A purine scaffold HSP90 inhibitor BIIB021 has selective activity against KSHV-associated Primary Effusion Lymphoma and blocks vFLIP K13-induced NF-κB

Ramakrishnan Gopalakrishnan, Hittu Matta, and Preet M. Chaudhary

From Jane Anne Nohl Division of Hematology and Center for the Study of Blood Diseases, University of Southern California Keck School of Medicine, Los Angeles, California, United States of America

Running title: BIIB021 specifically targets PEL

Key Words: BIIB021, KSHV, HSP90, K13, NF-κB

Request for reprints: Preet M. Chaudhary, M.D., Ph.D. Jane Anne Nohl Division of Hematology and Center for the Study of Blood Diseases, University of Southern California Keck School of Medicine, Los Angeles, CA, 90033; Email: preet.chaudhary@med.usc.edu; Phone: 323-865-3916; Fax: 323-865-0060

Conflict of Interest: The authors declare no competing financial interests

Translational relevance word count: 975

Abstract word count: 225

Manuscript word count (excluding references): 5625

Total number of Figures: 6

Total number of Tables: 1
Translational Relevance: Primary Effusion Lymphoma (PEL) is an aggressive form of non-Hodgkin's lymphoma commonly seen in HIV-positive patients that is universally associated with infection by Kaposi's 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 heat shock protein90 inhibitor BIIB021 blocks constitutive NF-κB activity present in PEL cells and demonstrate in vitro and in vivo activity against this disease. These results provide the basis for clinical testing of BIIB021 in PEL.
Abstract

Purpose: Kaposi’s sarcoma associated herpesvirus (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 Heat shock protein 90 (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 demonstrate that BIIB021, a purine scaffold based orally administrable HSP90 inhibitor, shows preferential cytotoxicity towards PEL cells as compared to 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 demonstrate preferential anti-tumor activity against PEL in vitro and in vivo. BIIB021 may be a promising agent for treatment of PEL.
Introduction

Primary effusion lymphoma (PEL) is a variant of non-Hodgkin’s lymphoma that is characterized by infection with Kaposi sarcoma associated herpesvirus (KSHV; also known as Human Herpes virus 8)(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 3-6 months upon diagnosis(2). Thus, there is an urgent need for safer and more effective therapeutic options for PEL.

Nuclear factor-κB (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 two 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).

Heat shock protein 90 (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 proteasomal 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 one of the following three categories: 1) Geldanamycin derivatives (e.g. 17-AAG and 17-DMAG); 2) Purine scaffold-based HSP90 inhibitors (e.g. BIIB021 and PU-H71) and 3) 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 carried out a comparative analysis of the three main classes of HSP90 inhibitors against PEL cells. We demonstrate that while all three classes of HSP90 inhibitors are effective against PEL, the purine-scaffold inhibitor BIIB021 shows preferential cytotoxicity towards PEL as compared to the other lymphoma cells. We further demonstrate that the cytotoxicity of BIIB021 against PEL is associated with inhibition of classical and alternative NF-κB pathways induced by KSHV vFLIP K13.

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 sub-cloning 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-FT cells. Post-infection, cells were cultured in normal growth media containing the appropriate drugs to select positive clones.

**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 x 10³ cells/well, treated with an increasing concentration of the drugs and subsequently assessed for cell viability using the MTS reagent (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) following the manufacturer's instructions (Promega). Percent cell survival was calculated based on the reading of cells grown in the presence of DMSO control. IC₅₀ for the individual drugs were calculated using GraphPad Prism 5 software. DNA content analysis was performed as described previously(17). Apoptosis was analyzed using BD AnnexinV-FITC apoptosis detection kit I as per manufacturer instructions.

**Statistical analysis**
Two-tailed unpaired Student's $t$ test was used to test for differences between two groups using GraphPad Prism 5 software. Differences with a $p$ value $\leq 0.05$ were considered statistically significant. All experiments were repeated a minimum of three times.

Additional information regarding materials and methods is provided in the supplementary information file.

