Vol. 174, No. 3, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1991 Pages 1070-1076

Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent

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Received January 4, 1991

The anti-angiogenic activity of AGM-1470, a new synthetic analog of fumagillin isolated from *Aspergillus fumigatus*, was extensively examined both *in vitro* and *in vivo* using four different types of assay and compared to that of the fumagillin parent. Locally administered AGM-1470 inhibited the angiogenesis in the chick embryo chorioallantoic membrane assay and the rat corneal assay. In the rat sponge implantation assay, systemically administered AGM-1470 inhibited angiogenesis induced by basic fibroblast growth factor. Furthermore, in the rat blood vessel organ culture assay, AGM-1470 (1-1,000 ng/ml) was found to selectively inhibit the capillary-like tube formation of endothelial cells with a minimal effect on the non-endothelial cell growth. AGM-1470 showed more potent anti-angiogenic activity and less toxicity than the fumagillin parent. Therefore, AGM-1470 is much better than the fumagillin parent as anti-angiogenic compound.

Angiogenesis, formation of new blood vessels, plays important roles in a variety of physiological states such as wound healing, corpus luteum formation and embryonic development, and participates in many pathological states such as diabetic retinopathy, arthritis and inflammation (1,2). It has also been shown that angiogenesis is essential for the growth of solid tumors. Therefore, it is thought that anti-angiogenic agents might be clinically useful for therapy of these diseases. Several anti-angiogenic agents have been reported in recent years. However, only a few of them have been shown to have pharmacological effects in animal models in association with anti-angiogenic activity; protamine (3,4), angiostatic steroids in combination with heparin (5,6), sulfated polysaccharide-peptidoglycan complex (7,8) and platelet factor-4 (9,10) are reported to exhibit antitumor activity besides anti-angiogenic activity. Although these compounds are expected

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Abbreviations: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified minimum essential medium; FBS, fetal bovine serum; EVA, ethylene-vinylacetate copolymer; CAM, chorioallantoic membrane; sc, subcutaneously.

0006-291X/91 $1.50

to be applied clinically, they still have problems with regard to efficacy, toxicity or structural heterogeneity.

Recently, Ingber *et al.* (11) reported a novel angiogenesis inhibitor, fumagillin, a natural product of *Aspergillus fumigatus*. They also demonstrated that AGM-1470 (O-(chloroacetyl-carbamoyl)fumagillol), one of the new synthetic analogs of fumagillin, exhibited potent inhibitory activity on endothelial cell and solid tumor growth. However, anti-angiogenic activity of AGM-1470 was not described. We therefore studied extensively the anti-angiogenic potency of AGM-1470 using four different assay systems *in vitro* and *in vivo* and compared to the fumagillin parent.

Materials and Methods

Materials: Basic fibroblast growth factor (bFGF) from bovine brain was purchased from R & D Systems (Minneapolis). Methylcellulose (4,000 centipoise) was obtained from Fisher Scientific (Fair Lawn). Hemoglobin assay kits were purchased from Wako Pure Chemicals (Osaka). Bovine fibrinogen and thrombin were obtained from Sigma (St. Louis). Dulbecco's modified minimum essential medium (DMEM) was obtained from Flow Laboratories (Irvine). Fetal bovine serum (FBS) was purchased from Whittaker Bioproducts (Walkersville). Ethylene-vinylacetate copolymer (EVA) was obtained from Takeda Chemical Ind.(Osaka).

Chorioallantoic membrane (CAM) assay: CAM assay was carried out according to the procedure described previously (6) with a minor modification using the CAM of a chick embryo cultured without the shell. A mixture of an ethanolic solution of AGM-1470 and an aqueous solution of methylcellulose was dried, and the resulting pellet was placed on the CAM of 6-day embryos. After 2 or 3 days, formation of avascular zones was examined using a stereomicroscope.

Rat corneal micropocket assay: Rat corneal micropocket assay was carried out as described previously (12). Both an EVA pellet containing bFGF (250 ng) and an empty EVA pellet or an EVA pellet containing AGM-1470 were implanted into rat corneas. Ten days later, the anti-angiogenic activity of AGM-1470 was examined using a stereomicroscope.

