How to starve a tumor

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The formation of new blood vessels, termed angiogenesis, is a central process in the evolution of a tumor and its subsequent metastasis. Angiogenesis is potently inhibited by the fumagillins, a family of epoxide-containing natural products. The recent identification of a protein target for these compounds may help decipher the mechanisms underlying endothelial cell growth. The fact that binding is irreversible may inspire the design of electrophilic inhibitors of other, poorly understood cellular processes.

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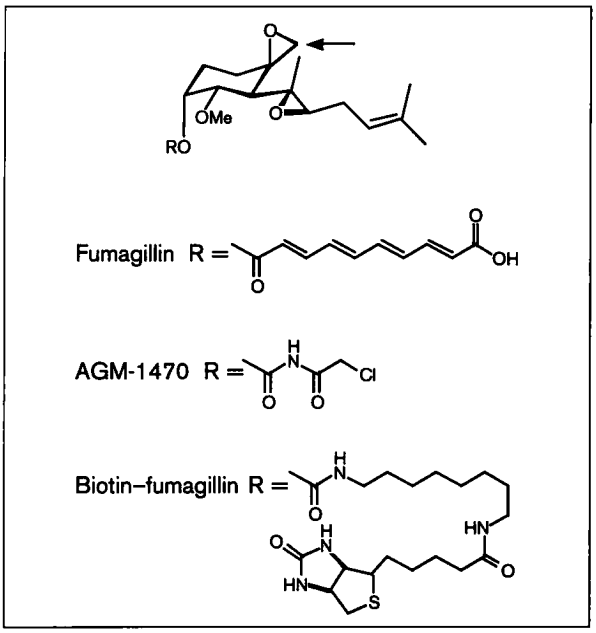
When an unwelcome fungal spore chances to settle down and start a family in a mammalian cell culture dish, most of us throw away the cells and start again. Not the cell biologists in Judah Folkman's laboratory. Rather than discard a contaminated endothelial cell culture as a botched experiment, Folkman and colleagues decided to pursue the curious contaminant in order to understand the basis for its remarkable inhibition of endothelial cell growth. Cells growing closest to the intruding fungal colony were rounded and in various stages of detachment from the culture dish, whereas cells more distant from the colony appeared healthy, suggesting that the fungus was secreting a diffusible growth inhibitory substance into the culture medium. Purification of this substance led to the rediscovery of fumagillin [1], a sesquiterpene diepoxide (Figure 1) originally described in the 1950's as an anti-amoebic agent.

Pure fumagillin, isolated from the fungal culprit *Aspergillus fumigatus*, inhibits cultured endothelial cell growth at subnanomolar concentrations, but does not affect many other cell types, including a variety of tumor cell lines, at these low levels. Presumably reflecting its cytostatic effect on endothelial cells *in vitro*, fumagillin also inhibits angiogenesis *in vivo*, the process by which vascular endothelial cells develop into new blood vessels. Solid tumors require new blood vessels to deliver nutrients and oxygen to the inner recesses of the tumor mass, so inhibition of angiogenesis represents an indirect way of shutting down tumor growth and metastasis. After demonstrating that fumagillin suppresses tumor-induced angiogenesis *in vivo*, the Folkman laboratory went a step further and, in collaboration with scientists at Takeda Industries, synthesized over 100 fumagillin derivatives. One of these, called AGM-1470 (Figure 1; also known as TNP-470), is 50 times more potent as an inhibitor of endothelial cell growth than fumagillin and is less toxic to mice [1]. AGM-1470 is currently in phase II clinical trials for the treatment of certain types of cancer in humans.

