The Effects of Heat Shock Protein 90 Inhibitors on Apoptosis and Viral Replication in Primary Effusion Lymphoma Cells

Chizuka Higashi,[a,#] Chiaki Saji,[b,#] Koji Yamada,[b] Hiroki Kagawa,[b] Rie Ohga,[a] Takahiro Taira,[a] and Masahiro Fujimuro*[,a,†]

[a] *Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi; 1110 Shimokato, Chuoh 409–3898, Japan: and* [b] *Faculty of Pharmaceutical Sciences, Hokkaido University; Kita-ku, Sapporo 060–0812, Japan.* Received January 5, 2012; accepted January 26, 2012; published online February 14, 2012

Primary effusion lymphoma (PEL) is an aggressive neoplasm caused by Kaposi's sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients and human immunodeficiency virus (HIV)-infected homosexual males. We evaluated the cytotoxic effects of heat shock protein 90 (HSP90) inhibitors on PEL cells. The HSP90 inhibitors geldanamycin (GA), 17(allylamino)-17-demethoxygeldanamycin (17-AAG), and radicicol dramatically inhibited cell proliferation and induced apoptosis of PEL cells through caspase activation. Furthermore, GA induced the stabilization of inhibitor of κB (IκB)α and reduced the phosphorylation of IκBα in PEL cells. HSP90 inhibitors suppressed the transcriptional activity of nuclear factor-kappa B (NF-κB) in PEL cells. It is known that the constitutive activation of NF-κB signaling is essential for the survival of PEL cells and HSP90 contributes to promote activation of NF-κB signaling. The suppression of NF-κB signaling by HSP90 inhibitors may contribute to the induction of apoptosis in PEL cells. In addition, HSP90 activity is required for KSHV replication in KSHV latently infected PEL cells. GA, 17-AAG and radicicol reduced the production of progeny virus from PEL cells at low concentrations, which do not affect PEL cell growth. Our results suggest that HSP90 activity is required for both the survival of PEL cells and viral replication in PEL cells, and that pharmacologic inhibition of HSP90 may be an effective treatment for PEL and KSHV-related diseases.

Key words primary effusion lymphoma; heat shock protein 90; nuclear factor-kappa B; apoptosis; Kaposi's sarcoma-associated herpesvirus

Primary effusion lymphoma (PEL; also known as body cavity-based lymphoma) is classified as a non-Hodgkin's B cell lymphoma associated with immunocompromised patients, such as acquired immunodeficiency syndrome (AIDS) patients or those who have undergone organ transplantation.[1,2] In general, PEL presents as a lymphomatous effusion in body cavities. PEL cells are infected with Kaposi's sarcoma-associated herpesvirus (KSHV; also known as HHV-8) and are often co-infected with Epstein–Barr virus (EBV).[3] KSHV is a rhadinovirus of the γ-herpesvirus subfamily and is the causative agent of Kaposi's sarcoma and acquired immunodeficiency syndrome AIDS-related lymphoproliferative disorders, such as PEL and multicentric Castlemans disease.[4]

The KSHV genome circularizes and forms a double-stranded episome in the nucleus of PEL cells during latent infection. KSHV establishes a latent infection in PEL cells expressing several viral proteins (including LANA, v-FLIP, and v-cyclin) that achieve KSHV-associated malignancies by the manipulation of cell proliferation and apoptosis.[5,6] In particularly, v-FLIP contributes to anti-apoptosis by activating nuclear factor-kappa B (NF-κB) signaling in PEL cells.[7,8] KSHV alternates between lytic replication and latency by way of RTA expression. RTA, encoded by the KSHV immediate-early gene ORF50, is a critical switch molecule for initiating lytic replication. During lytic infection, progeny viruses are generated and released from host cells.

Heat shock protein 90 (HSP90), which acts as a molecular chaperone, requires ATP-hydrolysis to maintain its function. Hsp90 promotes the proper folding, assembly, and transportation of the substrate (also known as client protein). Hsp90 is regulated through cooperative binding of the co-chaperones that link Hsp90 to the client protein. The complex of HSP90 and the co-chaperone, such as Cdc37 and FKBP51, contribute to activation of NF-κB signaling.[9]

