Combination of Ponatinib with Hedgehog Antagonist Vismodegib for Therapy-Resistant BCR-ABL1–Positive Leukemia

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Abstract

Purpose: The Hedgehog signaling pathway is a key regulator of cell growth and differentiation during development. Whereas the Hedgehog pathway is inactive in most normal adult tissues, Hedgehog pathway reactivation has been implicated in the pathogenesis of several neoplasms including BCR-ABL1–positive leukemia. The clear link between the Hedgehog pathway and BCR-ABL1–positive leukemia led to an effort to identify small molecules to block the pathway.

Experimental Design: We investigated the combined effects of vismodegib and ponatinib, a pan-ABL1 kinase inhibitor, in nonobese diabetic/severe-combined immunodeficiency (NOD/SCID) repopulating T315I BCR-ABL1–positive cells in vitro and in vivo.

Results: We observed that combination with vismodegib and ponatinib helps to eliminate therapy-resistant NOD/SCID repopulating T315I BCR-ABL1–positive cells. The percentage of CD19-positive leukemia cells in peripheral blood was significantly lower in vismodegib þ ponatinib–treated mice than that of the vehicle or ponatinib alone (P < 0.001). Spleen weights were also lower in vismodegib þ ponatinib–treated mice than in ponatinib alone (P < 0.05). Overall tumor burden, as assessed by BCR-ABL mRNA from bone marrow cells, was significantly lower in vismodegib þ ponatinib–treated mice than in ponatinib alone (P < 0.005). We also found that vismodegib significantly reduced BCR-ABL1–positive leukemia cell self-renewal in vitro as well as during serial transplantation in vivo.

Conclusions: The combination with a Smo inhibitor and ABL1 tyrosine kinase inhibitors may help eliminate therapy-resistant T315I BCR-ABL1–positive leukemia cells. Our preclinical results indicate that vismodegib has potential as an important option for controlling minimal residual cells in BCR-ABL1–positive leukemia. 

Introduction

An emerging concept in cancer biology is that a rare population of cancer stem cells exists in the heterogeneous cell mass that constitutes a tumor (1). This concept also applies to BCR-ABL1–positive leukemia (2). Normal and leukemic hematopoietic stem cell functions are defined by a common set of critical stemness genes that regulate self-renewal (3). Hematopoietic stem cells and leukemic stem cells share common features, including self-renewal, the capacity to differentiate, resistance to apoptosis, and limitless proliferative potential (3). Despite these similarities, several stemness factors, such as Hedgehog, Wnt, Notch, and BMI-1, show differential activation in normal versus leukemia stem cells (4).

Hedgehog signaling is increased in BCR-ABL1–positive stem and progenitor cells becoming more active with disease progression (5–7). The Hedgehog signaling pathway consists of 3 closely related ligands, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), that can each bind to the transmembrane protein Patched (Ptc1; refs. 8–10). Upon ligand binding, Ptc1 inhibition of the positive effector Smoothened (Smoo) is released and leads to production of activating forms of glioma-associated oncoproteins 1–3 (Gli1–3; refs. 9–12). Gli1 is a positive effector of signaling, Gli3 is predominantly a transcriptional inhibitor, and Gli2 can function in both roles (9–12). Smo signals to a cytoplasmic...
Translational Relevance

Despite the great success with combination of high-dose ABL tyrosine kinases and intensive chemotherapy in BCR-ABL1–positive acute lymphoblastic leukemia, there are still drawbacks that need to be addressed. Above all, 40% of patients, even with hematopoietic stem cell transplantation, have relapse of the disease. Therefore, it is necessary to define targets in BCR-ABL1–positive leukemia stem cells that may be candidates for new treatment options. In the present study, we investigated the combined effects of vismodegib and ponatinib in mutant forms of BCR-ABL1–expressing leukemia cells. We observed that combination with a Smo inhibitor and pan-ABL1 tyrosine kinase inhibitors (TKI) helps to eliminate therapy-resistant T315I BCR-ABL1–positive leukemia cells. Thus, exploring a vast array of possible therapeutic combinations will be useful to simultaneously target these pathways. Our preclinical results indicate that vismodegib has potential as an important option for controlling resistance in BCR-ABL1–positive leukemia. The combined results of cell-based and in vivo studies suggest that vismodegib exhibits sufficient activity against mutant forms of BCR-ABL1 to warrant consideration for combined use with ABL TKIs.

