

Anti-Angiogenic Activities of Gliotoxin and Its Methylthio-Derivative, Fungal Metabolites

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In the search for new naturally occurring angiogenic inhibitor, we found that culture broths from two unidentified fungal strains exerted potent inhibitory activities on capillary-like tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro*. Two active compounds were isolated by bioassay-guided separation and their structures were identified as gliotoxin (1) and its derivative methylthiogliotoxin (2) by spectroscopic analyses. These compounds significantly inhibited the migration of HUVEC assessed by *in vitro* wounding migration assay and exhibited at least 10 times more potent inhibition of proliferation of HUVECs as compared with that of cancer cell lines such as HeLa, MCF-7, and KB 3-1 cells. Especially, gliotoxin having disulfide group exerted more potent activities than methylthiogliotoxin, suggesting that gliotoxin could be a useful compound for further study as an anti-angiogenic agent.

Key words: Gliotoxin, Methylthiogliotoxin, Anti-angiogenic activity, HUVECs. Wound migration, Cytotoxicity

INTRODUCTION

Angiogenesis is a fundamental process by which new blood vessels are formed and is composed of several steps including proteolytic degradation of basement membrane in the vessels, directed migration and proliferation of endothelial cells to provide cells for the new vessels. These steps are strictly regulated by the balance of angiogenic inhibitors and activators (Folkman *et al.*, 1971; McAuslan *et al.*, 1979). In adult, angiogenesis occurs in several physiological conditions including maturation of the corpus luteum, inflammation, wound healing and delayed hypersensitivity reactions (Ausprunk and Folkman, 1977). However, uncontrolled angiogenesis can contribute to a number of pathological processes such as rheumatoid arthritis, diabetic retinopathy and tumor growth and metastasis (Folkman, 1995). Thus anti-angiogenic therapy was postulated to be an attractive approach for the treatment of such diseases (Fidler and Ellis, 1994; Gastl *et al.*, 1997).

Several angiogenic inhibitors have been developed for targeting angiogenic factors or their receptors, extracellular matrix compounds, and vascular endothelial cell proliferation. Among them, thalidomide and TNP-470 are known to inhibit the growth of endothelial cells specifically and are in clinical trials (D'Amato *et al.*, 1994; Ingber *et al.*, 1990).

In the course of our screening to find new anti-angiogenic agents from natural resources, we identified two structurally related compounds gliotoxin (1) and methylthiogliotoxin (2) from two unidentified fungal strains by bioassay-guided fractionation and isolation. In this report, we describe the isolation, identification, and anti-angiogenic activity of compounds 1 and 2.

MATERIALS AND METHODS

General experimental procedures

Melting points were measured on a Electrothermal model 9100 and are uncorrected. UV spectra were obtained on a Milton Roy 3000 spectrometer. ¹H-NMR (300 MHz) and ¹³C-NMR (75.5 MHz) spectra were obtained on a Varian Unity NMR Spectrometer and chemical shifts were referenced to tetramethylsilane (TMS) as the

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internal standard. ESI-MS was measured on a JMS-HS 110A mass spectrometer. Kieselgel 60 (Merck No, 9385 and 7729) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography. Preparative HPLC was carried out on a DelataPak C18 column (φ 19 mm \times 300 mm, Waters) with UV detection at 245 nm. Gelatin and (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), Medium 199, and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO/BRL Laboratories (Grand Island, NY).

Purification of gliotoxin (1) and methylthiogliotoxin (2)

A slant culture of Y90086 strain or Y80805 strain grown on malt extract agar medium consisting of malt extract 2%, polypeptone 0.1%, glucose 2% and agar 2% was inoculated into 1000 ml baffled flasks containing 200 ml of medium consisting of glucose 2%, yeast extract 0.2%, polypeptone 0.5%, magnesium sulfate (MgSO_4) 0.05%, and potassium phosphate, monobasic (KH_2PO_4) 0.1% (pH 5.6~5.8). After 5 days of cultivation on a rotary shaker for 7 days at 28°C and 140 rpm, the cultured broth was extracted once with the same volume of acetone and the cell debris was removed by filtration. The aqueous phase was evaporated to small volume and then extracted twice with EtOAc. Combined extracts were concentrated to dryness *in vacuo*. The EtOAc soluble fraction (1.4 g) obtained from Y90086 culture broth was chromatographed on a silica gel column eluted with EtOAc - MeOH (5 : 1) and further purified by preparative HPLC eluted with a acetonitrile - water gradient system to give gliotoxin (1, 16 mg). The EtOAc soluble fraction (2 g) obtained from the strain Y80805 was chromatographed on Sephadex LH-20 eluted with MeOH. Active fraction was further chromatographed on silica gel eluted with EtOAc - MeOH (5 : 1) to give methylthiogliotoxin (2, 12 mg).