**Results**

**BIIB021 specifically targets KSHV-associated PEL**

We treated a panel of logarithmically growing PEL and non-PEL cells lines for 72 hours (h) 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 towards the PEL cell lines (Fig. 1B). Thus, the IC$_{50}$ values of BIIB021 for the PEL cell lines ranged from 41.5 nM to 71.5 nM, while its IC$_{50}$ for non-PEL cell lines ranged from 187 nM to 275 nM (Table 1). In contrast, the IC$_{50}$ of 17-DMAG for PEL and non-PEL cell lines ranged from 55 nM to 217 nM and from 24.2 nM to 1655 nM, respectively. Similarly, the IC$_{50}$ of NVP-AUY922 for PEL and non-PEL cell lines ranged from 19.3 nM to 60.5 nM and from 16.1 nM to 40.5 nM, respectively. The preferential toxicity of BIIB021 against PEL cells was also seen at 24 h and 48 h of drug treatment (Fig. 1C).

**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 percent 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). Additionally, BIIB021 depleted the levels of c-MYC, Cyclin A and Cyclin B1 (Fig. 2A). In contrast, BIIB021 up-regulated 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, poly ADP-ribose polymerase (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 PEL (BC-3, BCBL-1 and JSC-1) and non-PEL (Jurkat, BJAB, Namalwa, MM1S and U266) cell lines following treatment with BIIB021 (100 nM), 17-DMAG (100 nM) and NVP-AUY922 (50 nM) for 48 h. Consistent with the results of cell viability assay, BIIB021 resulted in increased PARP cleavage in all the three 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, while 17-DMAG increased PARP cleavage in all PEL cell lines and three 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.

**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 XIAP1, two known targets of the NF-κB pathway(23-24) (Fig. 3C).
To study the mechanism of classical NF-κB inhibition by BIIB021, we studied its effect on IκBα phosphorylation. Treatment with BIIB021 reduced the phosphorylation of IκBα 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/Ser180 and Ser177/Ser181, respectively (Fig. 3D). Collectively, the above results suggest that BIIB021 blocks classical NF-κB pathway in PEL by blocking the expression and activity of IKKα/β, which in turn results in inhibition of IκBα phosphorylation and degradation.

**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 nM) of BIIB021 and was not seen at the lower doses (50 nM and 100 nM). BIIB021 also down-regulated the expressions of LANA and vCyclin (Fig. 4A), two other KSHV latent proteins (25). A quantitative RT-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). Since 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 to 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 CHX-treated BC-1 and BC-3 cell lines (Fig. 4B and supplementary Fig. 1), 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. 1), 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 GAPDH stability.

BIIB021 blocks vFLIP K13 induced NF-κB activity

As the inhibition of K13 expression by BIIB021 was evident only at its highest dose, we next asked if inhibition of K13 activity could also contribute to its inhibitory effect on the NF-κB pathway. For this purpose, we generated a clone of 293 cells, designated 293A-pSLIK-TO-K13/NF-κB-Luc, stably expressing FLAG-tagged K13 from a tetracycline-inducible promoter along with a stably integrated copy of an NF-κB-driven luciferase reporter construct. Since 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 Figure 4C, induction of K13 expression with doxycycline resulted in a significant increase in NF-κB-Luc activity that was accompanied by an increase in phosphorylation and degradation of IκBα and up-regulation 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-κB 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-κB, we examined their effect on K13-IKK complex interaction in 293-pSLIK-TO-K13/NF-κB-Luc cells using a co-immunoprecipitation (co-IP) assay. BIIB021 reduced the amounts of HSP90, IKKα/β and IKKγ/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, IKKα/β and IKKγ/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 IKKα/β and IKKγ/NEMO that co-immunoprecipitated with K13 in the BIIB021-treated BCBL-1 cells expressing FLAG-tagged K13 cells as compared to 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-κB 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-κB pathway in PEL

PEL cells also demonstrate constitutively active alternate NF-κB pathway that involves proteasome-mediated processing of p100/NF-κB2 into the active p52 subunit (28). Treatment of BC-3 cells with 50nM BIIB021 blocked the processing of p100 into p52, suggesting inhibition of the alternate NF-κB pathway (Fig. 5C). The expression of both p100/NF-κB2 and RELB subunits of the alternative NF-κB pathway is under the transcriptional control of the classical NF-κB pathway(29-30). Consistent with above, treatment with higher doses of BIIB021 (100 and 200 nM) resulted in reduced expression of both p100 and RELB (Fig. 5C). Thus, BIIB021 blocks alternate NF-κB pathway in PEL directly by down-regulating IKK1/IKKα expression and indirectly by blocking the classical NF-κB pathway.

