A Purine Scaffold HSP90 Inhibitor BIIB021 Has Selective Activity against KSHV-Associated Primary Effusion Lymphoma and Blocks vFLIP K13-Induced NF-κB

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Abstract

**Purpose:** Kaposi sarcoma–associated herpes virus (KSHV)–associated primary effusion lymphomas (PEL) have extremely poor prognosis when treated with conventional chemotherapy. KSHV-encoded viral FLICE-inhibitory protein (vFLIP) K13 binds to the IkappaB kinase (IKK) complex to constitutively activate the NF-κB pathway, which has been shown to be essential for the survival and proliferation of PEL cells. The molecular chaperone HSP90 is a component of the IKK complex and is required for its activity.

**Experimental Design:** We have analyzed the effect of HSP90 inhibitors on the survival and proliferation of PEL cells and on the activity of the NF-κB pathway.

**Results:** We show that BIIB021, a purine scaffold–based orally administrable HSP90 inhibitor, shows preferential cytotoxicity toward PEL cells as compared with non-PEL cells. The cytotoxic effect of BIIB021 against PEL was associated with induction of cell-cycle arrest and apoptosis. BIIB021 blocked the expression of a number of cellular proteins involved in the regulation of cell cycle and apoptosis. BIIB021 also blocked constitutive NF-κB activity present in PEL cells, in part, by blocking the interaction of vFLIP K13 with the IKK complex subunits. In a xenograft model of PEL, BIIB021 significantly reduced tumor growth.

**Conclusion:** BIIB021 blocks constitutive NF-κB activity in PEL and shows preferential antitumor activity against PEL in vitro and in vivo. BIIB021 may be a promising agent for treatment of PEL. *Clin Cancer Res; 19(18); 5016–26. ©2013 AACR.*

Introduction

Primary effusion lymphoma (PEL) is a variant of non-Hodgkin lymphoma that is characterized by infection with Kaposi sarcoma–associated herpes virus (KSHV; also known as human herpes virus 8; ref. 1). Although administration of cytotoxic chemotherapeutic agents represents the current standard of care, the prognosis of PEL is extremely poor with a short median survival time of 3 to 6 months upon diagnosis (2). Thus, there is an urgent need for safer and more effective therapeutic options for PEL.

NF-κB is a critical transcription factor that controls the expression of several genes involved in the regulation of cellular survival, proliferation, and inflammatory response (3). The NF-κB pathway is constitutively active in a majority of PEL cells and is believed to be essential for their survival and proliferation (4–7). The constitutive activity of NF-κB pathway in the PEL cells is primarily due to the activity of KSHV-encoded viral FLICE-inhibitory protein (vFLIP) K13 (5–8). K13 activates the NF-κB pathway by activating a multi-subunit IkappaB kinase (IKK) complex that consists of 2 catalytic subunits, IKK1/IKKα and IKK2/IKKβ, and a regulatory subunit, NEMO/IKKγ (5, 8). K13, however, is not a kinase that activates the IKK complex by inducing its phosphorylation. Instead, K13 activates the complex by direct interaction via a mechanism believed to involve a conformational change of the IKK complex (9).

HSP90 is an abundant molecular chaperone that functions in the proper folding, assembly, and transportation of a wide range of client proteins (10). Inhibition of HSP90 function causes many oncogenic client proteins to adopt aberrant conformations followed by their ubiquitylation and proteosomal degradation. HSP90, along with CDC37, was also shown to be an essential component of the IKK complex (11). Treatment with geldanamycin, an HSP90 inhibitor, was shown to block TNFα-induced activation of IKK and NF-κB (11). A subsequent study revealed that HSP90 is also a component of the K13/IKK complex and K13-induced NF-κB activity could be blocked by geldanamycin (12).

Most of the currently available HSP90 inhibitors belong to 1 of the following 3 categories: (i) geldanamycin...
derivatives (e.g., 17-AAG and 17-DMAG), (ii) purine scaffold–based HSP90 inhibitors (e.g., BIIB021 and PLI-H71), and (iii) resorcinol derivatives (e.g., NVP-AUY922 and KW-2478). Although geldanamycin-derivative 17-AAG was the first to enter clinical trials, its development has been limited by hepatotoxicity and limited solubility and stability (13, 14). Subsequently, HSP90 inhibitors using purine as a scaffold were designed that lacked the above limitations of geldanamycin derivatives, particularly hepatotoxicity (15). In this study, we conducted a comparative analysis of the 3 main classes of HSP90 inhibitors against PEL cells. We show that while all 3 classes of HSP90 inhibitors are effective against PEL, the purine scaffold inhibitor BIIB021 shows preferential cytotoxicity toward PEL as compared with the other lymphoma cells. We further show that the cytotoxicity of BIIB021 against PEL is associated with inhibition of classical and alternative NF-κB pathways induced by KSHV vFLIP K13.

