Borrelidin is an Angiogenesis Inhibitor; Disruption of Angiogenic Capillary Vessels in a Rat Aorta Matrix Culture Model

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Borrelidin, an antibiotic from *Streptomyces rochei*, was found to be an angiogenesis inhibitor in a rat aorta matrix culture model which forms capillary vessels *in vitro*. Borrelidin strongly inhibited capillary tube formation with a 50%-inhibitory concentration value of 0.8 nM, and decreased the number of capillary tubes within 24 hours when added after maturation of tube formation. Borrelidin remarkably disrupted capillary tubes in a dose-dependent manner, by inducing apoptosis of the tube-forming cells.

Angiogenesis, the formation of new blood vessel capillaries, plays an important role in a variety of pathological conditions, such as tumor growth, diabetic retinopathy, rheumatoid arthritis and atherosclerosis[1]. Furthermore, the growth of solid tumors to beyond microscopic size, 1~2 mm in diameter, depends on the development of an adequate blood supply through angiogenesis[2]. On the basis that an anti-angiogenesis strategy might represent a new therapeutic approach for the treatment of solid tumors, several angiogenesis inhibitors are currently undergoing clinical trials[3~6].

Angiogenesis is a complex biological process whose regulatory mechanisms are incompletely understood: it is influenced not only by growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) secreted by tumor or mesenchymal cells[7], but also by extracellular matrix components and proteases[8]. Although it is possible to investigate proliferation, migration and proteolytic activity using endothelial cells isolated from the vascular wall, those cultures represent only a partial reconstruction of *in vivo* angiogenesis.

In contrast, fragments of rat thoracic aorta form microvessels (capillary tubes) in 7 days in a serum-free matrix culture within type I collagen gel[9]. This angiogenesis model (RATF model) is stimulated by VEGF, bFGF and PDGF[10], and reproduces more accurately *in vivo* angiogenesis than can be done with endothelial cells alone.

We have used this model to screen micobial metabolites for angiogenesis-inhibitory activity. Borrelidin was isolated as an antibiotic in 1949[11]. It shows high activity against certain micrococci, but not against most common test bacteria[12]. Furthermore, activities against spirochetes of the genus *Treponema*[13], viruses and tumor cells have been reported[14,15]. Its mode of antibiotic action in sensitive microorganisms involves selective inhibition of threonyl tRNA synthetase[16].

In this report we show that borrelidin has a strong inhibitory activity in the angiogenesis model, and also causes disruption of formed capillary tubes in a dose-dependent manner by inducing apoptosis of the capillary-forming cells.

Materials and Methods

Materials

Cycloheximide (CHX) and actinomycin D (AcD) were purchased from Sigma (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase (TdT), biotinylated dUTP and streptoavidin-peroxidase were obtained from Takara (Shiga, Japan), Boehringer Mannheim (Tokyo, Japan) and Seikagaku Industry (Tokyo, Japan), respectively. Human umbilical vein endothelial cells (HUVEC) and E-GM medium were purchased from Kurabo (Osaka, Japan). Rat smooth muscle cells were isolated by us and other cells were obtained from Dainippon Pharmaceuticals (Osaka, Japan) and American Type Culture Collection (Rockville, USA). MCDB 131 medium, RPMI 1640 medium and HANK's balanced salt solution were purchased from Kurorera (Tokyo, Japan), Nissui (Tokyo, Japan) and Gibco BRL (Gaithersburg, USA),

respectively. Collagen gels were obtained from Nitta Gelatin (Tokyo, Japan). Endothelial cell growth supplement (ECGS) was purchased from Becton Dickinson (Two Oak Park, MA, USA).

Rat Aorta Matrix Culture Assay

Rat aorta matrix gels were prepared by the method described previously[9]. 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-wells 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 borrelidin-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 borrelidin was added to the gels. Testing was conducted using four cultures per dose and results were confirmed by repeated experiments.

Cell Growth Assay

HUVEC (1×10^3 cells) were cultured with E-GM medium containing 2% fetal calf serum (FCS) in 96-well plates. At 3 days after addition of borrelidin, the ratios of surviving cells were measured by means of the MTT assay. Other cell lines were cultured with RPMI 1640 media containing 10% FCS. The cell toxicity assay was carried out as follows: 1×10^4 HUVEC per well (6-well plate) were cultured with borrelidin. After 24 hours, cells were recoved from the plate by trypsin-EDTA treatment and the cell numbers were counted under a microscope.

Macromolecular Biosynthesis Assay

HUVEC (1×10^4 cells) were cultured with 500 μl of E-GM medium containing 2% FCS in a 24-well plate. After 1 day, the culture medium was changed to fresh medium containing borrelidin. ^{3}H-Labeled thymidine, uridine or leucine was added at 1 hour after borrelidin treatment and the cells were cultured for 1 hour, then washed with PBS followed by 5% trichloroacetic acid, and solubilized with 0.25 M NaOH. The amount of radioisotope incorporated into the cells was measured with a liquid scintillation counter.