Materials and Methods

Antibodies and reagents

Anti-Gli1 Ab, anti-ABL Ab (11-23), anti-CD19 Ab, anti-Bcl2 Ab, anti-Cyclin D2 Ab, and anti-c-Myc Ab were purchased from Santa Cruz Biotechnology, Inc. Human Gli1 siRNA and Smo siRNA were also purchased from Santa Cruz Biotechnology, Inc. Vismodegib, ponatinib, sorafenib, and BEZ235 were obtained from Selleck.

Cells and cell culture

BaF3 cells expressing wild-type BCR-ABL and mutant forms of BCR-ABL (M244V, G250E, Q252H, Y253F, T315A, T315I, F317L, F317V, M351T, H396P) were described previously (25, 26). SK-9 was described previously (27). Briefly, SK-9 cell line was established from a BCR-ABL1–positive acute lymphoblastic leukemia (ALL) patient with T315I mutation (27). K562 cells were obtained from the American Type Culture Collection. OM;922 cells were described previously (28). TF-1 BCCR-ABL was described previously (29).

NOD/SCID repopulating BCR-ABL1–positive cells

We serially transplanted human leukemia cells from patients with chronic myeloid leukemia blast crisis (T315I BCR-ABL1: UPN1) or Ph-positive ALL (WT-BCR-ABL1: UPN5) into NOD/SCID/IL-2γc−/− mice. The cell fractions with CD34+/CD38−/CD19− and CD34+/CD38+/CD19+ could self-renew and transfer the leukemia in nonobese diabetic/severe-combined immunodeficiency (NOD/SCID) mice (30). UPN1 cells and UPN5 cells were used as the NOD/SCID repopulating cells.

Apoptosis assay

The incidence of apoptosis was determined by flow cytometric analysis with the fluorescein isothiocyanate (FITC)-conjugated APO2.7 monoclonal antibody (clone 2.7), which was raised against the 38-kDa mitochondrial membrane protein (7A6 antigen) and is expressed by cells undergoing apoptosis (28).

siRNA experiments

siRNA experiments were carried out as described previously (26).

Transfection

Activated Akt1 cDNA in pUSEamp was purchased from Millipore. Transfection experiments were carried out as described previously (31).

Immunoblotting

Immunoblotting was conducted as described previously (31). Nuclear extracts were prepared as described previously (32).

Colony-forming assay

T315I BCR-ABL BaF3, SK-9, UPN1, and UPN5 cells were treated with 1 or 10 μmol/L of vismodegib for 72 hours, washing free of drug and seeded in triplicate in condition complex that releases Gli1, which translocates to the nucleus where it activates the Hedgehog target genes (9, 10, 13). Leukemia cells are believed to rely on autocrine and paracrine Hedgehog signaling. The clear link between the Hedgehog pathway and BCR-ABL1–positive leukemias led to an effort to identify small molecules to block the pathway.

Vismodegib is a selective hedgehog pathway inhibitor with greater potency and more favorable pharmaceutical properties than cyclopamine (14–19). Vismodegib has antitumor activity in a mouse model of medulloblastoma and in xenograft models of primary human tumor cells, including colorectal cancer and pancreatic carcinoma, in which its effects correlate with blockade of the Hedgehog pathway (20–23).

