VOL. 53 NO. 8, AUG. 2000 THE JOURNAL OF ANTIBIOTICS pp. 799 — 806

cis-Fumagillin, a New Methionine Aminopeptidase (type 2) Inhibitor Produced by *Penicillium* sp. F2757

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(Received for publication December 27, 1999)

Selective inhibition against the yeast MetAP2 (methionine aminopeptidase type 2) was detected in the fermentation broth of a fungus F2757 that was later identified as *Penicillium janczewskii*. A new compound *cis*-fumagillin methyl ester (**1**) was isolated from the diazomethane treated fermentation extracts together with the known compound fumagillin methyl ester (**2**). The *cis*-fumagillin methyl ester, a stereoisomer of fumagillin methyl ester at the C2′-C3′ position of the aliphatic side chain, selectively inhibited growth of the *map1* mutant yeast strain (MetAP1 deletion strain) at a concentration as low as 1 ng. However, the wild type yeast *w303* and the mutant *map2* (MetAP2 deleted) strains were resistant up to 10 μg of the compound. In enzyme experiments, compound **1** inhibited the MetAP2 with an IC_{50} value of 6.3 nM, but it did not inhibit the MetAP1 (IC_{50} >200 μM). Compound **2** also inhibited the MetAP2 with an IC_{50} value of 9.2 nM and 105 μM against MetAP1.

Solid tumors require new blood vessels to supply nutrients and oxygen to the inner recesses of the tumor mass. Inhibition of angiogenesis represents an indirect way of shutting down tumor growth and metastasis[1,2]. In 1990, INGBER *et al.* rediscovered fumagillin as an inhibitor of endothelial cell growth and angiogenesis[3]. Two research groups proved that the methionine aminopeptidase type 2 (MetAP2) was a common molecular target for fumagillin, ovalicin, and AGM-1470 (TNP-470)[4,5], all of which are well known angiogenesis inhibitors. It was also reported that there is a correlation between their ability to inhibit the growth of cultured endothelial cells and to inhibit MetAP2 activity[5]. These results have suggested that specific inhibitors of MetAP2 might lead effective agents for the treatment of angiogenic related diseases.

In the course of a screening program for new types of anti-angiogenic agents from natural sources, we found that the strain of *Penicillium* sp. F2757, which was isolated from a soil sample, produced two structurally related compounds having inhibitory activity against a *map1* mutant (MetAP1 deleted) strain[6].

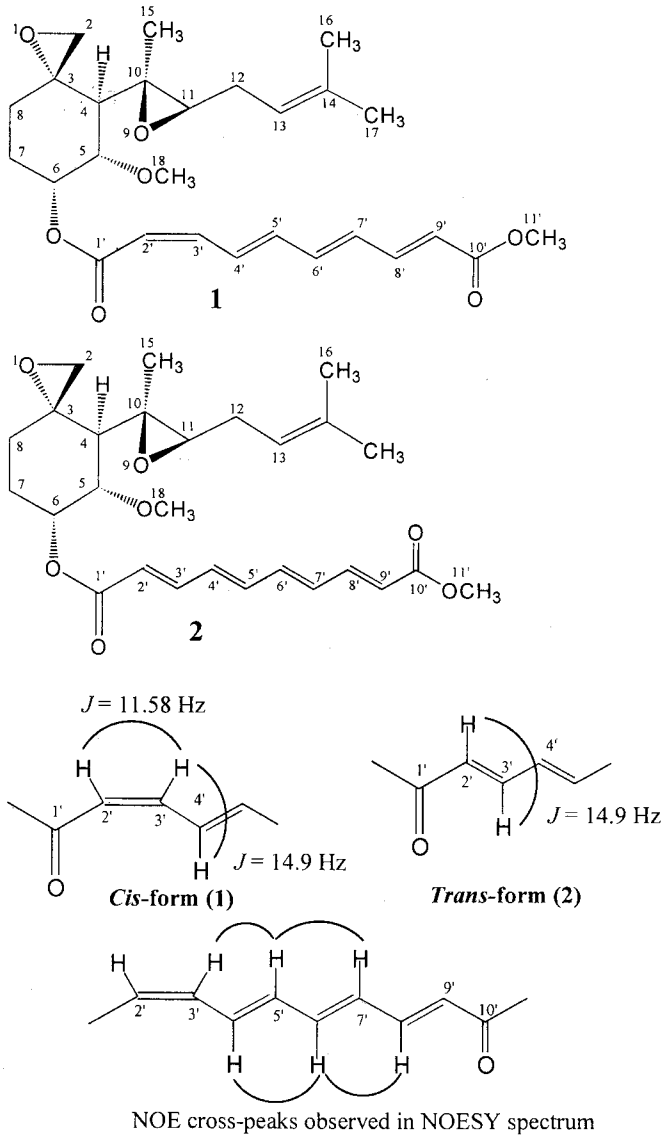
In this paper, we report on the taxonomy of the producing strain F2757, fermentation, isolation, physicochemical properties and biological activity of *cis*-fumagillin methyl ester.