Cell viability, cell-cycle, and apoptosis analysis

Cells from exponentially growing cultures were plated in untreated flat-bottom 96-well plates at a density of 10 × 10^3 cells per well, treated with an increasing concentration of the drugs and subsequently assessed for cell viability using the MTS reagent following the manufacturer’s instructions (Promega). Percent cell survival was calculated on the basis of the reading of cells grown in the presence of dimethyl sulfoxide (DMSO) control. IC50 for the individual drugs were calculated using GraphPad Prism 5 software. DNA content analysis was conducted as described previously (17). Apoptosis was analyzed using BD AnnexinV-FITC apoptosis detection kit I as per manufacturer’s instructions.

Translational Relevance

Primary effusion lymphoma (PEL) is an aggressive form of non–Hodgkin lymphoma commonly seen in HIV-positive patients that is universally associated with infection by Kaposi sarcoma–associated herpes virus. PEL has extremely poor prognosis when treated with conventional chemotherapy, and novel therapeutic strategies are urgently needed for the treatment of this disorder. The work presented in this article shows that HSP90 inhibitor BIIB021 blocks constitutive NF-κB activity present in PEL cells and also shows \textit{in vitro} and \textit{in vivo} activity against this disease. These results provide the basis for clinical testing of BIIB021 in PEL.

Materials and Methods

Lentivirus constructs

To construct a lentiviral vector expressing K13 from a tetracycline-inducible promoter, K13 cDNA with a C-terminal FLAG epitope was initially cloned in a modified pENT entry vector containing a tetracycline-inducible promoter (TO). Recombination-based subcloning was used to transfer the TO-K13-FLAG cassettes into pSLIK destination vector (16). A lentivirus construct expressing an NF-κB–driven luciferase reporter construct was generated in pLENTI6/V5-based vector (Invitrogen). Recombinant lentiviruses were generated in the HEK293-F cell line. Postinfection, cells were cultured in normal growth media containing the appropriate drugs to select positive clones.

Cell viability, cell-cycle, and apoptosis analysis

Two-tailed unpaired Student \(t\) test was used to test for differences between 2 groups using GraphPad Prism 5 software. Differences with a \(P \leq 0.05\) were considered statistically significant. All experiments were repeated a minimum of 3 times.

Additional information about materials and methods is provided in the Supplementary Information.

Results

\textbf{BIIB021 specifically targets KSHV-associated PEL}

We treated a panel of logarithmically growing PEL and non-PEL lines for 72 hours with increasing concentrations of HSP90 inhibitors BIIB021, 17-DMAG, and NVP-AUY922, respectively (Fig. 1A). While 17-DMAG and NVP-AUY922 inhibited the growth of both PEL and non-PEL cell lines equivalently, BIIB021 showed preferential cytotoxicity toward the PEL cell lines (Fig. 1B). Thus, the IC50 values of BIIB021 for the PEL cell lines ranged from 41.5 to 71.5 nmol/L, whereas its IC50 for non-PEL cell lines ranged from 187 to 275 nmol/L (Table 1). In contrast, the IC50 of 17-DMAG for PEL and non-PEL cell lines ranged from 55 to 217 nmol/L and from 24.2 to 1655 nmol/L, respectively. Similarly, the IC50 of NVP-AUY922 for PEL and non-PEL cell lines ranged from 19.3 to 60.5 nmol/L and from 16.1 to 40.5 nmol/L, respectively. The preferential toxicity of BIIB021 against PEL cells was also seen at 24 and 48 hours of drug treatment (Fig. 1C).

