Inhibition of Tumor Angiogenesis by Targeting Endothelial Surface ATP Synthase with Sangivamycin

Yusuke Komi1,2, Osamu Ohno3,5, Yasuhiro Suzuki1,6, Mariko Shimamura4, Kentaro Shimokado2, Kazuo Umezawa3 and Soichi Kojima1

1Molecular Cellular Pathology Research Unit, RIKEN, Wako, Saitama, 2Vascular Medicine and Geriatrics, Tokyo Medical and Dental University Graduate School, Tokyo, 3Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama and 4Medical R&D Center, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

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Background: Sangivamycin, an antibiotic with anti-tumor and anti-herpes virus activities by inhibiting both DNA/RNA synthesis and protein kinase C activity, was reported to suppress selectively DNA synthesis and growth of human umbilical vein endothelial cells and their tube formation in vitro. Here, to address the potential clinical use of sangivamycin in future, we investigated its anti-angiogenic effect in in vivo chicken chorioallantoic membrane (CAM) and mouse dorsal air sac (DAS) assays, and investigated underlying mechanism.

Methods: The effect of sangivamycin on blood vessel formation in CAM was observed under the microscope after treating for two days. For DAS assays, chambers fulfilled with tumor cells were implanted beneath mouse dorsal skin. After the mice were administered with sangivamycin, tumor-induced angiogenesis was observed under the microscope. The effect of sangivamycin on ATP synthesis on the endothelial cell surface was assayed by measuring ATP production with bioluminescence assay.

Results: Sangivamycin suppressed angiogenesis within CAM down to 94–71%, which was partially blocked by simultaneous addition of a 40-fold excess of adenosine. Sangivamycin also inhibited tumor-angiogenesis in the DAS assay by 61%, and suppressed ATP production on the endothelial cell surface by 75%.

Conclusion: Sangivamycin inhibits the in vivo angiogenesis within CAM and tumor-induced angiogenesis within mouse dorsal skin, at least in part via inhibiting endothelial cell surface ATP metabolism in addition to inhibition of DNA/RNA synthesis and/or protein kinase C activity, suggesting a potential clinical use of sangivamycin as a novel anti-cancer reagent capable of targeting not only cancer cells but also endothelial cells.

Key words: sangivamycin — tumor angiogenesis — ATP synthase

INTRODUCTION

Angiogenesis, formation of new blood vessels, is essential for development, reproduction and wound healing. It is also a key event in tumor cell growth, because angiogenesis is required for supplying nutrition to tumor cells and provision of the route for metastasis (1,2). Endothelial cells play an essential role in angiogenesis, and are activated by several angiogenic factors, e.g. vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), produced from tumor cells. Therefore, the endothelial cell is a good target for the treatment of solid tumors, and in fact, many inhibitors of angiogenesis have been reported to diminish tumors by suppressing the growth and functions of the endothelial cells (3,4). VEGF mainly binds to its receptor, KDR/Flik-1, and activates MAP kinase via protein kinase C (PKC). Therefore, PKC inhibitors were known to inhibit angiogenesis (5).
Inhibition of angiogenesis by sangivamycin

Sangivamycin is a potent PKC inhibitor (6). We previously reported that sangivamycin selectively inhibited the growth of human umbilical vein endothelial cells (HUVECs), while other protein kinase C inhibitors, such as calphostin C, H-7 and staurosporine, did not (7).

Moser et al. (8) reported that angiostatin, a potent angiogenesis inhibitor, binds to the α- or β-subunit of mitochondria-type F₁₋F₀-adenosine triphosphate (ATP) synthase on the surface of HUVECs, and inhibits endothelial cell proliferation and migration. Other groups reported that extracellular ATP synthase, F₁ catalytic domain is mandatory for endothelial cells proliferation (9). The presence of ATP synthase at the cell surface of lymphocytes, hepatocytes and some tumor cells has been reported (10,11). These reports suggest that inhibition of ATP synthase may selectively inhibit the growth of endothelial cells, implying the possibility of sangivamycin being such a novel angiogenic inhibitor.

In this study, we characterized the anti-angiogenic activity of sangivamycin in vivo, and investigated the underlying mechanism. We found that sangivamycin inhibited angiogenesis, at least in part, by suppression of ATP synthase activity on the endothelial cell surface, suggesting a promising, clinical application of this drug in near future.

MATERIALS AND METHODS

Reagents

Sangivamycin was isolated from the culture filtrate of Streptomyces as described elsewhere (7) or purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), and dissolved in ethanol or dimethyl sulfoxide (DMSO) to yield a stock solution of 60 mg/ml (194 mM). Adenosine was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA) and dissolved in DMSO to yield a stock solution of 300 mg/ml (1.1 M).

