Brief report

PI3Kδ inhibitor, GS-1101 (CAL-101), attenuates pathway signaling, induces apoptosis, and overcomes signals from the microenvironment in cellular models of Hodgkin lymphoma

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GS-1101 (CAL-101) is an oral PI3Kδ-specific inhibitor that has shown preclinical and clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. To investigate the potential role of PI3Kδ in Hodgkin lymphoma (HL), we screened 5 HL cell lines and primary samples from patients with HL for PI3Kδ isoform expression and constitutive PI3K pathway activation. Inhibition of PI3Kδ by GS-1101 resulted in the inhibition of Akt phosphorylation. Cocultures with stroma cells induced Akt activation in HL cells, and this effect was blocked by GS-1101. Conversely, production of the stroma-stimulating chemokine, CCL5, by HL cells was reduced by GS-1101. GS-1101 also induced dose-dependent apoptosis of HL cells at 48 hours. Reductions in cell viability and apoptosis were enhanced when combining GS-1101 with the mTOR inhibitor everolimus. Our findings suggest that excessive PI3Kδ activity is characteristic in HL and support clinical evaluation of GS-1101, alone and in combination, as targeted therapy for HL. (Blood. 2012;119(8):1897-1900)

Introduction

Hodgkin lymphoma (HL) is a malignant lymphoma of B-cell origin.1 The malignant cells, known as Reed-Sternberg (RS) cells, represent less than 2% of the tumor mass, the remainder composed of a mix of reactive inflammatory cells attracted by the RS cells. Patients usually present with lymphadenopathy, organomegaly, and constitutional symptoms.2 Chemotherapy with or without radiation cures most patients. However, some patients develop recurrent disease and are given sequential chemotherapeutic or immunotherapeutic agents to control tumor growth.3 Unfortunately, cumulative toxicities and progressive resistance limit benefits. Safer therapies with novel mechanisms of action are needed for patients with relapsed HL.

Phosphatidylinositol 3-kinases (PI3Ks) are enzymes that mediate signals from cell surface receptors. The 4 class I PI3K isozymes (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) regulate a variety of cellular functions through the production of phosphatidylinositol-3,4,5-triphosphate.4 Generation of phosphatidylinositol-3,4,5-triphosphate activates the downstream serine/threonine kinase, Akt, and the mammalian target of rapamycin (mTOR), both of which have positive effects on cell survival, proliferation, growth, and metabolism.5,6 Dysregulation of the PI3K/Akt/mTOR pathway is important in the etiology of human malignancies.5,7 Of the several PI3K isoforms, PI3Kδ has been shown to play a pivotal role in B-cell signaling in response to chemokines and cytokines.8,9,10 Past studies have indicated a role for the PI3K/Akt/mTOR pathway in the pathogenesis of HL11,12 and have suggested that PI3K might constitute an important therapeutic target in this disease. GS-1101 is a novel, oral, PI3Kδ-specific inhibitor that has shown activity in other types of B-cell cancers.8,9,13,14 We now characterize the activity of GS-1101 in cellular models of HL, providing translational support for PI3Kδ inhibition as a novel strategy for the treatment of HL.

Methods

Immunoblotting and ELISA analysis

Whole-cell lysates were analyzed on 10% polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF), blocked, probed with antibodies, and detected using the Odyssey from LiCor as previously described.15 The effect of GS-1101 on CCL5 secretion was measured as previously described.8 After 24 hours, supernatants were harvested and assayed for CCL5 by quantitative ELISA according to the manufacturer’s instructions (Quantiకine, R&D Systems).

Cell viability and apoptosis

Cell viability was assessed using Cell Titer Aqueous One Solution Cell Proliferation Assay reagent (Promega) following the manufacturer’s protocol. For cell-cycle analysis, cells were permeabilized, stained with propidium iodide, and subjected to FACS evaluation of labeled DNA. Apoptosis was measured by annexin V–FITC/7-amino-actinomycin D (7-AAD) labeling followed by fluorescence flow cytometry as previously described.9

Immunohistochemistry

Immunohistochemical methods to detect expression of the four PI3K isoforms were performed using routinely processed tissue microarray specimens.
appropriate antibodies. (D) GS-1101 selective inhibition of PI3K proteins were incubated for 2 hours with GS-1101. Proteins in cell lysates were resolved by SDS-PAGE electrophoresis, transferred onto PVDF membranes, and probed with appropriate antibodies. Each bar histogram is an average of 4 independent experiments. * p < .001 (t test).

