Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth

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NEOVASCULARIZATION is critical for the growth of tumours[1–3] and is a dominant feature in a variety of angiogenic diseases[4] such as diabetic retinopathy, haemangiomas, arthritis and psoriasis. Recognition of the potential therapeutic benefit of controlling unabated capillary growth[5] has led to a search for safe and effective angiogenesis inhibitors. We report here the synthesis of a family of novel inhibitors that are analogues of fumagillin, a naturally secreted antibiotic[6] of *Aspergillus fumigatus* fresenius. We first isolated this fungus from a contaminated culture of capillary endothelial cells. Purified fumagillin inhibited endothelial cell proliferation *in vitro* and tumour-induced angiogenesis *in vivo*; it also inhibited tumour growth in mice, but prolonged administration was limited because it caused severe weight loss. Synthesis of fumagillin analogues yielded potent angiogenesis inhibitors ('angioinhibins') which suppress the growth of a wide variety of tumours with relatively few side-effects.

During routine culturing of capillary endothelial cells we found a fungal contamination that produced a local gradient of endothelial cell rounding (Fig. 1). Cells that were only a few cell diameters away were normally spread, suggesting that the fungus was specifically inducing cell rounding rather than causing toxicity. Other fungal contaminants in our experience had produced total cell detachment and cell death. Cell rounding was in itself interesting as endothelial cells must spread in order to proliferate, even when grown in the presence of saturating amounts of soluble mitogens[7,8]. Also, other angiogenesis inhibitors cause endothelial cell rounding as part of their action *in vivo*[9].

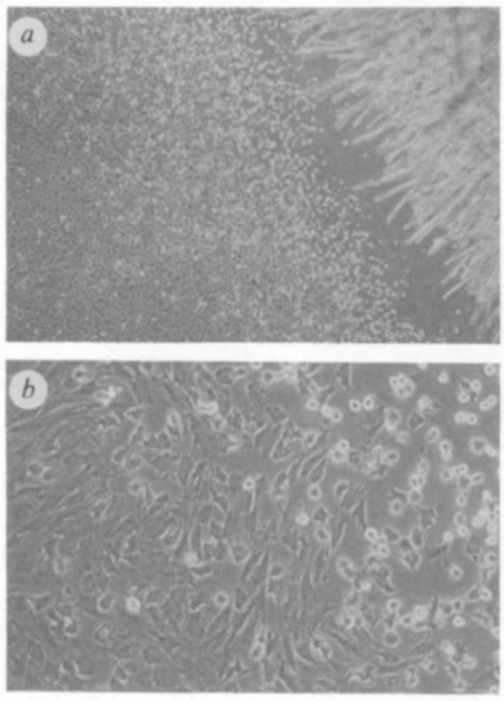

FIG. 1 *a*, A contaminated culture of bovine capillary endothelial cells showing fungal hyphae at the right. *b*, Higher magnification of *a* showing an apparent diffusion gradient resulting in cell detachment and rounding nearest the edge of the fungal colony; cells only a few cell diameters away have normal morphology.

We isolated this fungus and identified it as *Aspergillus fumigatus* fresenius. Conditioned medium from fungal cultures was found to have potent endothelial cell-rounding activity and to inhibit angiogenesis in the growing chick chorioallantoic membrane. The active fraction was purified from large-scale fungal cultures and identified as fumagillin (Fig. 2), an antibiotic used to treat amoebiasis in humans[10]. Purified fumagillin completely inhibited endothelial cell proliferation in the presence of saturating levels of basic fibroblast growth factor (half-maximal inhibition of human umbilical vein endothelial cells at 0.5 ng ml^{-1}). Cell rounding, as in our original contaminated cultures, was induced at concentrations greater than 10 μg ml^{-1}. Angiogenesis was inhibited in the chorioallantoic membrane model at concentrations of fumagillin above 2 μg per chorioallantoic membrane. Fumagillin also suppressed tumour-induced neovascularization in the mouse dorsal air sac (Fig. 3), but its effectiveness as an inhibitor of tumour growth was limited because it produced severe weight loss. This side-effect probably explains the low anti-tumour effect of fumagillin in humans reported previously[11].