Threonyl tRNA Synthetase Assay

Threonyl tRNA synthetase was prepared from KB cell lysate, and the assay was performed by the method described previously[17].

TUNEL Method

Labeling of nuclear DNA was performed by the method described previously[18]. Gels were fixed with 4% paraformaldehyde for 2 hours at 4°C, then rinsed with phosphate-buffered saline (PBS) containing 0.1% Tween 20, and endogenous peroxidase was inactivated by soaking them with 5% H_2O_2 for 5 hours. The gels were then rinsed and reacted with TdT and biotinylated dUTP for 30 minutes. Staining was performed with strepto-avidin-peroxidase.

Results

Among several thousand strains of actinomyces, broth of Mer-N7167 strain, a strain of *Streptomyces rochei*, showed a potent inhibitory activity in the RATF model at the concentration of 0.00065%. We isolated the active product by Shephadex LH-20 column chromatography followed by silica gel column chromatography and identified it as borrelidin[11,16] on the basis of NMR, IR and mass spectrometry (Fig. 1).

As shown in Fig. 2, borrelidin strongly inhibited the tube formation of rat aorta; the IC_{50} value was 0.4 ng/ml (0.8 nM). The anti-proliferative activity towards human umbilical vein endothelial cells (HUVEC) was 15-fold weaker than the anti-angiogenesis activity in the same medium (IC_{50} = 6 ng/ml). Anti-proliferative activities of borrelidin towards various other cell lines were examined, and the results (Table 1) suggest that this compound has a general activity towards various cell types.

In the aortic angiogenesis experiment, migrating cells appeared after 3~5 days, followed by microvascular sprouts, and vascular growth continued for 1 to 2 weeks after the beginning of the experiment. To study the mechanism of action, we treated aortic collagen gel culture with borrelidin (2 ng/ml) over three different

Fig. 1. Structure of borrelidin.

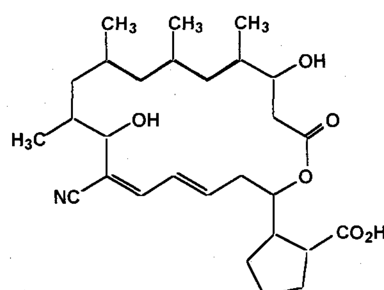
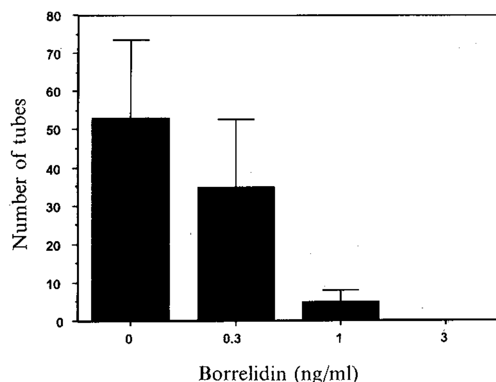


Fig. 2. Anti-angiogenesis activity of borrelidin.

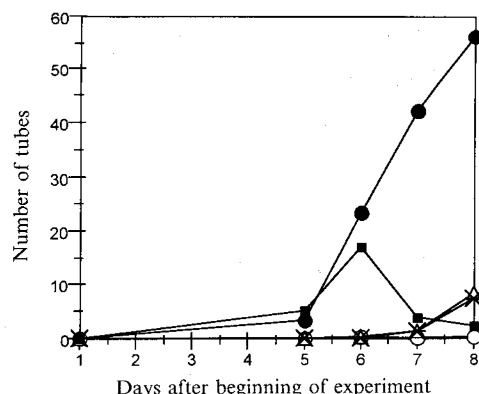


Rat aortic fragments were cultured with borrelidin at the indicated concentrations in collagen gel for 7 days. The number of capillary tubes was counted periodically under a microscope. Data are expressed as means (n=4).

Table 1. Effect of borrelidin on cell growth.

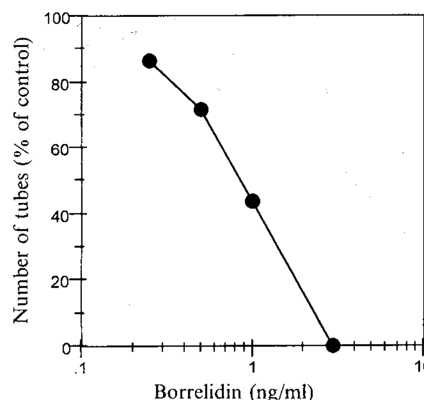
Cell line	Origin	IC_{50} (ng/ml)
HUVEC	Human umbilical vein	6.0
P388	Mouse leukemia	7.6
B16	Mouse melanoma	7.2
WiDr	Human colon carcinoma	49.3
KB	Human epidermoid carcinoma	80.0
H460	Human lung carcinoma	87.0
SD 6	Rat smooth muscle	32.5
NRK	Normal rat kidney	74.0

Fig. 3. Growth curves of capillary tubes in the presence of borrelidin.



