Kaposi’s Sarcoma-Associated Herpesvirus Positive Primary Effusion Lymphoma Tumor Formation in NOD/SCID Mice Is Inhibited by Neomycin and Neamine Blocking Angiogenin’s Nuclear Translocation

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ABSTRACT

Angiogenin (ANG) is a 14-kDa multifunctional pro-angiogenic secreted protein whose expression level correlates with the aggressiveness of several tumors. We observed increased ANG expression and secretion in endothelial cells during de novo infection with Kaposi’s Sarcoma-associated herpesvirus (KSHV), in cells only expressing latency-associated LANA-1 protein, in KSHV latently infected primary effusion lymphoma (PEL) BCBL-1 and BC-3 cells. Inhibition of PLCγ mediated ANG’s nuclear translocation by neomycin, an aminoglycoside antibiotic (not G418-neomycin), resulted in reduced KSHV latent gene expression, increased lytic gene expression, and increased cell death of KSHV+ PEL and endothelial cells. ANG detection in significant levels in KS and PEL lesions highlights its importance in KSHV pathogenesis. To assess the in vivo anti-tumor activity of neomycin and neamine (non-toxic derivative of neomycin), BCBL-1 cells were injected intraperitoneally into NOD/SCID mice. We observed significant extended survival of mice treated with neomycin or neamine. Markers of lymphoma establishment, such as increases in animal body weight, spleen size, tumor cell spleen infiltration, and ascites volume, were observed in non-treated animals which were significantly diminished by neomycin or neamine treatments. Significant decrease in LANA-1 expression, increase in lytic gene expression, and an increase in cleaved caspase-3 were also observed in neomycin or neamine-treated animal ascitic cells. These studies demonstrated that ANG played an essential role in KSHV latency maintenance and BCBL-1 cell survival in vivo and targeting ANG function by neomycin/neamine to induce the apoptosis of cells latently infected with KSHV is an attractive therapeutic strategy against KSHV associated malignancies.
INTRODUCTION

Kaposi’s Sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), is a γ-herpesvirus which is etiologically associated with the pathogenesis of Kaposi’s sarcoma (KS), an angioproliferative tumor of endothelial origin. KSHV is also associated with two B-cell proliferative neoplasms: body cavity-based lymphoma (BCBL) or primary effusion B-cell lymphoma (PEL), and multicentric Castleman’s disease (MCD) (1-3). PEL is a rare aggressive form of non-Hodgkin’s lymphoma that occurs most frequently in AIDS patients. This B-cell monoclonal malignancy is observed in various body cavities such as the pleura, pericardium and peritoneum (2, 4). Occasionally, PEL can be present as a solid mass in lymph nodes and other organs (5, 6). PEL is associated with a poor prognosis, resistance to conventional chemotherapy with a survival time of 2 to 6 months (7). Histologically, PEL cells are large B-cells having the appearance of anaplastic or immunoblastic cells (8). They express CD45, CD30 and immunoglobulin genes but lack B-cell differentiation antigens (8). Among the PEL B-cell lines isolated from patients, BC-1, HBL-6, and JSC carry both KSHV and Epstein-Barr (EBV) genomes, whereas BCBL-1 and BC-3 carry only the KSHV genome (9). Available treatment strategies to control HHV-8 infection associated malignancies are limited and of low efficacy. Hence, there is a vital requisite for designing therapies that target viral infection and tumor formation.

Similar to that of other members of the herpes virus family, the KSHV life cycle can be divided into latent and lytic cycles. In PEL cells, 50 to 150 copies of the viral genome are maintained as nuclear episomes (10). During the latent phase, no new viral particles are produced and the cells express KSHV latency associated genes, such as ORF 73 (LANA-1), ORF 72 (vCyclin), ORF 71 (vFlip), K12 (Kaposin), ORF 10.5 (LANA-2), 12 viral microRNAs, and...
occasionally vIL-6. The oncogenesis of PEL is predominantly mediated by latent KSHV genes. In PEL cells, proteins expressed from the latent genes are responsible for the maintenance of the episomal KSHV genome, inhibition of tumor suppressor p53, cell cycle regulation, inhibition of apoptosis, host gene regulation, stabilization of cytokine expression, anti-apoptosis, anti-autophagy, immune evasion, and proliferation (11-18). In addition, KSHV latency associated microRNAs are also involved in cell survival (19, 20), and recently, miR-K12-11 has been shown to promote B-cell expansion in vivo (21).

Only about 1 to 3% of PEL cells spontaneously enter the lytic cycle, induced by the KSHV lytic switch RTA (ORF 50) protein. However, about 10% to 25% of cells enter the lytic phase after chemical treatment such as phorbol esters or histone deacetylase inhibitors (sodium butyrate). The lytic non-structural genes mediate several functions such as immune evasion, inhibition of apoptosis, host gene modulation, host protein expression shutoff, and modulation of signal transduction (9). In contrast to PEL pathogenesis, both the latent and lytic cycles of KSHV, along with the infection induced angiogenic inflammatory network are involved in KS pathogenesis (9).

ANG, a 14-kDa multifunctional protein, was first isolated as an angiogenic secreted protein produced by HT-29 human colon adenocarcinoma (22, 23). The level of expression of ANG correlates with the aggressiveness of several tumors such as urothelial carcinoma and tumors of the pancreas, gastric system, colon, ovary, endometrium, cervix and breast (24-31). ANG is a multifunctional protein with different functions dependent on its localization. In addition to being a secreted protein, ANG has also been detected at the plasma membrane, in the cytoplasm, in the nucleus, and in the nucleolus of cells. Secreted ANG has been shown to interact with actin on the cell membrane and is involved in the migration of endothelial cells by
promoting the production of plasmin from plasminogen (32, 33). ANG activates several signaling pathways, including phospholipase C (PLCγ), phospholipase A2 (PLA2), protein kinase B (PKB/AKT), and extracellular signal-related kinase 1/2 (ERK1/2) (34-36). ANG is also called RNAse 5 as it contains 35% identity with the human pancreatic RNase 1 and is involved in the generation of 18S and 28S rRNA (37).