Sponge implantation assay: Sponge implantation assay was carried out using Sprague-Dawley rats or C57BL/6 mice (Charles River Japan, Kanagawa). A polyurethane sponge (10x10x6 mm for rats, 10x5x6 mm for mice) inserted with an EVA pellet containing bFGF (500 ng) or an empty EVA pellet was implanted subcutaneously (sc) into a rat or a mouse. AGM-1470 suspended in a vehicle of 1% ethanol and 5% gum arabic in saline was administered sc daily for 7 days. The sponge was soaked in 0.1 M ammonia solution to extract hemoglobin in the sponge (13), a marker of angiogenesis, and the extracted hemoglobin was measured using a hemoglobin assay kit. Histological analysis demonstrated an increase of new blood vessels in the sponge containing bFGF.

Rat blood vessel organ culture assay: Rat blood vessel organ culture assay was performed by a modified version of the method described previously (14,15) as follows. Thoracic veins were obtained from 7-9-week-old male Sprague-Dawley rats. After removal of fibroadipose tissue, the veins were washed with DMEM supplemented with 10% FBS. The veins were then cut into small fragments (about 2x2 mm) and cultured in fibrin gels (1.5ml) which were formed by addition of thrombin to the same medium containing fibrinogen in a 12-well plate. On the following day, AGM-1470 in the same volume of medium was added to the fibrin gel in the wells. After 9 days, tube formation and cell growth were examined using a microscope and graded from 0 (complete inhibition) to 4 (maximal growth).

Results and Discussion

In the CAM assay, a widely used anti-angiogenesis assay, AGM-1470 inhibited capillary growth by local administration and induced avascular zones in the CAM in a dose-dependent manner (Table 1). This result is compatible with the previous observation that fumagillin, a parent compound of AGM-1470, induced avascular zones in the CAM. No undesirable symptoms such as thrombosis, hemorrhage or marked distortion of large vessels were observed, unlike many other cytotoxic compounds. Empty pellets without AGM-1470 did not affect the formation of the vascular network of the CAM. Therefore, AGM-1470 seems to specifically inhibit the microvascular formation that occurs normally during embryogenesis.

In the rat corneal micropocket assay, the effect of AGM-1470 on angiogenesis induced by a potent angiogenic factor, bFGF, was evaluated by local administration. AGM-1470 at 20 μg per cornea suppressed the number and length of new blood vessels induced by bFGF in all tested rat corneas (Table 1). Swelling and inflammation of the corneas, often induced by toxic agents, were not observed during treatment with AGM-1470.

In both the CAM and corneal micropocket assays, anti-angiogenic activity of locally administered AGM-1470 was thus shown. However, these assays are not sufficiently quantitative, and the results do not yield information about the anti-angiogenic activity of AGM-1470 administered systemically. To determine the anti-angiogenic activity of AGM-1470 quantitatively by systemic administration, the sponge implantation assay was performed using rats and mice. The hemoglobin content of sponges containing bFGF was about three times higher than that in control sponges, indicating angiogenesis by bFGF (Fig.1). Subcutaneously administered AGM-

Table 1. Anti-angiogenic activity of AGM-1470 in CAM and rat corneal micropocket assays. In the CAM assay, pellets containing AGM-1470 were placed on the CAM of 6-day embryos and formation of avascular zones was examined by stereomicroscopy 2 days later. In the rat corneal micropocket assay, pellets containing bFGF (250 ng) were implanted into rat corneas to induce angiogenesis. Pellet containing AGM-1470 or empty pellet were also implanted close to the bFGF pellet. After 10 days, inhibition of angiogenesis was examined by stereomicroscopy.