It is known that the NF-κB signaling pathway is constitutively activated in PEL cells for anti-apoptosis and cell growth.[7,8,10] In fact, many NF-κB inhibitors induce apoptosis in PEL cells. NF-κB signaling is regulated by the inhibitor of κB (IκB)α. In the canonical pathway, NF-κB transcriptional factors, which are heterodimers of p50 and p65, are retained in the cytosol by interaction with IκBα. A stimulus, such as tumor necrosis factor-alpha (TNF-α), can induce activation of the IκB kinase (IKK) complex, consisting of IKKα, IKKβ, and the scaffold NEMO/IKKγ. A complex of HSP90 and the co-chaperone Cdc37 binds the IKK complex and promotes kinase activity of the IKK complex.[9] The activated IKK complex phosphorylates Ser32 and Ser36 of IκBα. Phosphorylated IκBα is then polyubiquitinated and subsequently degraded by the 26S proteasome. This degradation of IκBα releases active NF-κB from IκBα. Because HSP90 inhibitors induce apoptosis in PEL cells,[11,12] HSP90 may contribute to activation of the NF-κB signaling pathway in PEL cells. While cell-based pharmacological studies of NF-κB and HSP90 inhibitors have been extensively carried out, little is known about the molecular mechanisms and how HSP90 actually contributes to NF-κB signaling in PEL cells. Therefore, we investigated the mechanisms that cause HSP90 inhibitors geldanamycin (GA), 17(allylamino)-17-demethoxygeldanamycin (17-AAG),

The authors declare no conflict of interest.

[#] These authors contributed equally to this work.

[†] Present address: *Department of Cell Biology, Kyoto Pharmaceutical University; 1 Misasagi-Shichonocho, Yamashina-ku, Kyoto 607–8412, Japan.*

* To whom correspondence should be addressed. e-mail: fuji2@mb.kyoto-phu.ac.jp

and radicicol to induce apoptosis in PEL cells. We also evaluated the effect of HSP90 inhibitor on KSHV replication in PEL cells.

MATERIALS AND METHODS

Cell Lines and Inhibitors KSHV-positive, EBV-positive PEL cell lines (HBL6) and KSHV-positive, EBV-negative PEL cell lines (BC3 and BCBL1) were derived from patients with KSHV-mediated PEL. All PEL cells and KSHV-negative lymphoma cell lines (Ramos and BJAB) were maintained in a RPMI 1640 medium supplemented with 10% fetal calf serum. GA, 17-AAG, and radicicol were purchased from MERCK Japan (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO).

Cell Viability Assay Cells were seeded onto 96-well plates at 10^4 cells/well in 100 μL medium with or without various concentrations of GA, 17-AAG, or radicicol and then incubated at 37°C for 24 h. The number of viable cells was estimated by a Cell-Counting Kit-8 (Dojindo, Tokyo, Japan), as described previously.[10] The optical density of each sample was measured at 450 nm with a microplate spectrophotometer and expressed as a percentage of the value measured in untreated cells (defined as 100%). The value of CC_{50}, the 50% cytotoxic concentration of the drug, was calculated from the plot of drug concentration *versus* the percentage of live cells. Data are shown as the mean value±S.E.M. of three independent experiments.

Western Blotting and Antibodies For Western blotting, cells (1×10^6) were lysed in an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin and 5 μg/mL aprotinin and boiled for 5 min. The resulting lysate was subjected to SDS-PAGE gel followed by Western blot analysis, as described previously.[13] Primary antibodies used in these experiments included those against IκBα, Ser32/36-phospho-IκBα, active-caspase-3 and active-caspase-7 (Cell Signaling Technology, MA, U.S.A.), β-actin (Sigma, MO, U.S.A.), and K-bZIP (Santa Cruz Biotechnology, CA, U.S.A.).

Caspase-3/7 Assay and Immunofluorescence To measure caspase activity, 5×10^5 BC3 PEL cells/well were placed in 2 mL growth medium and incubated with HSP90 inhibitor for 6 h. Activity of caspase-3/7 in cell lysates was measured by the caspase-Glo assay kit (Promega, WI, U.S.A.), as described previously.[10] For immunofluorescence (IF) analysis, BC3 PEL cells were treated with 10 nM GA for 6 h and fixed in 50% methanol–acetone (1 : 1) on glass slides. Cells were incubated with anti-active caspase-7 rabbit polyclonal antibody for 1 h. After washing, the cells were incubated with rhodamine-conjugated donkey anti-rabbit immunoglobulin G. Immunofluorescent images were obtained with an invert confocal microscopy system (LSM510; Carl Zeiss, Tokyo, Japan).

Nucleofection and Luciferase Reporter Assay of PEL Cells HBL6 cells (5×10^6) were nucleofected with 4 μg luciferase reporter and 1 μg pRL-TK plasmid (for use as an internal control) using the Nucleofector as described previously.[10] The nucleofected cells were incubated in 2 mL medium with 25 nM GA, 1 μM 17-AAG, 75 nM radicicol, 20 μM BAY11-7082, or a vehicle control for 6 h. Cells were resuspended in 0.2 mL passive lysis buffer (Promega) for luciferase assays. Luciferase activity was measured by a luminescencer.

