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Research paper

Inhibition of hedgehog signaling by GANT58 induces apoptosis and shows synergistic antitumor activity with AKT inhibitor in acute T cell leukemia cells

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ABSTRACT

The hedgehog (Hh) signaling pathways have a crucial role in cell proliferation and survival, and the deregulation of these pathways can lead to tumorigenesis. Here we investigated the expression and function of these pathways in acute T lymphocytic leukemia cells (T-ALL). Profiling of Hh pathway members revealed common expression of key Hh signaling effectors in all T-ALL cells. We found that T-ALL cells were insensitive to specific Smoothened (SMO) inhibition following the use of low concentrations of the SMO antagonist cyclopamine. In contrast, treatment with the novel GLI antagonist GANT58 reduced expression of the target gene Patched 1 as well as GLI family zinc finger 1 (GLI1) and preferentially decreased the viability of T-ALL cells. We also found perifosine, a novel AKT inhibitor, down-regulated GLI1 protein by dephosphorylation of AKT and GSK3β dose-dependently and that pretreatment with PD98059, a MEK/ERK pathway inhibitor, enhanced this down-regulation by 20%–30%. Then we questioned whether use of both GANT58 and AKT inhibitor together could confer a synergistic effect to decrease T-ALL cell viability. By applying the Chou–Talalay method, low concentration of GANT58 induced T-ALL cell death in a synergism fashion with perifosine or GSK690693 when used simultaneously. These findings indicate that the combined use of GANT58 and AKT inhibitor could help treat a broad range of malignant tumors in conjunction with existing cancer treatments.

1. Introduction

Hedgehog (Hh) signaling plays a critical role in embryogenesis and adult tissue homeostasis [1,2]. The Hh ligands which include Sonic hedgehog (SHH), Desert hedgehog (DHH) and Indian hedgehog (IHH), bind to the receptor Patched (PTCH). This results in the activation of a second transmembrane protein Smoothened (SMO). Once activated, SMO results in stabilization and nuclear accumulation of GLI family members GLI family zinc finger 1 (GLI1), GLI family zinc finger 2 (GLI2) and GLI family zinc finger 3 (GLI3), defined as canonical Hh signaling [3]. Hh signaling regulates a host of genes including the Hh pathway regulators GLI1 and PTCH1, as well as key regulators of cell proliferation, survival and metastasis [4].

Acute T-cell leukemia (T-ALL) is an aggressive neoplastic disorder of developing T cells in the thymus and accounts for 10%–15% of pediatric and 25% of adult ALL cases [5,6]. Despite novel combination chemotherapy regiments, prognoses were still extremely poor. Among these patients, only 70–80% of children and as few as 40% of adults [7] reach long-term remission. SHH signaling regulates T-cell development and peripheral T-cell activation [8–10]. There is ample evidence to suggest that aberrantly activated Hh signaling is associated with the development of a variety of human tumors [11–17]. However, the mechanism by which Hh signaling acts during T-ALL is not understood, and Hh expression profiles in T-ALL patients are largely inconsistent. Furthermore, there exists little information on the effect of the SMO inhibitor cyclopamine in treating T-ALL [18–20].

An increasing body of evidence suggests that SMO-deficiency does not affect normal hematopoiesis or development of acute

Abbreviations: Hh, hedgehog; T-ALL, acute T cell leukemia; SMO, Smoothened; GLI1, GLI family zinc finger 1; SHH, Sonic hedgehog; DHH, Desert hedgehog; IHH, Indian hedgehog; PTCH1, Patched 1; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; SUFU, Suppressor of Fused.

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leukemia induced by either the MLL-AF9 fusion genes or an activation form of Notch [21,22]. GLI plays more important roles than SMO in the development of cancers [23]. The GLI antagonists GANT58 and GANT61 are more potent in inducing growth arrest and apoptosis compared to cyclopamine in a number of cancer cells. This has been found to be the case in myeloid leukemia cells, colon carcinoma cells, rhabdomyosarcoma cells, ovine squamous-cell carcinoma cells and chronic leukemia cells [24–29]. Despite these studies, information surrounding the role of Hh signaling and use of GLI inhibitors in T-ALL cell lines has been little investigated.

In addition, recent findings have highlighted constitutively active phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling as a common feature of T-ALL [30–33]. Non-canonical regulators of Hh signaling such as PI3K and RAS act downstream to promote expression of GLI transcription factors in various tumors [19,34–36], but the contribution of these signaling in activation of Hh signaling has not been explored in T-ALL. As preventing AKT membrane localization and phosphorylation, the new AKT inhibitor perifosine may be useful to target the Hh signaling pathway, yet no studies have demonstrated the effect of using perifosine on this pathway.

There is a growing interest in multicomponent chemotherapy; the combined delivery of multiple drugs in an attempt to overcome drug resistances and to improve clinical outcome. This strategy combines drugs with different targets of action to result in a more significant biological effect. Since over-activation of AKT signaling can result in the downstream expression of GLI transcription factors in various tumors [34–36], it remains to be tested whether simultaneously targeting GLI1 transcription factors with GANT58 and AKT signaling could have a synergistic effect on reducing malignant cell viability. Thus for the first time we set out to determine the potential cytotoxic effects of GANT58 in combination with AKT inhibitor perifosine or GSK690693 in T-ALL cells.

