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Sulfated polymannuroguluronate, a novel anti-AIDS drug candidate, inhibits HIV-1 Tat-induced angiogenesis in Kaposi's sarcoma cells

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ABSTRACT

Kaposi's sarcoma (KS), a neoplasm often associated with iatrogenic and acquired immunosuppression, is characterized by prominent angiogenesis. Angiogenic factors released from KS and host cells and HIV viral products—the protein Tat are reported to be involved in angiogenesis. Mounting evidence further suggests that multiple angiogenic activities of Tat contribute to AIDS-associated Kaposi's sarcoma (AIDS-KS). Herein, we report that sulfated polymannuroguluronate (SPMG), a novel anti-AIDS drug candidate now undergoing phase II clinical trial, significantly eliminated Tat-induced angiogenesis in SLK cells both in vitro and in vivo. SPMG significantly and dose-dependently inhibits proliferation, migration, and tube formation by SLK cells. SPMG also dramatically arrested Tat-driven KDR phosphorylation and blocked the interaction between Tat and integrin β1, thus inhibiting the phosphorylation of the downstream kinases of FAK, paxillin and MAPKs. In addition, SPMG was noted to block the release of bFGF and VEGF from ECM. All these collectively favor an issue that SPMG functions as a promising therapeutic against Tat-induced angiogenesis and pathologic events relevant to AIDS-KS, which adds novel mechanistic profiling to the anti-AIDS action of SPMG.

1. Introduction

Kaposi's sarcoma (KS), an angioproliferation disease, is a form of malignancy which occurs in elderly or immunosuppressed patients, and is often associated with acquired immune deficiency syndrome (AIDS). KS generally arises on the skin of the extremities as multiple patches, plaques, or nodular lesions, but it can also involve mucosa and visceral organs [1].

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Abbreviations: AIDS, acquired immune deficiency syndrome; AIDS-KS, AIDS-associated Kaposi's sarcoma; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; GAGs, glycosaminoglycans; GST-Tat, glutathione S-transferase-Tat; HIV-1, human immunodeficiency virus type 1; JNK, c-jun amino-terminal kinase; KS, Kaposi's sarcoma; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; RGD, arginine-glycine-aspartic acid; SPMG, sulfated polymannuroguluronate; Tat, transactivator of transcription; VEGF, vascular endothelial growth factor; KDR, kinase insert domain-containing receptor.

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Characteristic histological features of KS include the proliferation of spindle-shaped cells (KS cells, KSC) of vascular origin considered to be the tumor cells of KS, and of normal endothelial cells forming blood vessels (angiogenesis), inflammatory cell infiltration, and edema [2]. KS is much more frequent and aggressive in the setting of human immunodeficiency virus type 1 (HIV-1) infection, suggesting that HIV itself or molecules produced during HIV infection might play important roles in KS development and progress [3].

Tat (the HIV-1 transactivator of transcription) is a small regulatory polypeptide of 86–102 amino acids released by HIV-1-infected cells, which recently has been pointed out as a key progression factor of KS due to its involvement in all the biological steps of angiogenesis and progression [4,5]. In addition to the transactivation of HIV-1 gene expression, Tat can modulate the expression of many cellular genes including those for cytokines, adhesion molecules, major histocompatibility complex class I proteins and oncogenes, as well as cellular functions such as cell survival, growth and angiogenesis [6]. In its extracellular form, Tat stimulates proliferation, adhesion, migration and invasion of KSC and normal endothelial cells activated by inflammatory cytokines, and promotes the tube formation in vitro and angiogenesis in vivo as well [7,8]. Therefore, the inhibition of Tat production or prevention of its activity could be a useful way to inhibit the development and progression of KS in AIDS patients [9,10].

Glycosaminoglycans (GAGs), such as heparin, have been critically identified as pathological chaperons in Tat-mediated biological response in targeted cells [11]. And heparan sulfate proteoglycans (HSPGs) have been shown to act as Tat receptors. Conversely, free heparin inhibits the uptake of intracellular Tat and affects the cell surface interaction mediated by Tat [12]. Growing evidences show that heparin and heparin analogs represent potent extracellular Tat antagonists of possible therapeutic value [13].

Sulfated polymannuroguluronate (SPMG), a new heparin-like sulfated polysaccharide extracted from brown alga, is rich in 1,4-linked β-D-mannuronate, with an average of 1.5 sulfates and 1.0 carboxyl groups per sugar residue and an average molecular weight of 10 kDa. SPMG has entered phase II clinical trial in China, making it the first marine sulfated polysaccharide with the potential of becoming an anti-AIDS drug. Our previous in vitro and in vivo studies demonstrated that SPMG inhibits HIV replication and may exert this effect by interfering with HIV entry into host T lymphocytes [14,15]. We further found that SPMG was able to interact with Tat via binding to its basic domain. Of particular note was the highly selective affinity of SPMG for Tat ($K_D = 8.69 \times 10^{-10}$ M), as compared with heparin ($K_D = 1.86 \times 10^{-9}$ M) but not other GAGs including heparan sulfate (HS), dermatan sulfate C, chondroitin sulfates A and hyaluronic acid. This preferentially allows SPMG to serve as a specific scaffold for physically blocking the Tat basic domain-mediated actions facilitated by cell surface heparin/HS, conferring Tat-involved pathological events. In fact, our more recent data have showed that SPMG blocks HIV-1-associated neuroinflammatory signaling by targeting the Tat protein in THP-1 cells [16].