Real-Time Polymerase Chain Reaction (PCR) and Real-Time Reverse Transcription PCR Real-time PCR, real-time reverse transcription PCR and PCR conditions were preformed by the method described previously.[10] Briefly, BC3 cells (8×10^5) were treated with 20 ng/mL 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma) for 24 h to induce the production of viral particles. Cells stimulated by TPA were cultured in medium with or without HSP90 inhibitor. Then, 300 μL supernatant was treated with DNase I to obtain only enveloped and encapsidated viral genomes. Viral DNA was purified and extracted from 200 μL DNase-treated supernatant using the QIAamp DNA blood mini kit. To quantify viral DNA, SYBR green real-time PCR was performed using KSHV-encoded ORF21 specific primer set. The following primers were used to amplify a 140-bp amplicon internal to the ORF21 sequence: 5′-TCCACAACCAGCACGGATATGGAC-3′ and 5′-GAGGGAGTTGTCGTGCATTAAATG-3′.

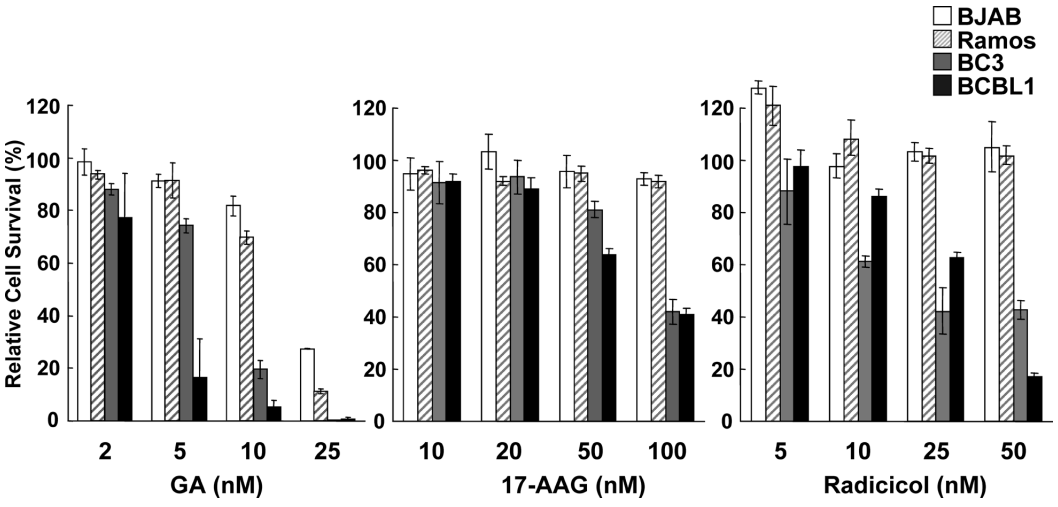

Fig. 1. Cytotoxic Effects of GA, 17-AAG, and Radicicol on KSHV-Infected and Uninfected Lymphoma Cell Lines

KSHV-infected cells (*i.e.*, BC3 and BCBL1 cells) and KSHV-uninfected cells (*i.e.*, DG75, BJAB, and Ramos cells) were incubated with various concentrations of HSP90 inhibitors for 24 h and then subjected to cell viability assays. For each experiment, viability was assessed in six replicate wells. Optical density was measured at 560 nm, and the values of the respective untreated cells were defined as 100%. Standard deviation was determined by three independent experiments and is indicated by error bars.

For real-time reverse transcription (RT), total RNA was purified and extracted from 5×10^6 BC3 cells using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, U.K.). First-strand cDNAs were synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR was performed with SYBR Master Mix Plus (Bio-Rad) and KSHV-encoded ORF50/RTA specific primer set. The following primers were used to amplify the ORF50: 5′-TTCGTCGGCCTCTCGGACGAACTGA-3′ and 5′-ATAATCCGAATGCACACATCTTCCACCAC-3′. For quantification, the expression levels of the ORF50 gene were normalized to that of the β-actin gene.

RESULTS AND DISCUSSION

The Cytotoxic Effects of HSP90 Inhibitors on PEL Cells

Table 1. Cytotoxic Effects of HSP90 Inhibitors on B Lymphoma Cells

Cell type	CC_{50}		
	GA (nM)	17-AAG (nM)	Radicicol (nM)
BJAB	18	4380	477
Ramos	15	772	346
BC3	7.2	92	19
BCBL1	3.3	82	31

CC_{50}, cytotoxic concentration of HSP90 inhibitor that reduces cell viability by 50%.

