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

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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 NF-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 rhabdovirus 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 Castleman’s 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.5,6) 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 transport 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 NF-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 IκBα 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 phosphorolates 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),

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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 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 100µL 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) 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 luminescence reporter assay kit (Promega, WI, U.S.A.)

Western Blotting and Antibodies  For Western blotting, cells (1×10^6) were lysed in 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 lysates were subjected to SDS-PAGE gel followed by Western blot analysis, as described previously. Primary antibodies used in these experiments included those against β-tubulin, 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. For immunofluorescence (IF) analysis, BC3 PEL cells were treated with 10 nM GA for 6 h and fixed in 50% methanol–aceton (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. The nucleofected cells were incubated in 2 mL 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 the ORF21 expression primer set. The following primers were used to amplify a 140-bp amplicon internal to the ORF21 sequence: 5'-TCCACAAAACAGCAGGATATGGAC-3' and 5'-GAGGGAGTGTGCATTAATG-3'.
For real-time reverse transcription (RT), total RNA was purified and extracted from $5 \times 10^6$ BC3 cells using the Illustra RNA spin 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'-TTCTCGGCTCTCTGGACGAATG-3’ and 5'-ATCTCGGATTCACATCTTCCACCCAC-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

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 24h, 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 CC50 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 6h (Fig.

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

<table>
<thead>
<tr>
<th>Cell type</th>
<th>GA (nM)</th>
<th>17-AAG (nM)</th>
<th>Radicicol (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJAB</td>
<td>18</td>
<td>4380</td>
<td>1772</td>
</tr>
<tr>
<td>Ramos</td>
<td>15</td>
<td>772</td>
<td>346</td>
</tr>
<tr>
<td>BC3</td>
<td>7.2</td>
<td>92</td>
<td>19</td>
</tr>
<tr>
<td>BCBL1</td>
<td>3.3</td>
<td>82</td>
<td>31</td>
</tr>
</tbody>
</table>

CC50, cytotoxic concentration of HSP90 inhibitor that reduces cell viability by 50%.

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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 6h 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 48h. (c) Immunofluorescence analysis using anti-active caspase-3 antibody. BC3 cells were cultured with 10 nM GA for 6h and fixed in methanol-acetone (1:1). Immunofluorescent images were obtained with an invert confocal microscopy system.
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.5,19 Hsp90-Cdc34 functions as an additional scaffold for IKK complex and promotes IKK kinase activity for IkBα, resulting in activation of NF-κB signaling.4) Therefore, we asked whether HSP90 inhibitors affect phosphorylation of Ser32 and Ser36 in IkBα. When BC3 cells were treated with 10 nM GA, a decrease in the phosphorylation of IkBα (p-IkBα) was detected (Fig. 3a). In addition, we asked whether HSP90 inhibitors affect the stabilization of IkBα, because phosphorylation of Ser32 and Ser36 of IkBα can be a trigger for polyubiquitination and degradation of IkBα. When BC3 cells were treated with 10 nM GA, the amount of IkBα was increased (Fig. 3a). Next, we confirmed the inhibition of IkBα phosphorylation by NF-κB inhibitor BAY11-7082 by comparing it to the GA-mediated inhibition. BAY11-7082, which inhibits IkB phosphorylation, is an irreversible and specific inhibitor of NF-κB. The phosphorylation of IkBα was strongly decreased in BC3 cells treated with BAY11-7082 (Fig. 3b). When IkBα 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 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, abolishes the constitutive activation of NF-κB, resulting in the apoptosis of PEL cells.

IKK complex phosphorylates IkBα and then phosphorylated IkBα is polyubiquitinated, which induces proteasomal degradation of IkBα. In the normal stage, IkBα 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 activates NF-κB signaling to achieves anti-apoptosis in KSHV-infected cells including PEL cells.7,8) The v-FLIP activates the IKK complex through interaction with NEMO of the IKK- HSP90 complex.13) 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,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 affect phosphorylation or stabilization of IkBα. In this study, we demonstrated that HSP90 inhibitors inhibit phosphorylation of Ser32 and Ser36 of IkBα and induce the stabilization of IkBα in PEL cells. This stabilization of IkBα 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 3d. 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
HSP90 activity is not essential for lytic gene expression for cells treated with GA and 17-AAG. These results indicate that 4c). However, K-bZIP protein levels did not change in BC3 and 17-AAG treatment had only a slight effect on 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.

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