In this report, we present data that Hh pathway members expressed commonly with different levels in different T-ALL cell lines, and that perifosine regulated GLI1 through PI3K/AKT and MEK/ERK signal pathways. We also investigated the cytotoxic effect of GANT58 with or without AKT inhibitor perifosine or GSK690693 on T-ALL cells in vitro, suggesting that GLI inhibitor alone and a combination of GANT58 and ATK inhibitor may have a therapeutic role in the treatment of T-ALL.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen, 10091148). Cell culture media RPMI 1640 and supplementary were from Thermo Scientific. Cyclopamine (Merck, 239803), GANT58 (BioVision, 1812-25) and PD98059 (Cell Signaling Technology, 9900), GSK690693 (Selleck Chemicals, S1113) were first dissolved in absolute dimethyl sulfoxide (DMSO, Sigma, D4540) and then diluted with RPMI 1640, the final DMSO concentration was lower than 0.1%. Perifosine (Selleck Chemicals, S1037) was dissolved in PBS at the storage concentration (100 mM). PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche) was dissolved in 1 ml distilled water. The human A549 cell lines were obtained from cell bank (Chinese Academy of Sciences) and have grown in our laboratory for less than 2 years.

2.2. Cell cultures

The human ALL cell lines CCRF–CEM and Jurkat were bought from cell bank (Chinese Academy of Sciences). Human CEM clonal cell lines C7–14 and C1–15 were kindly provided by Professor E. Brad Thompson, from Emeritus (U. of Texas Med. Branch) as well as Center for Nuclear Receptors and Cell Signaling (U. of Houston, TX). The human subclone C7–14 is sensitive to GC-evoked apoptosis and subclone C1–15 remains resistant to GC. Many properties of the two original clones are similar. The cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 humid atmosphere.

2.3. Real-time reverse transcriptase PCR

Total RNA of 1×10^6 T-ALL cells was extracted using TRIzol Reagent (TaKaRa, D9108A). A total of 500 ng RNA was transcribed into first strand cDNA using the primescript™ RT reagent kit (TaKaRa, DRR037A). Real-time PCR reaction mixture was added to a total volume of 20 μl according to the manufacture's protocol of SYBR Premix^{Ex} Taq™ (TaKaRa, DRR041A). Primer sequences used for real-time analysis are shown in Table 1. The amplification was performed using the Roche LightCycler®480 platform. The expression of each targeted gene was determined by the mRNA level quantified by the calibration curve of the standards and normalized to the level of the housekeeping gene, GAPDH.

2.4. Cell viability assays

The inhibitory effect on cell growth was evaluated using Cell Counting Kit-8 (CCK8, DOJINDO) and MTT (CT01-5, Millipore) assays. 1×10^5 cells were plated into 96-well plates in 0.1 ml of culture medium per well, allowed to recover and stabilize for 24 h, then treated with different concentrations of drugs for 24 h or 48 h. At the end of each drug exposure time point, CCK8 or MTT was transferred to each well according to the manufacture's protocol and incubated for an additional 1–4 h at 37 °C. The optical density (OD) was then measured using an enzyme-linked immunosorbent assay (ELISA) plate reader. Every test was repeated at least three times.

2.5. Detection of apoptosis

Early and late apoptotic cells were detected by the Annexin V-PI detection kit (KeyGEN BioTECH, KGA107). After exposure to 10 μM GANT58 for 48 h, cells were washed once with PBS, resuspended with 500 μl binding buffer and then incubated in the dark at room temperature for 15 min with 5 μl Annexin V-FITC and 5 μl PI. Prepared cells were analyzed with a FACScan flow cytometer and

Table 1
Primers for quantitative PCR.

SHH	5-CGGCTTCGACTGGGTGTACT-3	(forward)
	5-GCAGCCTCCCGATTTGG-3	(reverse)
IHH	5-GGCTTTGACTGGGTGTATTACGA-3	(forward)
	5-AGCAGCCGCCCGTCTT-3	(reverse)
DHH	5-TGTCAGTAGCAGGTCCTA-3	(forward)
	5-GCGTTCTTGTCCTCACTA-3	(reverse)
PTCH1	5-CCCCTGTACGAAGTGGACACTCTC-3	(forward)
	5-AAGGAAGATCACCACTACCTTGGCT-3	(reverse)
SMO	5-GCTACTTCCTCATCCGAGGAGTCA-3	(forward)
	5-GGCGCAGCATGGTCTCGTT-3	(reverse)
SUFU	5-GCTGCTGACAGAGGACCCACA-3	(forward)
	5-GTGCAGACACCAACGATCTGGA-3	(reverse)
GLI1	5-CCAACTCCACAGGCATACAGGAT-3	(forward)
	5-CACAGATTCAGGCTCACGCTTC-3	(reverse)
GLI2	5-AAGTCACTCAAGGATTCCTGCTCA-3	(forward)
	5-GTTTTCCAGGATGGAGCCACTT-3	(reverse)
GAPDH	5-CAACAGCGACACCCACTCCT-3	(forward)
	5-CACCCTGTTGCTGTAGCCAAA-3	(reverse)

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