The aim of the present study is to unravel the possible involvement of SPMG in Tat-mediated KS-associated angiogenesis, and to explore its possible mechanisms, in an additional hope to further our understanding of the unidentified roles of SPMG in AIDS-driven KS-associated angiogenesis.

2. Materials and methods

2.1. Reagents

SPMG, a new kind of sulfated polysaccharide extracted from brown algae, depolymerized by acidic hydrolysis, and then sulfatation ($M_W \sim 10{,}000$ Da), was provided by the Marine Drug and Food Institute (Ocean University of China, Qingdao, China). One hundred millimolar of SPMG was prepared by dissolving the powder in serum-free medium (SFM) and stored at 4 °C until use. Human bFGF/VEGF Quantikine immunoassay kits were obtained from R&D (R&D Systems, Minneapolis, MN). Flk-1/KDR (C-1158), integrin β1 (M-106), GST(Z-5), phosphorylated tyrosine (PY350), paxillin kinase (H-114) antibodies and protein A–agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-FAK (Tyr397) and FAK antibodies were obtained from Sigma Chemical Co. (St Louis, MO); phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-JNK (Thr183/Tyr185) and the HRP-linked anti-rabbit IgG were all obtained from Cell Signaling Technology (Beverly, MA). JNK antibody was purchased from Abcam (Cambridge, UK). CD31 (bs-0195R) and IgG-FITC (bsF-0295G) antibodies were purchased from Beijing Biosynthesis Biotechnology (Beijing, China).

2.2. Cells culture

Human Kaposi's sarcoma cell line (SLK), kindly provided by Dr. Jay A. Levy and Dr. Sophie Leventon-Kriss (The NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), was maintained in RPMI 1640 (Gibco Laboratories, Grand Island, New York, USA) supplemented with 10% foetal calf serum (FCS; Gibco), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

2.3. Purification of recombinant GST-Tat

GST-Tat was prepared as described previously [11]. Briefly, Plasmid pGEX-2T (Pharmacia, Uppsala, Sweden) and pGST-Tat were kindly provided by Profs. M. Giacca and M. Prestai (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy). Recombinant GST-Tat was expressed in *E. coli* as fusion proteins, the cells were lysed, and the lysates were mixed with 1 ml of 50% (v/v) slurry of glutathione-cross-linked agarose beads (Sigma, St. Louis, MO). The fusion protein was allowed to bind to the beads at 4 °C on a rotating wheel for 1 h. The fusion proteins were freed from nucleic acid contamination using a high-salt wash (3 M NaCl), and the purified fusion protein was eluted with 100 mM Tris containing 2 mM DTT and 20 mM reduced glutathione (Sigma, St. Louis, MO). Protein purity and integrity were routinely checked by SDS-PAGE and Coomassie blue staining. The bioactivity of recombinant proteins was evaluated using surface plasmon resonance (SPR), flow cytometry, confocal microscopy and western blotting, and the proteins were stored at −70 °C until use [16].

2.4. Cell proliferation

SLK cells (4×10^3 cells/well) were plated in 96-well plates (Costar, MA, USA) in serum-containing medium. After 24 h, the medium was replaced with SFM and cells were stimulated with GST-Tat (20 nM) in the absence or presence of SPMG (3.12 μM, 6.25 μM and 12.5 μM, respectively). Forty-eight hours later 10 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromidemide) was added to the cell medium for 4 h at 37 °C. Then the medium was removed and crystals were dissolved in DMSO. Absorbance was measured at 570 nm using a fluorescence plate reader (Fluoroskan; Tecan, Research Triangle Park, NC) and expressed as mean ± standard deviation (S.D.) ($n = 4$) per sample.

2.5. Cell adhesion assay

Ninety-six well plates were coated overnight at 4 °C with GST-Tat (40 nM), washed, and then incubated for an additional 1 h at room temperature with 1% BSA in PBS to saturate nonspecific binding sites [17]. Cells (suspended at 5×10^4/0.1 ml in SFM) were seeded in the absence or presence of SPMG (1.56 μM, 3.12 μM, 6.25 μM and 12.5 μM, respectively) and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. Plates were washed with PBS, and adherent cells were fixed with 4% paraformaldehyde and stained with crystal violet. The absorbance was read at 630 nm and expressed as mean ± S.D. S.D. ($n = 4$) per sample.

2.6. Migration and invasion assays

Cell migration and invasion assays were performed in 24-transwell chambers (Costar, MA, USA) with a polystyrene membrane (6.5 mm diameter, 10 μm thickness, and 8 μm pore size) as described previously [7]. Cells (suspended at 7×10^4/0.1 ml in RPMI 1640 containing 1% FCS) were seeded in the upper chamber. 600 μl RPMI 1640 containing GST-Tat (20 nM) were added to the lower chamber in the absence or presence of SPMG (3.12 μM, 6.25 μM and 12.5 μM, respectively). For the invasion assay, the upper chamber was pre-coated with Matrigel (Collaborative Research) (diluted to a final concentration of 1 mg/ml) which polymerizes at 37 °C for 4 h forming a basement membrane. After incubation for 8 h (for migration) or 24 h (for invasion) at 37 °C in 5% CO_2, the migrated or invaded cells to the lower side of the transwell were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells on the upper surface of the filter were removed by wiping with a cotton swab. The number of cells that had moved to the lower surface of the filter was counted in four random fields under a microscope. Each assay was conducted in triplicate and similar results were repeated at least three times.