\textbf{BIIB021 induces cell-cycle arrest in PEL}

We next examined the effect of BIIB021 on cell-cycle progression. Treatment of BC-1 with BIIB021 resulted in G1 arrest as observed by a marked increase in the number of cells in the G1 phase and concomitant decreases in cells in the S-phase (Fig. 2A). In contrast, treatment of BC-3 cells with BIIB021 resulted in cell-cycle arrest in G2–M phase as observed by an increase in the percentage of cells in the G2–M phase (Fig. 2A). BIIB021 also significantly increased the proportion of cells with reduced DNA content (sub-G0/G1) suggestive of apoptosis (Fig. 2A). BIIB021-induced cell-cycle arrest was accompanied by reduced expression of several HSP90 clients critical for cell-cycle progression, including CDK2 (cyclin-dependent kinase 2), CDK4, CDK6, and CDK9 (Fig. 2A). In addition, BIIB021 depleted the levels of c-MYC, cyclin A, and cyclin B1 (Fig. 2A). In contrast, BIIB021 upregulated the expression of CDK inhibitor p21 (Fig. 2A), which is a negative regulator of cell-cycle progression. Finally, BIIB021 decreased the expression of HSP90 clients AKT, GSK3β, and survivin (Fig. 2A), which have been implicated in the pathogenesis of PEL (18–20).
BIIB021 induces apoptosis in PEL with the cleavage of PARP, caspases-9, -7, and -3

We next examined the effect of BIIB021 on the induction of apoptosis upon staining with nuclear dye Hoechst 33258. Treatment of BC-1 and BC-3 cells with BIIB021 resulted in appearance of cells with condensed and fragmented nuclei suggestive of apoptosis, which was confirmed by staining with AnnexinV/propidium iodide (Fig. 2B). Induction of apoptosis by BIIB021 involved activation of caspases-9, -7, and -3 and cleavage of their downstream substrate, PARP (Fig. 2C). However, BIIB021 had no significant impact on the levels of expression of BCL-2, BCL-XL, and MCL-1 and on the expression or cleavage of BID (Fig. 2C). Similarly, BIIB021 had no significant effect on the level of p53 (Fig. 2C).

To provide further evidence of the preferential toxicity of BIIB021 against PEL cells, we analyzed the cleavage of PARP (as a measure of caspase activation) in a panel of cell lines, as shown in Table 1. We found that BIIB021 had significant effects on the expression and cleavage of PARP in PEL cells, as compared to non-PEL cells, indicating a preferential toxicity of BIIB021 against PEL cells. These findings suggest that BIIB021 has potential as a therapeutic agent for the treatment of PEL.

Table 1. List of cell lines, the associated viruses, diseases, and IC50 doses of HSP90 inhibitors for 72 hours

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Associated virus</th>
<th>p53 status</th>
<th>BIIB021 (IC50, nmol/L)</th>
<th>17-DMAG (IC50, nmol/L)</th>
<th>NVP-AUY922 (IC50, nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>PEL</td>
<td>KSHV and EBV</td>
<td>WT</td>
<td>41.5</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>BC-3</td>
<td>PEL</td>
<td>KSHV</td>
<td>WT</td>
<td>62.6</td>
<td>74.8</td>
<td>28.1</td>
</tr>
<tr>
<td>BCBL-1</td>
<td>PEL</td>
<td>KSHV</td>
<td>Mut</td>
<td>53.6</td>
<td>217</td>
<td>60.5</td>
</tr>
<tr>
<td>JSC-1</td>
<td>PEL</td>
<td>KSHV and EBV</td>
<td>WT</td>
<td>71.5</td>
<td>63.8</td>
<td>40.6</td>
</tr>
<tr>
<td>UMPERL-1(c)</td>
<td>PEL</td>
<td>KSHV and EBV</td>
<td>N/A</td>
<td>59.6</td>
<td>92.1</td>
<td>19.3</td>
</tr>
<tr>
<td>BJAB</td>
<td>Burkitt lymphoma</td>
<td>None</td>
<td>Mut</td>
<td>187</td>
<td>33.3</td>
<td>16.1</td>
</tr>
<tr>
<td>Namalwa</td>
<td>Burkitt lymphoma</td>
<td>EBV</td>
<td>Mut</td>
<td>275</td>
<td>1655</td>
<td>31.2</td>
</tr>
<tr>
<td>Jurkat</td>
<td>T-cell acute lymphoblastic leukemia</td>
<td>None</td>
<td>Mut</td>
<td>214</td>
<td>410.5</td>
<td>40.5</td>
</tr>
<tr>
<td>MM1S</td>
<td>Multiple myeloma</td>
<td>None</td>
<td>WT</td>
<td>203</td>
<td>41.8</td>
<td>24</td>
</tr>
<tr>
<td>U266</td>
<td>Multiple myeloma</td>
<td>None</td>
<td>Mut</td>
<td>238</td>
<td>24.2</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Abbreviations: EBV, Epstein–Barr virus; Mut, mutated; N/A, not available; WT, wild-type.
PEL (BC-3, BCBL-1, and JSC-1) and non-PEL (Jurkat, BJAB, Namalwa, MM1S, and U266) cell lines following treatment with BIIB021 (100 nmol/L), 17-DMAG (100 nmol/L), and NVP-AUY922 (50 nmol/L) for 48 hours. Consistent with the results of cell viability assay, BIIB021 resulted in increased PARP cleavage in all 3 PEL cell lines but in none of the non-PEL cell lines tested (Fig. 2D). In contrast, NVP-AUY922 increased PARP cleavage in all cell lines, whereas 17-DMAG increased PARP cleavage in all PEL cell lines and 3 non-PEL cell lines (BJAB, MM1S, and U266). Thus, while PEL cells are sensitive to all HSP90 inhibitors, they show preferential sensitivity to BIIB021 at concentration that is not cytotoxic to non-PEL cell lines.