Materials and Methods

General

Optical rotation was measured on a JASCO DIP-371 digital polarimeter. Mass spectra were supplied by the Mass Spectrometer Facility, Department of Chemistry, University of California at Riverside. NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker ARX-400 NMR.

Fig. 1. Structure of *cis*-fumagillin methyl ester (**1**) and *trans*-fumagillin methyl ester (**2**).



Yeast Growth Inhibition Assay

Inhibition of the MetAP2 activity was determined on an agar plate containing the MetAP1 deletion strain, *Sacchromyces cerevisiae* (map1::HIS3) that was gift from YIE-HWA CHANG (St Louis Univ.). Sterile filter disks impregnated with 10 μl of culture extracts were placed on the assay plate with recombinant yeast and the plate was incubated at 30°C for 3 days[4,5]. Control plates were prepared by wild strain *S. cerevisiae w303* and recombinant strain null MetAP2 (map2::URA3).

Producing Organism and Taxonomy

Fungal strain F2757 was isolated on YM agar (Difco) from a soil sample collected at Taejon, Korea. The strain F2757 was selected among the colonies by its MetAP2 inhibitory activity on the selective assay plate containing the MetAP1 deleted *S. cerevisiae*. The morphological and cultural properties were examined on MEA, CYA, G25N and CzA media. The characterization media MEA (malt extract agar) contained malt extract 20 g, peptone 1 g, glucose 20 g, agar 20 g in 1 liter of distilled water; CYA (Czapek yeast extract agar) contained K_2HPO_4 1 g, Czapek concentrate 10 ml (Czapek concentrate consisted of $NaNO_3$ 30 g, KCl 5 g, $MgSO_4 \cdot 7H_2O$ 5 g, $FeSO_4 \cdot 7H_2O$ 0.1 g in 100 ml of distilled water), yeast extract 5 g, sucrose 30 g, agar 15 g in 1 liter of distilled water; G25N (25% glycerol nitrate

agar) contained K_2HPO_4 0.75 g, Czapek concentrate 7.5 ml, yeast extract 3.7 g, glycerol 250 g, agar 12 g in 750 ml of distilled water; CzA (Czapek agar) contained Czapek concentrate 10 ml, sucrose 30 g, agar 15 g in 1 liter of distilled water. The strain was observed and analyzed under light and scanning electron microscopes by the method of PITT's group[7]. The culture was examined after incubation at 25°C for 14 days.

Fermentation

The frozen stock of strain F2757 was inoculated into two 1 liter Erlenmeyer flasks (Bottom baffled) containing 100 ml of YM broth (yeast extract 0.3 g, malt extract 0.3 g, peptone 0.5 g and dextrose 1.0 g) and cultivated on a rotary shaker at 170 rpm and at 28°C for 48 hours. This seed culture of 200 ml was transferred into a 14 liter fermentor that contained 8 liters of the production medium; maltose 80 g, soluble starch 224 g, cottonseed flour 80 g, *NZ*-amine 40 g and $Mg_3(PO_4)_2 \cdot 8H_2O$ 24 g. Antifoam A (Sigma Chem. Co.) was added by a foam sensing probe. The fermentation was carried out at 26°C for 96 hours with agitation of 180 rpm and an aeration of 4 liter per minute.