Chicken Chorioallantoic Membrane (CAM) Assay

The formation of new blood vessels within chicken CAM was assessed as described previously (12). In brief, fertilized Dekalb chicken eggs (Omiya Kakin, Saitama, Japan) were placed in a humidified egg incubator. After a 4.5-day incubation at 38°C, a 1% solution of methylcellulose containing sangivamycin at various concentrations was loaded inside a silicon ring that was placed onto the surface of the CAM. After further incubation for 2 days, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid. Anti-angiogenic responses were evaluated under a stereomicroscope and photographed with a 7.25× objective. Quantitative analyses were performed with the angiogenesis-measuring software (version 2.0; KURABO, Osaka, Japan). Six eggs were analyzed in each treatment group, and all experiments were repeated three times.

Histology of CAM Tissues

CAM tissues removed from chicken embryos were fixed with 4% paraformaldehyde for 1 h, gradually dehydrated with alcohol and embedded in paraffin (McCormick Scientific, LLC, St Louis, MO, USA). Vertical sections (5 µm) were mounted on slides, stained with hematoxilin and eosin, and observed under a Leica model DM IRB microscope (Leica Microsystems, Wetzlar, Germany).

Mouse Dorsal Air Sac (DAS) Assay

Tumor-induced blood vessel formation was assessed as described previously (13). In brief, Millipore chambers (Millipore Co., Billerica, MA) were filled with either Roswell Park Memorial Institute (RPMI)-1640 medium alone or a suspension of 4 × 10⁶ Lewis lung carcinoma (LLC) cells in the medium and sealed with membrane filters (0.45 µm pores). Chambers were implanted subcutaneously in dorsal air sacs, created surgically by injection of an appropriate volume of air, in 7-week-old female ICR mice (Charles River Co. Ltd, Yokohama, Japan). These mice were intraperitoneally administered every other day with sangivamycin (15 mg per kg body weight) dissolved in a solution of 5% DMSO, 15% Cremophor EL (Sigma-Aldrich, Inc., St Louis, MO, USA) and 5% glucose in saline. One and 3 days after, mice were killed with an overdose of diethyl ether. The skin was carefully removed and angiogenesis that had been induced around the chamber was examined under a stereomicroscope and photographed with a 5.6× objective. Quantitative analyses were performed with angiogenesis-measuring software (version 2.0; KURABO, Osaka, Japan). Each experimental group included six mice, and each experiment was repeated at least twice. No obvious adverse effect appeared after treatment of animals with sangivamycin. All surgical procedures were performed under pentobarbital (Dainabot, Osaka, Japan) anesthesia. All animal experiments were performed according to the guidelines of the Animal Experiments Committee of RIKEN.

Cell Cultures

HUVECs were purchased from Cell Systems (Kirkland, WA, USA) and were cultured on dishes coated with type I-C collagen (Nitta Gelatin Inc., Osaka, Japan) in MCDB-131 medium (Sigma-Aldrich, Inc., St Louis, MO, USA) supplemented with 10% fetal calf serum (Invitrogen Co., Carlsbad, CA, USA) and 10 ng/ml recombinant human basic FGF (bFGF; R&D Systems Inc., Minneapolis, MN, USA).

In Vitro Tube Formation Assay

Tube formation by HUVECs on matrigel was assessed as described previously (7). Unpolymerized Matrigel (Becton Dickinson, Bedford, MA, USA) was diluted to a final concentration of 5 mg/ml with MCDB-131 medium, aliquoted 150 µl each into 24-well plates, and allowed to polymerize...
for 30 min at 37°C. HUVEC were seeded onto the polymerized gel at 2 x 10^5 cells/well; then 20 ng/ml bFGF, 10 ng/ml sangivamycin and/or 10 μg/ml adenosine were added, and incubated for 6 h. *In vitro* tube formation was examined under a phase-contrast microscope and photographed with a 40x objective.

**Measurement of ATP Generation on the Cell Surface**

The effect of sangivamycin on ATP generation on the surface of HUVECs was measured by bioluminescent luciferase assay as described by Moser et al. (8). Confluent HUVECs grown on 24-well plates were washed and the medium was changed to serum-free α-modified minimum essential medium (αMEM; Invitrogen Co., Carlsbad, CA, USA) containing 10 mM potassium phosphate (Sigma-Aldrich Inc., St Louis, MO, USA). After cells had been untreated (control cells) or treated with sangivamycin for 1 h, all cells were incubated with 50 μM ADP for 1 min, and supernatants were harvested. After centrifugation to remove cell debris, the amount of ATP generated and released into each supernatant was determined from the activity of luciferase that is emitted depending on the amount of ATP in the sample, using an ATP bioluminescence assay kit (Sigma-Aldrich Inc., St Louis, MO, USA).