Figure 2. BIIB021 induces cell-cycle arrest and apoptosis in PEL cells. A, left, cell-cycle analysis of control and BIIB021-treated BC-1 and BC-3 cell lines. BIIB021 (200 nmol/L for 48 hours) results in G1 arrest in BC-1 cells and G2–M arrest in BC-3 cells. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. Data are a representative of 3 individual experiments. Right, Western blot analysis showing the effect of BIIB021 on the expression of HSP90 client proteins involved in cell-cycle regulation. Lack of effect on COX-2, a protein that is not an HSP90 client, shows the specificity of the observed effect. B, left, BC-1 and BC-3 cells were treated with 200 nmol/L BIIB021 or DMSO control for 48 hours. Cells were then stained with Hoechst 33258 and photographed. Right, BC-1 and BC-3 cells were treated with 200 nmol/L BIIB021 or DMSO control for 48 hours, stained with AnnexinV-FITC/PI, and analyzed for apoptosis by flow cytometry. C, Western blotting showing the effect of BIIB021 on the cleavage of PARP, activation of caspases, and expression of BCL-2 family members and p53. D, Western blotting showing the effect of BIIB021 (100 nmol/L, 48 hours), 17-DMAG (100 nmol/L, 48 hours), and NVP-AUY922 (50 nmol/L, 48 hours) treatment on the cleavage of PARP in the indicated cell lines. Cl, cleaved; FL, full length.
Inhibition of NF-κB pathway in PEL by BIIB021

NF-κB pathway has been shown to be essential for the survival of PEL cells. To check whether BIIB021 inhibits the NF-κB pathway in PEL cells, we took advantage of BC-1 and BC-3 cells engineered to express a stably integrated copy of an NF-κB–driven luciferase reporter construct (NF-κB-Luc). BIIB021 decreased the NF-κB-Luc activity in a dose-dependent manner (Fig. 3A), which was accompanied by a reduction in the level of nuclear NF-κB as measured by a p65/RelA DNA-binding assay (Fig. 3A). Interleukin 6 (IL-6) is an NF-κB target gene and a known growth factor for PEL cells (21, 22). Consistent with its inhibition of the NF-κB pathway, BIIB021 resulted in reduced secretion of IL-6 secretion in the supernatants of BC-1 and BC-3 cells (Fig. 3B). Finally, BIIB021 blocked the expression of A20 and XIAP, 2 known targets of the NF-κB pathway (refs. 23, 24; Fig. 3C).

To study the mechanism of classical NF-κB inhibition by BIIB021, we studied its effect on IkBα phosphorylation. Treatment with BIIB021 reduced the phosphorylation of IkBα on conserved Ser32/36 residues in both BC-1 and BC-3 cells as measured by Western blot analysis (Fig. 3C). BIIB021 resulted in a dose-dependent reduction in the expression of IKKα/β but had no significant effect on the expression of NEMO/IKKγ (Fig. 3C). Furthermore, BIIB021 blocked the phosphorylation of IKKα and IKKβ on Ser176/Ser177/Ser181, respectively (Fig. 3D). Collectively, the above results suggest that BIIB021 blocks classical NF-κB pathway in PEL by blocking the expression and