Figure 1. GS-1101 selective inhibition of PI3K inhibits Akt phosphorylation. (A) PI3K isoform expression is confirmed in HL cell lines. Proteins from 10⁶ cells were separated by SDS-PAGE, transferred onto PVDF membranes, and analyzed by immunoblotting using antibodies specific for the α, β, δ, and γ isoforms. Purified recombinant PI3K proteins were used as controls (data not shown). Antiactin antibodies were used to assess equal loading of the samples. (B) Expression of PI3K isoforms in HL tumor samples. The number and percentage of cases positive for each isoform are shown. The intensity of expression is indicated as negative (−), weak (+), or strong (++). PI3Kα and δ were the isoforms more frequently expressed in tumor samples; they were detected in 97.2% and 80.6% of the cases, respectively. Strong expression of PI3Kα and δ was detected in 37.5% and 26.4% of cases, respectively. Protein expression was scored as +, +, or ++ depending on the staining signal intensity. Expression of each isoform in the Hodgkin RS cells was compared with that seen in positive control; if higher or equal, then expression of each p110 isoform was considered ++ and if lower, then expression was considered + (supplemental Figure 1). (C) GS-1101 selective inhibition of PI3K reduces constitutive Akt phosphorylation in HL cell lines. Serum-starved cells were incubated for 2 hours with GS-1101. Proteins in cell lysates were resolved by SDS-PAGE electrophoresis, transferred onto PVDF membranes, and probed with appropriate antibodies. (D) GS-1101 selective inhibition of PI3K overcomes phosphorylation of Akt in HL cell lines cocultured with HS-5 stromal cells. HL cells were cultured for 24 hours at 37°C with or without HS-5 stromal cells and GS-1101. Proteins from HL cell lysates were resolved by SDS-PAGE electrophoresis, transferred onto PVDF membranes, and probed with appropriate antibodies. Each bar histogram is an average of 4 independent experiments. * p < .001 (t test).

These tissue microarrays were constructed with triplicate cores from selected areas of 103 formalin-fixed, paraffin-embedded classic HL samples. The tissue sections were incubated for 60 or 90 minutes at room temperature with antibodies specific for PI3Kα (dilution 1:400; Cell Signaling), β (3 μg/mL; Abcam), and γ (1:100) and δ (1:100; Santa Cruz Biotechnology). Protein expression levels were scored as negative, weakly positive, or strongly positive, depending on the staining signal intensity. Sections of lung adenocarcinoma were used as a positive control for PI3Kα, β, and γ, and sections of a case of diffuse large B-cell lymphoma were used as positive control for the δ isoform.

Results and discussion

PI3K isoform expression and pathway activation in HL cell lines and primary patient samples

We first investigated the level of expression of each PI3K isoform in 5 HL cell lines. In all cases, we detected high levels of PI3Kδ. The majority of the cells also expressed PI3Kα and PI3Kβ, but at lower levels (Figure 1A). PI3Kγ was only detected in one cell line (L428). Immunohistochemical analysis in HL tumor samples revealed expression of PI3Kα and PI3Kδ in 97.2% and 80.6% of the cases, respectively (Figure 1B; supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Staining for the PI3Kβ and γ isoforms was found to be weak or absent in most HL samples tested (Figure 1B; supplemental Figure 1). These data demonstrate that PI3Kδ is highly expressed in HL cell lines and in tumor samples.

Previous studies have reported high constitutive levels of the active, phosphorylated form of Akt (pAkt) in RS cells in primary lymph node sections of HL. To evaluate constitutive PI3K pathway activation, we screened HL-derived cell lines for pAkt. We found that, in all HL cell lines tested, pAkt was readily detected. To determine whether Akt phosphorylation was dependent on PI3Kδ, unstimulated cell lines were incubated with GS-1101. GS-1101 induced a dose-dependent reduction in pAkt. These findings confirm a functional role for the PI3Kδ isoform in HL cell lines (Figure 1C).