**Statistical Analysis**

Data are expressed as means ± SD or ± SE. Statistical significance was assessed by one-way analysis of variance followed by Sheffe’s t-test.

**Results**

**Effect of Sangivamycin on In Vivo Blood Vessel Formation**

To assess the effect of sangivamycin on *in vivo* angiogenesis, we first performed CAM assay (Fig. 1). Sangivamycin inhibited the angiogenesis within CAM in a dose-dependent manner at 1–6 μg/egg in the methylcellulose solution (Fig. 1A). Quantitative analyses indicated 29% inhibition in area and 53% inhibition in number of branches following treatment with 6 μg/egg sangivamycin. Figure 1B shows the result of hematoxylin and eosin staining of vertical sections of CAM tissues. As seen in the left panel, 6.5-day-old CAM was composed of four different layers, including a thin chorionic epithelium (ce), microvasculatures (mv), a thick mesenchymal layer (fb) consisting of sparsely distributed fibroblasts and a few small blood vessels and a thin allantoic epithelium (ae). Capillaries were filled with nucleated erythrocytes. Exposure to 6 μg/ml sangivamycin for 48 h remarkably reduced the number of capillaries developed underneath the chorionic epithelium as compared with the untreated control vehicle (Fig. 1B, right panel).

Next, to see whether sangivamycin might affect tumor angiogenesis, we performed DAS assays using LLC (Fig. 2). Tumor-induced blood vessel formation, which is normally characterized by strikingly disorganized and tortuous vessels, was essentially absent in mice that had been implanted with a chamber that contained only RPMI-1640 medium without tumor cells (Fig. 2a). When mice were implanted with a chamber that contained LLC cells and then vehicle was administered intraperitoneally, blood vessels were induced towards the chamber from pre-existing blood vessels beneath the epidermis (Fig. 2b). Such tumor-induced blood vessel formation was, however, reduced by the administration of sangivamycin at 15 mg/kg body weight (Fig. 2c). Quantitative analysis indicated that the area of blood vessels induced by tumors was reduced to 39 ± 12% after treatment with sangivamycin as compared with the control value in mice treated with the vehicle only.

**Relief of Sangivamycin-inhibited Blood Vessel Formation by Adenosine**

We previously reported that adenosine neutralized the inhibition of DNA synthesis by sangivamycin in HUVECs (7). Therefore, we examined whether adenosine also might cancel the inhibitory effect of sangivamycin on angiogenesis, in both CAM (Fig. 3A) and an *in vitro* tube formation (Fig. 3B). As shown in Fig. 3A(a), angiogenesis was induced in CAMs treated with only vehicle (control eggs). Suppression of angiogenesis by sangivamycin (Fig. 3A(b)) was partially rescued by simultaneously treating eggs with a combination of sangivamycin and a 40-fold excess of adenosine (Fig. 3A(d)). Quantitative analyses indicated that the 74% inhibition in area seen following treatment with 6 μg/egg sangivamycin was restored to 89% inhibition by simultaneous incubation with 240 μg/ml adenosine. Moreover, to determine whether adenosine relieved inhibition of *in vitro* tube formation by sangivamycin in HUVECs, we examined the effect of treatment of sangivamycin together with adenosine on *in vitro* tube formation by HUVECs. HUVECs seeded on the Matrigel formed a closely knit meshwork of capillary-like structures 6 h after stimulation with 20 ng/ml bFGF (Fig. 3B(b)). Ten nanograms per milliliter (10 ng/ml) sangivamycin inhibited tube formation in HUVECs induced by bFGF (Fig. 3B(e)), which was partially canceled by addition of 10 μg/ml adenosine (Fig. 3B(f)).

**Inhibition of ATP Synthase**

F1→F0 ATP synthase on the endothelial cell surface has been reported to be important for endothelial cell survival and proliferation (8,9). Therefore, to determine whether sangivamycin might inhibit ATP synthesis on the HUVEC surface and this inhibition might result in apoptosis of the cells, leading to avascularization, we measured the effect of sangivamycin on the ATP production in the extracellular medium using a bioluminescence assay as described in the Materials and Methods section. Sangivamycin inhibited ATP synthesis in a dose-dependent manner (Fig. 4A). We further
found that efrapeptin, a specific inhibitor of ATP synthase, also inhibited angiogenesis within CAM in a dose-dependent manner at the same concentration range as sangivamycin, namely 1–6 μg/egg in the methylcellulose solution (Fig. 4B). Quantitative analyses indicated that 23% inhibition in the number of newly formed branches and 29% inhibition in area were seen following treatment with 6 μg/egg efrapeptin.
DISCUSSION