2.7. In vitro tube formation assay

SLK tube-structure formation on Matrigel was also conducted as described previously [18]. Briefly, 70 μl Matrigel at 4 °C was added to 96-well plates and then allowed to polymerize at 37 °C, 5% CO_2 for 1 h. SLK cells were suspended at the concentration of 2×10^4/0.1 ml in RPMI 1640 with 1% FCS containing GST-Tat (20 nM) and SPMG (0 μM, 3.12 μM, 6.25 μM and 12.5 μM, respectively), negative controls were plated in RPMI 1640 with 1% FCS alone. Cells were carefully layered on top of the polymerized gel and incubated for 8 h at 37 °C 5% CO_2. Tube formation was observed and photographed under a microscope (CK40-F200, Olympus, Tokyo, Japan). The degree of tube formation was assessed by counting the number of closed tubes in four random fields from each well.

2.8. Immunoprecipitation and western blot analyses

To analyze cellular kinase activation, immunoprecipitation (IP) or western blot (WB) were performed alternatively. SLK cells, grown to confluence, were serum starved for 16–18 h and washed twice with PBS before Tat treatment. The cells were treated with SFM RPMI 1640 containing GST-Tat (20 nM) in the absence or presence of SPMG (3.12 μM, 6.25 μM and 12.5 μM, respectively) for different periods. Controls included SFM RPMI 1640 alone. The cells were rapidly washed with ice-cold PBS and lysed with ice-cold RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate) or 1% Nonidet P-40 (for the assay of interaction between Tat and integrin β1) for 20 min at 4 °C. Total-cell lysates were sonicated for 5 s and clarified by centrifugation at 10,000 × *g* for 10 min. Protein concentrations were determined using Bradford assay. For the immunoprecipitation studies, identical amounts of protein from each sample were incubated for 2 h at 4 °C with different primary antibodies for each experiment. The antibody–antigen complexes were immunoprecipitated by incubation for 2 h to overnight at 4 °C with 20 μl of the protein A–agarose. Nonspecific proteins were removed by washing the agarose beads three times with the RIPA buffer and once with PBS. Bound proteins were solubilized in 40 μl of lysis buffer (62.5 mM Tris/HCl, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol) and further analyzed by immunoblotting. Samples were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline plus Tween-20 (TBS-T) and probed with primary antibody overnight at 4 °C. Immunoreactive bands were visualized by using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

2.9. bFGF and VEGF quantification by ELISA

bFGF and VEGF proteins released into the supernatants of cultured SLK cells were measured using a commercial ELISA kit for bFGF or VEGF [8], following the manufacturer's instructions. SLK cells (8×10^4/well) were grown in 12-well plate for 24 h, and then rinsed with PBS twice. 0.5 ml RPMI 1640 with 5% FCS, containing GST-Tat (20 nM) and SPMG (0 μM, 0.78 μM, 1.56 μM, 3.12 μM, 6.25 μM and 12.5 μM, respectively) was added and incubated at 37 °C 5% CO_2 for 3 h. Negative controls were plated in RPMI 1640 with 5% FCS alone. The supernatants were collected for analysis. Assays were done in triplicate and expressed as mean ± S.D. ($n = 3$).

2.10. *In vivo angiogenesis*

We used the Matrigel sponge model of angiogenesis described previously [9]. PBS alone (as control), GST-Tat (20 nM) alone or GST-Tat (20 nM) with SPMG (12.5 μM) were added to unpolymerized liquid Matrigel at 4 °C and the mixture brought to a final volume of 600 μl. The Matrigel suspension was then injected slowly s.c. into the flanks of C57BL6 male mice with a cold syringe. At body temperature in vivo, the Matrigel quickly polymerizes to form a solid gel. Different groups of animals (three mice in each group) were used for the different treatments. All groups were provided plain drinking water *ad libitum*. After 10 days, gels were recovered by dissection, fixed in formaldehyde, and embedded in paraffin. To visualize endothelial cell infiltration, a total of 10-μm-thick sections were stained with rabbit anti-mouse CD31 antibody and then anti-rabbit IgG-FITC as secondary antibody. Immunofluorescence images were captured using a laser confocal microscope (Zeiss, Germany) equipped with a digital camera using software. Sections were also stained with hematoxylin and eosin (HE) for analysis of blood vessel infiltration. Images of histological sections were captured using a divert microscope described previously.

2.11. *Statistics*

All of the values are presented as mean ± S.D. Statistical significance was evaluated by unpaired Student's *t*-test for comparison between two means. Differences were considered statistically significant at $P < 0.05$.

3. Result

3.1. *SPMG inhibits Tat-stimulated SLK proliferation and adhesion*

Tat exerts an essential role in KS cell proliferation. In our study, Tat significantly stimulated SLK proliferation, while SPMG dose-dependently blocked SLK cell proliferation stimulated by Tat. 3.12 μM of SPMG markedly inhibited the proliferation of SLK with the percentage inhibition 56.0%, while the percentages were as high as 78.6% and 91.6% at doses 6.25 μM and 12.5 μM, respectively (Fig. 1A).

Given that Tat is also capable of inducing the adhesion of KS cells [19], we thus evaluated the effect of SPMG on cell adhesion. We found that Tat-induced the adhesion of SLK cells in a dose-dependent manner (data not shown), while SPMG at the chosen concentrations significantly and dose-dependently blocked Tat-induced SLK adhesion. The percentage inhibition was 48.9% at doses as low as 1.56 μM (Fig. 1B).