In the present study, we investigated the combined effects of vismodegib and ponatinib, a pan-ABL1 kinase inhibitor (24), in mutant forms of BCR-ABL1–expressing BaF3 cells and the T315I-expressing human leukemia cells. We observed that combination with a Smo inhibitor and ABL1 tyrosine kinase inhibitors (TKI) helps to eliminate therapy-resistant T315I BCR-ABL1–positive leukemia cells. Our preclinical results indicate that vismodegib has potential as an important option for controlling minimal residual cells in BCR-ABL1–positive leukemia. The combined results of cell-based and in vivo studies suggest that vismodegib exhibits sufficient activity against mutant forms of BCR-ABL1 to warrant consideration for combined use with pan-ABL1 TKIs.
medium MethoCult GF H4434 (Stem Cell Technologies). At 14 days, the leukemic colonies (>50 cells) were counted as initial plating. The representative plate was then washed and cells were resuspended and replated. After an additional 14 days, colonies were counted as secondary replating. Clone-forming recovery of untreated cells was normalized to 100%, and plating results from all treatment groups were expressed as percentage of control.

**Nude mice xenografts model**

Twelve-week-old nude mice were injected with 5 × 10⁵ cells of mixture of BaF3 cells expressing wild-type BCR-ABL and mutant forms of BCR-ABL (M244V, G250E, Q252H, Y253F, T315A, T315I, F317L, F317V, M351T, H396P). At 24-hour injection of the leukemia cells, these mice were treated with either vehicle or vismodegib (20 mg/kg; every day) or ponatinib (30 mg/kg; every day), or vismodegib (20 mg/kg; every day) + ponatinib (30 mg/kg; every day). Mice were observed daily, and body weight as well as signs of stress (for example, lethargy, ruffled coat, or ataxia) was used to detect possible toxicities.

**NOD/SCID mouse models**

On day 1, 5-week-old female NOD/SCID mice were injected intravenously with 1 × 10⁶ cells of BCR-ABL1–positive leukemia cell line, SK-9 with T315I mutation, or NOD/SCID re-populating T315I BCR-ABL1–positive cells (UPN1). On day 2, these mice were treated with vehicle (n = 6) or vismodegib (20 mg/kg per os; every day; n = 6) or ponatinib (30 mg/kg; every day; n = 6) or vismodegib (20 mg/kg per os; every day) + ponatinib (30 mg/kg; every day; n = 6). On day 28, mice were sacrificed for evaluation.

**Second transplantation**

NOD/SCID mice were injected with UPN1 cells then treated with vismodegib for 28 days. All mice showed engraftment of leukemia by flow cytometry. We isolated human CD45⁺ cells from the spleen of mice from each treatment group and injected equivalent numbers of leukemia cells into secondary recipients. On 28 days, all mice were sacrificed for evaluated.

**Results**

**Interactions between Hedgehog activation and BCR-ABL1 signaling pathways**

Major advances have been made in understanding the interactions between Hedgehog signaling and other pathways during carcinogenesis (9). Several pathways are implicated in expression of Gli1 by Ras, TGFb, JUN, SCL/TAL1, and EWS-FLI1 oncoproteins (9). Therefore, we examined expression of Gli1 and BCR-ABL1 signaling pathways (Fig. 1A and B). K562, T315I BCR-ABL, and TF-1 BCR-ABL cells were treated with ponatinib or of vismodegib or ponatinib + vismodegib for 72 hours and then cytoplasmic cell lysates and nuclear extracts were immunoblotted with anti-Gli1 antibodies. Treatments with ponatinib and vismodegib reduced the expression of Gli1 in the cytoplasmic extracts and the nuclear extracts (Fig. 1A). Next, we treated SK-9 cells with several kinds of inhibitors, including ponatinib, vismodegib, sorafenib (Raf-1 kinase inhibitor), and BEZ235 (mTOR/P13K inhibitor). Activated Akt1-transfected SK9 cells were also treated with ponatinib (Fig. 1B). Treatments with ponatinib, vismodegib, and BEZ235 reduced the expression of Gli1 in the nuclear extracts in SK-9 cells; however, Gli1 expression was partially reduced in the activated Akt1-transfected SK-9 cells treated with ponatinib and SK-9 cells treated with sorafenib (Fig. 1B). These results suggest that Gli1 is regulated by BCR-ABL1 signaling especially, in part, mTOR/P13K signaling pathways. To investigate the entire Hedgehog signaling pathway, cell lysates from SK-9 cells treated with ponatinib, vismodegib, and ponatinib + vismodegib were immunoblotted with anti-Bcl2 Abs, anti-cyclin D2 antibodies, anti-c-Myc antibodies, or anti-ABL antibody. Treatments with ponatinib and vismodegib reduced the expression of Bcl2, cyclin D2, and c-Myc (Fig. 1C). Next, WT-p210 BCR-ABL BaF3 cells and T315I BCR-ABL BaF3 cells were treated with indicated concentrations of vismodegib for 48 hours, and the cytoplasmic extracts and the nuclear extracts were immunoblotted with anti-Gli1 antibody (Fig. 1D). The nuclear fraction of Gli1 was suppressed by 10 μmol/L of vismodegib (Fig. 1D). SK-9 cells were also incubated with nilotinib or dasatinib or ponatinib for 72 hours, the cytoplasmic extracts and the nuclear extracts were immunoblotted with anti-Gli1 antibody (Supplementary Fig). Ponatinib reduced the expression of Gli1 in both cytoplasmic extracts and nuclear extracts (Supplementary Fig).