AGM-1470 (μg/pellet)	CAM assay	Corneal assay
	No. of CAM avascular/total	No. of corneas inhibited/total
0	0/10	0/6
10	2/10	
20	6/10	6/6
50	9/9	

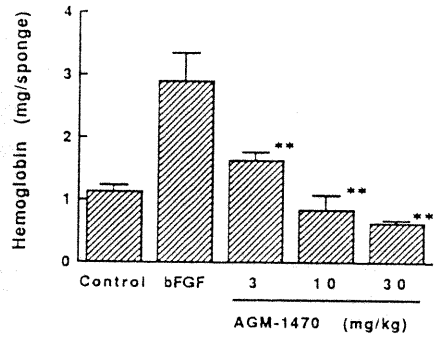

Fig. 1. Anti-angiogenic activity of AGM-1470 in the rat sponge implantation assay. A bFGF (500 ng)-containing pellet inserted into a sponge was implanted sc into a rat. AGM-1470 was administered sc for 7 days from the day after implantation. Hemoglobin content of the sponge was determined as a marker of angiogenesis using a commercial assay kit (Mean ± SEM. N=5). Analysis of variance followed by Dunnett's test. **:P<0.01 (compared with the bFGF control).

1470 dose-dependently decreased the hemoglobin content of the bFGF-containing sponge, and the resulting hemoglobin content reached a level even lower than that in the control sponge by administration of 30 mg/kg. The hemoglobin content of the control sponge indicates angiogenesis which may be caused by inflammation due to the implantation of the sponge as a foreign substance. Thus, AGM-1470 seems to inhibit also the angiogenesis induced by unknown angiogenic factors involved in inflammation in addition to bFGF. In the mouse sponge implantation assay, AGM-1470 showed similar anti-angiogenic activity to that in rats (data not shown).

All of the three *in vivo* assays described above are complicated by inflammatory reactions caused by direct contact with foreign substances. Therefore, we tested the anti-angiogenic potency of AGM-1470 by rat blood vessel organ culture assay *in vitro* using a modified version of the original method (15), which is quantitative and free from inflammatory complications. In our modified assay system, rat thoracic vein fragments were cultured in fibrin gels supplemented with 10% FBS. Under these conditions, both cell growth and capillary-like tube formation were observed, allowing the specific effect on tube formation to be examined. On day 2 or 3 of culture, outgrowth of cells was observed around the fragments of a thoracic vein. On day 9, non-endothelial cells proliferated as single cells and a monolayer (Figs. 2A, C). On the other hand, endothelial cell sprouts formed capillary-like tubes in the fibrin gel (Fig. 2E). AGM-1470 (100 ng/ml) inhibited tube formation by endothelial cells (Fig. 2F) while inhibition of cell growth by AGM-1470 was minimal (Figs. 2B, D). The effects of AGM-1470 and the fumagillin parent at various concentrations on tube formation and cell growth are shown in Fig. 3. AGM-1470 inhibited tube formation at only 1 ng/ml. On the other hand, inhibition of cell growth was minimal until 1,000 ng/ml. This selective inhibition of tube formation by AGM-1470 is a feature distinct from the effects of cytotoxic antitumor drugs, which inhibited both tube formation and cell growth at the same concentration (unpublished data). As to the cause of the selective inhibition, there are two possibilities: AGM-1470 may inhibit specifically endothelial cell growth or alternatively