Isolation

The culture broth (30 liters) was filtered with a Whatman No.1 filter and the mycelial cake was extracted with acetone (3 liters). The extract was concentrated *in vacuo* to an aqueous solution that was extracted with ethyl acetate. The organic layer was concentrated *in vacuo* to dryness. The broth filtrate part was also extracted with ethyl acetate (30 liters) and the extract was concentrated. Both ethyl acetate extracts were combined for concentration under reduced pressure. The concentrate (7.8 g) was applied to a 500 ml of silica gel column, which was pre-filled with chloroform. The active fraction was eluted with 3% to 10% methanol in chloroform. The active oily material (4.6 g) was dissolved in 5 ml of methanol, and applied to a 200 ml of ODS column. The column was eluted with 50% to 90% aqueous methanol. The active fractions showed many spots with long tailing on TLC (ethyl acetate : *n*-hexane = 6 : 4). We thought that probably they had fatty acid group, therefore, the active substance (1.8 g) was dissolved in methanol and treated with diazomethane. The methylated compounds was applied to a 200 ml of silica gel column, which was pre-filled with *n*-hexane and the column was eluted with *n*-hexane - ethyl acetate (8 : 2). From the silica gel column chromatography, two active factions A and B, were collected and each fraction was concentrated under reduced pressure to yield yellow oily material. Both of the active fraction A (80 mg) and B (350 mg) were further purified on preparative TLCs with CH_2Cl_2 - *n*-hexane - ethyl acetate (1 : 6 : 3) and then the active parts were subjected to HPLC (YMC-Pack PVA-Sil). The column was eluted with *n*-hexane - ethyl acetate (8 : 2), to give 15 mg of *cis*-fumagillin methyl ester (**1**) and 135 mg of *trans* - fumagillin methyl ester (**2**) from fraction A and fraction B, respectively (Figure 2).

MetAP Enzymatic Assay

Recombinant human MetAP2 was expressed and purified from insect cells in a method described by ZUO *et al.*[8]. Various concentrations of *cis*-fumagillin methyl ester (**1**) in methanol and methanol control were each incubated with 15 nM recombinant human MetAP2 or yeast MetAP1 in buffer A (20 mM HEPES, pH 7.5, 150 mM KCl and 1.5 mM $CoCl_2$) for 10 minutes at 37°C. To begin the enzymatic reaction, Met-Gly-Met-Met was added to a final concentration of 2 mM and incubated at 37°C. After 15 minutes, EDTA was added to a concentration of 10 mM in order to quench the reaction. Released methionine was quantified as reported previously[5,8].

