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Gli inhibitor GANT61 causes apoptosis in myeloid leukemia cells and acts in synergy with rapamycin

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ABSTRACT

Aberrant reactivation of Gli signaling has been described in a wide variety of human cancers and rapamycin can down-regulate Gli pathway in some solid tumors. In this study, we attempt to define the cytotoxic effect of Gli inhibitor on AML cells. And the regulation action of rapamycin on Gli in AML cells also has been assessed. Gli inhibitor GANT61 caused growth arrest and apoptosis in AML cells. Rapamycin decreased not only the Gli protein and mRNA expressions but also expression of the Gli-luciferase reporter in AML cells. Synergism effect between GANT61 and rapamycin was found in Kasumi-1, HL-60 and U937 cell lines. The results suggest that aberrant Gli activation is a feature of some myeloid leukemic cells and Gli activiation can be down-regulated by rapamycin.

1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder characterized by accumulation of malignant hematopoietic progenitor cells with impaired differentiation program. Despite important progress in the therapy of AML and high rates of complete remission after induction chemotherapy, most patients will relapse and die from the disease. Therefore, new agents or novel drug combinations are needed urgently [1].

The hedgehog (Hh) pathway plays a critical role during development of embryos and cancer including leukemia, lymphoma and multiple myeloma [2–6]. Recent studies have demonstrated that some inhibitors of this pathway can be used as a cancer therapeutic strategy [7,8]. Moreover, growing evidence suggest that Gli is more important than smo in the development of cancer [9]. While cyclopamine, a widely used smo inhibitor, has been reported to be effective in B-cell malignancies in vitro and in vivo, its effects on leukemia are not obvious [3,5]. In chronic lymphocytic leukemia, Gli inhibitor – GANT61 appears to be more powerful than cyclopamine, but its effect on AML is not well documented [10].

The mammalian target of rapamycin (mTOR) is a key regulator of growth and survival in many cell types. Its constitutive activation has been involved in the pathogenesis of various cancers, including AML [11]. Moreover, antileukemic activity of rapamycin, a mTOR inhibitor, in acute myeloid leukemia has been studied in various preclinical models [12,13]. And inhibition of mTOR or p70S6K2 down-regulates Hh/Gli pathway in non-small cell lung cancer and inhibition of AKT down-regulates Gli in various tumors including anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALCL) [14–16]. The regulation of rapamycin on Gli in AML and the potential cytotoxic effects of GANT61 in combination with rapamycin have not yet been determined in hematological malignancies.

In order to better define the antileukemic activity of GANT61, we investigated the cytotoxic effect of GANT61 on AML cells in vitro. Meanwhile, we assessed the regulation of rapamycin to Gli in AML cells and the cooperation of GANT61 and rapamycin. Our results suggest that aberrant Gli activation is a feature of some myeloid leukemic cells and Gli activiation can be regulated by rapamycin. Gli inhibitor with or without rapamycin may have a therapeutic role in the treatment of AML.

2. Materials and methods

2.1. Materials

Anti-smo, anti-Gli1, anti-Gli2 and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-phospho-p70S6K (Thr389) and anti-p70S6K were purchased from Cell

Abbreviations: Hh, hedgehog; smo, smoothened; rShh, recombinant sonic hedgehog; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; GSK3β, Glycogen synthase kinase 3β; AML, acute myeloid leukemia.

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Table 1
Primers for quantitative PCR.

GLI1 F	5-CCCAATCACAAGTCAGGTTCCT-3
GLI1 R	5-CCTATGTGAAGCCCTATTTGCC-3
GLI2 F	5-TGGCCGCTTCAGATGACAGATGTTG-3
GLI2 R	5-CGTTAGCCGAATGTCAGCCGTGAAG-3
ABL F	5-CGAGAGCCTGGCCTACAACAA-3
ABL R	5-CTAGCAGCTCATACACCTGGGACA-3

Signaling. Cell culture reagents and media were obtained from Invitrogen. Kasumi-1 cell line was kindly provided by Dr. Wang jianxiang. Fugene HD was from Roche. Cyclopamine, tomatidine, GANT61 and rapamycin were purchased from Merck. rShh was from Peprotech. The Luciferase Reporter Assay System was from Promega. 8* GLI-binding site luciferase reporter vector was kindly provided by Dr. Olivier Nolan-Stevaux.

2.2. Cell culture

Human myeloid leukemic cell line Kasumi-1 was cultured in DMEM containing 20% heat-inactivated FCS, 2 mM/L L-glutamine, 0.1% penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified 5% CO_2 atmosphere. K562, HL60 and U937 were cultured in PMI1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM/L L-glutamine, 0.1% penicillin (100 U/mL) and streptomycin (100 mg/mL).

2.3. Agent cytotoxic assays and apoptosis assay

Leukemic cells were washed once and resuspended in each adequate complete medium, and then 2×10^5 cells/mL were added to each well of a 12-well plate. The surviving cells were assessed by Annexin V-FITC Apoptosis Detection Kit and Premix WST-1 assay Cell Proliferation Assay System. After the AML cells (2×10^5/mL) were incubated in the presence of the indicated concentrations of the agents for indicated hours in 5% CO_2/air at 37 °C, 1 mL of this cell suspension was placed in a 1.5 mL tube, and the cells were washed twice in cold phosphate-buffered saline (pH 7.2). Cells in each tube were resuspended with 89 μL of phosphate-buffered saline containing 1% fetal bovine serum, and 1 μL of annexin V-FITC solution and 10 μL of propidium iodide (PI) solution were added to the tube (total volume was 100 μL). Then, the cells were incubated for 15 min at room temperature in the dark. Subsequently, 400 μL of binding buffer was added to each tube, and the cells were analyzed by flow cytometry within 1 h after staining. The data were analyzed with a FACSCant flow cytometer (Becton Dickinson) using FACS Diva software (Becton Dickinson). The fluorescence intensity of the FITC was detected on FITC channel (515–545 nm) of the FACSCant. PI fluorescence was detected on PerCP-Cy5.5 channel (more than 670 nm). The WST-1 assay is based on mitochondrial conversion of WST-1 to yellowish formazan, being indicative of the number of viable cells according to the manufacturer's instructions. The number of viable cells was evaluated by absorbance OD450 nm.