The nuclear translocation of ANG is necessary for its angiogenic potential as both the inhibition and mutation of the nuclear localization sequence inhibits angiogenic activity (38, 39). In the nucleolus, ANG binds to CT repeats of rRNA promoters and promotes their transcription (40). Several studies have elucidated the role of nuclear ANG in cancer cell proliferation and angiogenesis (38, 41-43). Treatment of cancer cells with the aminoglycoside antibiotic neomycin (distinct from neomycin G418) mediated anti-proliferative and anti-angiogenic effects which was shown to be due to the inhibition of ANG nuclear translocation (44). Investigation regarding the mechanism by which neomycin inhibits ANG nuclear translocation revealed that the phospholipase Cγ (PLC-γ)-inhibiting activity of neomycin was involved (44). Neomycin inhibited PLC-γ by binding to phosphatidylinositol 4,5-bisphosphate (PIP2) (45). The inhibition of ANG nuclear translocation was also observed with U-73122, a PLC-γ inhibitor. Other members of the aminoglycoside antibiotic family, such as streptomycin, kanamycin, gentamicin, paromomycin, and amikacin, did not inhibit ANG nuclear translocation and consequently were unable to inhibit ANG-induced proliferation or angiogenesis (44). In particular, paromomycin is structurally very similar to neomycin as the difference between these two drugs is a positive charged amino group (present in neomycin) replacing a neutral hydroxyl (present in paromomycin). However, it has been shown that paromomycin does not inhibit ANG nuclear translocation and ANG-induced proliferation (44). ANG nuclear translocation was also
unaffected by inhibitors of tyrosine kinases, phosphotyrosine phosphatase and protein kinase C (44). In normal cells, though neomycin inhibits the nuclear translocation of ANG by inhibiting PLC-γ activation, it did not affect the viability of the cells, and even a concentration of 1 mM is non-toxic (46).

We have previously reported a novel role of ANG in the biology of KSHV. ANG expression and secretion was increased upon de novo KSHV infection of human dermal microvascular endothelial cells (HMVEC-d) and was elevated in long-term KSHV-infected endothelial cells (Telomerase-immortalized human umbilical vein endothelial Long-term-infected cells TIVE-LTC) (47). Expression of KSHV latency protein LANA-1 and lytic protein vGPCR induced ANG gene expression and ANG protein secretion. In addition, we have shown that ANG expression and secretion was increased in PEL cells (BCBL-1 and BC3) which was not observed however in EBV+ lymphoma and lymphoblastoid cells (46). Our studies suggested that ANG plays important roles in KSHV pathogenesis through its antiapoptotic, cell proliferation, migration and angiogenic properties (46, 47). We have also shown that ANG addition induced KSHV ORF 73 (LANA-1) gene expression (46). Inhibition of its nuclear translocation with neomycin, reduced latent ORF 73 gene expression and increased the lytic ORF 50 gene both during de novo infection and in latently infected TIVE-LTC and PEL cells. The role of ANG was confirmed, as silencing ANG with shRNA had a similar effect on viral gene expression as that of neomycin treatment. A greater quantity of infectious KSHV was detected in the supernatants of neomycin-treated BCBL-1 cells than 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated cells (46, 48). This suggested a role for ANG in the regulation of KSHV latent and lytic cycles (In vitro model Fig. 2A).
In addition, we observed that ANG is crucial for the anti-apoptotic effect of KSHV observed after serum starvation of endothelial cells (47). Whereas, KSHV infection protected endothelial cells from apoptosis, blocking nuclear translocation of ANG with neomycin allowed apoptosis to proceed. We also observed a role for ANG in KSHV oncogenesis of PEL cells as nuclear ANG was essential for BCBL-1 cell survival in vitro (46). Indeed, treatment with neomycin significantly decreased the viability of KSHV positive lymphoma cells (BCBL-1, BJAB-KSHV, BC-3, and JSC-1 cells) as well as latently infected endothelial TIVE-LTC cells, but had no effect on EBV positive cells (LCL or Raji) or KSHV and EBV negative cells (BJAB, Akata, Ramos, and Loukes) (46). Similarly, knocking down ANG with shRNA decreased PEL cell viability, thus confirming the role of ANG in PEL cell survival (46) (In vitro model Fig. 2A).

Treatment of normal endothelial cells with ANG also induced PLC-γ and AKT phosphorylation while treatment with neomycin and ANG silencing inhibited PLC-γ and AKT phosphorylation (46). Our studies demonstrated that blockage of PLC-γ activation by neomycin mediated the inhibition of latent gene expression and the conventional PLC-γ inhibitor U73122 showed similar results. Collectively, these studies suggested that KSHV has evolved to exploit ANG for its advantage via the PLC-γ pathway for maintaining its latency (In vitro model Fig. 2A).

Correlation of ANG’s expression level with the aggressiveness of several tumors and inhibition of progression and metastasis of human cancer cells by anti-ANG monoclonal antibodies in athymic mice suggested that actively proliferating cancer cells could be inducing ANG for inhibiting apoptotic pathways (24-31, 49, 50). However, how ANG regulates cell survival and apoptosis was not known. We have recently demonstrated that ANG interacts with p53 and colocalizes in the nucleus of KSHV negative cancer cells (51). Silencing endogenous ANG induced p53 promoter activation and p53 target gene expression, downregulated the
expression of anti-apoptotic Bcl-2 gene and increased p53-mediated cell death. In contrast, ANG expression blocked pro-apoptotic Bax and p21 expression, induced Bcl-2 and blocked cell death. ANG also co-immunoprecipitated (co-IPed) with Mdm2, a p53 regulator protein. ANG expression inhibited p53 phosphorylation, increased p53-Mdm2 interaction, and increased p53 ubiquitination. These studies demonstrated that ANG inhibits p53 functions to promote anti-apoptosis and cell survival of cancer cells, and suggested that targeting ANG could be an effective therapy for several cancers.

In the context of KSHV infected cells, we observed that LANA-1 and ANG colocalized and co-IPed in de novo infected endothelial cells and in latently infected PEL BCBL-1 and BC-3 cells (48). LANA-1 and ANG interaction occurred in the absence of the KSHV genome and other viral proteins. ANG coeluted with LANA-1, p53, and Mdm2, while LANA-1, p53, and Mdm2 also co-IPed with ANG. LANA-1, ANG, and p53 colocalized in KSHV-infected cells. Silencing ANG or inhibiting its nuclear translocation resulted in decreased nuclear LANA-1 and ANG levels, decreased interactions between ANG-LANA-1, ANG-p53, and LANA-1-p53, the induction of p53, p21, and Bax proteins, the increased cytoplasmic localization of p53, the downregulation of Bcl-2, the increased cleavage of caspase-3, and the apoptosis of cells. Together, these studies suggested that the anti-apoptosis observed in KSHV-infected cells and the suppression of p53 functions are mediated in part by ANG, and KSHV has probably evolved to utilize ANG's multiple functions for the maintenance of its latency and cell survival. These studies also suggested that targeting ANG to induce the apoptosis of cells latently infected with KSHV is a potential therapeutic strategy against KSHV infection and associated malignancies.

