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Luminacins: A Family of Capillary Tube Formation Inhibitors from *Streptomyces* sp.

II. Biological Activities

TOSHIAKI WAKABAYASHI*, RENA KAGEYAMA-KAWASE, NOBUAKI NARUSE, YASUHIRO FUNAHASHI and KENTARO YOSHIMATSU

Tsukuba Research Laboratories, Eisai Co. Ltd.,
5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan

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Twelve of the fourteen isolated components of the luminacin family were assayed for activity to inhibit capillary tube formation *in vitro*. Seven of them showed potent activity with IC_{50} values of less than 0.1 μg/ml in a rat aorta matrix culture model. Luminacin D, the strongest inhibitor, inhibited both endothelial cell proliferation and capillary tube formation. Morphological observation suggested that luminacin D inhibited the rearrangement of endothelial cells in the initial stage of tube formation. Luminacins and their derivatives are good candidates for application as angiogenesis inhibitors with a novel mechanism of action.

Angiogenesis consists of capillary formation from pre-existing vessels and is stringently controlled under normal circumstances in the adult[1]. However, angiogenesis can be activated in response to tissue injury or inflammation, which are closely related to pathological situations such as rheumatoid arthritis and diabetic retinopathy[2]. Solid tumors also stimulate angiogenesis, and the concept that the tumor growth depends on angiogenesis is well established[3]. Several angiogenesis inhibitors are currently undergoing clinical trials as antitumor agents.

The initiation of capillary formation involves focal reduction of intercellular interactions and interactions between endothelial cells and the surrounding extracellular matrix (ECM). This is associated with a loss of pericytes and smooth muscle cells. In tissues, many angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and hepatocyte growth factor (HGF) are induced[4~7], and stimulate both endothelial cells and mesenchymal cells to form new capillary vessels. Such capillary formation is a complex biological process involving cell migration, proliferation and tube formation of endothelial cells. Although many phases of capillary formation overlap, it is clear that complex orchestration is required in order for angiogenesis to proceed. The mechanisms of tube formation are not yet well understood at the molecular level.

Fragments of rat thoracic aorta within type I collagen gel provide a good model of the angiogenic process, including cell migration, proliferation and tube formation as a whole[8]. The capillaries in this model (rat aorta tube formation: RATF model) branch, develop lumina and form networks. The aortic fragments contain several types of cells, such as endothelial cells, smooth muscle cells and fibroblast cells, which co-ordinate to form capillary vessels in the collagen gel. Furthermore, angiogenic factors such as bFGF and VEGF have been detected in the culture medium of aortic fragments, and may play a key role in this model[9]. By using this model, we previously found a novel angiogenesis inhibitor, borrelidin, among microbial metabolites[10].

In this report, we describe the anti-angiogenesis activity in the RATF model of a novel microbial metabolite complex, luminacin, which includes fourteen structurally related components[11]. Luminacin inhibited branching and tube formation without decreasing the number of migrating cells. This activity was confirmed in another angiogenesis model using human umbilical vein endothelial cells

(HUVEC) sandwiched between two Type I collagen gel layers, in which the interaction of type I collagen fibrils with the apical cell membrane is important for the induction of tube formation[12].

Materials and Methods

Materials

MCDB 131 medium, RPMI 1640 medium and Hank's balanced salt solution were purchased from Kurorera (Tokyo, Japan), Nissui (Tokyo, Japan) and Gibco BRL (Gaitherburg, USA), respectively. Type I collagen gels were obtained from Nitta Gelatin (Tokyo, Japan).

HUVEC were isolated according to the method of JAFFE *et al.* with modification[13] and cultured with E-GM medium (Kurabo; Osaka, Japan) containing 2% fetal calf serum (FCS). Cells were obtained from the following sources: WI-38 and WiDr, Dainippon Pharmaceuticals; H-520 and MDA-MB-231, American Type Culture Collection; MDA-MB-435, Eisai Research Inc., USA; SD 6, described previously[10]. Cells were cultured in RPMI 1640 media containing 10% FCS.