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**Figure 3.** Inhibition of NF-κB pathway by BIIB021 in PEL cells. A, left, BC-1/NF-κB-Luc and BC-3/NF-κB-Luc cells were treated with increasing concentrations of BIIB021 or vehicle control for 15 hours and cell lysates used for measurement of luciferase activity. Values shown are the mean ± SEM from 1 representative experiment of 3 carried out in duplicate. Asterisks (*** and ****) indicate significance at levels of P ≤ 0.001 and P ≤ 0.0001, respectively. Right, an ELISA-based NF-κB binding assay showing reduced p65/RelA DNA-binding activity in the nuclear extracts of BC-1 and BC-3 cells treated with BIIB021 (200 nmol/L for 48 hours). Values shown are the mean ± SEM from 1 representative experiment of 3 carried out in duplicate. Asterisks (*** and ****) indicate significance at levels of P < 0.0001. B, BIIB021 blocks interleukin (IL)-6 secretion. IL-6 levels were measured using ELISA in the supernatants of BC-1 and BC-3 cells treated with 200 nmol/L of BIIB021 or DMSO control for 24, 48, and 72 hours. The values (mean ± SEM) shown are from a representative of 3 independent experiments carried out in triplicate. C, Western blot analyses showing the effect of BIIB021 on the expression of phospho-IκBα and total IκBα, IKKα/β, and NEMO, and NF-κB target proteins A20 and XIAP. D, PathScan ELISA for phospho-IKKα (Ser176/180) and phospho-IKKβ (Ser177/181) showing inhibition of IKKβ phosphorylation by BIIB021 (200 nmol/L, for 48 hours) in BC-1 and BC-3 cells. A representative of 2 independent experiments carried out in triplicate. Statistically significant differences are shown by asterisks (****) at a level of P ≤ 0.0001 and (*****) at level of P ≤ 0.00001.


Clinical Cancer Research
BIIB021 blocks vFLIP K13 expression

To examine the role of vFLIP K13 in the inhibitory effect of BIIB021 on NF-κB pathway, we examined its expression in BIIB021-treated cells. We observed a reduction in K13 protein level upon BIIB021 treatment (Fig. 4A). However, reduction in K13 was evident only at the highest dose (200 nmol/L) of BIIB021 and was not seen at the lower doses (50 and 100 nmol/L). BIIB021 also downregulated the expressions of LANA and vCyclin (Fig. 4A), 2 other KSHV latent proteins.
proteins (25). A real-time quantitative reverse transcript-polymerase chain reaction (qRT-PCR) analysis revealed that BIIB021 led to robust suppression of K13 and vCyclin and modest suppression of LANA at the mRNA levels (Fig. 4A). HSP90 inhibitors are known to promote degradation of HSP90 client proteins (26). As we observed a much greater reduction in the levels of LANA, and to a lesser extent K13 and vCyclin, at the protein level as compared with the mRNA level, we examined the effect of BIIB021 on the stability of these proteins. For this purpose, we examined the effect of BIIB021 on the expression of K13, LANA, and vCyclin in BC-1 and BC-3 cells after blocking protein synthesis with cycloheximide (CHX). We used AKT, a known HSP90 client protein, as a positive control and COX-2 as a negative control (27). BIIB021 reduced the half-life of LANA by several hours in cycloheximide-treated BC-1 and BC-3 cell lines (Fig. 4B and Supplementary Fig. S1), confirming the results of a recent study showing that LANA is an HSP90 client protein (26). However, BIIB021 did not have any significant effect on the half-lives of K13 and vCyclin (Fig. 4B and Supplementary Fig. S1), indicating that these proteins are not clients of HSP90 (Fig. 4B). Finally, BIIB021 reduced the half-life of AKT but did not significantly affect COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) stability.

**BIIB021 blocks vFLIP K13 induced NF-kB activity**

As the inhibition of K13 expression by BIIB021 was evident only at its highest dose, we next asked whether inhibition of K13 activity could also contribute to its inhibitory effect on the NF-kB pathway. For this purpose, we generated a clone of 293 cells, designated 293A-pSLIK-TO-K13/NEK-kB-Luc, stably expressing FLAG-tagged K13 from a tetracycline-inducible promoter along with a stably integrated copy of an NF-kB-driven luciferase reporter construct. As K13 is being expressed from an ectopic promoter, this cell line afforded us the opportunity to examine the effect of BIIB021 on K13 activity independent of its effect on K13 expression. As shown in Fig. 4C, induction of K13 expression with doxycycline resulted in a significant increase in NF-kB-Luc activity that was accompanied by an increase in phosphorylation and degradation of IkBz and upregulation of A20 expression. However, all the above effects were effectively blocked by BIIB021 in a dose-dependent manner (Fig. 4C). A Western blot analysis confirmed that inhibition of NF-kB activity by BIIB021 is not due to a block in K13 expression (Fig. 4C). Essentially similar results were obtained upon treatment with 17-DMAG and NVP-AUY922 (Fig. 4C).