In the present study, we tested the in vivo antitumor activity of the ANG nuclear translocation inhibitor neomycin as well as neamine, a derivative of neomycin known to have
fewer adverse side effects (41-43). Our studies show that in vivo treatment of BCBL-1 cell injected NOD/SCID mice with neomycin and neamine significantly prolong their survival by inhibiting tumor establishment. At the time of initial tumor detection, the weight, ascites development and BCBL-1 infiltration in the animals’ spleens were reduced in neomycin and neamine treated animals compared to PBS treated mice. At the cellular level, we observed a decrease of KSHV latent gene expression and an increase of lytic gene expression in BCBL-1 injected and treated animals. In addition, we observed increased BCBL-1 cell apoptosis in neomycin and neamine treated mice. These findings suggest that neomycin and neamine could be used as potential therapeutic candidates for the treatment of KSHV associated PEL.
MATERIALS AND METHODS

Reagents. Neomycin, paromomycin and CD19 antibody (IFA: 100) were from Sigma-Aldrich, St. Louis, MO. Neamine was a generous gift from Dr G.F. Hu, Sackler School of Graduate Biomedical Sciences, Tufts University, MA. ANG antibody (IFA: 1:100) was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Total caspase-3 and cleaved caspase-3 antibodies (WB 1:1000; IFA 1:100) were from Cell Signaling Technology, Danvers, MA. Human CD19 antibody (WB 1:1000) is from GeneTex, Irvine, CA. Rabbit polyclonal gB (UK-218) (IFA 1:100) and rabbit polyclonal LANA-1 (WB 1:1000; IFA 1:80) and mouse monoclonal LANA-1 (IFA: 1:50) antibodies were generated in our laboratory (52). Horseradish peroxidase linked antibodies (WB 1:5000) were from KPL Inc., Gaithersburg, MD. Alexa-488 (IFA 1:500) and Alexa-594 (IFA 1:1000) secondary antibodies and DAPI were from Molecular Probes, Invitrogen, Grand Island, NY.

Cells and animals. BCBL-1 cells were propagated and maintained as per procedures described previously (53-55). BCBL-1 cells were routinely tested for mycoplasma by the Lonza Mycoalert kit (LT37-618) (Lonza, New Jersey) as per the manufacturer’s instructions and were found to be negative. NOD.CB17-Prkdscid/J (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) were kept at the Biological Resource Facility at Rosalind Franklin University of Medicine and Sciences, North Chicago, IL. NOD/SCID mice were housed in micro-isolator cages. All animal experiments were approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Sciences (IACUC protocol No.10-06). Mice were weighed as a criterion for ascites growth and tumorigenesis. Animals were monitored and euthanized when signs of distress were clearly visible, according to our protocol. For the engraftment of BCBL-1 cells, BCBL-1 cells were injected intraperitoneally (i.p.) into
NOD/SCID mice at $10^7$ cells per mouse.  

**Statistical analysis of the survival curves.** Comparison of survival curves was done using the log rank test (56).

**Soft agar assay and proliferation measurement.** The assay was performed in a 48-well plate format. The base agar matrix layer was prepared as per manufacturer’s protocol (Cell Transformation Assays Soft Agar with Cell Recovery, CBA 135, Cell Biolabs, CA). BCBL-1 cells, resuspended at $5 \times 10^5$ cells per ml were added to the agar matrix layer. After solidification, media containing 200 µM neomycin was added on top of the cell/agar matrix layer. 6 days later, the colonies were viewed under a Nikon eclipse TE2000-5 microscope using the Nikon MetaMorph Digital Imaging System. Quantification of anchorage-independent growth was performed as per the manufacturer’s guidelines, using an MTT-based assay. Briefly, the cell-containing matrix was solubilized, MTT solution added, and the absorbance was read at 570 nm in a Synergy HT Microplate reader (BioTek Instruments) after the addition of detergent solution.

**Spleen sectioning and hematoxylin and eosin (H&E) staining.** The tissue samples were excised and fixed in 4% paraformaldehyde (PFA) for 7 days and kept in 20% sucrose in PBS. The samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) at the Northwestern University Mouse Histology and Genotyping Core, Chicago, IL.

**Immunofluorescence staining of paraffin embedded tissue sections.** Sections of skin biopsy samples from healthy subjects or KS patients as well as sections from healthy lung or PEL solid lung lesions were obtained from the AIDS and Cancer Specimen Resource (ACSR). The sections were deparaffinized and hydrated with water before antigen retrieval using DAKO target retriever solution in a steamer for 20 min. Slides were cooled, rinsed, blocked using 1% BSA in 0.025% Triton X-100-PBS for 30 min and used for staining of ANG alone, double-
staining with anti-ANG and mouse monoclonal anti-CD19 antibodies, or double-staining with anti-ANG and mouse monoclonal anti-LANA-1 antibodies. Sections were washed and incubated with 1:200 dilution of Alexa 488-coupled anti-rabbit antibody or Alexa 594-coupled anti-mouse antibody (Molecular Probes) for 1h at RT. Nuclei were visualized using DAPI and stained cells were viewed with the appropriate filters under a fluorescent microscope (Nikon 80i) with a 20x objective and the Nikon MetaMorph Digital Imaging System.

**Immunofluorescence staining of Ascites cells.** The ascites fluids recovered from the different animals were centrifuged. Cell pellets were washed in PBS, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 for 10 min, blocked with Image-iTFX signal enhancer (Invitrogen) for 20 min and incubated for 2.5 hours with the primary antibodies indicated in the respective figures. After three washes, the cells were incubated for 1.5 hours with the secondary anti-rabbit antibodies. Nuclei were visualized using DAPI (Molecular Probes, Invitrogen) and stained cells were viewed with the appropriate filters under a fluorescent microscope with a 20x objective.

**Immunoblotting.** Cells were harvested in RIPA lysis buffer (125 mM NaCl, 0.01 M sodium phosphate pH7.2, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, and 50 mM sodium fluoride) with protease inhibitor and phosphatase inhibitor cocktails (Sigma). Cellular debris was removed by centrifugation at 13,000 x g for 5 min at 4°C and equal amounts of protein samples were resolved by 10% SDS-PAGE and subjected to western blotting with the antibodies as indicated in each figure. To confirm equal protein loading, blots were also probed with antibodies against human tubulin or actin. Secondary antibodies conjugated to horseradish peroxidase were used for detection. Immunoreactive bands were visualized by enhanced chemiluminescence.
RNA extraction, reverse transcription and real-time RT-PCR. Total RNA was extracted by using TRIzol reagent (Invitrogen), quantified by densitometric analysis at 260 nm and analyzed by real-time RT-PCR using primers to ORF 73 (57). PCR was performed using an ABI Prism 7500 real-time PCR system utilizing Taqman EZ RT-PCR Core reagents (Applied Biosystems).
RESULTS

Angiogenin expression is increased in human Kaposi’s Sarcoma and PEL lesions.

In our previous studies, we have shown that \textit{de novo} KSHV infection of HMVEC-d cells resulted in increased secretion of ANG (47, 58). In addition, we have shown that ANG expression and secretion were increased in KSHV-associated B-lymphoma cell lines (46). To determine whether ANG is expressed in KSHV associated tumors, we analyzed skin sections from healthy subjects and KS positive patients with anti-ANG and anti-LANA-1 antibodies in immunofluorescence assays (IFA) (Fig. 1A). In contrast to healthy tissues, intense ANG staining colocalizing with LANA-1 staining was observed in KS lesions (Fig. 1A, compare upper and lower panels). Similarly, we analyzed the expression of ANG in tissues from healthy lung and lung with solid PEL lesions (Fig. 1B). We observed a striking increase in ANG expression in PEL lesions. ANG staining in PEL lesions was specific to the B-cell lymphoma as it colocalized with the B-cell marker, CD19 (Fig. 1B). In addition, we performed a co-staining with ANG and LANA-1 antibodies in the solid PEL lesions of lungs (Fig. 1C). We observed increased ANG staining in the areas of cells expressing LANA-1. These results suggested that the expression pattern of ANG is consistent with the presence of latent KSHV in the lesions. Taken together, increased detection of ANG in KSHV-associated malignancies highlighted the importance of ANG in KSHV pathogenesis.