3.2. *SPMG reduces Tat-stimulated SLK migration and invasion*

As HIV-1 Tat has been consistently correlated with the metastatic potential of AIDS-KS [20], the migration and invasive activities of SLK cells were assessed in transwell chambers in the presence and absence of Matrigel coating, respectively. To determine whether SPMG interferes with the migrating and invasive properties of SLK in response to Tat, GST-Tat (20 nM) was used as chemoattractant in the lower compartment in the presence of different concentrations of SPMG. As shown in Fig. 2, the SLK cells abundantly migrated or invaded to the lower chamber upon the stimulation with Tat. However, SPMG markedly inhibited both the migration of SLK (Fig. 2A) and the invasion induced by Tat (Fig. 2C). The percentage inhibitions were strong (>50%) and significant ($P < 0.01$) (Fig. 2B and D).

3.3. *SPMG suppresses Tat-induced tube formation of SLK cells*

SLK cells form capillary-like structures when cultured on polymerized Matrigel at 37 °C. Tat increases the formation of this anastomosed cellular network as compared with untreated cells. Treatment with SPMG suppressed the effect of Tat significantly, and the inhibitory effects were obvious and potent, with the inhibition rate almost approaching to 50% (Fig. 3A). The number of tubes was calculated and shown in Fig. 3B.

3.4. *SPMG blocks Tat-mediated activation of KDR and the downstream signals*

VEGFR-2 (also known as KDR or Flk-1) is a high-affinity receptor for VEGF-A. It is expressed in both vascular endothelial and several other cell types including KS cells.

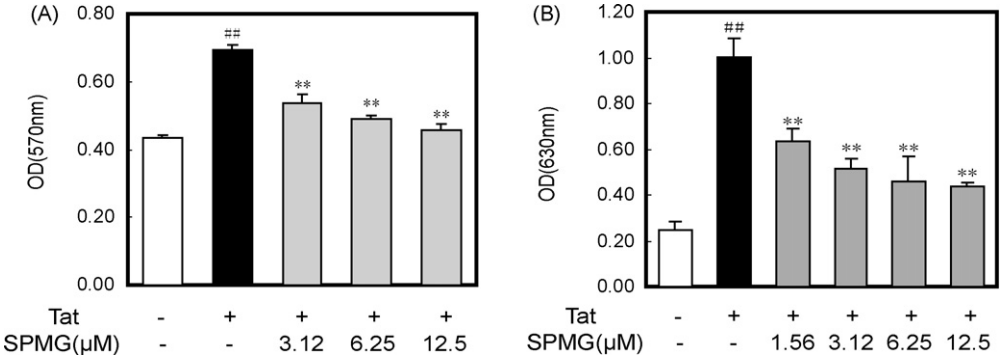

Fig. 1 – Effects of SPMG on Tat-induced SLK proliferation and adhesion. (A) The inhibitory effect of SPMG on Tat-induced SLK proliferation was measured by MTT assay. (B) The inhibitory effect of SPMG on Tat-induced SLK adhesion was measured as described in Section 2. Data are means ± S.D. ($n = 4$). $^{\#\#}P < 0.01$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Tat-treated alone.