Aortic gel culture was treated with borrelidin (2 ng/ml) for different periods: not treated (●), treated during days 2~8 (○), days 2~4 (△), days 4~6 (▲), or days 6~8 (■). Data are expressed as means (n=4).

Fig. 4. Capillary tube disruption by borrelidin.

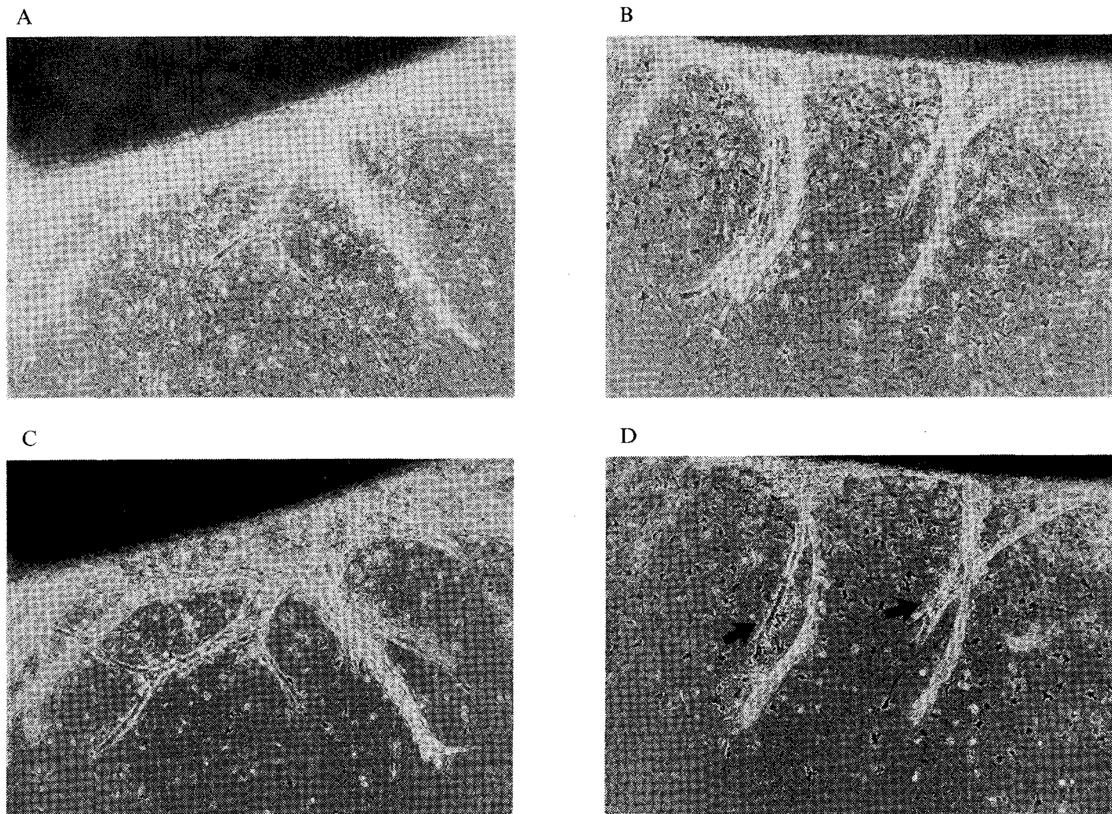


On day 6, borrelidin was added to the aortic gel culture, and capillary tubes were counted after 24 hours. Data are expressed as means (n=4) and by percent of control.

periods, days 2~4, 4~6 and 6~8, and counted the number of tubes on day 8. Borrelidin showed similar effects in each case, and day 6~8 treatment actually decreased the number of capillary tubes (Fig. 3). To investigate the action of borrelidin on capillary tubes in detail, borrelidin was added to aortic gel culture when the aorta had formed 20~30 capillary tubes (on day 6). Within 24 hours borrelidin disrupted the capillary tubes in a dose-dependent manner, and the tubes completely disappeared at the dose of 3 ng/ml; the IC_{50} was about 1 ng/ml (Fig. 4). During this time, the cell death of endothelial cells composing the tubes was observed under a microscope (Fig. 5-B, C). Other migrating cells, perhaps smooth muscle cells[19], were unaffected (Fig. 5-C), indicating that borrelidin selectively acted toward endothelial cells. In order to clarify whether or not the mechanism of cell death induced by borrelidin involves apoptosis, we applied TUNEL method[18], which detects nicked DNA. At 4 hours after treatment, the gels containing capillary tubes and migrating cells were clearly stained (data not shown). Therefore, it seems that borrelidin induces apoptosis of capillary-forming cells, leading to the disruption of capillary tubes. Borrelidin inhibited protein synthesis as well as DNA synthesis in HUVEC, with IC_{50} values of about 10 ng/ml (Fig. 6), suggesting that these activities may account for the anti-proliferative activity.