Neomycin reduces the foci formation of KSHV positive BCBL-1 cells.

We have previously shown that ANG localized predominantly in the nuclei and nucleoli of KSHV infected cells (47). In addition, blocking ANG nuclear translocation by neomycin treatment decreased the survival of latently-infected endothelial cells and BCBL-1 cells (46). The results of our extensive previous \textit{in vitro} studies are summarized in Fig. 2A. A characteristic
of tumor development is the ability of the cells to proliferate independently of anchorage and the oncogenic capacity of BCBL-1 cells to form colonies on soft agar has been previously shown (59, 60). Hence, we examined the growth of BCBL-1 cells in soft-agar in the absence or presence of neomycin (Fig. 2). We chose a 200 µM concentration of neomycin as it has previously been used and showed no toxicity on normal endothelial cells, KSHV negative TIVE, BJAB, Akata cells as well as EBV+ cells, whereas it reduced survival of KSHV + cells. We observed loose, disaggregated BCBL-1 cell colonies in soft agar (Fig. 2B, two lefts panels). The morphology of these colonies is similar to that of the colonies observed with the BCP-1 cell line (61). However, in the presence of 200 µM neomycin, the quantity and the size of the colonies formed in soft agar were reduced (Fig. 2B, two right panels). As manual counting of colonies was less quantitative and does not reflect colony size, we used the assay developed by Cell Biolabs to quantify the anchorage-independent growth. Following the manufacturer’s protocol, the semisolid media was solubilized and the anchorage-independent growth was quantified by an MTT solution. We observed a significant decrease in BCBL-1 cell viability after growth in soft agar in neomycin treatment conditions with roughly 65% decrease in MTT assay (Fig. 2C). These results suggested that nuclear translocation of ANG plays an important role for the survival and tumorigenic properties of BCBL-1 cells.

Neomycin and neamine treated NOD/SCID mice with KSHV+ BCBL-1 induced tumors survive longer.

Transfer of KSHV infected PEL cells to immunodeficient mice leads to tumor engraftment without any spread of KSHV infection to murine tissues (61, 62). After intra-peritoneal (i.p.) injection of 10⁷ BCBL-1 cells into NOD/SCID mice, we observed tumor development starting at day 28, and all animals developed tumors with a mean survival time of 44
days (Fig. 3A). To determine the \textit{in vivo} effect of inhibiting the nuclear transport of ANG by neomycin, we injected the drug after BCBL-1 cell injection. Mice were injected with $10^7$ cells followed by the injection of 10 mg/kg of neomycin every 2 days for 1 week and once a week thereafter. We observed a significant delay ($p<0.004$) in tumor development in the neomycin-treated mice (Fig. 3B). The mean survival time was improved from 56 days in non-treated animals to 96 days in neomycin-treated mice. The effect of blocking ANG was confirmed using neamine, a derivative of neomycin known to have fewer adverse side effects (41-43). We observed an even greater delay in tumor development in the neamine treated mice (Fig. 3C). The mean survival time was increased from 56 days in non-treated animals to 118 days in neamine-treated mice ($p<0.0015$).

To determine that these effects were specific to blocking the nuclear localization of ANG, we used paromomycin as negative control. Paromomycin, an analogue of neomycin, does not affect the nuclear transport of angiogenin. When mice were injected with paromomycin, BCBL-1 tumor development was not significantly inhibited. Indeed, the survival of paromomycin-treated mice was comparable to PBS injected animals, with a mean survival time of 60 and 56 days, respectively (Fig. 3D). All together, these results suggested that agents that block ANG nuclear translocation in BCBL-1 cells \textit{in vitro} are also effective \textit{in vivo} resulting in protection from BCBL-cell tumor development with increased survival time of mice, and neamine had a greater protective effect than neomycin.

\textbf{Neomycin and neamine treatments prevent KSHV+ BCBL-1 tumor formation in NOD/SCID mice.}

To determine the effect of ANG inhibitors early during tumor development, all mice were injected i.p. with $10^7$ BCBL-1 cells followed by the injection of the corresponding drugs (10 mg/kg) every 2 days for 1 week and once a week thereafter. 7 weeks after the injection of tumor
cells, all the animals were euthanized at the same time. At this time, we observed some abdominal
distention in the PBS-treated animals but none in the neomycin- or neamine-treated animals (Fig.
4A a and b, respectively). Abdominal distention is a well-established sign of ascites development. In
addition, the PBS-treated animals were significantly heavier compared to the animals treated with
neomycin and neamine (Fig. 4Ac). Whereas the average weight of an NOD/SCID mouse at 7 weeks
was 20 g, the weight of BCBL-1 injected mice treated with PBS was around 29 g. However, the
body weight of the mice injected with BCBL-1 cells and treated with neomycin was significantly
reduced to 24 g and the weight of neamine-treated animals was comparable to the average weight of
NOD/SCID mice at the same age (20 g) (Fig. 4Ac). An increase in body weight is a second sign
indicating tumor formation.

To confirm that the abdominal distension and gain of weight were due to tumor formation,
we extracted the ascites cells from these mice for further analysis (Fig. 4B). Animals not injected
with BCBL-1 cells did not show any ascites formation (data not shown). However, all of the
mice injected with BCBL-1 cells and treated with PBS developed ascites (5/5). In contrast,
ascites formation was observed in 3 of the 5 neomycin-treated mice and only in 1 mouse of the 5
neamine-treated mice (Fig. 4B). In addition, we collected the ascites and measured the volume
produced in each mouse. We collected an average of 1.5 ml of ascites from PBS-treated animals,
and the volumes of ascites from neomycin- and neamine-treated animals were reduced to an
average of 0.35 and 0.05 ml, respectively (Fig. 4B). The presence and quantity of ascites
correlated with the increased weight observed in Fig. 4Ac, confirming that the weight gain
observed in Fig. 4A was due to tumor establishment. These data demonstrated a significant delay
in tumor formation in neomycin and neamine treated animals and indicated that neamine
treatment was more potent in inhibiting BCBL-1-tumor formation.
Neomycin or neamine treatment prevents spleen infiltration of BCBL-1 cells in NOD/SCID mice.