First, we examined the cytotoxic effects of HSP90 inhibitors on KSHV-infected lymphoma (PEL) cell lines (*i.e.*, BC3 and BCBL1 cells) and KSHV-uninfected lymphoma cell lines (*i.e.*, Ramos and BJAB cells). These B lymphoma cells were cultured in the presence of the HSP90 inhibitors GA, 17-AAG, or radicicol for 24 h, and the cytotoxicity was assessed by analyzing the viability of HSP90 inhibitor-treated *versus* untreated cells. GA, 17-AAG, and radicicol significantly decreased the viability of KSHV-infected PEL cells compared to KSHV-uninfected cells (Fig. 1). In particular, the absolute numbers of BC3 and BCBL1 cells were remarkably decreased after treatment with 10 nM GA. The cytotoxic effects of HSP90 inhibitors are summarized in Table 1. All inhibitors prevented the proliferation of KSHV-infected BC3 and BCBL1 cells at lower concentrations than required for KSHV-uninfected Ramos and BJAB cells. GA was active against both BC3 and BCBL1 cells, with CC_{50} values of 7.2 and 3.3 nM, respectively. BC3 and BCBL1 cells were less sensitive to 17-AAG and radicicol than GA.

HSP90 Inhibitors Induce Apoptosis through the Activation of Caspase-3 and -7 in PEL Cells We next investigated whether cytotoxic effects of HSP90 inhibitors were due to apoptotic cell death. To monitor HSP90 inhibitor-mediated apoptosis, the activation of executioner caspases (*i.e.*, caspase-3 and -7) were analyzed by colorimetric assay, immunoblotting, and IF. We measured the peptidase activity of caspase-3/7 in BC3 cells pretreated with 25 nM GA, 1 μM 17-AAG, 75 nM radicicol, or 5 μM MG132 for 6 h (Fig.

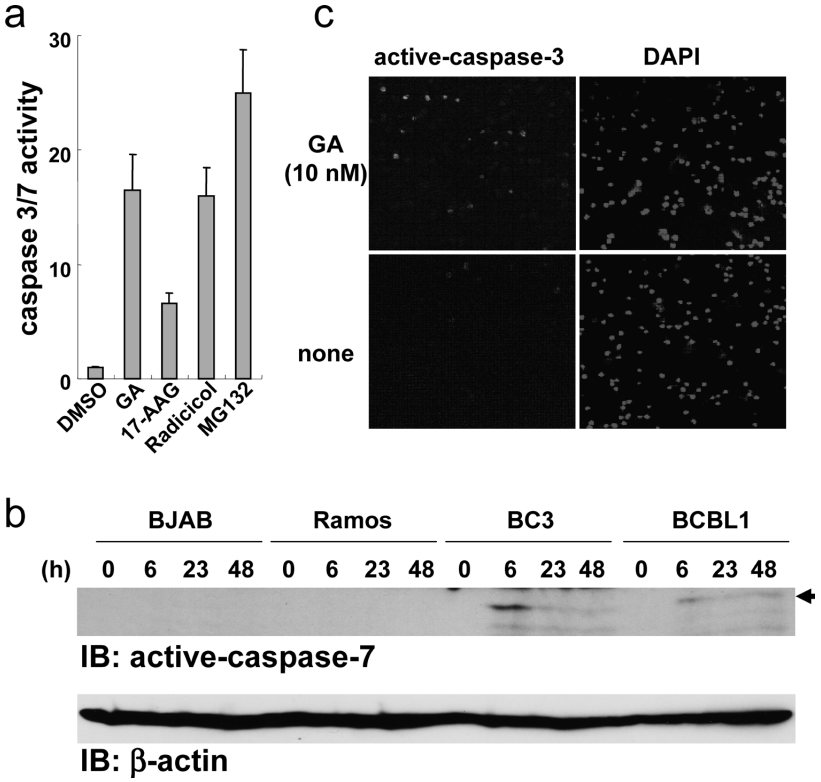

Fig. 2. Apoptosis-Inducing Effects of GA, 17-AAG, and Radicicol on PEL and KSHV-Uninfected Cells

(a) Changes in the activity of caspase-3/7 in BC3 cells. BC3 cells were cultured in the presence or absence of 25 nM GA, 1 μM 17-AAG, 75 nM radicicol, or 5 μM MG132 for 6 h before harvesting. The activity of caspase-3/7 was measured using the caspase-Glo assay with luciferin-conjugated DEVD polypeptide as a substrate. Caspase activity in untreated cells was defined as 1.0 relative light unit. The error bar indicates standard deviation. (b) Immunoblot analysis with anti-active caspase-7 antibody. KSHV-infected (BC3 and BCBL1) and KSHV-uninfected (BJAB and Ramos) cells were cultured with 25 nM GA for 6, 23, or 48 h. (c) Immunofluorescence analysis using anti-active caspase-3 antibody. BC3 cells were cultured with 10 nM GA for 6 h and fixed in methanol–acetone (1 : 1). Immunofluorescent images were obtained with an invert confocal microscopy system.