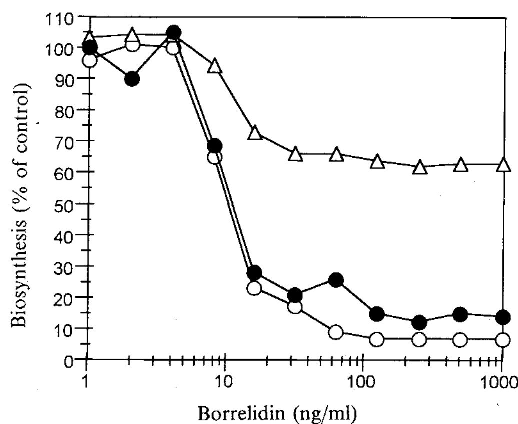
Borrelidin also showed potent inhibition in mouse dorsal air sac model[20], a model of tumor-angiogenesis, at the dose of 1.8 mg/kg by intraperitoneal injection (in preparation), confirming that borrelidin is a potent angiogenesis inhibitor.

Fig. 5. Photomicrographs of capillary tube disruption by borrelidin.



Rat aortic fragments were cultured in the absence (A; 0 hour, C; 24 hours) or presence (B; 0 hour, D; 24 hours) of borrelidin (1 ng/ml). In the culture treated with borrelidin for 24 hours, capillary tubes were disrupted (arrow) and migrating cells around tubes remained without morphological change (D). Magnification, ×100.

Fig. 6. Effect of borrelidin on macromolecular synthesis in HUVEC.



HUVEC were plated into 24-well plates at a density of 1.3×10^4 cells/well. [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]-leucine was added at 1 hour after the borrelidin treatment. After incubation for 1 hour, the amount of DNA (○), RNA (△) or protein (●) synthesis was measured.

Discussion

Borrelidin strongly inhibited angiogenesis in the RATF model including cell migration, cell growth, capillary tube formation[9] and it disrupted formed capillary tubes within a short time. This activity only appeared within the collagen gel matrix culture, since cultured endothelial cells treated with borrelidin did not show growth inhibition at the same drug concentration. Borrelidin reported to be a bacterial threonyl tRNA synthetase inhibitor[16], inhibited DNA and protein synthesis (Fig. 6) as well as the mammalian threonyl tRNA synthetase (data not shown).

It has been reported that cycloheximide (CHX) and actinomycin D (AcD), inhibitors of protein and RNA synthesis, respectively, inhibited tube formation of endothelial cells grown on Matrigel, which were derived from extracellular matrix of mouse ESH cells[21]. CHX also disrupted capillary tubes during 24 hours treatment in the rat aorta model, but the effective dose was higher than that required to inhibit protein synthesis (IC_{50} = 1 μg/ml for capillary tubes, 100 ng/ml for protein

synthesis; data not shown). CHX is known to inhibit protein synthesis by inhibiting the translocation of peptidyl tRNA and the release of deacylated tRNA[22]. CHX and its derivatives were also reported to selectively inhibit ras protein synthesis[23]. The above results imply that the survival of capillary tubes may require some protein synthesis.

Borrelidin induces the disruption of capillary tubes at a lower dose concentration than that required to inhibit protein synthesis ($IC_{50} = 1$ ng/ml for capillary tubes, 10 ng/ml for protein synthesis) and selectively acts to endothelial cells. The activity of capillary tube disruption is 100-fold stronger than that of CHX compared with the activity for protein synthesis, indicating that the mechanism of action in borrelidin is different from that of CHX. Using the TUNEL method, we found that the capillary tube disruption by borrelidin resulted from apoptosis of tube-forming cells. Apoptosis is an important homeostatic mechanism that maintains correct cell numbers in the body by balancing cell production with cell death. BROOKS *et al.* reported that monoclonal antibody against integrin induces tube disruption accompanied with apoptosis in a CAM model[24]. They proposed that when angiogenesis begins, individual vascular cells divide and begin to move toward the angiogenic source, then $\alpha v \beta_3$ integrin is induced especially by bFGF, and suppresses the apoptosis of endothelial cells involved in the angiogenic process. It may be that borrelidin inhibits a signal in the pathway from integrin, or the synthesis of a regulatory protein. However, further investigation is necessary to understand the molecular basis of the anti-angiogenic mechanism.

Borrelidin may be useful as a tool for studying endothelial cell apoptosis.

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