BIIB021 does not induce expression of KSHV lytic genes

K13 is required for maintenance of KSHV latency and inhibition of K13-induced NF-κB 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) is not only 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. 2). The effect of BIIB021 on the transcripts of other KSHV genes is presented in Supplementary
Fig. 3. 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 anti-proliferative effect of BIIB021 observed *in vitro* can be translated *in vivo*, we injected the BC-1 cells subcutaneously into 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 ~ 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 6/6 vehicle-treated mice but in only 2/7 BIIB021-treated mice, 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 6D). 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/quinoneoxidoreductase I) gene that encodes for an enzyme required for bio-reduction 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 over-expressing cells(14, 38). Over-expression 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).

Due to the above limitations of the geldanamycin-based HSP90 inhibitors, we compared the three main classes of HSP90 inhibitors currently under development. We demonstrate that while all three classes of HSP90 inhibitors have activity against PEL, BIIB021, a novel purine-based HSP90 inhibitor, is preferentially toxic to PEL as compared to other lymphoma subtypes. BIIB021 holds several additional advantages over geldanamycin-based HSP90 inhibitors. BIIB021 has improved pharmacological profile as compared to 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 does not require activation by NQO1 and is not a substrate of P-glycoprotein and MRP-1 drug efflux pumps(38). Indeed, no
case of acquired resistance to BIIB021 has been reported in the literature so far and it has shown activity against a wider panel of tumors(37). BIIB021 has also been tested in a number of phase I clinical trials and at least two phase II clinical trials, where it appears to be well tolerated(10).

Treatment of PEL cell lines with BIIB021 resulted in cell-cycle arrest and induction of apoptosis. Since NF-κB pathway 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 alternate NF-κB pathways through multiple mechanisms. First, BIIB021 reduced the levels of IKKα/IKKβ, 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. Since 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 down-regulating the expression of IKKα/IKKβ 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 down-regulation 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 anti-proliferative and cytotoxic effects of BIIB021 against PEL.

The NF-κB pathway has been shown to keep KSHV in a latent state (41). Since 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 $C_{\text{max}}$ for BIIB021 observed in clinical trial is 3.6 µM (44) which is 60-fold greater than the IC$_{50}$ values of this compound for PEL cell lines observed in the present study. As PEL are relatively refractory to conventional chemotherapy, the observed pre-
clinical activity of BIIB021 against PEL cells at low nano-molar doses suggests that it may be a promising compound for the treatment of PEL.

Acknowledgements

The authors thank Dr. Jae Jung for his generous gift of PEL cell lines, Dr. Izidore S Lossos for UMPEL-1 cells and Dr. Ellen Vitetta for Myeloma cell lines.

Grant Support

This work was supported by grants from the National Institutes of Health (CA139119, DE019811 and P30CA014089) and Stop Cancer Foundation. Flow Cytometry was performed in the USC Flow Cytometry Core Facility that is supported in part by the National Cancer Institute Cancer Center Shared Grant award P30CA014089 and the USC Provost Office Dean’s Development Funds.

Supplementary information

Supplementary Information accompanies the paper on the Clinical Cancer Research website.

References

23. Matta H, Gopalakrishnan R, Punj V, Yi H, Suo Y, Chaudhary PM. A20 is induced by Kaposi sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 and
34. Wen KW, Damania B. Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. Oncogene. 2010;29:3532-44.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Associated virus</th>
<th>p53 status</th>
<th>BIIB021 (IC$_{50}$, nM)</th>
<th>17-DMAG (IC$_{50}$, nM)</th>
<th>NVP-AUY922 (IC$_{50}$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>Primary effusion lymphoma</td>
<td>KSHV$^a$&amp;EBV$^b$</td>
<td>WT</td>
<td>41.5</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>BC-3</td>
<td>Primary effusion lymphoma</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>Primary effusion lymphoma</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>Primary effusion lymphoma</td>
<td>KSHV &amp; EBV</td>
<td>WT</td>
<td>71.5</td>
<td>63.8</td>
<td>40.6</td>
</tr>
<tr>
<td>UMPLE-1(c)</td>
<td>Primary effusion lymphoma</td>
<td>KSHV &amp; 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>

a-KSHV: Kaposi’s Sarcoma Herpes Virus; b-EBV: Epstein Barr Virus; WT: wild-type; Mut: Mutated; N/A: Not Available
Figure legends