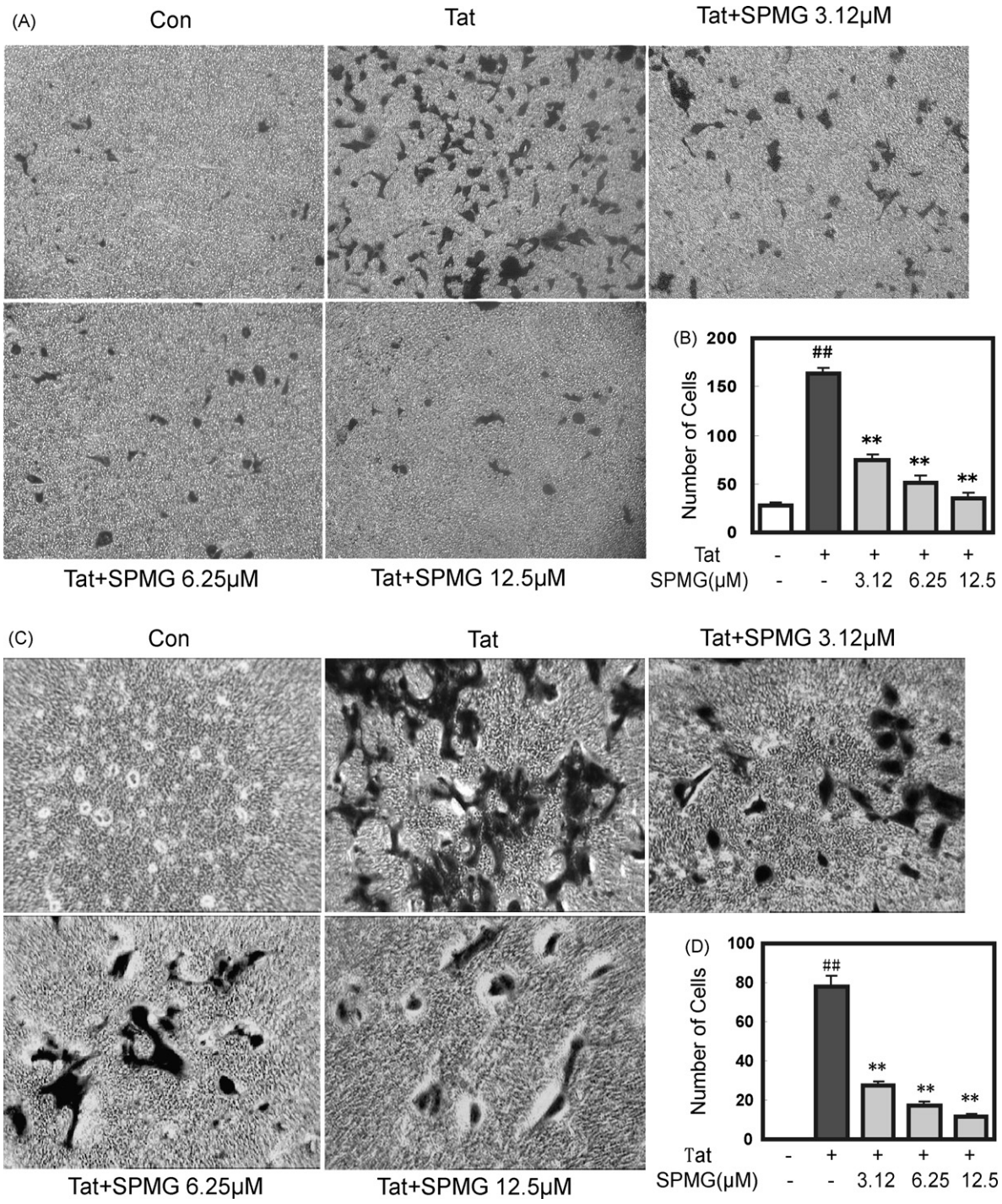

Fig. 2 – SPMG inhibits Tat-induced SLK migration and invasion. SLK were seeded in the upper chamber of the transwell and 600 μl RPMI 1640 containing GST-Tat (20 nM) in the absence or presence of SPMG were added to the lower chamber, incubating for 8 h for migration and 24 h for invasion at 37 °C in 5% CO_2. The migrated or invaded cells to the lower side of the transwell were photographed and counted in four random fields under a microscope as described in Section 2. (A, B) The inhibitory effect of SPMG on Tat-induced SLK cells migration (magnification 40×). (C, D) The inhibitory effect of SPMG on Tat-induced SLK cells invasion (magnification 100×). $^{\#\#}P < 0.01$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Tat-treated alone.

The phosphorylation of KDR triggers several intracellular signalling pathways and mediates the physiological and pathological effects of VEGF-A, including proliferation, migration and permeability [21,22]. It is noteworthy that Tat binds and activates KDR which mediates most of the VEGF angiogenic effects in vascular endothelial cells and KS cells [22]. We therefore investigated whether SPMG blocked Tat-mediated KDR activation and thus its downstream molecules

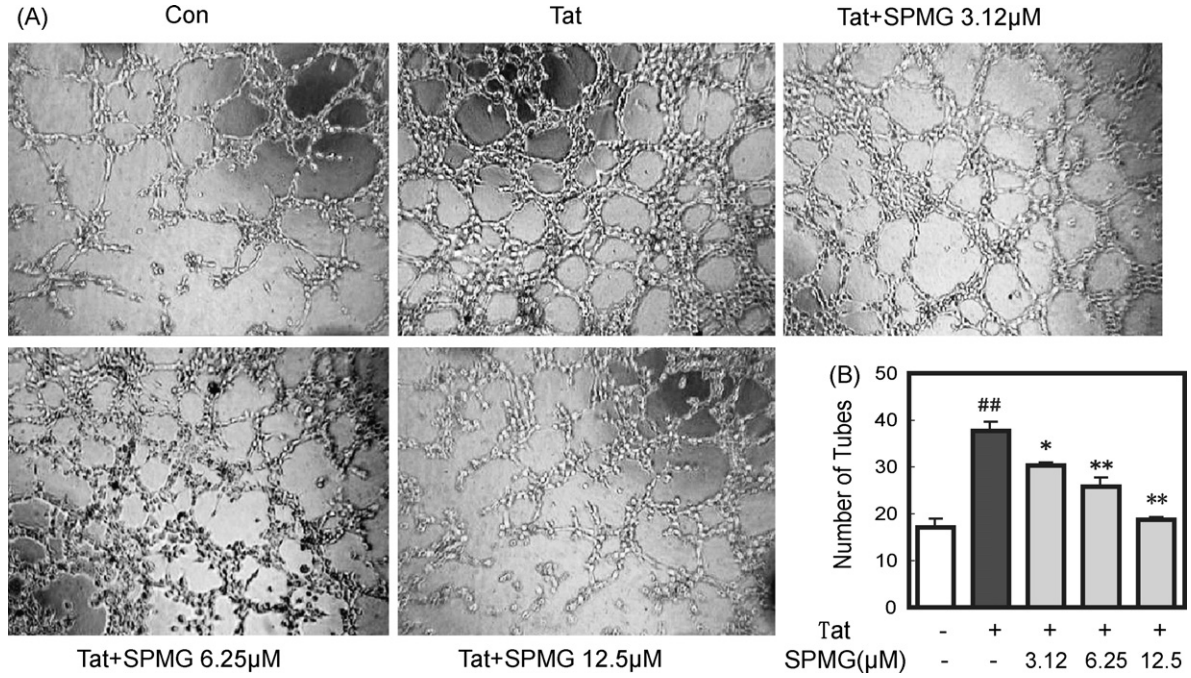

Fig. 3 – Inhibitory effect of SPMG on tube formation induced by Tat. (A) SLK were seeded in Matrigel-coated plates in RPMI 1640 with 1% FCS containing GST-Tat (20 nM) and indicated concentrations of SPMG for 8 h at 37 °C in 5% CO_2, then observed and photographed under a microscope (magnification 40×). (B) Tubes were quantified in four random fields. ##P < 0.01 vs. control, *P < 0.05, **P < 0.01 vs. Tat-treated alone.

including extracellular-signal regulated kinase (ERK)1/2 [23] and c-jun amino-terminal kinase (JNK) [24] in SLK cells.

