression of genes involved in the G1–S (Cyclin E, Cyclin A, CDK2, and CDC25A) transition in HT29 (and GC3/c1) human colon carcinoma cell lines treated with GANT61 for 24 hours (12).

Using bivariate flow cytometric analysis, accumulation of p21(Cip1), cyclin E, and cyclin A was observed in the G1 and S-phases in contrast to cyclopamine-treated cells. Incorporation of BrdU also showed accumulation of GANT61-treated cells at the G1–S boundary and delay in early S-phase without further progression. Previously, it was shown in GANT61-treated HT29 cells that the mRNAs of genes involved in DNA replication, including thymidylate synthase, thymidine kinase, topoisomerase2, E2F, and DNA polymerases, were also downregulated at this time (12), supporting the lack of progression of cells through S-phase. p21(Cip1) binds to and inhibits cyclin–Cdk complexes with a preference for those containing Cdk2 (39) and plays an essential role in growth arrest after DNA damage (40, 41). Overexpression of p21(Cip1) can lead to G1 and G2 (42) or S-phase (43) arrest. However, stable p21(Cip1) knockdown had no effect on GANT61-induced cell death in HT29 cells, supporting a p21(Cip1)-independent mechanism.

We have previously reported that GANT61-treated cells showed modifications in genes involved in DNA damage response signaling including H2AFX, MDC1, BRC1, FANC, CDC45L, DDL, and RAD genes (12). The present study characterized the DNA damage response elicited by GANT61-mediated inhibition of HH signaling activity in human colon cancer cells. In mammalian cells, there are 2 parallel pathways that respond to stress-induced DNA damage: the ATM pathway, which responds to DSBs, and ATR, which responds to DSBs and to agents that interfere with replication forks (44, 45). Both ATM and ATR are kinases that phosphorylate several target proteins, are early transducers of the DNA damage response (reviewed in refs. 46–48), and are recruited to DNA break sites following activation (49). Checkpoint functions of ATM are primarily mediated by the effector kinase Chk2, and of ATR by Chk1, following phosphorylation (reviewed in refs. 46, 49). Efficient transduction of DNA damage signals downstream of ATM and ATR also requires a class of checkpoint mediators and adaptors whose mechanisms are not yet completely defined (50). One of the earliest modifications of chromatin in the DNA damage response is phosphorylation of H2AX (γH2AX), a direct phosphorylation target of ATM and ATR (46, 49), located at the sites of DNA strand breaks as immunoreactive foci. Expression of γH2AX was detected by both Western analysis and confocal microscopy by 24 hours in GANT61-treated cells upstream of cell death. This was not observed in cyclopamine-treated cells. A differential DNA damage response evaluated in single cells in GANT61-treated
versus cyclopamine-treated cells was also determined by COMET assay. The involvement of DNA damage in GANT61-induced cytotoxicity was further substantiated from the protective effect of nucleoside supplementation during exposure of HT29 cells to GANT61, which would elevate the pool of dATP, dGTP, dCTP, and dTTP required for DNA replication. Subsequent examination of the early response genes, the activated forms of ATM and Chk2, showed the appearance of p-ATM and p-Chk2 (but not p-ATR or p-Chk1) at 4 hours following GANT61 treatment, which was sustained; p-Chk2 nuclear foci were also determined in individual cells by confocal microscopy (summarized in Fig. 7). No activation of ATM, ATR, Chk1, or Chk2 was detected in cyclopamine-treated cells.

The role of the Gli proteins in colon cancer cell survival was further confirmed using the C-terminus deleted repressor Gli3R to inhibit Gli1 and Gli2 activity. Transient expression of Gli3R over a period of 72 hours parallelled the effects of GANT61 by decreasing growth and expression of Gli1 and Gli2 in HT29 cells, inducing cell death, γH2AX expression, and cleavage of PARP and caspase-3. Multiple human colon carcinoma cell lines (HT29, SW480, HCT116) respond to the exogenous expression of Gli3R (a C-terminus truncated mutant of the Gli3 protein that localizes to the nucleus; ref. 3), by induction of immunoreactive γH2AX nuclear foci in the same cells expressing nuclear Gli3R. These data show the far-reaching consequences of Gli3R expression in human colon carcinoma cells that express active HH signaling. The GANT61- or Gli3R-induced DNA damage response is also independent of p53, because expression HT29 and SW480 express mutant p53, whereas HCT116 is p53 wild-type.

GANT61 functions in the nucleus to abrogate Gli function, blocks both Gli1- and Gli2-mediated transcription, reduces expression of Gli1 and HIP1 mRNA (qRT-PCR) in contrast to cyclopamine in SUFU-null mouse embryonic fibroblasts, and inhibits Gli1 DNA binding activity (electrophoretic mobility shift assay; ref. 26). Further confirmation of the specificity of Gli1 and Gli2 as targets for GANT61 is provided by ChIP analysis, luciferase reporter assays, and inhibition of the transcriptional regulation of BCL-2.

Figure 6. Specificity of GANT61 for targeting Gli1 and Gli2 in human colon cancer cells. Binding between Gli1 or Gli2 and the promoter regions of HIP1 and BCL-2, but not FAS, was inhibited in GANT61-treated cells. HT29 cells treated with GANT61 (20 μmol/L) for 24 hours (A) or 1 hour (C) were employed for ChIP analysis using antibodies specific for Gli1, Gli2, IgG (negative control), or histone H3 (positive control, used for normalization). Subsequent qPCR used primers that flanked the promoter regions of the Gli target genes HIP1 and BCL-2 or FAS (negative control and not a direct Gli target). B, GANT61 treatment (20 μmol/L; 24 hours) of HT29 cells showed specific decreased reporter activity of a Gli-luc reporter. In contrast, exposure to GANT61 did not affect luciferase activity in HT29 cells transfected with p65-luc (NF-κB) or AP1-luc reporters. D, GANT61 treatment (20 μmol/L; 1 hour) of HT29 cells inhibits the transcriptional regulation of BCL-2.
specificity of GANT61 in targeting Gli transcriptional activity in human colon carcinoma cells.

In summary, inhibition of the HH signaling pathway by targeting the transcription factors Gli1 and Gli2 is highly effective at inducing cell death in human colon carcinoma cells in contrast to targeting Smo upstream of Gli. Inhibition of Gli1 and Gli2 by GANT61 induced inhibition of DNA replication in early S-phase leading to DNA damage signaling involving an ATM–Chk2 axis and induction of cell death. Pharmacologic (GANT61) and genetic (Gli3R) downregulation of Gli1 and Gli2 by Gli3R reduced Gli1 and Gli2 expression, reduced cell proliferation, and induced changes in cellular morphology, DNA damage, γH2AX nuclear foci, cleavage of PARP and caspase-3, and cell death (schematically represented in Fig. 7). The mechanisms underlying the induction of Gli1/Gli2-regulated DNA damage, the significance of an early S-phase response, and the inability to repair damaged DNA are currently under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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