**HSP90 inhibitors disrupt the interaction between K13 and the IKK complex**

To understand the mechanism by which HSP90 inhibitors block K13-induced NF-kB, we examined their effect on K13/IKK complex interaction in 293-pSLIK-TO-K13/NEK-kB-Luc cells using a co-immunoprecipitation (co-IP) assay. BIIB021 reduced the amounts of HSP90, IKKc/a/b, and IKKg/NEMO that co-immunoprecipitated with K13, suggesting that HSP90 function is essential for the structural integrity of the K13/IKK complex (Fig. 5A). Essentially similar results were obtained upon treatment with 17-DMAG (Fig. 5A). Finally, treatment with NVP-AUY922 resulted in a near-complete disappearance of HSP90, IKKc/a/b, and IKKg/NEMO bands in the co-IP samples, although a reduction in the expression of these proteins probably also contributed to this effect (Fig. 5A). Similarly, we observed a reduction in the levels of IKKc/a/b and IKKg/NEMO that co-immunoprecipitated with K13 in the BIIB021-treated BCBL-1 cells expressing FLAG-tagged K13 cells as compared with the control-treated cells (Fig. 5A). Thus, HSP90 activity is required for the assembly of the K13/IKK complex and BIIB021 blocks K13-induced NF-kB activity by disrupting this complex.

**K13 interacts with HSP90 via NEMO**

NEMO is essential for the interaction of K13 with the IKK complex (5, 12). To check whether NEMO is required for the interaction of K13 with HSP90, we used FLAG-tagged K13-expressing wild-type and NEMO-deficient Jurkat cells. While we readily detected an interaction between K13 and HSP90 in the wild-type Jurkat cells using the co-IP assay, we failed to observe this interaction in the NEMO-deficient cells (Fig. 5B). Thus, NEMO is essential for the interaction of K13 with HSP90.

**BIIB021 blocks alternate NF-kB pathway in PEL**

PEL cells also show constitutively active alternate NF-kB pathway that involves proteasome-mediated processing of p100/NEK-kB2 into the active p52 subunit (28). Treatment of BC-3 cells with 50 nmol/L BIIB021 blocked the processing of p100 into p52, suggesting inhibition of the alternate NF-kB pathway (Fig. 5C). The expression of both p100/NEK-kB2 and RELB subunits of the alternate NF-kB pathway is under the transcriptional control of the classical NF-kB pathway (29, 30). Consistent with above, treatment with higher doses of BIIB021 (100 and 200 nmol/L) resulted in reduced expression of both p100 and RELB (Fig. 5C). Thus, BIIB021 blocks alternate NF-kB pathway in PEL directly by downregulating IKK1/IKKc/a/b expression and indirectly by blocking the classical NF-kB pathway.

**BIIB021 does not induce expression of KSHV lytic genes**

K13 is required for maintenance of KSHV latency and inhibition of K13-induced NF-kB results in KSHV lytic reactivation through the induction of expression of KSHV replication and transcription activator (RTA), the master regulator of KSHV lytic replication (31, 32). BIIB021 not only failed to induce the expression of RTA in BCBL-1 cells but also blocked TPA-induced RTA expression in a dose-dependent manner (Fig. 5D). KSHV-encoded viral IL6 (vIL6) not only is an autocrine growth factor for KSHV-infected PEL cells but also contributes to immune evasion and angiogenesis (33). Similar to its effect on RTA, BIIB021 not only failed to induce vIL6 expression in BCBL-1 cells but also blocked vIL6 expression induced by treatment with TPA.
Essentially similar results were observed in BC-1 and BC-3 cell lines (Supplementary Fig. S2). The effect of BIIB021 on the transcripts of other KSHV genes is presented in Supplementary Fig. S3. Thus, the cytotoxicity of BIIB021 against PEL is not accompanied by lytic reactivation of KSHV.