2a). MG132, which inhibits the 26S proteasome and induces apoptosis in PEL cells, was used as the positive control for comparing apoptosis-inducing activity.[10] Compared to treatment with the control vehicle (DMSO), treatment with GA, 17-AAG, radicicol, or MG132 increased caspase-3/7 activity by approximately 17-, 7-, 16-, and 25-fold, respectively. To obtain further evidence of the induction of apoptosis, we monitored the cleavage of caspase-7 by Western blotting using lysates of cells pretreated with 25 nM GA for 6, 23, or 48 h (Fig. 2b). The 20 kDa active-caspase-7 was detected in both BC3 and BCBL1 cells. In contrast, the cleavage of caspase-7 was not detected in either GA-treated Ramos or BJAB cells. In addition to the activation of caspase-7, IF assay using anti-active-caspase-3 antibody disclosed that caspase-3 activation was induced in approximately 15% of BC3 cells after 6 h of GA treatment (Fig. 2c). These results indicate that HSP90 inhibitors suppressed the growth of PEL cells by apoptosis *via* activation of executioner caspases.

HSP90 Inhibitors Suppress NF-κB Signaling in PEL Cells The NF-κB signaling pathway is constitutively activated in PEL cells and this constitutive NF-κB activation is necessary for PEL to achieve anti-apoptosis and cell growth.[7,8,10] Hsp90-Cdc34 functions as an additional scaffold for IKK complex and promotes IKK kinase activity for IκBα, resulting in activation of NF-κB signaling.[14] Therefore, we asked whether HSP90 inhibitors effect phosphorylation of Ser32 and Ser36 in IκBα. When BC3 cells were treated with 10 nM GA, a decrease in the phosphorylation of IκBα (p-IκBα) was detected (Fig. 3a). In addition, we asked whether HSP90 inhibitors effect the stabilization of IκBα, because phosphorylation of Ser32 and Ser36 of IκBα can be a trigger for polyubiquitination and degradation of IκBα. When BC3 cells were treated with 10 nM GA, the amount of IκBα was increased (Fig. 3a). Next, we confirmed the inhibition of IκBa phosphorylation by NF-κB inhibitor BAY11-7082 by comparing it to the GA-mediated inhibition. BAY11-7082, which inhibits IκB phosphorylation, is an irreversible and specific inhibitor of NF-κB. The phosphorylation of IκBα was strongly decreased in BC3 cells treated with BAY11-7082 (Fig. 3b). When IκBα is stabilized, the transcriptional activity of NF-κB is suppressed. Next, to verify the inhibition of the transcriptional activity of NF-κB by HSP90 inhibitors, we performed a reporter assay with the NF-κB reporter plasmid. HBL6 cells were nucleofected with the NF-κB-specific luciferase reporter plasmid, followed by incubation with the HSP90 inhibitor and BAY11-7082 (Fig. 3c). The NF-κB activity of cells treated with GA, 17-AAG, radicicol, or BAY11-7082 was decreased to 20, 54, 34, and 4% of vehicle control, respectively. These data indicate that HSP90 inhibitors, as well as NF-κB specific inhibitor BAY11-7082, abolished the constitutive activation of NF-κB, resulting in the apoptosis of PEL cells.

IKK complex phosphorylates IκBα and then phosphorylated IκBα is polyubiquitinated, which induces proteasomal degradation of IκBα. In the normal stage, IκBα binds NF-κB in cytoplasm and inhibits nuclear translocation of NF-κB and the NF-κB-dependent transcriptional activation. Hsp90-Cdc34 interacts with IKK complex and enhances kinase activity of IKK complex. KSHV targets and utilizes HSP90 including cell surface HSP90 to manipulate the cell signaling pathway and apoptosis. KSHV-encoded v-FLIP activate NF-κB signaling to achieves anti-apoptosis in KSHV-infected cells