Results

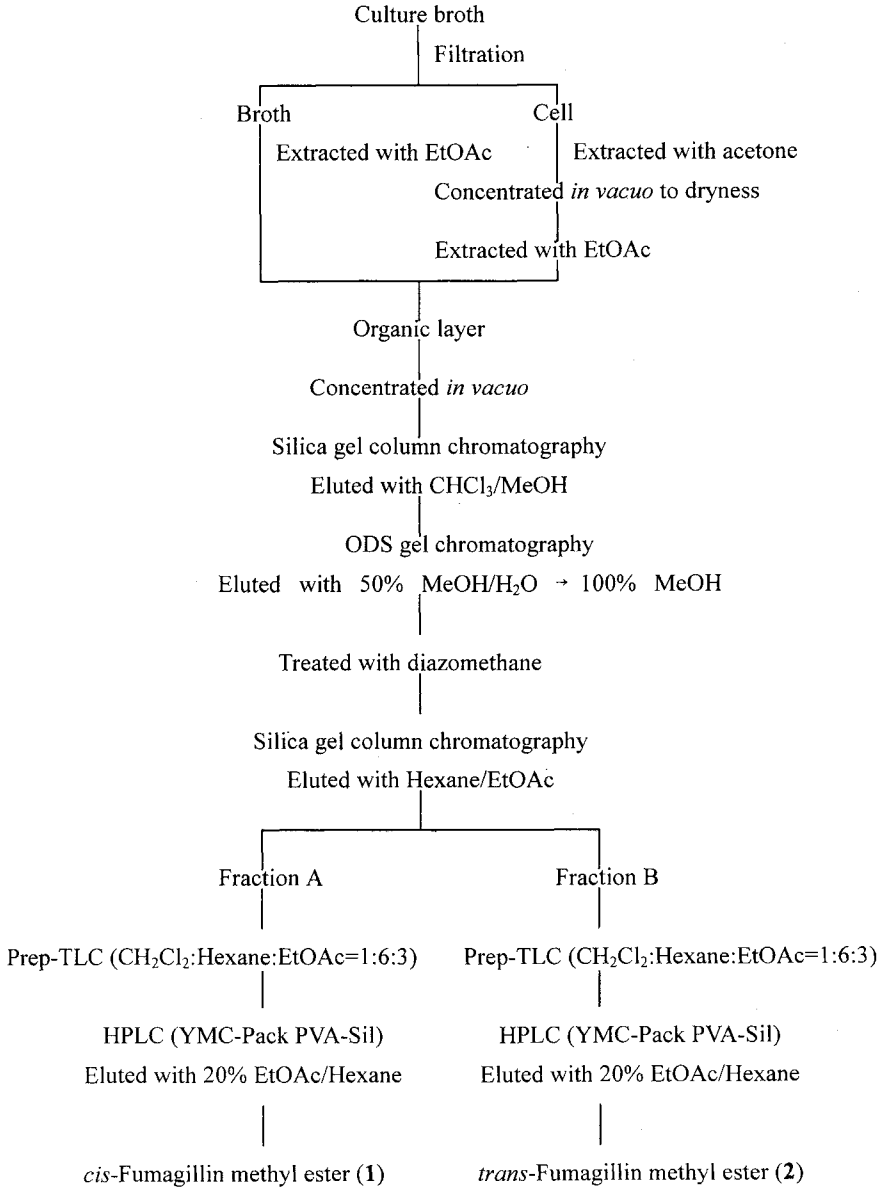
Taxonomy of the *cis*-Fumagillin Producing Strain F2757

The morphological characteristics are summarized in Table 1 and Fig. 3. The conidiophores were borne from aerial mycelium and penicilli were typically strongly devaricate. The stipes were thin with smooth walls and were 50~200 μm long. The metulae were strongly divergent and variable with size ranging from 8~15× 2.0~3.0 μm and apices commonly inflated. The phialides were ampulliform, usually borne in clusters of 6 to 12, more or less divergent and were 6~8 μm long. The conidia were 2.5~3.5 μm in diameter, globose, echinulate of spiny and appeared olive brown in color. Based on these characteristics, the strain F2757 was identified as *Penicillium janczewskii* and the strain has been deposited in the Korean Collection for Type Cultures.

Physicochemical Properties

cis-Fumagillin methyl ester (**1**) was soluble in methanol, $CHCl_3$, and dimethyl sulfoxide but insoluble in hexane, benzene, and H_2O (Table 2). The molecular formula of *cis*-fumagillin methyl ester was established as $C_{27}H_{36}O_7$ by EI-MS spectrum and by information from NMR. *cis*-Fumagillin methyl ester showed characteristic ultraviolet absorption at 335 nm and 354 nm in methanol due to double

Fig. 2. Isolation and purification of the *cis*-fumagillin methyl ester.



bonds in the side chain. The spectra of ^1H and ^{13}C NMR are shown in Table 3. These findings suggested that **1** and **2** are analogs of fumagillin.