BIIB021 exhibits in vivo growth inhibitory potential against PEL in a mouse xenograft model

To check whether the antiproliferative effect of BIIB021 observed in vitro can be translated in vivo, we injected the BC-1 cells subcutaneously in to the right flank of athymic NCr-nu/nu mice. The mice were monitored daily for development of palpable tumors and treatment with BIIB021 (or control vehicle) was initiated when the tumors reached approximately 75 mm³. BIIB021 resulted in a significant reduction in tumor volume (Fig. 6A), which was accompanied by a corresponding decline in the levels of circulating human IL-6 (Fig. 6A). BIIB021 also prevented the development of splenomegaly, which was seen in 6 of 6 vehicle-treated mice but in only 2 of 7 BIIB021-treated mice.

Figure 5. Effect of HSP90 inhibitors on K13/IKK complex interaction and KSHV lytic proteins. A, left, co-IP assay showing disruption of K13/IKK complex interaction by HSP90 inhibitors. 293-pSLIK-TO-K13/NF-κB-Luc cells were treated with BIIB021 (200 nmol/L), 17-DMAG (200 nmol/L), NVP-AUY922 (100 nmol/L), and DMSO control for 2 hours followed by treatment with doxycycline (DOX, 500 ng/mL) for 15 hours to induce K13 expression. Whole-cell lysates (WCL) were immunoprecipitated (IP) using a control antibody (C) or FLAG antibody (F) and subjected to SDS-PAGE and immunoblotting (IB) with the indicated antibodies. UT, Untreated. Right, a co-IP assay showing reduced interaction between K13 and IKK complex in FLAG-tagged K13 expressing BCBL-1 cells upon treatment with 200 nmol/L BIIB021 for 24 hours. B, a co-IP assay showing K13 interacts with HSP90 in wild-type Jurkat cells but not in NEMO-deficient Jurkat cells. WCLs were prepared from wild-type Jurkat cells and NEMO-deficient Jurkat cells expressing FLAG-K13 and immunoprecipitated (IP) using a control antibody (C) or FLAG antibody (F) and immunoblotted (IB) with the indicated antibodies. C, Western blot analyses showing downregulation of RELB and p100 expression and p100 to p52 processing in PEL cells upon treatment with BIIB021 for 48 hours. D, BIIB021 fails to induce expression of KSHV lytic proteins RTA and vIL6 and blocks their expression induced by TPA (12-O-tetradecanoylphorbol-13-acetate).
respectively (Fig. 6B). However, we failed to detect LANA-positive cells in the spleens of vehicle or BIIB021-treated mice, suggesting that the observed splenomegaly may be due to the stimulatory effect of cytokines produced by PEL cells on splenic cells rather than direct infiltration of spleen by PEL cells. Tumor tissues from the animals treated with BIIB021 also exhibited reduced expression of IKKα/β and LANA (Fig. 6C) and increased apoptosis, as assessed by the staining for cleaved caspase-3 and the number of TUNEL-positive nuclei (Fig. 6C and D). Taken collectively, the above results indicate that BIIB021 exerts a potent in vivo inhibitory effect on PEL by inducing tumor cell apoptosis.

Discussion

A number of preclinical studies have documented the activity of geldanamycin-based HSP90 inhibitors, such as 17-AAG and 17-DMAG, against PEL cells (12, 34). However, several issues intrinsic to the chemical structure of geldanamycin-based HSP90 inhibitors have limited their full clinical development (35). 17-AAG contains a benzoquinone moiety that is believed to be responsible for elevation of liver enzymes and liver toxicity observed in clinical trials (35, 36). Intrinsic and acquired resistance to 17-AAG has been associated with low expression or an inactivating polymorphism of the NQO1 (NAD(P)H/quinone oxidoreductase 1) gene that encodes for an enzyme required for bioreduction of 17-AAG to a more potent hydroquinone (37). 17-AAG and 17-DMAG are also substrates of P-glycoprotein/multidrug resistance protein 1 (MDR1) and multidrug resistance–associated protein 1 (MRP-1), and their cytotoxicity is dramatically reduced in MDR1 and/or MRP-1–overexpressing cells (14, 38). Overexpression of BCL-2 has been also linked to resistance to 17-AAG (38). Finally, for unclear reasons, 17-AAG is also less potent in several types of tumor sensitive to other HSP90 inhibitors (14).