Rat Aorta Matrix Culture Assay

Rat aorta matrix gels were prepared by the method described previously[8]. Briefly, thoracic aortas were obtained from 8- to 12-week-old male Sprague-Dawley rats. The fibroadipose tissue around the aorta was carefully removed and rinsed with Hank's solution. The aortas were cut into small fragments (2×2 mm) and transferred to wells of a 24-well culture plate containing collagen gel (0.5 ml). After collagen gelation, MCDB 131 medium (0.5 ml) was added on top of the gel in the wells. The medium was changed to extract- or luminacin-containing medium on day 2 and on day 5. At 7 days after the beginning of the experiment, (day 8), microvessels were counted under a light microscope. For assay of tube disruption, 1 ml of medium containing luminacin was added to the gels. Testing was conducted using four cultures per dose and results were confirmed by repeated experiments.

Cell Growth Assay

Cells were seeded on a 96-well culture plate at 1×10^3 cells/well for HUVEC and 2×10^3 cells/well for other cells, followed by addition of luminacin on the next day. At 3 days after addition of luminacin, the ratios of surviving cells were measured by means of the MTT assay[14]. All experiments were done at least in duplicate.

Tube Formation Assay

HUVEC were suspended in Human Endothelial-SFM Basal Growth Medium (Gibco BRL) supplemented with 10 ng/ml EGF and 20 ng/ml bFGF, seeded on 0.4 ml of collagen gel in a 24-well culture plate (1.2×10^5 cells/well) and allowed to spread overnight. The same volume of collagen gel (0.4 ml) was overlaid after removal of the culture media. After collagen gelation for 90 minutes, serial concentrations of luminacin were added (1.5 ml/well). At 4 days after the addition of luminacin, cells and capillary tubes were photographed under a light microscope.

Results

In the RATF model, new microvessels originate from the ends of aortic fragments within type I collagen gel under a serum-free medium. Migrating cells appear after 3 to 5 days, followed by microvascular sprouts, and vascular growth continues for 1 to 2 weeks. Luminacin D remarkably decreased the number of capillary tubes in a dose-dependent manner in 7 days (Fig. 1). The inhibitory activity was very strong and the IC_{50} value was 0.017 μg/ml. Nevertheless, the number of migrating cells was hardly decreased and the cells were located around the aortic fragment, with cell-cell contact. Twelve components of the luminacin inhibitor complex were investigated, and the results are shown in Table 1. Two components, E_3 and G_2, were not available in sufficient amount to examine the activity. The potency was classified into high, moderate and low categories. The highly active group consisted of luminacins C_1, C_2, D, E_1, E_2, F and G_1. Luminacin H was moderately active. Luminacins A_1, A_2, B_1 and B_2 were in the low activity group, showing no inhibition at less than 1 μg/ml. These groups correspond with the structural classification according to the C-1 substituent of benzene chromophore (see our previous report[11]). Since luminacin derivatives showed anti-angiogenesis activity in the RATF model, the growth-inhibitory activity toward endothelial cells was also investigated (Table 1). All the luminacins inhibited the growth of HUVEC, though the activities of the four components in the low activity group were as weak as they were in the RATF model. However, in the high activity group, the potencies of endothelial cell growth inhibition did not coincide with the activities in the RATF model. Luminacin D was less active (IC_{50} 0.18 μg/ml) and E_2 was the most potent (IC_{50} 0.06 μg/ml), suggesting that the anti-angiogenesis activity in the RATF model resulted from both endothelial cell growth inhibition and another mechanism of action.

Fig. 1. Anti-angiogenesis activity of luminacin D.

Rat aortic fragments were cultured with luminacin D at the indicated concentrations in collagen gel for 7 days. The number of capillary tubes was counted periodically under a microscope. Data are expressed as means (n=4).

