POSSIBLE FUNCTIONAL GROUPS RESPONSIBLE FOR INHIBITION OF *IN VIVO* ANGIOGENESIS BY HERBIMYCIN A

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Six herbimycin A (HBM) derivatives were examined for their anti-angiogenic effects in a bioassay system involving chorioallantoic membranes (CAMs) of growing chick embryos on the basis of our previous observation that HBM is a potent angiogenesis inhibitor. 17-Cyclopropylamino-HBM dose-dependently inhibited embryonic angiogenesis. The ID₅₀ value was 0.1 μ g (160 pmol) per egg and thereby lower than that of the parent compound HBM (ID₅₀=0.15 μ g (260 pmol) per egg). In contrast, 19-dimethylamino-, *N*-acetyl-, 2,3,4,5-tetrahydro- and 7-decarbamoyle-HBM at doses of 0.01-10 μ g/egg failed to affect angiogenesis in CAMs. These results strongly suggest as follows: (1) C-19 position, amino group between positions C-1 and C-20 and carbamoyl group in C-7 are essential for the anti-angiogenic action of HBM; (2) HBM needs certain fixed conformation for expression of angiogenesis inhibition; (3) it is expected that the modification of C-17 with a suitable functional group results in increased anti-angiogenic potency of HBM - - that is, a more potent angiogenesis inhibitor than the parent compound would be developed.

KEYWORDS herbimycin A derivative; anti-angiogenic activity; embryonic angiogenesis

Angiogenesis involves a dynamic cascade reaction, during which differentiation of vascular endothelial cells is a fundamental event.²⁾ Namely, in the process of angiogenesis vascular endothelial cells have both migrative and proliferative abilities, and subsequently differentiate into vessel-forming quiescent phenotype cells. In addition to that neovascularization occurs during normal physiological processes, such as embryogenesis and the corpus luteum formation, angiogenesis, which is not self-controlled, is closely associated with various disorders, including tumor growth and psoriasis, which are called angiogenic diseases.³⁾ Progressive interest in angiogenesis was mainly triggered by the phenomenon of tumor-induced angiogenesis. Through newly formed vascular networks, tumors receive nutrients and oxygen required for progressive growth, and also metastasize in other distant organs, suggesting that tumor-related angiogenesis would be a potential target of cancer therapy. Indeed, a variety of types of angiogenesis inhibitors have the potency to inhibit the growth and/or metastasis of several experimental animal tumors. And some of these inhibitors are undergoing clinical trials.⁴⁾

We previously found that an antibiotic herbimycin A (HBM) exerted a dose-dependent angiogenesis-inhibitory effect in an *in vivo* assay system involving chorioallantoic membrane (CAM) of growing chick embryo, and proposed that microbial metabolites would be an important source of useful angiogenesis inhibitors.⁵⁾ Subsequently, our and other groups have shown that various microbial products, including a sulfated polysaccharide-peptidoglycan compound, fumagillin analogs, eponemycin and radicicol analogs, have anti-angiogenic activity.⁶⁻⁸⁾ Here we examined the anti-angiogenic actions of several derivatives of HBM for the purpose of finding a derivative showing more potent anti-

angiogenic activity than the parent compound, as well as determining a functional group in a HBM molecule which is responsible for its anti-angiogenic action.

HBM derivatives used in this study were synthesized as described previously.⁹⁾ Ethylene-vinyl acetate copolymer 40 (EV), a generous gift from Mitsui-DuPont Polychemicals, Tokyo, Japan, was exhaustively washed with an excess of EtOH before use. Fertilized eggs were obtained from Ohmiya Kakin Lab., Ohmiya, Japan.

Anti-angiogenic activity was assessed in a CAM assay as described previously.^{10,11)} Briefly, fertilized eggs were incubated in a humidified egg incubator at 37°C for 4.5 days and an EV pellet containing various doses of HBM or its derivatives was placed on the surface of the 4.5-day-old CAM. After an additional 2-day incubation under the same condition, approximate volume of 20% fat emulsion was injected into the chorioallantois to show the vascular network better. When the test sample produced an avascular zone of 3-mm diameter or over in the CAM examined, the anti-angiogenic response was scored as effective. At least 7 eggs were used for each experimental group. Data on anti-angiogenic activity were analyzed by Fisher's exact probability test, with $P < 0.05$ as the level of significance.