Because of the above limitations of the geldanamycin-based HSP90 inhibitors, we compared the 3 main classes of HSP90 inhibitors currently under development. We show that while all 3 classes of HSP90 inhibitors have activity against PEL, BIIB021, a novel purine-based HSP90 inhibitor, is preferentially toxic to PEL as compared with other lymphoma subtypes. BIIB021 holds several additional advantages over geldanamycin-based HSP90 inhibitors. BIIB021 has improved pharmacologic profile as compared with 17-AAG especially with regard to availability through chemical synthesis, metabolic stability, water solubility, and ease of administration via both oral and intravenous routes (14, 15). BIIB021 also exhibits reduced expression of IL-6 in the plasma of animals treated with either vehicle control or BIIB021. Asterisks indicate significance at levels of P < 0.05. Circulating levels of IL-6 on day 14 in the plasma of animals treated with either vehicle control or BIIB021. Asterisks indicate significance at levels of P < 0.05. B, gross representative images of mice, tumor, and spleen from groups of animals treated with either vehicle control or BIIB021. C, immunohistochemical staining showing decreased expressions of IKKα/β, LANA along with positive staining for cleaved caspase-3, indicative of apoptosis, in the tumors of mice treated with BIIB021. D, TUNEL staining showing increased apoptosis in tumor tissues extracted from animals treated with BIIB021. **, significance at levels of P < 0.001.
Treatment of PEL cell lines with BIIB021 resulted in cell-cycle arrest and induction of apoptosis. As NF-κB pathway activation has been shown to be essential for the survival and proliferation of PEL cells (4, 6), we examined the effect of BIIB021 on the status of this pathway in detail. Our results suggest that BIIB021 blocks both the classical and alternative NF-κB pathways through multiple mechanisms. First, BIIB021 reduced the levels of IKKa/IKkB, which is consistent with a previous report indicating that these are HSP90 client proteins (39). Second, BIIB021 at higher doses resulted in a modest reduction in the mRNA and protein levels of vFLIP K13, a protein that is believed to be primarily responsible for activation of the classical and alternative NF-κB pathways in PEL cells (6, 7). Most importantly, BIIB021 blocked the activity of K13, which was associated with a disruption of the K13-IKK complex. It is important to point out that the inhibition of K13 activity by BIIB021 was independent of its effect on K13 expression as it was also observed in the 293-pSLIK-K13 cell line in which K13 expression was unaffected by the BIIB021 treatment. As HSP90 is a known component of the K13/IKK complex (12), the inhibitory effect of BIIB021 on K13-induced NF-κB activity probably reflects the key role played by HSP90 in the assembly and function of this complex. Taken collectively, our results suggest that BIIB021 blocks NF-κB activity in PEL by downregulating the expression of IKKa/IKKb and K13 and by interfering with the assembly and function of the K13/IKK complex. It is important to point out, however, that inhibition of the NF-κB pathway is unlikely to be the sole mechanism of action of BIIB021 against PEL as several other cellular and viral proteins are dependent on the chaperone function of HSP90. For example, HSP90 is known to enhance AKT signaling (40) and, consistent with these results, we observed downregulation of AKT expression in BIIB021-treated cells. HSP90 has been also shown to be required for the expression of KSHV-encoded K1 protein and for its apoptotic activity (34). Thus, we favor the hypothesis that the inhibition of NF-κB pathway works in conjunction with the inhibition of other signaling pathways to contribute to the antiproliferative and cytotoxic effects of BIIB021 against PEL.

The NF-κB pathway has been shown to keep KSHV in a latent state (41). As lytic replication of KSHV and expression of lytic genes is believed to promote KSHV tumorigenesis (42), lytic reactivation represents a potential safety concern with the use of HSP90 inhibitors. However, we observed that treatment with BIIB021 not only failed to induce the expression of lytic genes but also blocked their expression induced by TPA, thereby allaying these safety concerns. Essentially similar results have been reported recently using 17-AAG (43). While the exact mechanism for the inhibition of lytic reactivation by HSP90 inhibitors is not clear at the present, modulation of NF-κB–independent signaling pathways may be responsible for this effect.

In summary, we provide strong in vitro and in vivo data showing the promising activity of BIIB021 against PEL. The mean Cmax for BIIB021 observed in clinical trial is 3.6 μmol/L (44) which is 60-fold greater than the IC50 values of this compound for PEL cell lines observed in the present study. As PEL are relatively refractory to conventional chemotherapy, the observed preclinical activity of BIIB021 against PEL cells at low nanomolar doses suggests that it may be a promising compound for the treatment of PEL.

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

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Conception and design: R. Gopalakrishnan, H. Matta, P.M. Chaudhary
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A Purine Scaffold HSP90 Inhibitor BIIB021 Has Selective Activity against KSHV-Associated Primary Effusion Lymphoma and Blocks vFLIP K13-Induced NF-κB

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