Structure of *cis*-Fumagillin Methyl Ester (**1**)

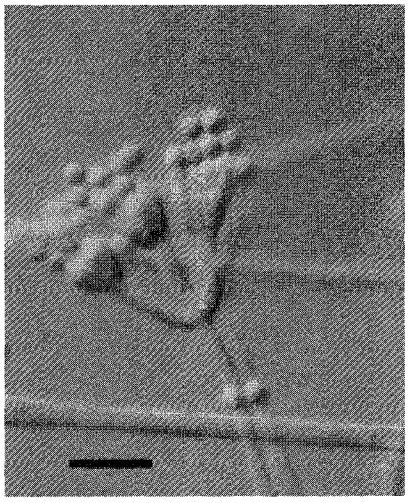
Analysis of HR-MS ($[M]^+$, *m/z* 472.2459, cald.472.2462) and the ^{13}C NMR spectrum of **1** led to molecular formula $C_{27}H_{36}O_7$. The ^{13}C NMR showed 27 carbons, which were identified as five methyls, four methylenes, four methines (three bearing oxygen), nine olefinic methines, three quaternary carbons, and two carbonyl carbons by HMQC and DEPT spectra. Three spin systems were easily delineated in ^1H-^1H COSY spectrum as related to substructures H-4 through H-8, H-11 through H-13, and H-2′ through H-9′ in Figure 1. In the HMBC experiment, correlations of a quaternary carbon C-3 (61.07 ppm) with H-2 and H-4, and C-10 (59.20 ppm) with H-11 were observed. Based on the long-range correlations in the HMBC experiment revealed that compound **1** has fumagillol nucleus and alkenyl moiety. The structure was confirmed by the comparison its spectral data with that of

Table 1. Colony characteristics of *P. janczewskii* sp. F2757.

Media	CYA	MEA	G25N	CzA
Diameter (mm)	20~32	13~25	12~14	13~20
Texture	Radially sulcate floccose	Velutinous or floccose	Velutinous or floccose	Floccose
Myceloium color	White of pale yellow	White to pale yellow	White to pale yellow	White
Conidial color	Light green	Grayish green	Light green	Light green
Exudate	Clear	-	-	-
Conidiogenesis	Moderate	Heavy	Moderate	Moderate
Reverse	Brown	Brown	Vivid yellow	Brown
Soluble pigment	-	-	Red	-

Fig. 3. Picture from differential interface microscopy of *P. janczewskii* sp. F2757 (×1,000; conidiosphore and conidia).

Bar represents 10 μm.



fumagillol moiety of FR-111142[9]. The stereochemistry of H-2′ and H-3′ was determined as *cis*-form based on the coupling constant (J=11.58 Hz) and the configuration of H-2′ and H-3′ was confirmed by the NOE interactions between H-3′ and H-5′, and also between H-4′ and H-6′ in NOESY spectrum as shown in Figure 1. Compound 2 was identified by the comparison the spectral data with that of the diazomethane treated authentic sample, purchased from Sigma Co.

Biological Activity

To identify selective inhibition of the *cis*-fumagillin methyl ester on MetAP2, the purified *cis*-fumagillin methyl ester was placed on MetAP1 deleted strain and the wild type strain *w303*. The summaries of the agar diffusion assay of *cis*-fumagillin methyl ester on the MetAP1 deleted recombinant yeast strain are shown in Fig. 4. To determine IC_{50} value, the *cis*-fumagillin methyl ester (**1**) was tested on the enzymes MetAP1 and MetAP2. The IC_{50} of *cis*-fumagillin methyl ester (**1**) on MetAP2 was 6.3 nM and the IC_{50} on MetAP1 was over 200 μM. Compound **2** also inhibited the MetAP2 with an IC_{50} value of 9.2 nM and 105 μM against MetAP1.

Discussion

The tumor growth could be inhibited by cutting off its blood supplies[10]. Antiangiogenic agents such as angiostatin, endostatin, fumagillin, ovalicin and the fumagillin analogue TNP-470 are known to cut off the blood supply. Among the antiangiogenic agents, fumagillin and its analogues were known to bind to MetAP2 covalently and eventually to inhibit its enzyme activity. In 1998, the crystal structure of MetAP2 was determined and three research groups independently reported the interactions

Table 2. Physico-chemical properties of *cis*-fumagillin methyl ester.