Figure 1.
HSP90 inhibitors efficiently target KSHV associated primary effusion lymphoma. A, Chemical structures of BIIB021, 17-DMAG and NVP-AUY922. B, The indicated PEL and non-PEL cell lines were treated with increasing concentrations of BIIB021, 17-DMAG and NVP-AUY922 for 72 h and cell viability was measured using an MTS assay as described in methods section. A grey circle shows preferential toxicity of BIIB021 on PEL cell lines at 100 nM. C, BC-1, BC-3, BJAB and Namalwa cells were treated with increasing concentrations of BIIB021 for 24 and 48 h and cell viability was measured by MTS assay. The values shown are mean±SEM of two independent experiments performed in triplicate.

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 nM for 48 h) 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. The data is a representative of three 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 Cyclooxygenase-2 (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 nM BIIB021 or DMSO control for 48 h. Cells were then stained with Hoechst 33258 and photographed. Right, BC-1 and BC-3 cells were treated with 200 nM BIIB021 or DMSO control for 48 h, stained with Annexin-V-FITC/PI and analyzed for apoptosis by flow cytometry. C, Western blot showing the effect of BIIB021 on the cleavage of PARP, activation of caspases and expression of BCL-2 family members and p53. D, Western
blot showing the effect of BIIB021 (100 nM, 48h), 17-DMAG (100 nM, 48h) and NVP-AUY922 (50 nM, 48h) treatment on the cleavage of PARP in the indicated cell lines. FL – Full length; Cl - Cleaved.

**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 h and cell lysates used for measurement of luciferase activity. Values shown are the mean ± SEM from one representative experiment out of three performed in duplicate. Asterisks (****) indicate significance at levels of $p \leq 0.0001$. 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 nM for 48 h). Values shown are the mean ± SEM from one representative experiment out of three performed in duplicate. Asterisks (****) indicate significance at levels of $p \leq 0.0001$. B, BIIB021 blocks IL-6 secretion. IL-6 levels were measured using ELISA in the supernatants of BC-1 and BC-3 cells treated with 200 nM of BIIB021 or DMSO control for 24, 48 and 72 h. The values (mean ± SEM) shown are from a representative of three independent experiments performed in triplicate. C, Western blots 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 XIAP1. D, PathScan ELISA for phospho-IKKα (Ser176/180) and phospho-IKKβ (Ser177/181) showing inhibition of IKKα/β phosphorylation by BIIB021 (200 nM for 48 h) in BC-1 and BC-3 cells. A representative of two independent experiments performed in triplicate. Statistically significant differences are shown by asterisks (*** at a level of $p \leq 0.001$ and (**** at level of $p \leq 0.0001$.
**Figure 4.**

BIIB021 down-regulates vFLIP K13 expression and blocks K13-induced NF-κB activation. *A*, left, Western blots showing a reduction in K13, LANA and vCyclin protein levels in BC-1 and BC-3 cells treated for 48 h with the indicated concentrations of BIIB021. Right, qRT-PCR analysis showing a decline in *K13, LANA and v-Cyclin* mRNA expression in BC-1 and BC-3 cells following treatment with 200 nM BIIB021 for 24 h. Real-time PCR reactions were performed in triplicate and the data is presented as fold change in target gene expression (mean±SEM) from a representative of two independent experiments. *B*, Effect of BIIB021 on the protein stability. BC-1 and BC-3 cells were treated with vehicle or BIIB021 in the presence of 5 μg/ml cycloheximide (CHX) for 0, 3, 6, 12, 18 and 24 hours, respectively. Whole cells lysates were immunoblotted for indicated proteins. Semi-quantitative analysis of the immunoblots is presented in Supplementary Fig.1. *C*, upper panel, luciferase-based reporter assay showing inhibition of K13-induced NF-κB transcriptional activity by HSP90 inhibitors. 293-pSLIK-TO-K13/NF-κB-Luc cells were treated with increasing concentrations of BIIB021, 17-DMAG and NVP-AUY922 for 2 h prior to induction of K13 expression by addition of doxycycline (DOX, 500 ng/ml). After 15 h, cell lysates were prepared to measure the NF-κB luciferase activity. The values (mean±SEM) shown are from a representative of three independent experiments performed in triplicate. Asterisks indicate statistical significance as explained for Figure 3D, ns-not significant. Lower panel, Western blot analysis from the cell lysates confirmed that inhibition of NF-κB activity by BIIB021 is accompanied by inhibition of IκBα phosphorylation and downregulation of NF-κB target gene (i.e. A20) expression but is not due to a block in K13 expression.
Figure 5.