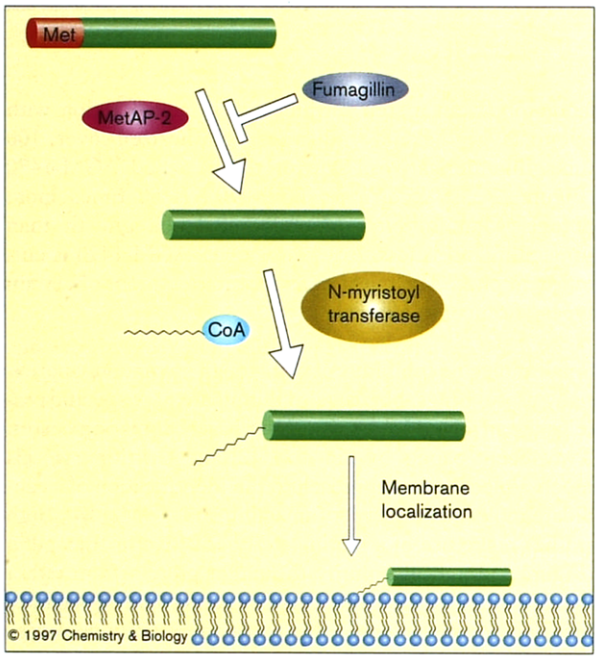
Small-molecule inhibitors of cell growth are not only potentially useful drugs, but can also illuminate how specific proteins are involved in the control of basic cellular processes. Two recent papers by Sin *et al.* [2] and Griffith *et al.* [3] report a biochemical approach to the identification of a candidate molecular target for fumagillin and AGM-1470. Both studies make use of a biotinylated derivative of fumagillin (Figure 1) to affinity purify the target protein from either mouse embryo or bovine brain extracts. The fumagillin-binding protein is identified as methionine aminopeptidase type 2 (MetAP-2), a cobalt-dependent enzyme that hydrolytically excises the amino-terminal methionine from

Figure 1



The chemical structures of fumagillin, AGM-1470 and biotin–fumagillin. The arrow indicates the exocyclic epoxide that is susceptible to nucleophilic attack. The biotin conjugate is used to affinity purify fumagillin-binding proteins from cell extracts.

Figure 2



Indirect inhibition of protein myristoylation by fumagillin. MetAP-2, in a reaction that is inhibited by fumagillin, removes the terminal methionine (red) from certain proteins (green). Only once this has occurred can N-myristoyl transferase catalyze the ligation of myristate to the amino terminus of the prqtein. Many important signaling proteins require myristoylation in order to be localized to specific membrane compartments within the cell. Improper localization of these proteins may be responsible for fumagillin's cell cycle arrest properties.

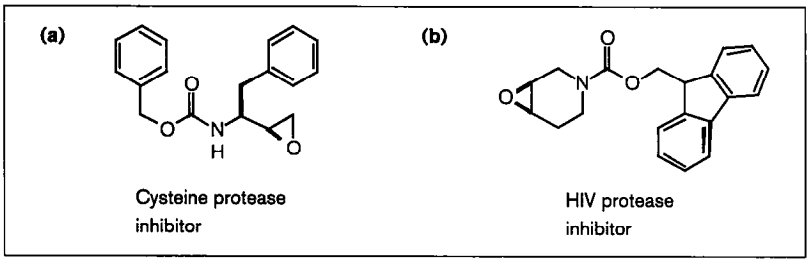
certain proteins. Griffith *et al.* [3] also show that the aminopeptidase activity of purified, recombinant MetAP-2 is abolished by 1 nM fumagillin.

The biology of methionine aminopeptidases

Budding yeast have two related methionine aminopeptidases, map1p and map2p. Their functions are partially redundant; deletion of either *map1* or *map2* results in a slow-growth phenotype, but deletion of both genes is lethal [4]. Of the two enzymes, map2p clearly shows more similarity than map1p to the human MetAP-2 (59% in the catalytic domain compared to 21% for map1p). Sin *et al.* [2] and Griffith *et al.* [3] show that yeast lacking *map1* are unable to grow in the presence of fumagillin. Conversely, a yeast strain that lacks the *map2* gene is resistant to fumagillin. This result is consistent with the idea that fumagillin specifically inhibits type 2 methionine aminopeptidase activity in the living yeast cell, thereby resulting in growth inhibition.