2.4. RNA isolation and quantitative PCR

Total RNA was isolated with Trizol-Reagent followed by further purification using a DNase treatment to remove genomic DNA. cDNA was synthesized with AMV reverse transcriptase according to the manufacturer's instructions. Real-time PCR analysis was done on a Rotor-gene 6000 (Corbett Research) using SYBR Green PCR Mix. Primer sequences used for real-time analysis are shown in Table 1 [17,18]. The mRNA expression of ABL gene was used as an internal standard [19].

2.5. Western blotting

For immunoblotting of total and phosphorylated p70s6k, Gli1 and Gli2, cell lysate was extracted from AML cells with a RIPA lysis buffer. The extracted 200 μg of total protein was subjected to 10% SDSPAGE analysis, and transferred to a NC membrane (Gli1 2.5h, Gli2 3h, p70s6k and p-p70s6k 1.5h, 100v). After blocking the membrane at room temperature for 3 h, the membrane was incubated overnight at 4 °C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1.5 h at 25 °C, the signals were visualized using enhanced chemiluminescence.

2.6. siRNA experiments

HL60 cells were transfected with a pool of Gli1-specific siRNA or with pooled non-specific siRNA duplexes (100 nM; SMARTpool, Dharmacon) using Jet-PEI (Polyplus transfection) according to the manufacturer's instructions. Silencing was confirmed 48 h post-transfection by Western blotting.

2.7. Transfection and luciferase assay

GLI reporting vector transfection was obtained using FuGENE HD Transfection Reagent according to the manufacturer's instructions and the ratio of plasmid (μg) to lipid (μl) was 2:3 [20]. HL-60 cells were grown and transfected with Gli reporting vector. For luciferase reporter assays, 10^6 cells were distributed in triplicate in 24-well plates and treated with rapamycin for 48 h. Samples were harvested and prepared for luciferase assays following the manufacturer's recommendations (Promega). Total proteins quantitation was used to control for intersample variations in transfection efficiency.

2.8. Statistical analysis

Each dataset was first evaluated for normality of distribution by the Komolgorov–Smirnov test to decide whether a non-parametric rank-based analysis or a parametric analysis should be used. Two groups were compared by either the Wilcoxon signed-rank test or the Student's *t*-test (two-tailed test). Effects were considered significant at $P < 0.05$.

3. Results

3.1. GANT61, but not cyclopamine, inhibits growth and induces apoptosis in AML cell lines

GANT61 is a Gli specific inhibitor which interferes with cellular DNA binding of Gli and has effect on chronic lymphocytic leukemia primary cells [21]. Hence, we first examined whether Gli inhibition using GANT61 has effect on AML. Kasumi-1, K562, HL60 and U937 cells were treated with different concentrations of GANT61. Subsequently, survival of leukemic cells was analyzed by mitochondrial conversion of WST-1 to yellowish formazan. When GANT61 was used, percent survival of all four kinds of leukemic cells decreased in a dose-dependent manner (Fig. 1A). Since it is reported that cyclopamine, a steroidal alkaloid from the corn lily that binds to and inactivates smo, decreases colony formation of K562 cells, we next tested whether GANT61 and cyclopamine have different effects on AML cells [22]. It is documented that 5–20 μM cyclopamine can inhibit Hedgehog pathway in different cell lines, therefore 20 μM cyclopamine was used [3,17,23–26]. Annexin V/PI assays for AML cells after exposure to 20 μM cyclopamine or certain concentration of GANT61 were conducted. And no apparent changes were observed after cyclopamine treatment except for K562 cells. In contrast, the percentage of annexin V and PI+ cells further increased after 48 h of GANT61 treatment (Fig. 1B). And features of apoptosis also could be detected by morphologic analysis (Fig. 1C). These results indicate that Gli inhibition could be a more optimal choice for AML than cyclopamine. To further ascertain that the effects of GANT61 on AML cells should be due to inhibition of Hh/Gli pathway, we used the expression of Gli transcription factor as surrogate indexes of Hh/Gli activation level. And Kasumi-1 and HL-60 cells were treated with GANT61 (30 μM) for 24–48 h, the expression of Gli1 and Gli2 mRNA were measured. In both cells treated with GANT61 for 48 h, expression of the target genes Gli1 and Gli2 were both down-regulated as determined by qRT-PCR (data not shown).

3.2. Silencing of Gli1 with siRNA inhibits HL-60 cell growth

To further confirm the specific role of Gli in AML cells survival, we induced Gli1 silencing with specific siRNA in HL60 cells. Gli1 siRNA-transfected HL60 cells showed a reduction in Gli1 protein expression levels compared with non-specific siRNA-transfected or untransfected cells (Fig. 2A). Subsequently, survival of leukemic cells was analyzed by mitochondrial conversion of WST-1 to yellowish formazan. And decreased cell survival was found in Gli1 silencing HL-60 cells (Fig. 2B). Annexin V/PI assays for AML cells after Gli1 silencing were also conducted (Fig. 2C). The results suggest silencing of Gli1 induced with siRNA inhibits HL-60 cell growth.

3.3. Smo-independent Gli activation exists in HL-60 cells

It is postulated that smo is absent in some AML cell line. To prove that assumption, we selected two AML cell lines, K562