We observed that mice injected i.p. with BCBL-1 cells presented significantly enlarged spleens compared to normal NOD/SCID mice (picture not shown). We next evaluated the effect of neomycin and neamine on the spleens of BCBL-1 cell injected mice euthanized 7 weeks post-injection. We observed significantly smaller spleens in neomycin- and neamine-treated mice compared with those from PBS-treated animals. Representative pictures of the spleens are shown in Fig. 5Aa. The spleens from un.injected animals weighted around 0.05 g whereas BCBL-1 injected and PBS-treated mice weighted approximately 0.2 g. Interestingly, the spleens were significantly smaller in neomycin- and neamine-treated animals, with an average weight of 0.1 g and 0.05 g, respectively (Fig. 5Ab).

To determine the cause of the enlarged spleens, we performed histologic analysis using H&E staining of the spleen sections (Fig. 5Ba). In BCBL-1 injected mice treated with PBS, we observed the presence of infiltrating cells (Fig. 5Ba, upper panel; enlarged in the right upper panel). These infiltrated cells are large and have the appearance of anaplastic cells. This morphology is similar to the morphology of PEL cells (8). However, the numbers of infiltrating cells were significantly reduced in neomycin and neamine treated animals (Fig. 5Ba, middle and lower panels, respectively). We observed an average of 15, 6, and 4 infiltrating cells per field in PBS-, neomycin-, and neamine-treated animals, respectively (Fig. 5Bb). The number of infiltrating cells is proportional to the weight of the spleens, suggesting that these cells are responsible for spleen enlargement.

To confirm that enlargement of the spleens was due to BCBL-1 cell infiltrations, we quantified the expression of KSHV latency ORF 73 gene from the spleen RNA. In mice injected
with BCBL-1 cells and treated with PBS, we observed significantly more ORF 73 expression than in mice injected with BCBL-1 cells and treated with neomycin or neamine (Fig. 5C). The ORF 73 expression is proportional to the weight of the spleen and to the number of infiltrating cells observed in the histologic analysis, indicating that enlargement of the spleens is likely due to BCBL-1 cell infiltration. All together, these results demonstrated that neomycin and neamine treatment decreased BCBL-1 cell dissemination into the spleens of NOD/SCID mice.

Neomycin and neamine treatments decrease KSHV latency gene expression in BCBL-1 cells injected into NOD/SCID mice.

Our earlier *in vitro* studies have shown that the decrease of BCBL-1 viability after neomycin treatment was partially due to a decrease in KSHV latency gene expression and ANG plays a role in the maintenance of KSHV latency (46). Because we observed a decrease of BCBL-1 oncogenesis *in vivo*, we analyzed the recovered ascites cells for the expression of the latency protein LANA-1. In western blot analysis of ascites cells, we observed a reduction in LANA-1 expression (bands at 220, 130, and 110-kDa) in cells isolated from animals treated with neomycin or neamine compared with the cells isolated from PBS-treated animals (Fig. 6Aa). We observed about 39% and 52% reduction of LANA-1 expression in the cells from neomycin- and neamine-treated animals, respectively. Actin was used as loading control. In addition, we performed a western blot analysis using an antibody against the human B cell marker CD19. We did not observe significant changes in CD19 indicating that the decrease in LANA-1 is not due to an increase in mouse cells collected with the ascites. To confirm the decrease in LANA-1 expression, ascites cells were analyzed by IFA with anti-LANA-1 antibodies (Fig. 6Ab). We observed a decrease in the expected nuclear punctate LANA-1 staining in the ascites cells from neomycin- and neamine-treated animals. We quantified the level of LANA-1 in the IFA
experiment by counting the number of LANA-1 puncta per cell (Fig. 6Ac). Whereas 30 puncta were observed in the ascites cells from PBS treated animals, only 17 and 7 puncta were observed in the neomycin and neamine treated animals, respectively (43% and 77% reduction, respectively).

Neomycin and neamine treatments increase KSHV lytic gene expression in BCBL-1 cells injected into NOD/SCID mice.

*In vitro* treatment of BCBL-1 cells with neomycin increased lytic gene expression with an increase in the early lytic ORF 50 mRNA levels after 3 days of neomycin treatment (46). In addition, the early and late lytic protein ORF 59 and K8.1A protein, respectively, were also increased after 3 days of neomycin treatment (46). To determine if the reduction of the observed latent gene expression in NOD/SCID mice was associated with a concomitant *in vivo* increase in the KSHV lytic cycle, the ascites cells from the different mice were stained with anti-KSHV envelope glycoprotein gB antibodies (Fig. 6Ba). In PBS treated animals, 3% of the ascites were expressing gB which is consistent with the estimated 3-5% of BCBL-1 cells that undergo spontaneous lytic reactivation. In contrast, about 37% and 22% of the ascites cells were positive for gB staining in neomycin- and neamine-treated mice, respectively (12- and 7-fold increase, respectively) (Fig. 6Bb). Taken together, these results indicated that *in vivo* treatment of BCBL-1 injected NOD/SCID mice with neomycin and neamine results in a decrease of the latent gene expression with a concomitant increase in KSHV lytic gene expression.

Neomycin and neamine treatments induce apoptosis in BCBL-1 cells injected into NOD/SCID mice.

*In vitro* neomycin treatment of BCBL-1 cells resulted in reduced viability (46). Our studies have demonstrated an anti-apoptotic role for ANG. It is well established that the
expression of KSHV latency proteins, such as vFlip and LANA-1, are essential for BCBL-1 cell survival. To further elucidate the consequence of neomycin/neamine treatment (blocking ANG nuclear translocation) and the decrease of viral latency protein expression on ascites cell apoptosis, we examined the activation of caspase-3, a crucial executioner of apoptosis. Like all caspases, caspase-3 activation requires its proteolytic cleavage. The induction of apoptosis in the ascites cells was measured by western blot using an antibody specific for the cleaved form of caspase-3 (Fig. 7Aa). Whereas cleaved caspase-3 was absent (mouse # 1 and 2) or low (mouse # 3 and 4) in the ascites recovered from PBS-treated animals, we observed the presence of active caspase-3 in all the ascites recovered from neomycin- and neamine-treated mice (mouse #5-8). We quantified the western blot and estimated a 3.3- and 2.9-fold increase in caspase-3 activation in neomycin and neamine treated mice, respectively (Fig. 7Ab). Actin and total pro-caspase-3 western blot were used as loading control. This result was confirmed by an IFA experiment, wherein cleaved caspase-3 staining was increased in ascites cells from neomycin and neamine treated animals compared with the staining in cells from PBS-treated animals (Fig. 7Ba). The percent of cells stained with cleaved caspase-3 antibody was quantified, and we observed 34% of the ascites cells stained by cleaved caspase-3 isolated from PBS treated animals (Fig. 7Bb). However, apoptosis was increased to 93% and 97% of the ascites cells isolated from neomycin and neamine treated animals, respectively (Fig. 7Bb). Taken together, these results indicated that the delay of BCBL-1 induced tumorigenesis observed in neomycin and neamine treated animals was collectively due to a reduction of KSHV latency, an increase in the lytic cycle, and concomitant increase in apoptosis of BCBL-1 cells.
We observed in the present study a higher expression of ANG in Kaposi’s sarcoma lesions compared with healthy skin as well as an increase of ANG expression in lung PEL compared with healthy lungs (Fig. 1). We have also previously shown that human B-cell lines isolated from PEL expressed higher levels of ANG and we demonstrated in vitro that ANG was a determinant factor in PEL cell proliferation and survival (46, 48). Indeed, blocking ANG nuclear translocation with neomycin treatment significantly decreased the viability of KSHV+ lymphoma cells as well as latently infected endothelial cells, but had no effect on EBV+ cells or KSHV- and EBV- cells (46, 48). Our present studies extended these observations and demonstrate reduction in the in vitro growth of BCBL-1 cells in soft-agar by blocking ANG nuclear translocation (Fig. 2). Finally, the studies here demonstrate for the first time that blocking ANG nuclear translocation significantly decreased the pathology of BCBL-1 induced tumors in NOD/SCID mice. In neomycin and neamine treated animals, tumor establishment was reduced and the life span of the animals was significantly increased (Model Fig. 8 A-B).