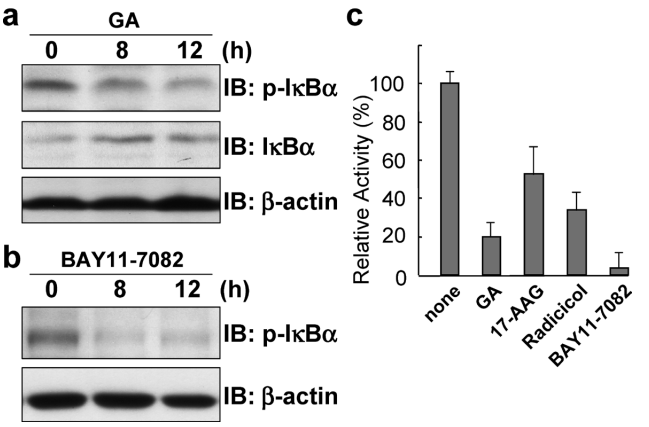

Fig. 3. HSP90 Inhibitors Suppress NF-κB Signaling in PEL Cells

(a) Immunoblot analysis of IκBα and phospholylated IκBα. BC3 cells were cultured with 10 nM GA for 0, 8 or 12 h and harvested. To detect phosphorylation of IκBα, cell lysates were subjected to blotting using anti-Ser32/36-phospho-IκBα or anti-IκBα antibody. (b) Inhibition of IκBα phosphorylation by BAY11-7082. BC3 cells were cultured with 20 μM BAY11-7082, and whole cell lysates were subjected to blotting with anti-Ser32/36-phospho-IκBα. (c) Inhibition of the transcriptional activity of NF-κB by HSP90 inhibitors and BAY11-7082 in PEL cells. HBL6 cells were nucleofected with the NF-κB reporter plasmid. Nucleofected cells were treated with 25 nM GA, 1 μM 17-AAG, 75 nM radicicol, 20 μM BAY11-7082, or a vehicle control for 6 h and lysed in a lysis buffer. The NF-κB activity of untreated HBL6 cells was defined as 100%.

including PEL cells.[7,8] The v-FLIP activates the IKK complex through interaction with NEMO of the IKK- HSP90 complex.[11] Furthermore, extracellular HSP90 serves as a co-factor for activation of NF-κB and MAPK signaling for cell proliferation, anti-apoptosis, and regulation of gene expression.[15,16] KSHV-encoded K1, viral glycoprotein, interacts with HSP90 for K1 dependent anti-apoptotic function.[17] Described above, NF-κB activation and HSP90 function are essential for the survival and growth of KSHV-infected lymphoma cells,[8,18,19] which is consistent with our data (Fig. 1). We also demonstrated that KSHV-infected PEL cells are more sensitive to the anti-proliferative effects of HSP90 inhibitors than KSHV-uninfected cells. While there are several studies showing the importance of NF-κB signaling and HSP90 for the survival of PEL cells, it has been unclear how HSP90 inhibitor suppresses NF-κB signaling in PEL cells: whether HSP90 inhibitors can effect phosphorylation or stabilization of IκBα. In this study, we demonstrated that HSP90 inhibitors inhibit phosphorylation of Ser32 and Ser36 of IκBα and induce the stabilization of IκBα in PEL cells. This stabilization of IκBα then induces the inhibition NF-κB and the subsequent inhibition of NF-κB-mediated transcription, which, in turn, may result in apoptosis of PEL cells.

HSP90 Activity Is Required for KSHV Replication in PEL Cells It is known that activity of HSP90 is essential for replications of many viruses such as poliovirus, rhinovirus, and coxsackievirus.[20] Therefore, we investigated whether the inhibition of HSP90 affects KSHV lytic replication in PEL cells. BC3 cells were cultured in TPA-containing media to induce lytic viral replication in the presence or absence of GA, 17-AAG, and radicicol for 3 d. Culture medium containing capsidated viral particles were subjected to real-time PCR to quantify viral DNA. GA, 17-AAG, and radicicol suppressed viral particle production at low concentrations, which do not affect the cell growth of BC3 (Fig. 4a). To our knowledge, this is the first report showing abrogation of KSHV replication by