Growth-factor-stimulated endothelial cells arrest late in the G1 phase of the cell cycle when treated with fumagillin. How can the removal of a single methionine residue be so important for cell cycle progression and why are endothelial cells more sensitive than other cell types to the inhibition of this process? Providing definitive answers to these questions will be a challenging task, yet it seems likely that a primary cause for cell cycle arrest lies not with inhibition of methionine processing *per se*, but rather with a second cotranslational modification, myristic acid attachment. Amino-terminal ligation of the 14-carbon fatty acid myristate occurs only when the first amino acid is glycine [5]. Hence, N-myristoyl transferase (NMT) will only act on nascent polypeptides that have had their amino-terminal methionine removed. As many important signaling proteins need to be myristoylated in order to function in the cell, inhibition of protein myristoylation is predicted to have dire consequences on cell growth and, indeed, the gene encoding yeast NMT is essential for viability [6]. Proteins known to be myristoylated include the src family tyrosine kinases, the small GTPase ARF, the HIV protein nef and the α subunit of heterotrimeric G proteins, among others. The recent report that hypoxic induction of vascular endothelial growth factor (VEGF), an obligate angiogenic factor, requires src tyrosine kinases [7] is therefore potentially relevant to the study of fumagillin's anti-angiogenic effect. Myristoylation of these proteins is necessary (but often not sufficient; see [5]) for their localization to specific membrane compartments. It will therefore be important to test whether fumagillin results in the aberrant localization of various candidate proteins. The idea that fumagillin indirectly causes cell cycle arrest by preventing

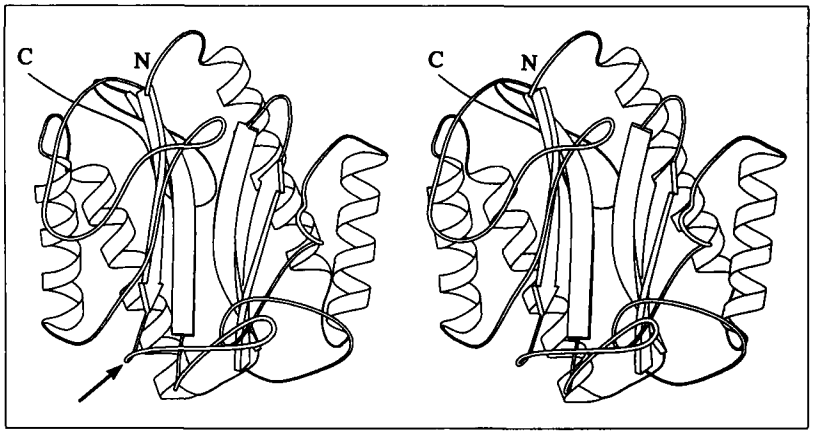
Figure 3



The structure of two epoxide-containing protease inhibitors. **(a)** A cysteine protease inhibitor [10]. **(b)** An HIV protease inhibitor [11].

Figure 4

A stereo view of a methionine aminopeptidase from *E. coli*. Figure adapted with permission from [12]. © 1993 American Chemical Society. The 64 amino acid insertion that is found only in the type 2 MatAPs is indicated by the arrow.



subcellular localization of important cell proteins (Figure 2) has a parallel in the action of the farnesyl transferase inhibitors; these inhibitors prevent membrane localization of Ras-family small GTPases [8] and are also in clinical trials as anticancer drugs.

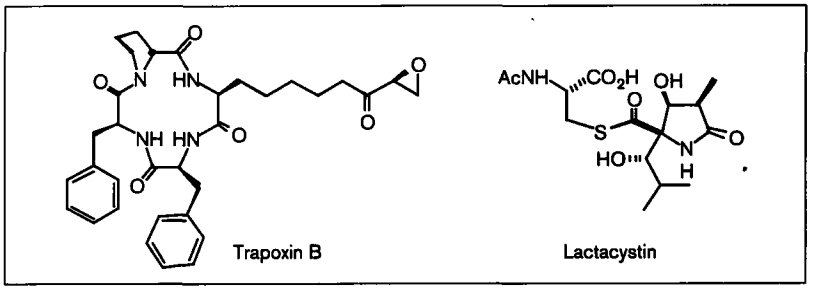
The apparent cell type specificity of fumagillin is puzzling and still requires explanation. It may be that mammalian type 1 and 2 MetAPs have nonredundant functions that vary with cell type, resulting in varying degrees of sensitivity to MetAP-2 inhibition. One must also consider the possibility that MetAP-2 is the most abundant and therefore most easily detected fumagillin-binding protein in the cell, but that it does not, in fact, mediate fumagillin's anti-proliferative effects. A BLAST search for proteins related in sequence to human MetAP-2 retrieves a nuclear protein p38 (smallest sum probability 10^{-17}) of unknown function; expression of this protein varies with the cell cycle and is detected only in cells that are actively proliferating [9]. Might this protein also be targeted by fumagillin? Regardless, the data presented by Griffith *et al.* [3] and Sin *et al.* [2] clearly show that fumagillin targets MetAP-2 with high, if not absolute, specificity.