Total KDR in cell lysates was immunoprecipitated with anti-KDR antibody and phospho-KDR was immunoblotted with anti-phosphorylated tyrosine antibody. The activation of ERK1/2 and JNK was analyzed by western blot analysis using anti-phosphoERK and anti-phosphoJNK antibodies. As shown in Fig. 4, Tat resulted in an increase in tyrosine phosphorylation of the KDR, ERK1/2 and JNK. SPMG at concentrations of 6.25 and 12.5 μM apparently dephosphorylated KDR, and subsequent ERK1/2 and JNK, respectively.

3.5. *SPMG interferes the interaction between Tat and integrin β1 and inactivates FAK and paxillin induced by Tat*

It is known that Tat can enhance angiogenesis by mimicking the effects of ECM proteins (fibronectin and vitronectin) on cell migration, invasion and adhesion through binding to α5β1 and αvβ3 integrins via its RGD domain [25]. Of note, β1 integrins are critically implicated in growth-factor-induced angiogenesis and αvβ3-mediated endothelial cell migration and angiogenesis depend on the ligation state of α5β1 to fibronectin [26]. For this reason, we preferentially investigated whether SPMG is capable of interfering the interaction of Tat with integrin β1. SLK cells were treated with GST-Tat (20 nM) in the absence or presence of SPMG at different concentrations. Total β1 was immunoprecipitated with anti-β1 antibody, and then Tat that interacted with β1 was immunoblotted with anti-GST antibody. Our studies revealed that SPMG interfered the interaction of Tat with β1 (Fig. 5, upper two panels).

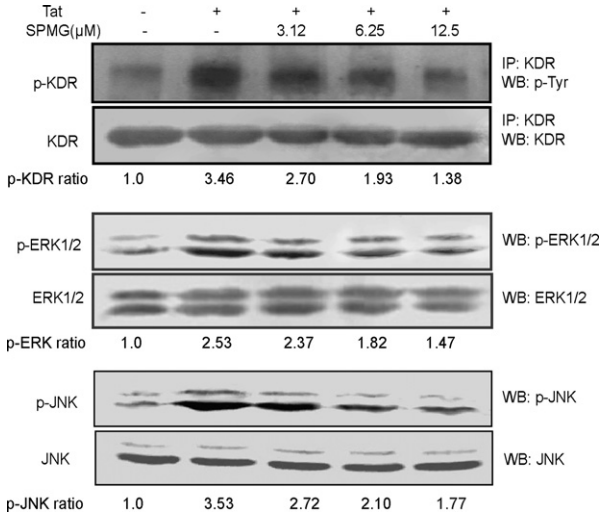

Fig. 4 – Effects of SPMG on Tat-induced activation of KDR, ERK1/2 and JNK. Serum-starved cells were stimulated with GST-Tat (20 nM) in the absence or presence of indicated concentrations of SPMG for 5 (for KDR) or 30 min (for ERK1/2 and JNK). Cell lysates were subjected to IP or WB using KDR and phospho-Tyrosine, phospho-specific and control ERK1/2, phospho-JNK and JNK antibodies. Densitometry of the developed blots was done, and the ratios between the density of phosphorylated KDR, ERK1/2, JNK and control KDR, ERK1/2, JNK were determined as relative protein band intensity, respectively. Data shown are typical from three independent experiments with similar results.

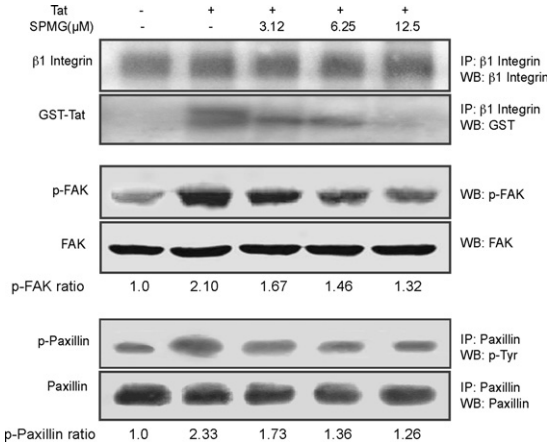

Fig. 5 – Effects of SPMG on the interaction of Tat with β1integrin and activation of integrin-associated focal adhesion kinases FAK and paxillin. Serum-starved cells were stimulated with GST-Tat (20 nM) in the absence or presence of indicated concentrations of SPMG for 30 min. Cell lysates were subjected to IP or WB using β1integrin and GST, phospho-specific or control FAK, phospho-tyrosine and paxillin antibodies. Densitometry of the developed blots was done, and the ratios between the density of phosphorylated FAK, paxillin and control FAK, paxillin were determined as relative protein band intensity, respectively. Data shown are typical from three independent experiments with similar results.

Extracellular Tat has been shown to induce the phosphorylation of tyrosine kinases localized at cellular focal adhesion plaques, which is activated by integrin triggering and has a major role in cellular adhesion and migration [27]. In particular, phosphorylation of the p125FAK induced by Tat results in the generation of downstream intracellular signals. Paxillin is a focal adhesion protein that serves as a binding site for a number of important signaling molecules, especially for FAK [28]. As a cytoskeletal protein, paxillin have been shown to be modulated during KS cell functions related to migration and adhesion [29]. We therefore investigated whether SPMG exert its inhibition effects on the FAK and paxillin activation induced by Tat. As shown in Fig. 5, treatment of SLK with GST-Tat (20 nM) resulted in an extensive phosphorylation of FAK and paxillin, when compared with the control. SPMG apparently decreased the degree of phosphorylation of FAK and paxillin, respectively.