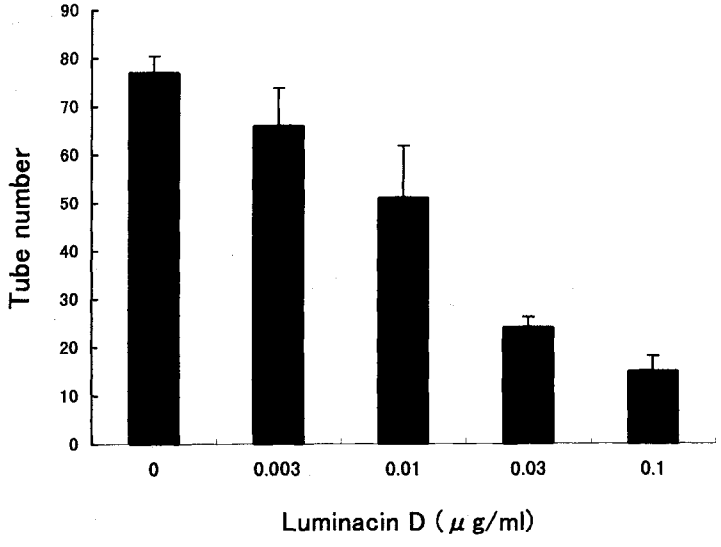


Table 1. Anti-angiogenesis and endothelial cell-antiproliferative activity of luminacins.

Luminacin	Anti-angiogenesis activity IC_{50} (μg/ml)	Endothelial cell-anti-proliferative activity IC_{50} (μg/ml)
A_1	> 1	6.5
A_2	> 1	7.1
B_1	> 1	17
B_2	> 1	18
C_1	0.053	0.24
C_2	0.050	0.08
D	0.017	0.18
E_1	0.047	0.21
E_2	0.067	0.06
F	0.065	0.11
G_1	0.038	0.07
H	0.38	0.52

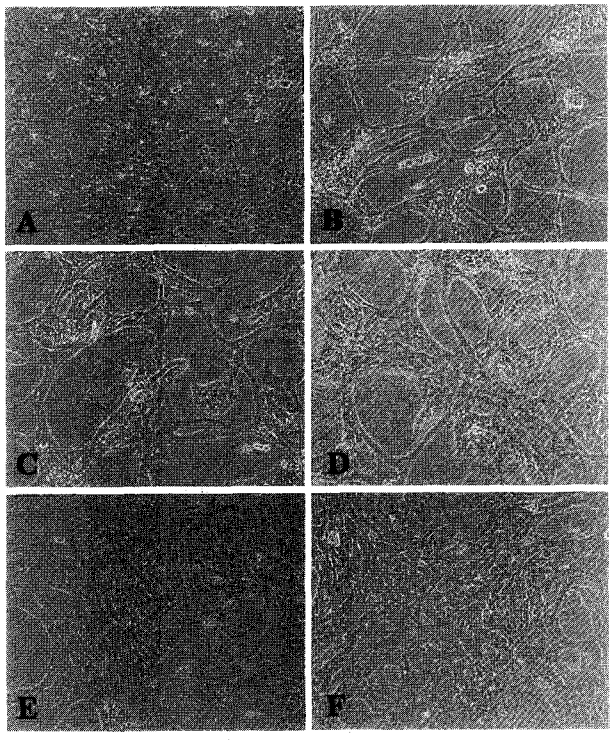
The anti-proliferative activity of luminacin D was examined with several cell lines, including tumor cell lines. Though smooth muscle cells and fibroblast cells are involved, as well as endothelial cells, in the angiogenesis process in the RATF model, the results indicated that luminacin D selectively inhibited endothelial cell growth (Table 2). The anti-proliferative activity of luminacin D towards non-endothelial cells including tumor cells is more

Table 2. Anti-proliferative activity of luminacin D on various cell lines.

Cell line	Origin	IC_{50} (μg/ml)
SD 6	rat smooth muscle	17.4
WI 38	human fibroblast	3.9
WiDr	human colon cancer	4.8
H-520	human lung cancer	8.0
MDA-MB-435	human breast cancer	8.0
MDA-MB-231	human breast cancer	5.6

Fig. 2. Morphological changes of HUVEC in the collagen gel sandwich model.

(A) HUVEC formed a confluent monolayer on collagen gel before being overlaid with a second gel. Cell morphology of capillary tubes 4 days after application of the collagen gel: without luminacin D (B), with luminacin D 0.1 μg/ml (C), 0.3 μg/ml (D), 1 μg/ml (E), 3 μg/ml (F). Magnification, ×100.



than 100-fold lower than the anti-angiogenesis activity, as shown in Table 1.