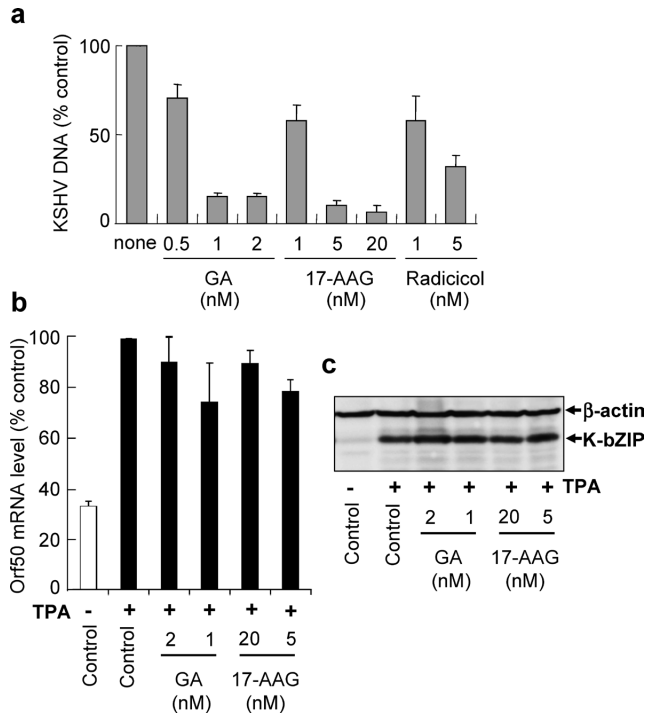

Fig. 4. Inhibition of HSP90 Activity Suppresses KSHV Replication in PEL Cells

(a) Effects of HSP90 inhibitors on KSHV lytic replication. BC3 cells were incubated with TPA for 24 h to induce the production of new virus particles, and then incubated with or without GA, 17-AAG, or radicicol for 3 d. KSHV genomes purified from culture medium were quantified by real-time PCR using a KSHV ORF21 standard curve to calculate viral genome copy numbers. The KSHV genome copy number in inhibitor-untreated BC3 cells induced by TPA was defined as 100%. (b) Effects of GA and 17-AAG on the mRNA expression of the immediate-early gene ORF50/RTA. BC3 cells were treated with TPA in the presence or absence of GA or 17-AAG for 24 h. Total RNA was extracted from harvested cells and subjected to real-time RT-PCR. The amount of ORF50 mRNA in inhibitor-untreated BC3 cells induced by TPA was defined as 100%. (c) Effects of GA and 17-AAG on protein expression of the early gene K8/K-bZIP. BC3 cells were treated with TPA in the presence or absence of inhibitor for 24 h. Treated cells were harvested and subjected to blotting with anti-K-bZIP and anti-β-actin antibodies using the same membrane.

GA, 17-AAG, and radicicol. We next examined the effects of GA and 17-AAG on the lytic viral gene expression for viral replication in BC3 cells. BC3 cells were treated with TPA and GA (or 17-AAG) for 24 h. Total RNA was extracted from cells and subjected to real-time RT-PCR to quantify ORF50/RTA expression, an immediate-early, lytic gene of KSHV. GA and 17-AAG treatment had only a slight effect on the mRNA expression of ORF50 (Fig. 4b). We also tested the protein expression of K8/K-bZIP (an early gene) in BC3 cells treated with TPA in the presence or absence of HSP90 inhibitors (Fig. 4c). However, K-bZIP protein levels did not change in BC3 cells treated with GA and 17-AAG. These results indicate that HSP90 activity is not essential for lytic gene expression for KSHV replication.

GA and 17-AAG reduced the production of progeny virus from PEL cells at low concentrations, which do not affect PEL cell growth. HSP90 activity is required for both cell survival and viral replication in PEL cells. These findings suggest a novel application of Hsp90 inhibitors in the treatment of KSHV infection. Not only NF-κB signaling but also several cell signaling pathways, such as hypoxia inducible factor (HIF), mitogen-activated protein kinase (MAPK), and Akt signaling,[21,22] regulate the initiation of KSHV lytic replication, and these pathways regulator molecules, such as HIF-1a, Raf-1, and Akt have been identified as clients of HSP90.[20] Inhibition of these signaling pathways by HSP90 inhibitors may contribute to the suppression of KSHV replication. On the other hand, HSP90 inhibitors did not significantly affect the mRNA (ORF50) or protein (K-bZIP) expression of KSHV lytic genes. These data indicate that HSP90 is important for the subsequent activities of expressions of lytic genes, such as viral protein maturation, assembly, transport, and the budding process. How HSP90 associates and regulates the KSHV replication-process, which is a planned future study, should be proved.

Acknowledgments This work was partly supported by Health and Labour Sciences Research Grants (Grant No. H23-AIDS-Ippan-002) from the Ministry of Health, Labour and Welfare of Japan, the Takeda Science Foundation, the Japan Health Sciences Foundation, and New Energy and Industrial Technology Development Organization (NEDO) of Japan.