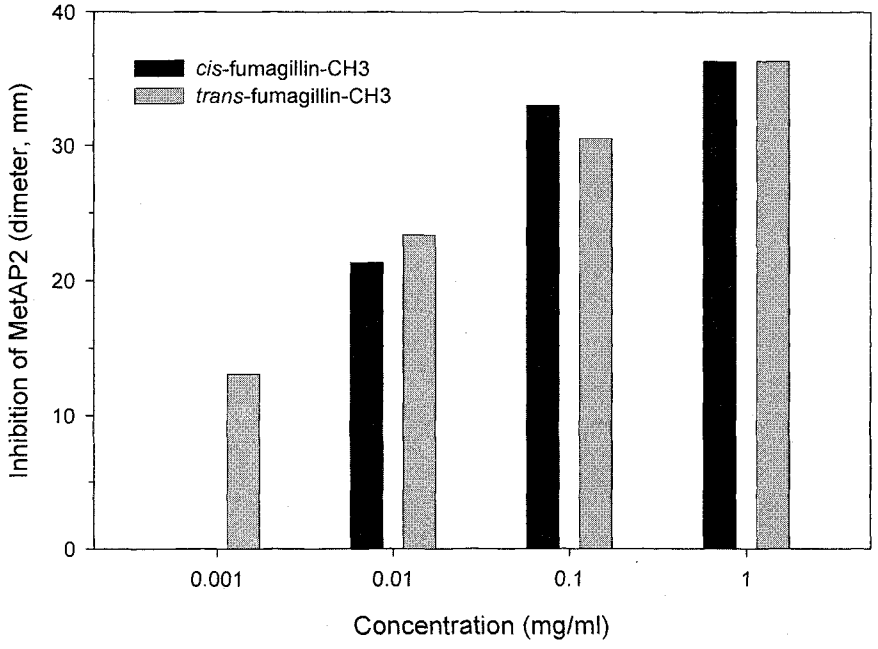
Appearance	Yellowish mass
Molecular formula	$C_{27}H_{36}O_7$
Melting point (℃)	52~55
Optical rotation [α]	+0.356 (in MeOH)
EI-MS (m/z)	471 $(M+H)^+$
Solubility	
Soluble	$CHCl_3$, CH_2Cl_2, EtOAc, MeOH
Insoluble	H_2O, n-Hexane, Benzene
Rf[a]	0.59

[a] Silica gel 60 F_{254} plates (Merck 1.05715): Solvent EtOAc-hexane (25:75); detection: UV or anisaldehyde dip and heating, Rf value of *trans*-fumagillin methyl ester (**2**) is 0.51.

Table 3. ^{13}C and ^1H NMR spectral data for *cis*-fumagillin methyl ester (**1**) in $CDCl_3$.

Position	^1H (ppm) (Multiplicity, *J*=Hz)	^{13}C (ppm)	Position	^1H (ppm) (Multiplicity, *J*=Hz)	^{13}C (ppm)
1			16	1.69 (s, 3H)	18.66
2	3.03 (d, 2H, *J*=3.96)	51.54	17	1.77 (s, 3H)	26.37
3		61.07	18	3.48 (s, 3H)	57.34
4	2.01 (d, 1H, *J*=10.81)	49.03	1'		166.25
5	3.72 (dd, 1H, *J*=14.03, 2.5)	79.86	2'	5.81 (d, 1H, *J*=11.58)	120.10
6	5.72 (m, 1H)	66.99	3'	6.63 (t, 1H, *J*=14.90)	144.18
7	1.91 (m, 2H,)	26.1	4'	7.72 (dd, 1H, *J*=13.6, 3.6)	133.20
8	1.12 (m, 2H)	30.04	5'	6.55 (dd, 1H, *J*=14.8, 3.7)	140.36
9			6'	6.79 (dd, 1H, *J*=15.6, 3.6)	140.59
10		59.20	7'	6.50 (t, 1H, *J*=14.4)	134.08
11	2.61 (t, 1H, *J*=16.68)	61.61	8'	7.38 (dd, 1H, *J*=14.8, 3.6)	144.59
12	2.23 (m, 1H), 2.42 (m, 1H)	28.05	9'	5.99 (d, 1H, *J*=15.6)	122.79
13	5.24 (s, 1H)	119.25	10'		167.87
14		135.56	11'	3.79 (s, 3H)	52.30
15	1.26 (s, 3H)	14.53			