3.6. SPMG combats the release of bFGF and VEGF from SLK retrieved by Tat

Tat is believed, by competing for heparin-binding sites, to retrieve extracellularly bound bFGF and VEGF into soluble forms which promote angiogenesis [8], and this is the most important mechanism underlying Tat-promoted angiogenesis both in vitro and in vivo. So we examined the effects of SPMG on the release of bFGF and VEGF from the SLK cells induced by Tat. ELISA measurements revealed that cells treated with 20 nM GST-Tat showed significantly higher levels of bFGF and VEGF in the media as compared with that of the control. SPMG significantly and dose-dependently blocked Tat-induced bFGF release and even occurred at concentration as low as 0.78 μM (Fig. 6A). However, the similar effect on VEGF (Fig. 6B) release needed much more SPMG at concentration as high as 12.5 μM.

3.7. SPMG inhibits angiogenesis induced by Tat in vivo

Thus far, our data showed the ability of SPMG to combat Tat-induced angiogenesis in vitro. To determine whether these in vitro activities were applicable in vivo, we employed the Matrigel plug model. This model can mimic very well normal, physiological conditions of neo-angiogenesis with quantitative assessment [30]. Matrigel suspensions containing GST-Tat (20 nM) in the presence or absence of SPMG (12.5 μM) were injected s.c. into mice as described in Section 2. After 7–10 days, the plugs were removed and assessed by HE staining (Fig. 7 upper panel) and CD31 immunostaining (Fig. 7 lower panel). The presence of Tat resulted in a strong angiogenic response with formation of new vessels that grew into the Matrigel within 10 days. SPMG showed noticeable inhibitory effect, whereas no reaction is induced by Matrigel with buffer alone. Quantification of the extent of angiogenesis by immunofluorescence staining of endothelial marker CD31

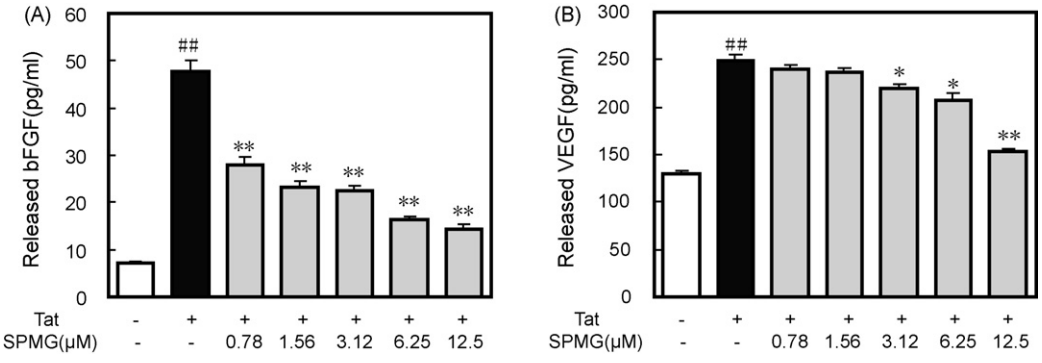

Fig. 6 – ELISA assay of released bFGF and VEGF. SLK cells were plated in 24-well plates. After 24 h of incubation, the medium was changed to RPMI 1640 with 5% FCS, containing the indicated concentrations of Tat and SPMG. Medium was collected after 3 h and released bFGF (A) and VEGF (B) were measured by ELISA assay. Results are representative of three independent experiments. $^{\#\#}P < 0.01$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Tat-treated alone.