We therefore set out to design fumagillin analogues that would retain the potent anti-angiogenic activity of fumagillin without its side-effects. Alkaline hydrolysis of fumagillin yields fumagillol, from which over 100 derivatives were synthesized and tested. Among these analogues we identified a subset of compounds that represent a new class of angiostatic antibiotics which we term angioinhibins, one of the most potent of which is *O*-(chloroacetylcarbamoyl)fumagillol or AGM-1470 (Fig. 2). This angioinhibin produced half-maximal cytostatic inhibition of endothelial cell proliferation at ~10 pg ml^{-1}, which is 50 times more active than the fumagillin parent. Cytotoxicity (reduction of cell number below the initial plating density) was only observed at much higher concentrations (>1 μg ml^{-1}). Although the sensitivity of a normal non-endothelial cell line (human embryonic lung fibroblasts) was not significantly different from human umbilical vein endothelial cells, doses of AGM-1470 had to be at least ten times higher for comparable inhibition of the

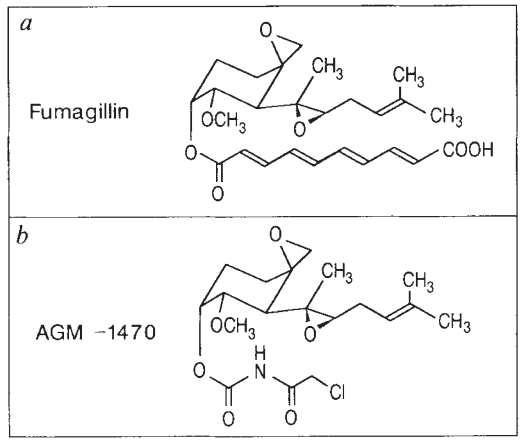

FIG. 2 Structure of *a*, fumagillin, the parent compound isolated from the culture contaminant described in Fig. 1, and *b*, the synthetic analogue AGM-1470, a related angioinhibin used in these antitumour studies.

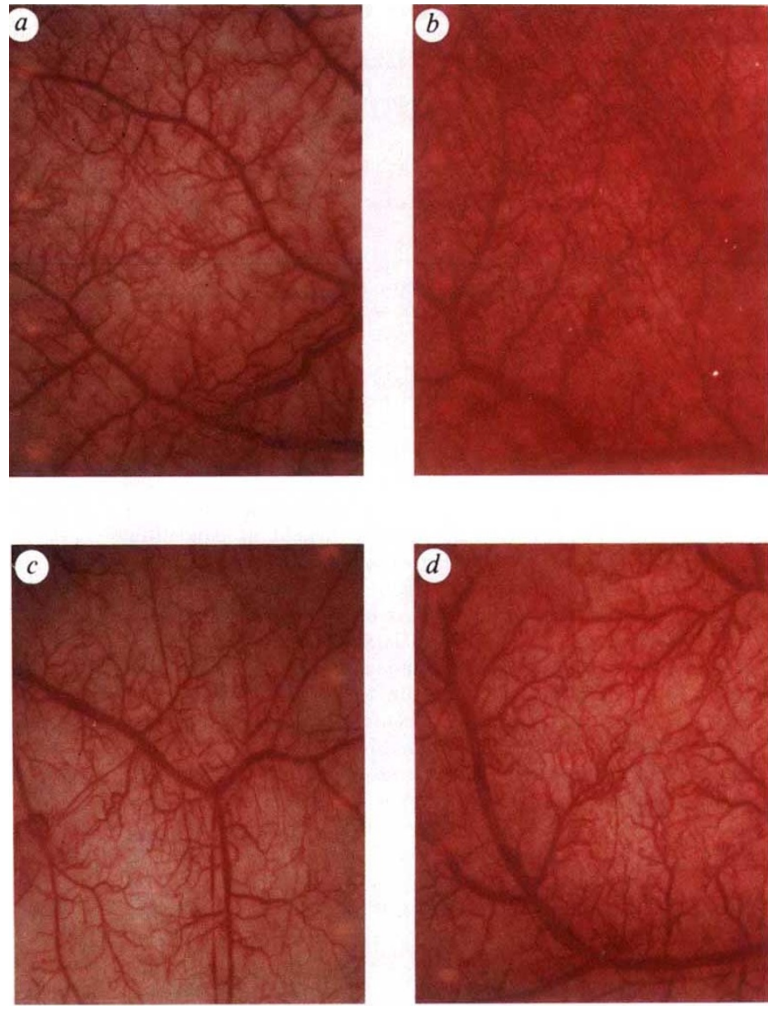

FIG. 3 Inhibition of tumour-induced angiogenesis in the subcutaneous dorsal air sac of the mouse by fumagillin. Chambers containing Millipore filters (0.45 μm) were filled either with saline (*a*, *c*) or 5×10^6 ascites sarcoma-180 cells in saline (*b*, *d*) and implanted in dorsal air sacs created surgically in mice. Animals containing the implanted chambers were treated systemically with fumagillin (100 mg kg^{-1}, subcutaneously) for 3 days (*c*, *d*). On day 4, angiogenesis within the subcutaneous fascia of fumagillin-treated animals (*c*, *d*) was compared with that of controls (*a*, *b*).

human tumour cell lines (squamous cell HSC-1, colon COLO-205, leukaemia HL-60 and squamous cell LS-180).