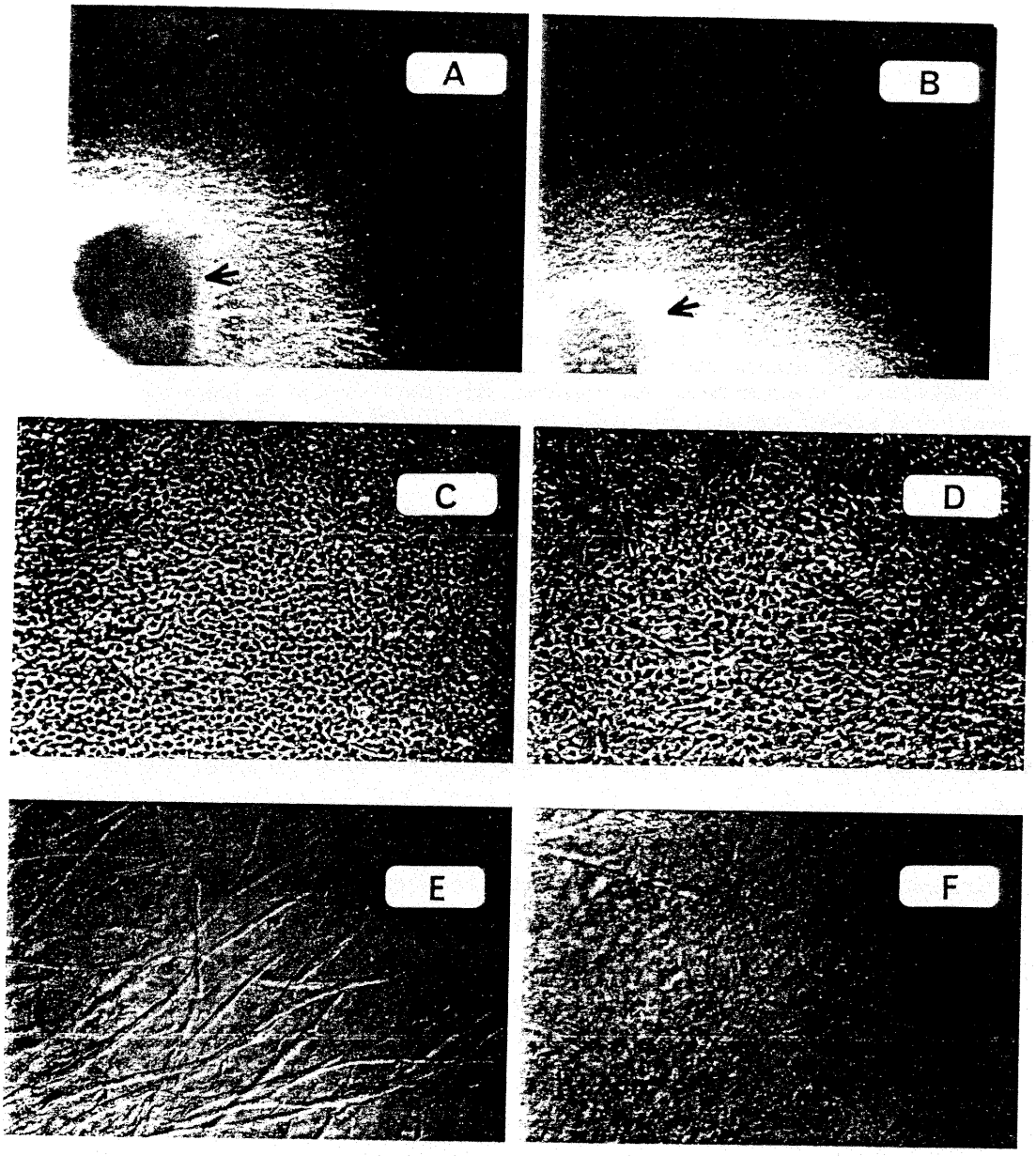

Fig. 2. Anti-angiogenic acitivity of AGM-1470 in the rat blood vessel organ culture assay. Rat thoracic vein fragments (arrows in A, B) were cultured in the absence (A, C, E) or presence (B, D, F) of AGM-1470 (100 ng/ml) in a fibrin gel for 12 days. In the control culture, growth of cells (A, C) and capillary-like tube formation (E) were observed. On the other hand, in AGM-1470-containing cultures, tubes were not observed (F) while growth of the cells ramained unchanged (B, D). (A, B, x10 ; C, D, x60 ; E, F, x150).

AGM-1470 may inhibit morphologic 'tube' formation. The concentration required for inhibition of tube formation (1 ng/ml) is comparable to that necessary for complete inhibition of human umbilical vein endothelial cell growth (in preparation). Therefore, selective inhibition of tube formation may be exerted by inhibition of endothelial cell growth.