The effects of HBM and its derivatives on embryonic angiogenesis were examined by placing EV pellets impregnated with increasing doses of these compounds on growing chick embryo CAMs. The chemical structure of HBM and dose-response relationships for the appearance of an avascular zone are shown in Fig. 1. HBM inhibited angiogenesis in a dose-dependent manner with the ID_{50} value of 150 ng (260 pmol) per egg, which reconfirmed our previous observation.^{5,10)}

Fig. 1. Inhibitory Effects of HBM and Its Derivatives on Embryonic Angiogenesis

The 4.5-day CAM was treated with an EV pellet impregnated with increasing doses of each test sample for 2 days, after which their anti-angiogenic effects were determined by measuring the avascular zone. HBM (open circles), 17-cyclopropylamino-HBM (closed circles), 19-dimethylamino-HBM (open triangles), *N*-acetyl-HBM (closed triangles), 2,3,4,5-tetrahydro-HBM (open squares), and 7-decarbamoyl-HBM (closed squares) were used. An empty EV pellet did not affect angiogenesis in any of 31 control CAMs used.

Similarly, treatment with 17-cyclopropylamino-HBM resulted in inhibition of angiogenesis in CAMs (Fig. 1). Its potent anti-angiogenic activity was dose-dependent, and the ID_{50} value was 100 ng (160 pmol) per egg, which implies that 17-cyclopropylamino-HBM shows more potent anti-angiogenic activity than the parent compound on a molar basis. At a dose of 10 µg/egg, HBM and the derivative with anti-angiogenic activity affected the growth of both CAMs and embryos. A comparison of anti-angiogenic potency of the present two angiogenesis inhibitors with that of the angiostatic agents we previously identified⁷⁾ reveals that HBM and 17-cyclopropylamino-HBM have moderate anti-angiogenic effects; these two angiostatic compounds appear to exhibit weaker anti-angiogenic activity than four synthetic retinoids, Re 80 (ID_{50} =6.3 pmol/egg), Ch 55 (ID_{50} =22 pmol/egg), Am 580 (ID_{50} =23 pmol/egg) and Am 80

(ID₅₀=28 pmol/egg), 22-oxa-1 α ,25-dihydroxyvitamin D₃ (ID₅₀=96 pmol/egg), eponemycin (ID₅₀=0.25 pmol/egg) and staurosporine (ID₅₀=71 pmol/egg), and seem to show more potent angiogenesis-inhibitory activity than medroxyprogesterone acetate (ID₅₀=260 nmol/egg), retinol (ID₅₀=4.2 nmol/egg), 15-deoxyspergualin (ID₅₀=960 pmol/egg), erbstatin (ID₅₀=450 pmol/egg) and radicicol (ID₅₀=540 pmol/egg). On the other hand, five other derivatives of HBM at doses of 0.01-10 μ g/egg did not affect embryonic angiogenesis (Figs. 1 and 2). At present we cannot conclusively exclude the possibility that the anti-angiogenic abilities of HBM and its six derivatives depend on the differences in their physical properties, such as the difference in the ease of their release from an EV pellet, a sustained support, and the difference in their solubility on the CAM. Taking all the results together in consideration, it may be possible that preferable modification of C-17 leads to an increase in anti-angiogenic ability of HBM. Also, it might imply the possibility that C-19, the amino group between positions C-1, and C-20 and the carbamoyl group in C-7 have fundamental functions in expression of anti-angiogenic action of HBM.

Fig. 2. Effects of HBM and Its Derivatives on Angiogenesis in CAMs

Note the presence of an avascular zone (indicated by arrows) in CAM treated with an EV pellet containing HBM (B) or 17-cyclopropylamino-HBM (C) and the absence of an avascular zone in CAM treated with an EV pellet alone (A), 19-dimethylamino-HBM (D), 19-methylpiperazino-HBM (E), *N*-acetyl-HBM (F), 2,3,4,5-tetrahydro-HBM (G) or 7-decarbamoyl-HBM (H), a dose of each sample being 1 μ g/egg. magnification, $\times 1.2$.

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