In this paper, we reported that sangivamycin, known to be a potent inhibitor of PKC, suppressed angiogenesis in two different in vivo models. We further showed that the anti-angiogenic effect of sangivamycin was abrogated by adenosine. Moreover, treatment of endothelial cells with sangivamycin resulted in reduced activity of cell surface ATP synthase, a target of angiostatin (8), implying that this unique drug might suppress in vivo angiogenesis through inhibition of endothelial cell surface ATP synthase activity. Sangivamycin did not affect the expression levels of ATP synthase in the same cells (data not shown). We previously reported that sangivamycin inhibited DNA synthesis more clearly in HUVECs compared with WI-38 human lung fibroblasts, and this inhibition was canceled by adenosine (7). This tendency was not seen in PKC inhibition (7). Because it is known that only endothelial cells and a couple of other blood cells express ATP synthase on their surface (10), a reduction in its activity might partially account for the cell-type selective growth inhibition with sangivamycin, leading to suppression of in vitro tube formation and in vivo angiogenesis with this compound. The mechanism, by which sangivamycin inhibits cell surface ATP synthase remains to be elucidated. Inhibition of extracellular ATP synthase with efrapeptin, an F1 catalytic domain-targeting drug as well as antibody to this domain has been reported to suppress ATP synthesis (9,14), inhibit proliferation of HUVECs (9), and now shown to inhibit angiogenesis in vivo (Fig. 4). Based upon this result and structural analogy between sangivamycin and adenosine, it is suggested that sangivamycin would target the F1 catalytic domain and directly inhibit its activity as a substrate analog. Monoclonal antibody directed against the beta-catalytic subunit of ATP synthase inhibits the F1 catalytic domain, and selectively inhibits ATP synthase and tube formation in low-pH environment as well as in tumor microenvironment (15). Inhibition of angiogenesis by sangivamycin might be more effective in tumor angiogenesis.

The newly discovered sangivamycin’s action as a cell surface ATP synthase inhibitor raises another question about the mechanism of its anti-angiogenic effect. It is reported

Figure 3. Adenosine neutralizes the ability of sangivamycin to suppress angiogenesis in CAM. (A) The 4.5-day-old CAMs were treated with or without 6 μg/egg sangivamycin and/or 240 μg/egg adenosine for 48 h and then patterns of angiogenesis were photographed. Scale bar, 1 mm. (a) vehicle (8% DMSO); (b) 6 μg/egg sangivamycin; (c) vehicle plus 240 μg/egg adenosine; and (d) 6 μg/egg sangivamycin plus 240 μg/egg adenosine. The total area of blood vessels was analyzed with angiogenesis-measuring software and is shown under each panel. Values are means ± SD (n = 6). Relative changes are shown as a percentage of control. Asterisks indicate significant differences (P < 0.05) from controls. A representative result from two independent experiments with similar results is shown. (B) Human umbilical vein endothelial cells (HUVECs) were seeded onto polymerized Matrigel at 2 × 10⁵ cells/well; then 20 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml sangivamycin and/or 10 μg/ml adenosine were added, and incubated for 6 h. Patterns of tube formation were photographed. Scale bar, 250 μm. (a) Vehicle (1% methanol); (b) vehicle plus 20 ng/ml bFGF; (c) vehicle plus 20 ng/ml bFGF and 10 μg/ml adenosine; (d) 10 ng/ml sangivamycin; (e) 10 ng/ml sangivamycin plus 20 ng/ml bFGF; and (f) 10 ng/ml sangivamycin plus 20 ng/ml bFGF and 10 μg/ml adenosine.
that extracellular ATP and adenosine modulate neutrophil function, including chemotaxis (16). Released ATP from the leading edge of migrating neutrophils is rapidly metabolized to ADP, AMP, and adenosine by ecto-ATP-diphosphohydrolase-1 and ecto-5'-nucleotidase. Metabolized ATP and adenosine initiate and accelerate directional chemotaxis via P2Y2 and A3 adenosine receptors, respectively, on neutrophils (16). Tumor cells are known to incorporate neutrophils and this contributes to induction of angiogenesis (17). Furthermore, it is reported that stimulation of mast cells via their adenosine receptors results in releasing angiogenic factors (18). Therefore, it could be possible that sangivamycin might inhibit neutrophil and/or mast cell surface adenosine receptors and that these would contribute to suppression of tumor angiogenesis by sangivamycin in vivo.

In summary we found that sangivamycin inhibited in vivo tumor-angiogenesis, at least in part, via suppression of ATP synthase on the endothelial cell surface. This result suggests that sangivamycin might selectively suppress in vivo angiogenesis via selective inhibition of endothelial cell proliferation. This unique property of sangivamycin promises that sangivamycin will be used in a combinational therapy with hitherto examined angiogenic inhibitors under clinical trials in near future.

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Conflict of interest statement

None declared.
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