Analysis of ascites cells from treated mice demonstrated that neomycin and neamine disrupted KSHV latency, induced the induction of the viral lytic cycle and increased apoptosis in these cells (Model Fig. 8C), validating our finding that ANG plays a critical role in the maintenance of KSHV latency (46, 48).

Our previous in vitro studies demonstrated that silencing ANG or inhibition of its nuclear translocation with neomycin inhibited latent ORF 73 gene expression and increased the lytic switch ORF 50 gene both during de novo infection and in latently infected cells (46, 48). Interestingly, ANG treatment activated PLC-γ and AKT whereas neomycin inhibited the activation of both proteins. In addition, the PLC-γ inhibitor U73122 induced KSHV reactivation,
similar to neomycin, suggesting that KSHV has evolved to exploit ANG for its advantage via the PLC-γ pathway for maintaining its latency (46, 48). The therapeutic effect of neomycin and neamine could be due to a direct effect on ANG nuclear translocation and ANG cellular function but also to a cumulative effect on viral gene expression. For better understanding, we have summarized the potential implications of the multiple roles that ANG could play in KSHV biology and KSHV associated malignancies below.

The anti-apoptotic role of ANG:

The observation that neomycin and neamine treatment resulted in an increase in apoptosis of the in vivo injected KSHV+ BCBL-1 cells (Fig. 7) likely reflects the in vivo inhibition of ANG nuclear translocation by these drugs. ANG has been shown to prevent apoptosis induced by serum withdrawal in human endothelial and mouse carcinoma cells (47, 63). A potential anti-apoptotic mechanism of ANG during serum withdrawal was the inhibition of the nuclear translocation of apoptosis inducing factor (AIF), thereby preventing AIF induced chromatin condensation and DNA fragmentation (64). Another anti-apoptotic mechanism of ANG is the upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes (63). These effects were dependent on Bcl-2 and NF-kB (63). Interestingly, we have shown that ANG is upregulated during KSHV infection through an NF-kB dependent pathway (47, 58). At 8 and 24 h post-infection of endothelial cells, ANG mediated mRNA levels were significantly reduced with the NF-kB inhibitor Bay11-7082. NF-kB is a well established anti-apoptotic protein and is constitutively active in PEL (65). Similar to our results, blocking the NF-kB pathway with Bay11-7082 has been shown to prevent or delay PEL tumor growth in NOD/SCID mice and prolong their disease-free survival (66). The therapeutic potential of blocking the NF-kB pathway has been confirmed by blocking the proteosome with Bortezomib, using the new NF-kB...
inhibitor dehydroxymethylepoxyquinomicin (DHMEQ), or using the biscoclaurine alkaloid cepharanthine (67-71). In all these studies, blocking the NF-kB pathway induced the apoptosis of PEL. We postulate that the observed effect of neomycin and neamine could be due to blocking an anti-apoptotic regulatory loop between NF-kB and ANG.

We have also shown that ANG activated the AKT pathway and neomycin treatment decreased AKT activation in BCBL-1 cells (46, 48). Interestingly, the inhibition of AKT with miltefosine and perifosine, two alkylphospholipids, inhibited PEL cell growth, induced apoptosis \textit{in vitro} and delayed PEL tumor progression \textit{in vivo} (72, 73). All together, these studies indicated that ANG could also be protecting the PEL cells from apoptosis in part through the regulation of crucial anti-apoptotic pathways such as NF-kB and AKT.

To better understand the role of ANG in KSHV biology, we previously performed a proteomic analysis of ANG-interacting proteins. We observed that 28 cellular proteins, with diverse functions, interacted with both ANG and LANA-1 (74). We further analyzed the interaction between ANG and annexin A2. We observed that silencing annexin A2 by siRNA resulted in significant cell death of KSHV+ BCBL-1 cells but had no effect on KSHV- B cell lines such as Ramos or BJAB. In addition, silencing annexin A2 impaired cell cycle progression specifically in BCBL-1 cells by decreasing some cell cycle-associated proteins (74). These results indicate a role for ANG in cell cycle and apoptosis regulation through its interaction with annexin A2.

Furthermore, we demonstrated that ANG decreased p53 mediated cell death (51). The expression of ANG correlated with p53 levels in several cancer cell lines and we observed a co-localization between ANG and p53 in human colon carcinoma. The silencing of ANG induced p53 target gene expression and increased p53-mediated cell death whereas its overexpression had
the opposite effect (51). In a recent study, we also confirmed that ANG participated in the anti-
apoptosis state of PEL cells by the suppression of p53. Suppressing ANG nuclear translocation
activated p53 and increased the expression of its target genes such as p53 itself, p21 and Bax in
KSHV+ BCBL-1 cells but not in KSHV– BJAB cells, leading to selective cell death (48).

In addition to a direct role for ANG in oncogenesis, ANG could regulate cell viability
through the regulation of KSHV gene expression. We observed that blocking ANG nuclear
translocation induced a decrease in KSHV latent gene expression and an increase in lytic gene
expression (Fig. 6). As several latency proteins have anti-apoptotic roles, a decrease of these
proteins would likely be associated with an increase in apoptosis. For example, it has been
shown that LANA-1 interacts with and inhibits p53 whereas vFlip inhibits apoptosis through the
activation of the transcription factor NF-kB (12, 15, 75-78). KSHV microRNAs have also been
shown to contribute to the inhibition of apoptosis in infected cells. For example, miR-K12-1,
K12-3 and K12-4-3p regulate caspase-3 expression (79). More recently, KSHV microRNAs
were shown to target numerous pro-apoptotic factors (80, 81). ANG could be protecting PEL
cells from apoptosis through multiple pathways, including upregulation of the latency gene
cluster, and the observed apoptosis of KSHV+ cells by blocking ANG’s nuclear translocation
could be due to the cumulative effects of reduction in latent gene expression and consequent
reduction in anti-apoptotic functions of viral gene products as well as ANG.