AGM-1470 also inhibited growth of solid tumours when it was delivered systemically in mice. These tumours included Lewis lung carcinoma (Fig. 4*a*, *b*), B16 melanoma (Fig. 4*c*, *d*), M5076 reticulum cell sarcoma, Meth A sarcoma, colon 26 carcinoma, and Engelbreth-Holm Swarm sarcoma (and others not shown). There was a sustained inhibition of tumour growth

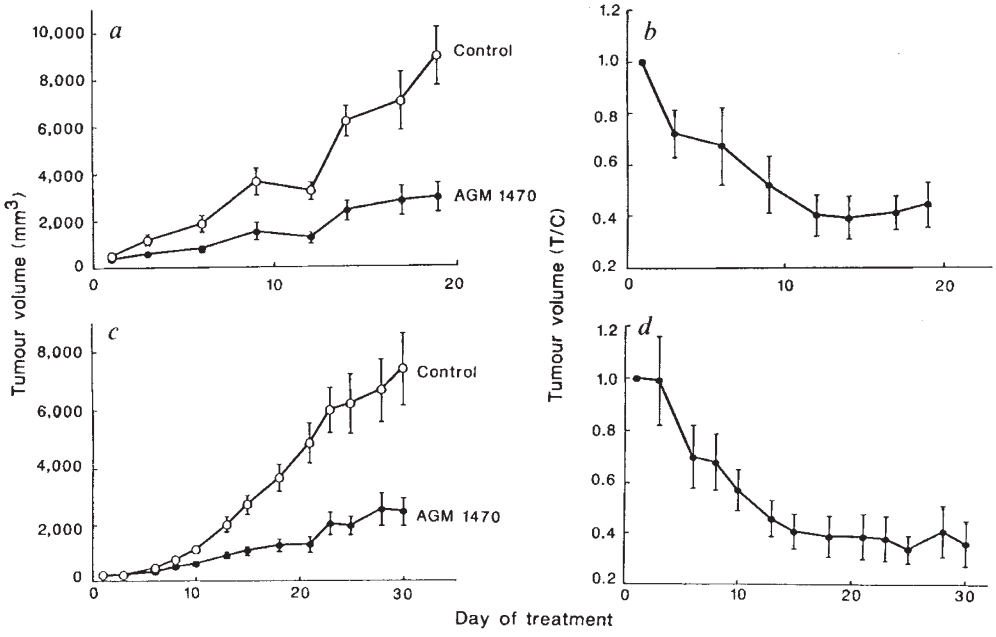

FIG. 4 Inhibition of growth of Lewis lung carcinoma (*a*, *b*) and B16 melanoma (*c*, *d*) by systemic administration of AGM-1470. Tumour cells (1×10^6) were inoculated subcutaneously in 0.1 ml saline in the same position along the dorsal midline in C57B1/6 mice. Treatment was not initiated until tumours were at least 100 to 250 mm^3 in volume. Tumours of similar size were matched for use in control and experimental groups. AGM-1470 was administered subcutaneously at a remote site at 30 mg per kg in body weight in saline once every other day. Tumours were measured in two dimensions and volume was calculated using a standard formula: width^2 *x* length ×0.52. *T*/*C* = tumour volume of treated/mean tumour volume of control (untreated) for each day. Lewis lung carcinoma, *N* = 29 animals per group; B16 melanoma, *N* = 16 animals per group. Error bars indicate standard error of the mean.

regardless of whether administration of AGM-1470 was started one day after tumour inoculation (data not shown) or once the tumour had become fully established (when tumour volume exceeded 100 mm^3) (Fig. 4). Whereas the application of fumagillin in animals was restricted by their unacceptable weight loss (>15% of starting weight), animals treated with effective doses of AGM-1470 gained weight slowly but consistently. Daily therapy against the tumour was unnecessary in any animal, with less frequent regimens of once every three days, for example, being highly successful (in the case of M5076-bearing animals, survival time increased by up to 260% over untreated controls). Yet cells from the M5076 tumour cell line, which were very sensitive to AGM-1470 *in vivo*, were found to be refractory to AGM 1470 *in vitro*. These tumour cells *in vitro* required 100 times more AGM-1470 than vascular endothelial cells for comparable inhibition of growth. Taken together, these results strongly suggest that AGM-1470 exerts its anti-tumour effects primarily by acting on the tumour vasculature. This hypothesis is further supported by the finding that AGM-1470 did not prolong the survival time of mice injected intraperitoneally with P388 leukaemia. This tumour grows in an ascites (non-solid) form and should therefore be less dependent on neovascularization for its development.