REFERENCES

1) Russo JJ, Bohenzky RA, Chien MC, Chen J, Yan M, Maddalena D, Parry JP, Peruzzi D, Edelman IS, Chang Y, Moore PS. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 14862–14867 (1996).
2) Nador RG, Cesarman E, Chadburn A, Dawson DB, Ansari MQ, Sald J, Knowles DM. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood*, **88**, 645–656 (1996).
3) Drexler HG, Uphoff CC, Gaidano G, Carbone A. Lymphoma cell lines: *in vitro* models for the study of HHV-8+ primary effusion lymphomas (body cavity-based lymphomas). *Leukemia*, **12**, 1507–1517 (1998).
4) Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science*, **266**, 1865–1869 (1994).
5) Fujimuro M, Wu FY, ApRhys C, Kajumbula H, Young DB, Hayward GS, Hayward SD. A novel viral mechanism for dysregulation of β-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat. Med.*, **9**, 300–306 (2003).
6) Fujimuro M, Hayward SD, Yokosawa H. Molecular piracy: manipulation of the ubiquitin system by Kaposi's sarcoma-associated herpesvirus. *Rev. Med. Virol.*, **17**, 405–422 (2007).
7) Chaudhary PM, Jasmin A, Eby MT, Hood L. Modulation of the NF-κ B pathway by virally encoded death effector domains-containing proteins. *Oncogene*, **18**, 5738–5746 (1999).
8) Keller SA, Schattner EJ, Cesarman E. Inhibition of NF-kappaB induces apoptosis of KSHV-infected primary effusion lymphoma cells. *Blood*, **96**, 2537–2542 (2000).
9) Hinz M, Broemer M, Arslan SC, Otto A, Mueller EC, Dettmer R, Scheidereit C. Signal responsiveness of IkappaB kinases is determined by Cdc37-assisted transient interaction with Hsp90. *J. Biol. Chem.*, **282**, 32311–32319 (2007).
10) Saji C, Higashi C, Niinaka Y, Yamada K, Noguchi K, Fujimuro M. Proteasome inhibitors induce apoptosis and reduce viral replication in primary effusion lymphoma cells. *Biochem. Biophys. Res. Commun.*, **415**, 573–578 (2011).
11) Field N, Low W, Daniels M, Howell S, Daviet L, Boshoff C, Collins M. KSHV vFLIP binds to IKK-gamma to activate IKK. *J. Cell Sci.*, **116**, 3721–3728 (2003).
12) Wen KW, Damania B. Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. *Oncogene*, **29**,

3532–3544 (2010).
13) Fujimuro M, Liu J, Zhu J, Yokosawa H, Hayward SD. Regulation of the interaction between glycogen synthase kinase 3 and the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen. *J. Virol.*, **79**, 10429–10441 (2005).
14) Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell*, **91**, 243–252 (1997).
15) Qin Z, DeFee M, Isaacs JS, Parsons C. Extracellular Hsp90 serves as a co-factor for MAPK activation and latent viral gene expression during *de novo* infection by KSHV. *Virology*, **403**, 92–102 (2010).
16) Defee MR, Qin Z, Dai L, Toole BP, Isaacs JS, Parsons CH. Extracellular Hsp90 serves as a co-factor for NF-κB activation and cellular pathogenesis induced by an oncogenic herpesvirus. *Am. J. Cancer Res.*, **1**, 687–700 (2011).
17) Wen KW, Damania B. Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. *Oncogene*, **29**, 3532–3544 (2010).
18) Keller SA, Hernandez-Hopkins D, Vider J, Ponomarev V, Hyjek E, Schattner EJ, Cesarman ENF. NF-kappaB is essential for the progression of KSHV- and EBV-infected lymphomas *in vivo*. *Blood*, **107**, 3295–3302 (2006).
19) Dabaghmanesh N, Matsubara A, Miyake A, Nakano K, Ishida T, Katano H, Horie R, Umezawa K, Watanabe T. Transient inhibition of NF-kappaB by DHMEQ induces cell death of primary effusion lymphoma without HHV-8 reactivation. *Cancer Sci.*, **100**, 737–746 (2009).
20) Solit DB, Chiosis G. Development and application of Hsp90 inhibitors. *Drug Discov. Today*, **13**, 38–43 (2008).
21) Haque M, Davis DA, Wang V, Widmer I, Yarchoan R. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains hypoxia response elements: relevance to lytic induction by hypoxia. *J. Virol.*, **77**, 6761–6768 (2003).
22) Cohen A, Brodie C, Sarid R. An essential role of ERK signalling in TPA-induced reactivation of Kaposi's sarcoma-associated herpesvirus. *J. Gen. Virol.*, **87**, 795–802 (2006).