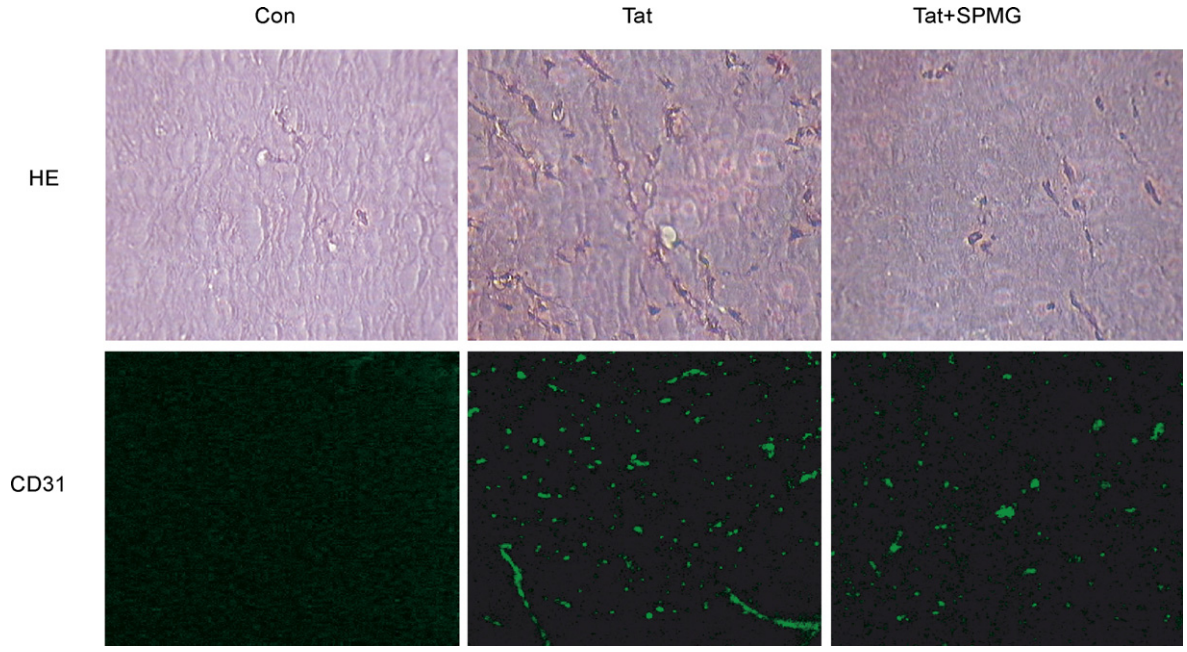

Fig. 7 – SPMG inhibits enhanced angiogenesis by Tat in Matrigel plug assay. Matrigel plug (600 μl) containing 20 nM GST-Tat alone or GST-Tat with SMPG (12.5 μM) were injected s.c. in the flank of C57BL6 mice, PBS as a control. Plugs were removed 7–10 days after their injection, fixed in formalin, and embedded in paraffin; and sections were stained with hematoxylin and eosin (HE) (upper panel). Immunofluorescence staining for endothelial marker CD31 was performed using anti-CD31 antibodies and images were taken at 40× magnification by confocal microscope (lower panel).

showed that SPMG treatment significantly reduced the neovascularization induced by Tat. All these findings indicated that SPMG is capable of inhibiting angiogenesis induced by Tat in Matrigel plug in vivo.

4. Discussion

Angiogenesis is a prerequisite for tumor growth and metastasis formation and represents a prominent feature of KS. The angiogenic properties of extracellular Tat have linked this HIV-1 protein to AIDS-KS pathogenesis since it enhances all the biological steps of angiogenesis and KS progression [6]. Here, we preferentially selected SLK cells since they are derived from a Kaposi's sarcoma tumor and in particular, they are of endothelial cell origin and capable of keeping the major characteristics of endothelial cell [31]. Expectedly, we found that the GST-Tat was able to induce SLK cell growth, adhesion, migration and tube formation in vitro, suggesting that SLK cells-based system could serve as a good surrogate in the subsequent experiments. Meanwhile, we also noted that GST moiety was unable to affect the biological function of Tat (data not shown), indicating that GST-Tat can be used instead of Tat in the relevant experiments [11,13,16].

The interaction of specific Tat domains with cellular receptors present on cell surfaces activates the signal cascades and thus a variety of functions [28]. Of the five distinct functional domains, the basic region and RGD sequence are critically involved. In particular, through the basic domain, Tat binds to KDR on KS cells and leads to its autophosphorylation which triggers several intracellular signaling pathways that mediate the proliferation and migration of KS cells [32]. In the current study, we found that Tat triggered a rapid KDR activation, which was markedly reversed by SPMG. The most reasonable explanation of this notion, together with the subsequent inactivation of downstream signaling of ERK1/2 and JNK, was likely the ultimate result of competitive inhibition of SPMG on KDR–Tat interaction due to the high and specific binding affinity of SPMG for the basic domain. These findings help uncover, at least partially, the mechanisms of the marked in vitro and in vivo anti-angiogenic action of SPMG in Tat-induced KDR-mediated events.

The released Tat by HIV-infected cells accumulates in the ECM as an immobilized protein and enables it to interact with integrins in KSC. This can promote the adhesion and trigger a complex signal transduction pathway [25,27,33]. Of this event, Tat RGD region, representing the principal cell attachment moiety [34], can bind with high-affinity to integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$, receptors for fibronectin and vitronectin, respectively. By molecular mimicry of ECM molecules fibronectin and vitronectin, Tat drives integrin-dependent activation of FAK and Paxillin phosphorylation thus involving the focal adhesion dynamics and cell migration [29,35,36]. All these cooperatively re-organize the cytoskeleton and activate diverse signaling pathways in the localized adhesion [8,19,25]. In the present study, we noted that SPMG significantly suppressed the interaction of Tat with integrin β1. Since SPMG is able to bind to the basic domain of Tat, this might permit the competitive blockage of SPMG on Tat RGD–integrin β_1 interaction probably via a conformation-based steric hindrance. The subsequent inactivation of FAK and paxillin is thus a resultant action of the above events, which together

account for the in vitro arrest of SPMG on Tat-induced adhesion to fibronectin [37,38].

In addition to the Tat-mediated events mentioned above, Tat can also compete with bFGF or VEGF and release bound bFGF and VEGF to soluble forms [8,39]. Being potent angiogenic factors, bFGF and VEGF both act as the key mediators of Tat-induced KS growth. One should take particular note that Tat is increasingly recognized not to synergize with VEGF in promoting angiogenesis, but by contrast, to enhance bFGF-driven endothelial mitogenesis [40]. In our study, SPMG exhibited significant but unequal contribution to the Tat-induced release of bound bFGF and VEGF from ECM, with bFGF being much more influenced. Though a precise mechanism underlying this disparity remains far from clear, the preferential inhibition on bFGF rather than VEGF favors the beneficial role of SPMG for bFGF-associated Tat-involved mitogenesis.

In sum, we herein show for the first time that SPMG, a novel anti-AIDS drug candidate, functions as a potent Tat antagonist to inhibit Tat-induced angiogenesis. These notions, together with the fact that SPMG is well-tolerated and effective in AIDS-infected individuals in both phase I and phase II clinical trials, provide important new insights into SPMG, which may prove to be a promising therapeutic intervention for AIDS-related Kaposi's sarcoma.

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