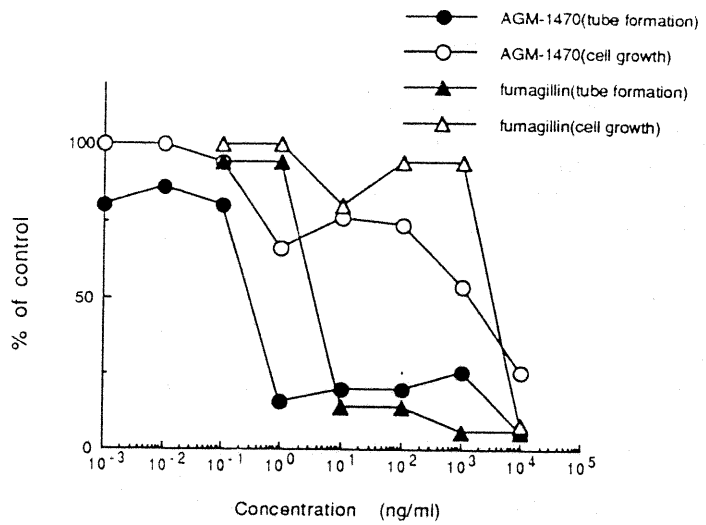

Fig. 3. Anti-angiogenic activity of AGM-1470 and fumagillin in the rat blood vessel organ culture assay. The culture was performed as described in the Materials and Methods. Each point represents the mean value of triplicate determination.

Since the CAM and rat corneal assay are not sufficiently quantitative, the anti-angiogenic potency of AGM-1470 was compared to that of the fumagillin parent in the rat sponge implantation assay and the rat blood vessel organ culture assay. In the rat sponge implantation assay, AGM-1470 was required for a sc administratin at 10 mg/kg to induce 70 % suppression of the hemoglobin content in the sponge (a level in the case of sponge without bFGF) against the fumagillin parent's 38 mg/kg. In the rat blood vessel organ culture, the dose of the fumagillin parent required for the suppression of tube formatin was at least ten times higher than that of AGM-1470 (Fig. 3). Furthermore, when both compounds were administered sc into mice daily for 12 days, a dose causing difference of 2 g in body weight from the control (about 10 % of whole body weight of control mice) was 18 mg/kg in AGM-1470 against only 2 mg/kg in the fumagillin parent.

In conclusion, AGM-1470 was found to inhibit more potently angiogenesis than the fumagillin parent both *in vivo* and *in vitro* regardless of any angiogenesis factors and administration mode. Then, AGM-1470 was less toxix than the fumagillin parent. Therefore, the anti-angiogenic action of AGM-1470 may be involved in its potent antitumor activity in animal models (11) and then, AGM-1470 may be clinacally useful for anti-angiogenic therapy against diseases which are contributed by the angiogenesis besides solid tumors.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Mr. Tsuneo Masaki, Miss Yumiko Akinaga and Miss Eiko Hashimoto.

References

1. Folkman, J. (1985) Adv. Cancer Res. 43, 175-203.
2. Folkman, J. (1990) J. Natl. Cancer Inst. 82, 4-6.
3. Hughes, L.E. (1964) Lancet 1, 408-409.
4. Taylor, S., and Folkman, J. (1982) Nature 297, 307-312.
5. Folkman, J., Langer, R., Linhardt, R.J., Haudenshild, C., and Taylor, S. (1983) Science 221, 719-725.
6. Crum, R., Szabo, S., and Folkman, J. (1985) Science 230, 1375-1378.
7. Inoue, K., Korenaga, H., Tanaka, N.G., Sakamoto, N., and Kadoya, S. (1988) Carbohydrate Res. 181, 135-142.
8. Tanaka, N.G., Sakamoto, N., Inoue, K., Korenaga, H., Kadoya, S., Ogawa, H., and Osada, Y.(1989) Cancer Res. 49, 6727-6730.
9. Maione, T.E., Gray, G.S., Petro, J., Hunt, A.J., Donner, A.L., Bauer, S.I., Carson, H.F., and Sharpe, R.J. (1990) Science 247, 77-79.
10. Sharpe, R.J., Byers, H.R., Scott, C.F., Bauer, S.I., and Maione, T.E. (1990) J. Natl. Cancer Inst.82, 848-853.
11. Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H., and Folkman, J. (1990) Nature 348, 555-557
12. Wakamatsu, K., Masaki, T., Itoh, F., Kondo, K., and Sudo, K. (1990) Biochem. Biophys. Res. Commun. 168, 423-429.
13. Davidson, J.M., Klagsbrun, M., Hill, K.E., Buckley, A., Sullivan, R., Brewer, P.S., and Woodward, S.C. (1985) J. Cell Biol. 100, 1219-1227.
14. Nicosia, R.F., and Ottinetti, A. (1990) In Vitro Cell. Dev. Biol. 26, 119-128.
15. Nicosia, R.F., and Ottinetti, A. (1990) Lab. Invest. 63, 115-122.