**Inhibition of the Hedgehog pathway linked the induction of apoptosis and reduced proliferation in BCR-ABL1–positive leukemia**

T315I BCR-ABL1–expressing SK-9 cells were cultured with ponatinib (1 nmol/L) or ponatinib (1 nmol/L) and sonic hedgehog (Shh: 30 ng/mL) for 72 hours. The incidence of apoptosis was determined by APO2.7 monoclonal antibody (Fig. 2A). Treatment with ponatinib and Shh prevented the induction of apoptosis in SK-9 cells (Fig. 2A). However, co-treatment with 10 μmol/L of vismodegib overcame Shh-mediated anti-apoptotic effects (Fig. 2A). These results indicate that vismodegib actually inhibits Hedgehog anti-apoptosis pathways in SK-9 cells. To assess the functional importance of Smo and Gli1, RNA interference was used to determine whether reductions in Smo and Gli1 affect proliferation after ponatinib treatment (Fig. 2B–D). K562 cells were transfected with control siRNA, Smo siRNA, and Gli1 siRNA (Fig. 2B). At 48 hours after transfection, K562 cells were treated with indicated concentrations of ponatinib for 48 hours. The number of cells in each well was counted by flow cytometry, and cell numbers were normalized by dividing the number of cells (Fig. 2C). In the presence of Smo siRNA or Gli1 siRNA, K562 cells increased antiproliferative activity with ponatinib (P < 0.01; Fig. 2C). Co-treatment with Smo siRNA or Gli1 siRNA and ponatinib also enhanced the induction of
Targeting the Hedgehog Pathway in BCR-ABL1–Positive Leukemia

apoptosis in K562 cells (Fig. 2D). We also carried out the above experiments using OM9;22 cells and TF1 BCR-ABL cells (Table 1). Similar results were obtained in these cell lines (Table 1). These results showed that inhibition of Smo and Gli1 can play an important role in the induction of apoptosis and the antiproliferative effects of ponatinib.

Cotreatment of vismodegib and ponatinib tends to prolong survival in mouse models of BCR-ABL1–mutant—induced leukemia

We investigate the in vivo efficacy of vismodegib and ponatinib in a BCR-ABL1 BaF3 xenograft (Fig. 3). Twelve-week-old nude mice were injected with 5 × 10^5 cells of a mixture of BaF3 cells expressing wild-type BCR-ABL1 and mutant forms of BCR-ABL1 (M244V, G250E, Q252H, Y253F, T315A, T315I, F317L, F317V, M351T, H396P). At 24 hours after injection of leukemia cells, these mice were treated with either vehicle, vismodegib (20 mg/kg; every day), ponatinib (30 mg/kg; every day), vismodegib (20 mg/kg; every day) + ponatinib (30 mg/kg; every day). Vehicle and vismodegib-treated mice died of a condition resembling acute leukemia by 16 days. The combination of vismodegib + ponatinib–treated mice survived for more than 40 days; tending to improve survival over ponatinib-treated mice (P = 0.1005; Fig. 3).
Combination with a Smo inhibitor and ABL tyrosine kinase inhibitors eliminates minimal residual BCR-ABL1–positive leukemia cells