Angioinhibins, such as the one reported here, represent a unique class of angiostatic antibiotics. Unlike other angiogenesis inhibitors, angioinhibins are not steroid[12,13], polysaccharide[13,14], retinoid[15] or peptide[15-17] structures. There are two other microbial products with angiostatic activity: one is a sulphated polysaccharide peptidoglycan complex[18] whose complete structure is still unknown; the structure of the other has been determined[19] but it is cytotoxic and its anti-tumour effects have not been reported.

Our new class of angiogenesis inhibitors, then, can suppress tumour growth without the usual toxic side-effects associated with conventional chemotherapy drugs. Specifically, we never observed hair loss, intestinal disturbance or infection although we administered AGM-1470 subcutaneously at 30 mg kg^{-1} every other day for >100 days (which is about one sixth of the life of a mouse). This absence of side-effects could be critical in treatment of tumours and other angiogenic diseases which may require long-term therapy in much the same way as diabetes and malaria do now. An example of prolonged anti-angiogenic therapy for a non-neoplastic disease has been reported in the treatment of haemangioma with alpha-interferon[20]. These findings in conjunction with ours provide a glimpse of what a cancer therapy based on anti-angiogenesis might be in the future. □

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No T-cell tyrosine protein kinase signalling or calcium mobilization after CD4 association with HIV-1 or HIV-1 gp120

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THE T lymphocyte surface protein CD4 is an integral membrane glycoprotein noncovalently associated with the tyrosine protein kinase $p56^{lck}$ (reviewed in ref. 1). In normal T cells, surface association of CD4 molecules with other CD4 molecules or other T-cell surface proteins, such as the T-cell antigen receptor, stimulates the activity of the $p56^{lck}$ tyrosine kinase, resulting in the phosphorylation of various cellular proteins at tyrosine residues[2-4]. Thus, the signal transduction in T cells generated through the surface engagement of CD4 is similar to that observed for the class of growth factor receptors possessing endogenous tyrosine kinase activity[5]. As CD4 is also the cellular receptor for the human immunodeficiency virus (HIV)[6,7], binding of the virus or gp120 (the virus surface protein responsible for specific $CD4^+$ T-cell association) could mimic the types of immunological interactions that have previously been found to stimulate $p56^{lck}$ and trigger T-cell activation pathways. We have evaluated this possibility and report here that binding of HIV-1 or the virus glycoprotein gp120 to $CD4^+$ human T cells fails to elicit detectable $p56^{lck}$-dependent tyrosine kinase activation and signalling, alterations in the composition of cellular phosphotyrosine-containing proteins, or changes in intracellular Ca^{2+} concentration.

The results presented in Fig. 1 demonstrate that crosslinking surface CD4 molecules with anti-CD4 monoclonal antibodies results in the stimulation of CD4-associated $p56^{lck}$ tyrosine protein kinase activity leading to the phosphorylation of T-cell proteins. Parallel immunoblots detecting $p56^{lck}$ or CD4 show that during the course of these experiments the abundance of $p56^{lck}$-CD4 complexes was not altered. These results imply that the specific activity of the CD4-associated $p56^{lck}$ was transiently increased around four- to fivefold as a consequence of antibody-mediated CD4 crosslinking. The results of anti-phosphotyrosine immunoblots indicate that cell proteins can be phosphorylated on tyrosine residues in response to the same CD4 crosslinking treatment. In addition, crosslinking of CD4 stimulated Ca^{2+} mobilization after the activation of $p56^{lck}$, and also led to the rapid appearance of interleukin-2 receptor α subunit (IL-2Rα) and transferrin receptor on the surface of the T cells.

To evaluate HIV interactions, purified HIV-1 (strain III-b) was added to the quiescent T cells on ice and binding was monitored by flow cytometry measuring the capacity of the virus to block subsequent binding of fluorescein-conjugated monoclonal antibody Leu3A. HIV-1 could saturate the available surface Leu3A sites within 30 min (Fig. 2*a*), but these conditions did not result in detectable HIV endocytosis and did not affect the subsequent binding of monoclonal antibody OKT4, which recognizes another CD4 surface epitope distinct from the HIV-binding domain. We found that HIV binding and prolonged

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