In order to clarify the presumed second mode of action of luminacin D, we investigated the activity in a collagen gel sandwich model (TF) which forms a tube-like network of endothelial cells in 4 days when HUVEC cultured on Type I collagen gel are overlaid with a second collagen gel (Fig. 2-A, B). In this model, proliferation is completely suppressed and the relevant event is capillary tube formation, including cellular reorganization or adhesion/dysadhesion. Luminacin D clearly inhibited the formation of tube-like network in a dose-dependent manner, as shown in Fig. 2. It was active at the concentration of 0.1 μg/ml of luminacin D (Fig. 2-D), and morphological change was dramatically observed at more than 1 μg/ml (Fig. 2-E, F). The observations were consistent with those in the RATF model. Cells did not begin to form a network, and the cell-cell contact remained tight. The cell number did not decrease even at the concentration of 3 μg/ml in 4 days (Fig. 2-F), indicating that luminacin D selectively inhibited tube formation, but was not cytotoxic.

Discussion

We previously isolated a complex consisting of fourteen components, named luminacins A_1 to H, from *Streptomyces*. The inhibitory activities toward tube formation (RATF model and TF model) and endothelial cell proliferation suggest that these compounds are angiogenesis inhibitors.

Luminacin contains an epoxide structure, like some other angiogenesis inhibitors derived from microbial metabolites, including fumagillin[15], eponemycin[16], radicicol[17] and rhizoxin[18]. It has been reported that TNP-470, a synthetic derivative of fumagillin, selectively inhibits endothelial cell proliferation through inhibiting methionine aminopeptidase[19], while eponemycin inhibits proliferation and cell migration of endothelial cells, radicicol inhibits some protein kinase activities[20] and rhizoxin exhibits anti-tubulin activity[21]. Thus, although an epoxide structure may be important for anti-angiogenesis activity, it does not seem to determine the target molecule. The results also suggest that the carbonyl structure in the benzene moiety is important for high activity. Further work is needed to establish the structure-activity relationship of luminacins in detail.

The RATF model permits us to observe the course of the angiogenesis process, *i.e.*, cell migration, proliferation and tube formation. The mode of inhibitory action of luminacin was different from those of other natural inhibitors tested,

such as borrelidin and fumagillin. Borrelidin decreased the number of both capillary tubes and migrating cells. Fumagillin inhibited the growth of endothelial cells, but did not affect the migration of cells, including smooth muscle cells[22]. Other angiogenesis inhibitors such as matrix metalloprotease (MMP) inhibitor[23], VEGF receptor kinase inhibitor[24], tyrosine kinase inhibitor[25] and integrin antagonist[26] also did not impair cell migration (data not shown). On the other hand, luminacin inhibited cell scattering and the cells formed a sheet-like structure around aortic fragments. These results strongly suggest that luminacin has an unique activity. This mode of action could be dramatically observed in the TF model, in which interaction of type I collagen fibrils with the apical cell membrane induces tube formation. Signals including angiogenic factors are transmitted into the cytoplasm and nuclei, leading to cell rearrangement[27]. It may be that luminacin D blocks one of the signaling pathways, because the cell morphology in the presence of a high concentration of luminacin D did not change to form a network structure after the second collagen gel was overlaid (Fig. 2). In other words, luminacin may modulate cell-cell and/or cell-ECM adhesion. Although little is known about the molecular mechanism of capillary tube formation, adhesion molecules such as cadherin 5[28], CD 31[29] and integrin $\alpha 2$[30] seem to play roles. Antibody experiments have demonstrated that inter- and intracellular interactions *via* these adhesion molecules are necessary for tube formation. Furthermore, the expression levels of these molecules change in the process of tube formation.

Molecules closely related or identical to luminacin C_1 and C_2 are known as mentioned in our previous report. However, this is the first report of anti-angiogenic activity of such compounds, and the other twelve components of luminacin are structurally novel. The reported compounds showed activities of immunosuppression[31] and low density lipoprotein (LDL) uptake enhancement[32]. The relationship, if any, of these activities to the angiogenic activity remains to be established.

We are currently planning to examine further the anti-angiogenic activity of luminacins in animal models, including solid tumor models.

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