We examined the NOD/SCID mouse to elucidate in vivo efficacy (Fig. 4). On day 1, 5-week-old female NOD/SCID mice were injected intravenously with 1 × 10⁶ cells of the BCR-ABL1–positive leukemia cell line, SK-9 with a T315I mutation (Fig. 4). We also used the NOD/SCID repopulating T315I BCR-ABL1–positive leukemia UPN1 cells for this study (Fig. 4). The next day, these mice were treated with either vehicle (n = 6), vismodegib (20 mg/kg per os; every day; n = 6), ponatinib (30 mg/kg; every day; n = 6), vismodegib (20 mg/kg per os; every day) + ponatinib (30 mg/kg; every day; n = 6). On day 28, mice were sacrificed for evaluation. The percentage of CD19-positive leukemia cells in peripheral blood was significantly lower in vismodegib + ponatinib–treated mice than that of the vehicle or ponatinib alone (P < 0.001; Fig. 4A). Spleen weights were also lower in vismodegib + ponatinib–treated mice than in ponatinib alone (P < 0.05; Fig. 4B). Overall tumor burden, as assessed by BCR-ABL mRNA from bone marrow cells, was significantly lower in vismodegib + ponatinib–treated mice than in ponatinib alone (P < 0.005; Fig. 4C). Histopathologic analysis of vehicle-treated mice revealed infiltration of bone marrow with leukemia cells; however, cotreatment with ponatinib and vismodegib showed normal hematopoiesis in the bone marrow cavity (Fig. 4D and E). These results suggest that combination with a Smo inhibitor and ABL tyrosine kinase inhibitors may help eliminate BCR-ABL1–positive leukemia cells.

Figure 2. Inhibition of the Hedgehog pathway enhanced the induction of apoptosis with ABL TKI and reduced proliferation in BCR-ABL1–positive leukemia.

A, SK-9 cells were cultured with ponatinib (1 nmol/L) or ponatinib (1 nmol/L) and sonic hedgehog (Shh; 30 ng/mL) for 72 hours. The incidence of apoptosis was determined by APO2.7 monoclonal antibody. B, K562 cells were transfected with control siRNA, Smo siRNA, and Gli1 siRNA. Cell lysates were immunoblotted with anti-Gli1 Ab, anti-Smo Ab, and anti-actin Ab. C, at 48 hours after transfection, K562 cells were treated with indicated concentrations of ponatinib for 48 hours. The number of cells in each well was counted by flow cytometry, and cell numbers were normalized by dividing the number of cells (*) by 100 compared with control. D, the incidence of apoptosis was determined by APO2.7 monoclonal antibody. Similar results were obtained in 2 independent experiments.
**Table 1.** Inhibition of the Hedgehog pathway enhanced the induction of apoptosis with ABL TKI and reduced proliferation in BCR-ABL1–positive leukemia

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<th>K562</th>
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<tr>
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<tr>
<td>Ponatinib (1 nmol/L)</td>
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<td>8.2</td>
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<td>36.6</td>
<td>29.6</td>
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<tr>
<td>Ponatinib (1 nmol/L) + Shh</td>
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<td><strong>Growth inhibition,</strong> %</td>
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<td>Control</td>
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*The number of cells in each well was counted by flow cytometry, and cell numbers were normalized by dividing the number of cells. The each cell line was cultured with ponatinib (1 nmol/L) or ponatinib (1 nmol/L) and Shh (30 ng/mL) for 72 hours. The incidence of apoptosis was determined by APO2.7 monoclonal antibody.