Evaluation of PI3Kδ function in HL cocultures

Inflammatory cells are composed of a large portion of HL tumors, and the tumor microenvironment appears to be essential for RS cell survival and growth. In models of non-Hodgkin lymphoma, the human bone marrow stromal cell line, HS-5, promotes malignant B-cell growth and protects lymphoma cells from cytotoxic drugs. To examine microenvironmental influences on PI3K/Akt signaling,
we cocultured HL cell lines with HS-5 cells. The presence of the HS-5 cells increased expression of pAkt in L1236, L428, and L591 cells. To determine the role of PI3K in this expression, we established L1236, L428, and L591 cells in coculture with HS-5 cells for 24 hours and then treated with GS-1101. In this setting, we observed a significant decrease in the level of stroma-induced pAkt (Figure 1D).

Elevated concentrations of circulating chemokines have been shown to contribute to RS cell proliferation and survival, and to the formation and maintenance of the tumor microenvironment. CCL5, a chemokine produced by RS cells, has both a direct effect on RS cell survival and on microenvironment formation. We measured levels of CCL5 in cell supernatants 24 hours after initiation of GS-1101 treatment. The L1236 and L591 cell lines produced CCL5, showing supernatant concentrations of 1500 pg/mL and 786 pg/mL, respectively. Incubation of L1236 and L591 cells with GS-1101 significantly reduced the detectable CCL5 levels ($P < .05$, n = 5), as displayed in Figure 2A, suggesting that CCL5 secretion can be PI3K-dependent. Interestingly, coculture with HS-5 cells induced the HL cells to secrete high concentrations of CCL5 into the supernatants. Treatment with GS-1101 was able to down-regulate the secretion of CCL5 in stromal cocultures (Figure 2A), indicating that the induction of CCL5 secretion in HL cocultures may be reliant on PI3K.

GS-1101 induces cell cycle arrest and apoptosis in RS cell lines

To determine whether inhibition of PI3K by GS-1101 affects the viability of HL cell lines, L1236 and L591 cells were treated with GS-1101 and cell-cycle analysis was performed.Twenty-four hours of GS-1101 treatment resulted in an accumulation of cells in G1 and a decrease in the S-phase population (Figure 2B). After 48 hours of GS-1101 exposure, a 2.5-fold increase in annexin V–positive cells was observed, indicating a significant ($P < .05$) increase in apoptosis (Figure 2B).
Combination PI3Kδ and mTOR inhibition

mTOR inhibitors have shown antitumor activity in non-Hodgkin lymphoma and HL. However, these drugs may not optimally inhibit the PI3K/Akt/mTOR pathway because they can increase pAkt by a feedback loop, thus suggesting that combination therapy may offer advantages. We assessed whether GS-1101 could enhance the antitumor effects of mTOR inhibition with everolimus (RAD001) in L1236 and L591 cell lines. After treatment with GS-1101 in the presence or absence of everolimus for 48 hours, the combination showed greater dose-dependent inhibition of cell viability compared with each agent alone (Figure 2C; supplemental Figure 2). In addition, GS-1101 increased the number of everolimus-induced annexin V–positive cells, indicating increased apoptosis (Figure 2C). In conclusion, our results confirm the potential importance of the PI3K/Akt/mTOR pathway in HL growth and survival and document that specific inhibition of PI3Kδ with GS-1101 can disrupt pathway activation. Our data suggest that PI3Kδ inhibition can overcome pro-survival signals from the microenvironment that maintain HL cells and reduce levels of CCL5, a chemokine with autocrine effects on RS proliferation and paracrine actions on inflammatory cell recruitment. Collectively, the data suggest a critical role for PI3Kδ in maintaining HL tumor lesions and provide strong preclinical rationale for clinical evaluation of GS-1101 in patients with HL.

Acknowledgments

The authors thank Jerry Evarts for supplying GS-1101 (CAL-101).

Authorship

Contribution: S.A.M. performed the experiments, analyzed the data, and designed the figures; F.V. and A.K. performed the experiments and analyzed the data; D.J. and L.L.M. planned components of the research and assisted with the writing and review of the manuscript; V.D. contributed vital reagents and reviewed the manuscript; A.Y. provided samples, helped with data interpretation, and assisted in writing the manuscript; and B.J.L. designed the research, supervised the study, analyzed the data, and assisted in writing the manuscript.


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References

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