Targeting ANG as an anti-tumor therapy:

As we have seen in our study, targeting ANG, by the use of blocking antibodies or
downregulation of ANG by siRNA or inhibitory drugs, has been proposed as an anti-cancer
therapy in other cancer models.
The role of ANG in tumor formation has been evaluated using RNAi technology to downregulate ANG expression, targeting ANG independently of its localization. ANG siRNA decreased the cell proliferation and colony formation of human lung adenocarcinoma A549 and PC-3 human prostate cancer in vitro and it significantly inhibited A549 and PC-3 tumor formation in mouse models (82, 83). In addition, downregulation of ANG has also been shown to prevent AKT-driven prostate intraepithelial neoplasia in murine prostate-restricted AKT transgenic mice (84).

The use of siRNA as a therapeutic is challenging, as all the cancerous cells need to be targeted. Therefore several pharmacologic approaches have been proposed to block the effect of ANG on oncogenesis. Mutagenesis analyses have shown that reducing the ribonucleotic activity of ANG also reduced its angiogenic properties (85-90). N65828, an inhibitor of ANG ribonucleotic activity, inhibited PC-3 prostate tumor cell oncogenesis as well as a model of AKT-induced prostate intraepithelial neoplasia in vivo (84, 91). Neomycin has been previously shown to inhibit ANG nuclear translocation and consequently to reduce ANG induced cell proliferation and angiogenesis (44). In vivo, neomycin inhibited lung adenocarcinoma development, human prostate cancer PC-3 cell tumor growth in athymic mice as well as the development of AKT-driven prostate intraepithelial neoplasia in murine prostate-restricted AKT transgenic mice (82-84). The use of neomycin as a chemotherapeutic agent was unfortunately accompanied with nephrotoxicity and ototoxicity. Interestingly, neamine, another member of the aminoglycoside antibiotic family and a derivative of neomycin, has been shown to present reduced toxicity compared to neomycin but retain the effects on ANG nuclear translocation, and ANG-induced angiogenesis and cell proliferation (38, 41-43). For example, neamine inhibited the proliferation, migration and invasion of the H7402 human hepatoma cell line in vitro (92).
vivo, neomycin and neamine decreased both the tumor weight and the formation of neovessels after injection of athymic mice with HT-29 human colon carcinoma and MDA-MB-435 breast cancer cells, or A431 human epidermoid carcinoma cells (43).

The role of ANG in tumor formation has also been evaluated using neutralizing antibodies, specifically targeting the functions dependent on the secreted form of ANG (33, 50, 93). In vitro, an anti-ANG polyclonal antibody inhibited ANG-induced endothelial cell invasiveness (33). The mouse monoclonal anti-ANG antibody mAb 26-2F inhibited the ribonucleotic, angiogenic and mitogenic activities of ANG and decreased in a dose-dependent manner the establishment of human colon adenocarcinoma after injection of HT-29 cells in athymic mice (49, 50). As the use of murine antibodies in human patients is problematic, a chimeric mouse/human antibody based on the structure of mAb 26-2F has been developed and it inhibited the formation of human breast cancer xenografts after injection of MDA-MB-435 and MCF-7 cells in athymic mice (93). The use of anti-ANG antibodies as a PEL therapeutic agent is beyond the scope of the present study and will be evaluated in the future.

Our earlier in vitro studies demonstrated that blocking nuclear transport of angiogenin disrupted KSHV latency resulting in apoptosis and cell death in KSHV+ PEL and endothelial cells. Our present in vivo studies extended our in vitro observations and demonstrate that neomycin and neamine inhibit the oncogenesis of PEL cells. Currently available clinically validated treatments for PEL include cytotoxic chemotherapy agents and mTOR inhibitors (94). Since no targeted agents have been added to the clinical practice even after 20 years of KSHV discovery, ANG’s specific associations with KSHV biology and latency, but not with EBV coupled with the relatively low adverse side effects of neamine, suggest that it could be considered as an attractive therapeutic candidate for PEL treatment.
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FIGURES LEGENDS

Figure 1. Expression of Angiogenin in Kaposi’s Sarcoma and PEL human samples. (A) Angiogenin expression in Kaposi’s Sarcoma samples. Sections of normal skin or KS tumors were analyzed by immunofluorescence staining for ANG (green) and LANA-1 (red) and counter stained with DAPI (Blue). Arrows indicate colocalization of ANG and LANA-1 in KS lesions. (B-C) Angiogenin expression in lung PEL human samples. Sections of normal lung and PEL solid lung metastasis were analyzed by immunofluorescence staining for ANG (green) and the B-lymphocyte antigen CD19 (red) in B, or LANA-1 (red) in C. Nuclei were visualized with DAPI staining (Blue). Arrows indicate colocalization of ANG with CD19 (B) or with LANA-1 (C) in PEL lesions. Magnification 20x.

Figure 2. Effect of Neomycin on the oncogenic properties of BCBL-1 cells. (A) Summary of previous findings on the in vitro role of ANG in KSHV positive endothelial and PEL cells: (a) In KSHV positive cells, we observed that: (1) ANG levels are increased, (2) ANG activated the PLC-γ pathway and consequently ERK1/2 and AKT, (3) PLC-γ activation is necessary for ANG nuclear translocation, (4) nuclear ANG participates in the maintenance of latency by upregulating latency gene expression, and (5) nuclear ANG participates in PEL cell survival. (b) Blocking ANG expression or ANG nuclear translocation has the following effects: (1) shRNA ANG and Neomycin inhibits PLC-γ activation as well as AKT activation in BCBL-1 cells, (2) Neomycin and PLC-γ inhibitor U73122 inhibits ANG nuclear translocation in BCBL-1 cells, (3) shRNA ANG or neomycin or PLC-γ inhibitor U73122 decreased ORF73 RNA levels by real-time PCR but increased ORF 50 RNA levels in BCBL-1 cells, and (4) shRNA ANG or neomycin or PLC-γ inhibitor U73122 decreased BCBL-1 cell survival by MTT. (B) Foci: BCBL-1 foci
formation was performed using a CytoSelect cell transformation assay. These were viewed under an inverted microscopy equipped with the Nikon Metamorph digital imaging system. Upper panels: Magnification 4x. Lower panels: Magnification 10x. (C) **Quantification of anchorage-independent growth:** Cells were recovered after solubilization of the agar matrix and their viability was measured by MTT assay. Each reading was done in triplicate and the data represent the mean of three independent wells ± S.E.M.. Statistical analysis was conducted using a two-tailed Student’s test. *** p<0.005.

**Figure 3. Effects of neomycin and neamine treatment in NOD/SCID mice injected with BCBL-1 cells.** (A) **BCBL-1 injected mice developed tumors:** PBS or $10^7$ BCBL-1 cells were injected i.p. into 6-weeks old mice SCID mice (Jackson). (B-D) **Angiogenin nuclear translocation inhibitors block BCBL-1 tumor development:** $10^7$ BCBL-1 cells were injected i.p. into 6 weeks old SCID mice (black arrows). Mice were injected i.p. with PBS, neomycin (10 mg/kg, 5 mice) (B), neamine (10 mg/kg, 5 mice) (C) or paromomycin (10 mg/kg, 5 mice) (D) every 2 days for 1 week (days 1, 3, 5 and 7) followed by once a week (grey arrows). The mice were euthanized by CO$_2$ after the tumor was established and before pain or distress was observed. A Kaplan-Meier curve is represented. Statistical analysis was performed using the log rank test.