*Each cell line was transfected with control siRNA, Smo siRNA, or Glil siRNA. At 48 hours after transfection, each cell line was treated with indicated concentration of ponatinib for 48 hours. The incidence of apoptosis was determined by APO2.7 monoclonal antibody.
Hedgehog inhibition with vismodegib limits self-renewal in vitro and in vivo

We further examined the effects of Hedgehog pathway modulation on in vitro clonogenic growth (Fig. 5A). T315I BCR-ABL1 BaF3, SK-9, activated Akt1-transfected SK9, the NOD/SCID repopulating BCR-ABL1–positive leukemia UPN1, and UPN5 cells were treated with 1 or 10 μmol/L of vismodegib for 72 hours, washed free of drugs, and plated in quadruplicate in methylcellulose. At 14 days, colonies were counted as initial plating (Fig. 5A and B). The representative plate was then washed and cells were resuspended and replated. After an additional 14 days, colonies were counted as secondary replating (Fig. 5A and B). Upon serial replating, secondary colony formations were significantly inhibited by vismodegib (P < 0.001; Fig. 5A and B). The NOD/SCID repopulating UPN1 and UPN5 cells were more sensitive to vismodegib than T315I BCR-ABL1 BaF3 cells (Fig. 5A and B). Also, no difference of clonogenic recovery was observed between SK-9 and activated Akt1-transfected SK9 cells (Fig. 5A). To further investigate the effects of Hedgehog inhibition on self-renewal and the relevance of the Hedgehog pathway as a therapeutic target in BCR-ABL1–positive leukemia, we examined the activity of vismodegib against UPN1 cells in vitro (Fig. 5C and D). NOD/SCID mice were injected with UPN1 cells and then treated with vismodegib on day 21 for 7 days (Fig. 5C). The treatments with vismodegib reduced the population of CD34+CD38- cells (Fig. 5C). Next, NOD/SCID mice were injected with UPN1 cells then treated with vismodegib on day 2 for 28 days (Fig. 5D). All mice showed engraftment of leukemia by flow cytometry. We isolated human CD45+ cells from the spleen of mice from each treatment group and injected equivalent numbers of leukemia cells into secondary recipients. Following 28 days, all mice received UPN1 cells from vehicle-treated mice engrafted with leukemia (Fig. 5D). In contrast, leukemia engraftment was not detected in recipient mice receiving UPN1 cells from initial vismodegib-treated donors (Fig. 5D). These results show the persistent effects of Hedgehog inhibition on long-term self-renewing BCR-ABL1–positive leukemia cells.

Discussion

The BCR-ABL1 oncoprotein is found in a subset of patients with ALL carrying the Philadelphia chromosome. This translocation is the most common cytogenetic abnormality in adults, with ALL occurring in 25% of patients (33). BCR-ABL1 defines a high-risk group and, as such, patients receive intensive chemotherapy in combination with ABL TKIs and are considered for hematopoietic stem cell transplantation (HSCT). Despite the great success with combination of high-dose ABL TKIs and intensive chemotherapy, there are still drawbacks that need to be addressed. Above all, 40% of patients, even with HSCT, have relapse of the disease. Furthermore, it is not clear whether responsive patients without HSCT cannot have relapse of the disease, as there is evidence that BCR-ABL1–positive leukemia stem cells remain present in the patient’s bone marrow even after years of therapy. Therefore, it is necessary to define targets in BCR-ABL1–positive leukemia stem cells that may be candidates for new treatment options.