Fig. 4. MetAP2 inhibition activities of *cis*-fumagillin methyl ester (**1**) and *trans*-fumagillin methyl ester (**2**).



of the enzyme's active site with various fumagillin compounds. J. CLARDY *et al.*[11] determined the structure of the human MetAP2 complexed with fumagillin. The structure showed that a covalent bond formed between a reactive epoxide group of fumagillin and a specific histidine residue (His-231) in the active site of the enzyme. They also showed why fumagillin was selective for MetAP2 over MetAP1. LOWTHER *et al.* showed fumagillin reacted with a bacterial enzyme EcMetAP1 that is similar to the human MetAP2[12]. LIU *et al.* found that only the ring epoxide rather than side chain epoxide of fumagillin was involved in the covalent bonding to MetAP2[13]. This fact raises the hope for a more potent analogue without the side chain epoxide on C-4. Furthermore, other groups in fumagillin structure could be changed to get more potent or selective analogues by implication of epoxide case. The comparison of the *cis*- and *trans*-isomer of the fumagillin might shed a light to the development of a new antiangiogenic drug for tumor treatment.

Acknowledgements

We thank the Ministry of Health and Welfare of Korea for financial supporting by a grant (HMP-98-D-4-0031) of the Good Health R&D Program.

Reference

1) ZETTER, B. R.: Angiogenesis and tumor metastasis. Ann. Rev. Med. 49: 407~427, 1998
2) KLAGSBRUN, M. & M. A. MOSES: Molecular angiogenesis. Chem. Biol. 6: R217~R224, 1999
3) INGBER, D.; T. FUJITA, S. KISHIMOTO, K. SUDO, T. KANAMARU, H. BREM & J. FOLKMAN: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature 348: 555~557, 1990
4) SIN, N.; L. MENG, M. Q. W. WANG, J. J. WEN, W. G. BORNMANN & C. M. CREWS: The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc. Natl. Acad. Sci. USA 94: 6099~6103, 1997
5) GRIFFITH, E. C.; Z. SU, B. E. TURK, S. CHEN, Y. H. CHANG, Z. WU, K. BIEMANN & J. O. LIU: Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem. Biol. 4: 461~471, 1997
6) LI, X. & Y. H. CHANG: Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc. Natl. Acad. Sci. USA 92: 12357~12361, 1995
7) PITT, J.: A Laboratory Guide to Common Penicillium Species, ISBN 0-643-03949-X, CSIRO, North Ryde, Australia, 1985
8) ZUO, S.; Q. GUO & Y. -H. CHANG: A protease assay *via* precolumn derivatization and high-performance liquid chromatography. Anal. Biochem. 222: 514~516, 1994
9) OTSUKA, T.; T. SHIBATA, Y. TSURUMI, S. TAKASE, M.

OKUHARA, H. TERANO & M. KOHSAKA: A new angiogenesis inhibitor, FR-111142. J. Antibiotics 45: 348~354, 1992

10) WELLS, W. A.: Starving cancer into submission. Chemistry & Biology 5: R87~R88, 1998

11) LIU, S.; J. WIDOM, C. W. KEMP, C. M. CREWS & J. CLARDY: Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science 282: 1324~1327, 1998

12) LOWTHER, W. T.; D. A. MCMILLEN, A. M. ORVILLE & B. W. MATTHEWS: The anti-angiogenic agent fumagillin covalently modifies a conserved active-site histidine in the *Escherichia coli* methionine aminopeptidase. Proc. Natl. Acad. Sci. USA 95: 12153~12157, 1998

13) GRIFFITH, E. C.; Z. SU, S. NIWAYAMA, C. A. RAMSAY, Y.-H. CHANG & J. O. LIU: Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. Proc. Natl. Acad. Sci. USA 95: 15183~15188, 1998