**Figure 4. Effect of neomycin and neamine treatments on BCBL-1 tumor formation in NOD/SCID mice.** $10^7$ BCBL-1 cells were injected i.p. into 6-weeks old SCID mice and euthanized by CO$_2$ 7-weeks post-injection. (A) **Neomycin and neamine treated animals show reduced abdominal distention.** The animals treated with neomycin and neamine did not
develop the abdominal distention observed in PBS treated animals (white arrows). Representative pictures of the animals are shown in Aa and b. The animal weights are indicated in Ac. n: the number of animals per group. (B) Neomycin and neamine treated animals show reduced ascites development. The number of animals developing ascites is reduced for treated animals which is indicated. When ascites is observed, the volume of the ascites is reduced in treated animals. The data represent the mean ± S.E.M. Statistical analysis was conducted using a two-tailed Student’s test. * p<0.05, ** p<0.01.

Figure 5. Effect of neomycin and neamine treatments on spleen infiltration of BCBL-1 cells. (A) The spleens from neomycin and neamine treated animals are smaller than non-treated animals: $10^7$ BCBL-1 cells were injected i.p. into 6-weeks old SCID mice, euthanized by CO$_2$ 7-weeks post-injection and the spleens were removed and weighed. The spleens from untreated animals are enlarged compared to treated animals. Representative pictures are shown in (Aa) and the weights of the spleens are shown in (Ab). n: the number of animals per group. (B) The quantity of infiltrated cells is decreased in neomycin and neamine treated animals. The spleens were sectioned and stained with H&E. Representative pictures are shown in (Ba). Infiltrated cells are indicated with black arrows. An enlarged picture of infiltrated cells is shown in the right panel. The number of infiltrated cells was counted in three fields/mouse (magnification 10x), averaged, and represented as infiltrated cells/field (Bb). n: the number of animals per group. (C) Enlarged spleens in PBS treated conditions are due to infiltration of BCBL-1 cells: RNAs were extracted from mouse spleens with Trizol reagent. RNA real-time PCR was performed using ORF 73 primers as previously described (57). n: the number of animal per group. The data represent the mean ± S.E.M. Statistical analysis was conducted using a two-
Figure 6. Effect of neomycin and neamine treatments on KSHV latency and lytic gene expression in BCBL-1 cells injected into NOD/SCID mice. (A) Ascites cells recovered from the different treated animals were analyzed for KSHV LANA-1 protein expression by western blot analysis (Aa) or IFA (Ab-c). The enlarged images of the boxed areas are shown in the right panels. Arrows indicate LANA-1 punctate staining. For quantification, the number of puncta was counted for 24 cells per animal. (B) KSHV lytic envelope glycoprotein gB expression was analyzed by IFA (Ba-b). The enlarged images of the boxed areas are shown in the right panels. Arrows indicate gB positive cells. For quantification, the cells in 4 different fields (total of 100-150 cells/sample) were counted per animal and the % of gB positive cells was calculated. n: the number of animals per group. The data represent the mean ± S.E.M.. Statistical analysis was conducted using a two-tailed Student’s test. *** p<0.005.

Figure 7. Induction of apoptosis in BCBL-1 cells injected into NOD/SCID mice by neomycin and neamine treatments. Ascites recovered from the different treated animals were analyzed for the activation of caspase-3 by western blot analysis (Aa and b) or IFA (Ba and b). The boxed areas in the IFA pictures are enlarged in the right panels. Arrows indicate cleaved caspase-3 positive cells. For IFA quantification, the cells in 4 different fields (total of 100-150 cells/sample) were counted per animal and the % of cleaved caspase-3 positive cells was calculated. The number of animals per group is indicated under each graph. The data represent the mean ± S.E.M.. Statistical analysis was conducted using a two-tailed Student’s test. * p<0.05, ** p<0.02 and *** p<0.005.
Figure 8. Schematic representation depicting the anti-tumor effect of neomycin and neamine on KSHV associated lymphoma. The results presented in the present studies demonstrate the following: (A) BCBL-1 injection in NOD/SCID mice induced the formation of ascites. 7-weeks post-injection, the animals’ weight is increased and abdominal distortion is observed due to ascites establishment. In addition, BCBL-1 cells infiltrated the animals’ spleens. The mice die from the tumor development 2 months post-injection. (B) Neomycin or neamine treatment of BCBL-1 injected mice reduce ascites development. 7-weeks post-injection, the number of mice and the volume of ascites were reduced in treated animals. BCBL-1 cell infiltration in the spleen was reduced. Consequently, neomycin and neamine prolonged the life span of the treated animals. (C) Blocking ANG nuclear translocation by neomycin and neamine blocked latent gene expression and induced lytic gene expression in BCBL-1 cells injected into NOD/SCID mice. In addition, the reduced ascites establishment at 7-weeks post-injection could also be due to increased apoptosis of KSHV+ BCBL-1 cells.
REFERENCES


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A. (a) Untreated KSHV+ endothelial and PEL cells

- LANA-1
- KSHV
- ANG
- PLCγ

1. ANG
2. LAN-1

Mediates ANG nuclear translocation
Latent gene expression
KSHV latency maintenance
Anti-apoptosis

(b) shANG or Neomycin or U73122 treated KSHV+ endothelial and PEL cells

- Neomycin
- U73122
- ANG
- PLCγ

1. ANG
2. LAN-1

Blocked ANG nuclear translocation
Latent gene expression
Lytic gene expression
Apoptosis

B. BCBL-1

- BCBL-1
- BCBL-1 Neomycin (200 µM)

C. MTT Assay on Foci cells

- Neomycin 200 µM

Figure 2

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Figure 4

A

B

C

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Figure 4
Figure 5

Aa

PBS

Neomycin

Neamine

Bb

BCBL-1 injection

Treatment

- n=3

+ PBS n=5

Neomycin n=5

Neamine n=5

Spleen Weight (g)

***

***

*

Infiltrated cells per field

***

***

PBS n=5

Neomycin n=5

Neamine n=5

ORF 73 expression

* * *
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Figure 7
A

BCBL-1 PBS

7 weeks
Abdominal distortion
Weight gain
Ascites establishment
PEL infiltration in the spleens

Early death
56 days

B

Neomycin or Neamine

BCBL-1

7 weeks
Reduced weight gain
Reduced ascites establishment
Reduced PEL infiltration in the spleens

Delayed death
Neomycin 96 days
Neamine 118 days

C

PLCγ

ANG

ANG nuclear translocation

KSHV

↓ Latent gene expression
↑ Lytic gene expression
↑ Apoptosis

Neomycin/Neamine

In vivo BCBL-1 Ascites

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Figure 8