Our study reveals that one candidate could be the Hedgehog pathway inhibitor, vismodegib. Vismodegib binds to and inhibits Smo, the 7-transmembrane Hedgehog pathway signaling protein (23). The activity of vismodegib was first shown in vivo in preclinical models of medulloblastoma, colon, and pancreatic tumors (20–22). In a phase I/II study for patients with advanced malignancies, vismodegib was well-tolerated, with pharmacodynamic evidence of Hedgehog pathway inhibition and tumor regression in patients with basal cell carcinoma and medulloblastoma (14–18). In the present study, we observed that cotreatment with vismodegib and Shh overcame Shh-mediated antiapoptotic effects (Fig. 2A and B and Table 1). Furthermore, the reduction of Gli1 or Smo by siRNA also enhanced the induction of apoptosis with ponatinib (Fig. 2C and D and Table 1). These results indicate the specificity of this compound confirmed by siRNA approaches (Fig. 2 and Table 1). We also observed that combination with vismodegib and an ABL1 TKIs helps to eliminate therapy-resistant T315I BCR-ABL1–positive leukemia cells (Fig. 4A–E). Overall tumor burden, as assessed by CD19 and BCR-ABL1 mRNA from bone marrow cells, was significantly lower in vismodegib + ponatinib–treated mice than in ponatinib alone (Fig. 4B and C). Also, cotreatment of vismodegib and ponatinib seemed to prolong survival in the mice model of BCR-ABL1–mutant–induced leukemia (Fig. 3). In a survival mouse model using BaF3 cells expressing WT BCR-ABL1 and mutant forms of BCR-ABL1 (M244V, G250E, Q252H, Y235F, T315A, T315I, F317L, F317V, M351T, and H396P),
Figure 4. Cotreatments with vismodegib and ponatinib eliminate minimal residual SK-9 cells. A, on day 1, 5-week-old female NOD/SCID mice were injected intravenously with $1 \times 10^6$ cells of the BCR-ABL1–positive SK-9 leukemia cell line or with $1 \times 10^8$ cells of the NOD/SCID repopulating T315I BCR-ABL1–positive leukemia UPN1 cells. On day 2, these mice were treated with either vehicle ($n = 6$), vismodegib (20 mg/kg per os; q.d.; $n = 6$), ponatinib (30 mg/kg; q.d.; $n = 6$), vismodegib (20 mg/kg per os; q.d.) + ponatinib (30 mg/kg; q.d.; $n = 6$) for each group. On day 28, mice were sacrificed for evaluation. CD19-positive cells from peripheral blood of each mouse have been shown. B, spleen weights from each treated mouse. C, BCR-ABL mRNA from bone marrow cells in each treated mouse was significantly lower in vismodegib + ponatinib–treated mice than in ponatinib alone ($P < 0.005$). D, histopathologic analysis of the bone marrow cavity from each treated mouse with SK-9 transplantation. Similar results were obtained in 2 independent experiments. E, histopathologic analysis of the bone marrow cavity from each treated mouse with UPN1 transplantation. Similar results were obtained in 2 independent experiments. H&E, hematoxylin and eosin.
Cotreatment with vismodegib and ponatinib tended to improve survival (Fig. 3). As the impact of Smo on short-term repopulating hematopoietic stem cells indicates that a combination of vismodegib with generally cytotoxic and hematotoxic chemotherapeutic reagents may induce prolonged neutropenias, anemias, and thrombocytopenias due to delayed bone marrow regeneration, our results from these studies suggest that combined use of vismodegib and ponatinib would be a viable strategy for preventing emergence of resistant clones in clinical setting.

Serial transplantation is widely accepted as an assay measuring long-term self-renewal in normal hematopoietic stem cells and neoplasms. We used a complementary in vitro assay with serial replating and colony formation, as well as serial in vivo transplantation to assess the effects of Hedgehog inhibition on long-term self-renewal (Fig. 5A–D). We found that vismodegib significantly reduced BCR-ABL1-positive leukemia cell self-renewal in vitro as well as during serial transplantation in vivo (Fig. 5A–D). This loss of serial transplantation ability is most consistent with self-renewal, as similarly seen in serial transplantation experiments with normal hematopoietic cells. It is notable that such long-lasting effects of Hedgehog inhibition were seen following only short exposure to vismodegib either 72 hours in colony formation assays (Fig. 5A and B) or after 28 days of treatment in primary recipient mice (Fig. 5D). On the basis of our in vitro clonogenic data, we believe that this loss of serial colony formation and transplantation ability is due to the