The chemistry of epoxide inhibitors

A fascinating chemical detail revealed by the recent papers is the fact that fumagillin binds irreversibly to MetAP-2, suggesting that an active site nucleophile attacks fumagillin at its less hindered exocyclic epoxide (see arrow in Figure 1). Note also that the more potent inhibitor, AGM-1470, with its chloroacetyl carbamoyl group, has two electrophilic sites. How is specific recognition achieved by this ensemble of 15 carbon atoms in a concentrated sea of thousands of competing nucleophiles and hydrophobic surfaces? The epoxide functionality is a finely tuned electrophile and does not yield readily to nucleophilic attack. Rather, reactions with physiological nucleophiles that open the epoxide ring are expected to be extremely slow in the absence of general acid or Lewis acid catalysis. An example is provided by the simple peptidic epoxide shown in Figure 3a, an irreversible cysteine protease inhibitor [10]. Reaction of this epoxide with the catalytic cysteine residue in papain occurs ~10^8 times faster than with cysteine itself. Furthermore, the rate of papain inactivation is strongly affected by additional structural elements in the inhibitor, including the absolute configuration of each chiral center. Another example is the

Figure 5

The chemical structures of trapoxin B and lactacystin, two natural products that are highly specific, irreversible inhibitors of their respective protein targets.



HIV protease inhibitor reported by Yu *et al.* [11] (Figure 3b). This epoxide alkylates the catalytic aspartic acid (Asp25), a reaction that is likely to be accelerated by epoxide protonation by Asp125. Shape-selective hydrophobic interactions are therefore predicted to orient fumagillin's epoxide in close proximity to an active site nucleophile as well as a general or Lewis acid, contributing substantially to the observed kinetic selectivity in the alkylation of MetAP-2. Consistent with this, Griffith *et al.* [3] report a fumagillin analog that lacks the terminal epoxide but still binds MetAP-2 and inhibits cell growth, albeit at micromolar rather than picomolar concentrations.

Although the atomic details of a fumagillin–MetAP-2 complex await a high resolution structure determination, the known crystal structure of a related methionine aminopeptidase from *Escherichia coli* offers some clues about structural elements that are involved in catalysis and that may be important in the covalent attachment of fumagillin to MetAP-2 (Figure 4) [12]. Catalysis by all MetAPs is metal-dependent and the crystal structure of *E. coli* MetAP reveals that the two active site cobalt atoms are ligated by two aspartic acids, a histidine, and two glutamic acids, all of which are conserved in human MetAP-2 [13]. Although the precise function of the two cobalt atoms and their ligands in promoting amide bond hydrolysis is unknown, it would be interesting to test whether alkylation of MetAP-2 by fumagillin is metal-dependent, implying a role for cobalt as a Lewis acid in the activation of the epoxide. It is also possible that one of the metal ligands (Asp, Glu or His) acts as a nucleophile, a scenario that has precedents in abiological metal-promoted epoxide reactions [14]. Finally, Sin *et al.* [2] suggest another plausible nucleophile, Cys416 (human MetAP-2 numbering), which lies in a 64 amino acid insertion (see arrow, Figure 4) found only in the type 2 MetAPs. Alkylation of this cysteine by fumagillin would be consistent with the observation that human MetAP-2 peptidase activity is sensitive to cysteine-modifying reagents and would also explain why yeast map1p is not inhibited by fumagillin. It is unclear whether this cysteine is located near the active site, however, and it is of course possible that fumagillin alkylates an amino acid that is not directly involved in catalysis, as is observed in the acetylation of cyclooxygenase by aspirin [15].

Fumagillin is not alone in its mode of inhibition. Lactacystin, which binds irreversibly to the proteasome [16], and trapoxin, an irreversible histone deacetylase inhibitor [17] (Figure 5), are similar to fumagillin in that they are chemically reactive natural products that nonetheless alkylate or acylate their protein targets with incredible specificity. These compounds are the product of the evolved ecosystems of diverse microbes, but might in the future inspire the design of synthetic small-molecule libraries that exploit electrophilic functionality, like that of the epoxide, as a molecular recognition element distinct from recognition elements based only on hydrogen bonding and hydrophobic/aromatic interactions. Such libraries might have particular advantages in the continuing hunt for drugs with higher potencies and selectivities.

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