Effect of HSP90 inhibitors on K13-IKK complex interaction and KSHV lytic genes. A, left, co-IP assay showing disruption of K13-IKK complex interaction by HSP90 inhibitors. 293-pSLIK-TO-K13/NF-kB-Luc cells were treated with BIIB021 (200 nM), 17-DMAG (200nM), NVP-AUY922 (100nM) and DMSO control for 2 h followed by treatment with doxycycline (DOX, 500 ng/ml for 15 h) 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 nM BIIB021 for 24 h. B, a co-IP assay showing K13 interacts with HSP90 in wild-type Jurkat cells but not in NEMO-deficient Jurkat cells. Whole cell lysates (WCL) 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 blots showing down-regulation of RELB and p100 expression and p100 to p52 processing in PEL cells upon treatment with BIIB021 for 48 h. 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). BCBL-1 cells were treated with the indicated doses of BIIB021 for 2 h followed by treatment with TPA (100ng/ml) for 48 h. Cell lysates were subjected to Western blot analysis using the indicated antibodies. NS- Non Specific.

Figure 6.
BIIB021 impairs *in vivo* growth of PEL in a mouse xenograft model. *A*, Athymic NCr-nu/nu mice with established subcutaneous BC-1 tumors were treated with BIIB021 (100 mg/kg body weight; n=7) or vehicle control (n=6) for 14 days. Tumor volumes are presented as mean±SEM from each group. Asterisks indicate significance at levels of *p*≤0.05. Tumor weight (in grams) of animals treated with either vehicle control or BIIB021 on day 14. Asterisks indicate significance at levels of *p*≤0.05. Circulating levels of hIL-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. Asterisks (***)) indicate significance at levels of *p*≤0.001.
Figure 1

A

B

C

Figure 1
Figure 3

A. Luciferase assay

BC-1/NF-κB-Luc
BC-3/NF-κB-Luc

Relative luciferase activity (10^3)

BIIB021 (nM) 0 50 100 200 0 50 100 200

BC-1

BC-3

Fold change

p65/RelA DNA binding activity

BC-1

BC-3

B. IL6 ELISA

BC-1

BC-3

IL6 (pg/mL)

Time (h) 24 48 72 24 48 72

C. Pathscan ELISA

BC-1

BC-3

A20 (82 kD)
XIAP (53 kD)

pIkBa (39 kD)

Total IkBa (39 kD)

IKKa/β (85 kD)

NEMO (48 kD)

Tubulin (55 kD)
Figure 5

(A) 293-pSLIK-TO-K13

IP:Flag

WCL

BCBL-1 K13

IP:Flag

WCL

IB: Tubulin (55 kD)
IB: HSP90 (90 kD)
IB: IKKα/β (85 kD)
IB: NEMO (48 kD)
IB: Flag-K13 (22 kD)

(B) Jurkat

IP: Flag

WCL

WT

NEMO Deficient

IB: Tubulin (55 kD)
IB: HSP90 (90 kD)
IB: NEMO (48 kD)
IB: Flag-K13 (22 kD)

(C) BC-1

BC-3

BIB021 (nM)

BCBL-1

TPA

IBB021 (nM)

RTA (180 kD)

p52 (52 kD)

REL (68 kD)

Tubulin (55 kD)

Downloaded from clincancerres.aacrjournals.org on April 16, 2017. © 2013 American Association for Cancer Research.
Figure 6

A) Tumor Volume

B) Vehicle vs. BIIB021 in mice

C) Immunohistochemistry images for Vehicle and BIIB021

D) TUNEL assay results for Vehicle and BIIB021
Clinical Cancer Research

A purine scaffold HSP90 inhibitor BIIB021 has selective activity against KSHV-associated Primary Effusion Lymphoma and blocks vFLIP K13-induced NF-κB

Ramakrishnan Gopalakrishnan, Hittu Matta and Preet M Chaudhary

Clin Cancer Res  Published OnlineFirst July 23, 2013.

Updated version

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-3510

Supplementary Material

Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/07/23/1078-0432.CCR-12-3510.DC1

Author Manuscript

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.