**Figure 5.** Hedgehog inhibition with vismodegib limits self-renewal in vitro and in vivo. Clonogenic recovery of T315I BCR-ABL BaF3, SK-9, activated Akt1-transfected SK9 (A); the NOD/SCID repopulating T315I BCR-ABL1-positive leukemia UPN1 cells and UPN5 cells (B). T315I BCR-ABL BaF3, SK-9, activated Akt1-transfected SK9, UPN1, and UPN5 cells were treated with 1 or 10 μmol/L of vismodegib for 72 hours, washed free of drugs, and plated in quadruplicate in methylcellulose. At 14 days, colonies were counted as initial plating. The representative plate was then washed and cells were resuspended and replated. After an additional 14 days, colonies were counted as secondary replating. Clonogenic recovery of untreated cells was normalized to 100% and plating results from all treatment groups were expressed as percentage of control. *, P < 0.001 compared with control. Similar results were obtained in 3 independent experiments. C, NOD/SCID mice were injected with UPN1 cells and then treated with vismodegib on day 21 for 7 days. The percentage of CD34⁺CD38⁻ cells was analyzed by flow cytometry. D, an initial cohort of mice was injected with UNP1 cells and treated with either vismodegib or vehicle. All mice were engrafted with leukemia. Following transplantation of harvested spleen cells, leukemia engraftment was not detected in recipient mice receiving UPN1 cells from initial vismodegib-treated donors. Similar results were obtained in 2 independent experiments.
effects of Hedgehog inhibition on the self-renewal properties of BCR-ABL1 leukemia-initiating cells. However, it is possible that the inhibition of engraftment during secondary transplantation is mediated by the effects of Hedgehog inhibition on quiescence of leukemia-initiating cells, their ability to interact with potential stem cell niches, and proper homing during transplantation.

Evidence suggests that BCR-ABL1–positive leukemia-initiating cell population persists despite BCR-ABL1 inhibition, and various mechanisms have been proposed to explain this observation. BCR-ABL1–positive leukemia-initiating cell population is independent of BCR-ABL1 activity and perhaps depend on other signaling pathways in vivo (34). Autocrine activation, in which the tumor cells produce and respond to own Hedgehog ligands, and paracrine Hedgehog activation have been reported in BCR-ABL1–positive leukemia (34). We have previously shown that Hedgehog ligands are produced by stroma cells in bone marrow and these allow survival and expansion of BCR-ABL1–positive cells (35). The activation of the Hedgehog pathway could be due to amplification of the Hedgehog ligands in BCR-ABL1–positive cells in Smo-dependent. In the same cells, BCR-ABL1 activates AKT/mTOR/S6K1 pathway, which phosphorylates and stabilizes the Gli1 protein in Smo-independent manner. Therefore, vismodegib alone had small effects in BCR-ABL1–positive leukemia-transplanted mice (Fig. 4A–E). Recent report showed that a Smo-independent activation of Gli1 by the mTOR/S6K1 pathway, which cannot be inhibited by Smo inhibitors but is sensitive to inhibitors of the mTOR pathways (36). Therefore, cotreatment with pan-ABL kinase inhibitor and Smo inhibitor, panatinib and vismodegib, indeed showed better inhibitory effects on T315I BCR-ABL leukemia in vivo than did single drug treatment (Fig. 4A–E). Our preclinical results indicate that vismodegib has potential as an important option for controlling resistance in BCR-ABL1–positive leukemia.

The combined results of cell-based and in vivo studies suggest that vismodegib exhibits sufficient activity against mutant forms of BCR-ABL1 to warrant consideration for combined use with ABL TKIs. Although several Hedgehog inhibitors have now entered clinical evaluation, it is expected that Hedgehog inhibitors may become extremely useful therapeutic interventions in a number of hematologic neoplasms where the persistence of cancer stem cells and the protective effect of the tumor microenvironment may be addressed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Combination of Ponatinib with Hedgehog Antagonist Vismodegib for Therapy-Resistant BCR-ABL1–Positive Leukemia

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