Gliotoxin (1) : white needle from MeOH, mp 221 - 224°C, UV λ_{max} (MeOH) 210, 266 nm, ESI-MS (negative-ion mode) m/z 325 $[\text{M}-\text{H}]^-$, $^1\text{H-NMR}$ (300 MHz, $\text{DMSO}-d_6$) δ ; 3.10 (3H, s, 2-Me), 3.12 (1H, d, $J=17.7$ Hz, H-10B), 3.33 (1H, 3a-OH, overlap), 3.64 (1H, d, $J=17.7$ Hz, H-10A), 4.22 (1H, dd, $J=12.8, 5.7$ Hz, H-3aA), 4.33 (1H, dd, $J=12.8, 5.7$ Hz, H-3aB), 4.54 (1H, d, $J=13.0$ Hz, H-6), 4.85 (1H, d, $J=13.0$ Hz, H-5a), 5.64 (1H, d, $J=9.3$ Hz, H-7), 5.72 (1H, s, 6-OH), 5.97 (1H, dd, $J=9.6, 3.0$ Hz, H-8), 6.00 (1H, br d, $J=5.7$ Hz, H-9), $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO}-d_6$) δ ; 28.0 (2-Me), 36.2 (C-10), 59.0 (C-3a), 69.6 (C-5a), 73.0 (C-6), 76.2 (C-10a), 78.6 (C-3), 119.4 (C-9), 124.2 (C-8), 129.6 (C-7), 132.9 (C-9a), 164.6 (C-4), 166.0 (C-1)

Methylthiogliotoxin (2) : white oil, UV λ_{max} (MeOH) 210, 273 nm, ESI-MS (positive-ion mode) m/z 379

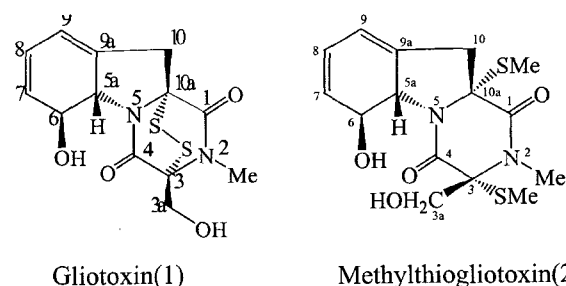


Fig. 1. Chemical structures of gliotoxin and methylthiogliotoxin

$[\text{M}+\text{Na}]^+$, $^1\text{H-NMR}$ (300 MHz, $\text{DMSO}-d_6$) δ ; 2.19 (3H, s, 11-Me), 2.20 (3H, s, 12-Me), 2.80 (1H, br d, $J=15.6$ Hz, H-10A), 2.99 (3H, s, 2-Me), 3.11 (1H, d, $J=15.6$ Hz, H-10B), 3.17 (1H, d, $J=5.6$ Hz, 3a-OH), 3.72 (1H, dd, $J=11.3, 5.6$ Hz, H-3aA), 4.06 (1H, dd, $J=11.3, 5.6$ Hz, H-3aB), 4.72 (1H, d, $J=13.7$ Hz, H-6), 4.83 (1H, d, $J=13.7$ Hz, H-5a), 5.48 (1H, s, 6-OH), 5.64 (1H, d, $J=9.8$ Hz, H-7), 5.91 (1H, dd, $J=9.8, 3.0$ Hz, H-8), 6.00 (1H, br d, $J=3.0$ Hz, H-9), $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO}-d_6$) δ ; 12.8 (11-Me), 14.7 (12-Me), 28.3 (2-Me), 38.4 (C-10), 63.0 (C-3a), 69.0 (C-5a), 73.8 (C-6), 71.5 (C-10a), 72.8 (C-3), 119.3 (C-9), 123.5 (C-8), 130.6 (C-7), 133.1 (C-9a), 166.3 (C-4), 165.2 (C-1)

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVECs) was purchased from American Type Culture Collection (Rockville, MD). The HUVECs were plated onto 0.3% gelatin-coated culture dishes and grown in medium 199 supplemented with heat-inactivated 20% (v/v) fetal bovine serum (FBS), 3 ng/ml basic fibroblast growth factor (bFGF), 100 ng/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C incubator in a humidified atmosphere containing 5% CO_2 . HUVECs were used between passage 18 and 23.

Cell proliferation assay

HUVECs were seeded at 5×10^3 cells/well in gelatin-coated 96-well plates. After one day, the medium was changed with the fresh one, and then various concentrations of compound 1 and 2 (300 ng, 100 ng, 30 ng, 10 ng/ml) were added to each well. After incubation for three days, MTT solution were added to each well, and incubated for further 4 hr. The formed formazan-dye was solubilized and then optical density was measured at 570 nm by a spectrophotometer (Tada *et al.*, 1986). Each experiment was performed in triplicate.

In vitro tube formation assay

HUVECs were seeded onto 96-well plate coated with

Matrigel at 2×10^4 cells/well in M199 medium supplemented with heat-inactivated 20% (v/v) fetal bovine serum (FBS), 3 ng/ml basic fibroblast growth factor (bFGF), 100 ng/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin. Immediately after the seeding, various concentrations of the tested compounds were added and the cells were incubated at 37°C for 16 hr. The morphological changes of HUVECs observed and photographed three different fields per well under a microscope ($\times 100$) (Pepper *et al.*, 1993). Inhibition of tube formation was assessed by the measurement of total tube-length formed in each photograph using Adobe Photoshop software.

Wound migration assay

Wound migration assay was performed as previously described (Shinji *et al.*, 1996). Briefly, HUVECs were plated on gelatin-coated 60mm culture dishes. At 90% confluency, the endothelial monolayer was marked an injury line and wounded 2 mm in width with a sterile razor blade. Plates were rinsed with serum-free medium to remove cellular debris, and added with fresh M199 medium supplemented with 10% serum and 1 mM thymidine to inhibit cell proliferation. Cells were allowed to migrate for 24 hr in the presence of compounds as indicated. Control cultures were exposed to medium alone. The migration of HUVECs was observed and photographed three different fields per dish through an inverted microscope ($\times 100$). The number of cells that moved beyond the reference line was counted.

RESULTS AND DISCUSSION

We identified two novel anti-angiogenic compounds, gliotoxin (**1**) and methylthiogliotoxin (**2**), from two unidentified fungal strains by bioassay-guided fractionation and isolation. Their structures were elucidated by the comparison of their physicochemical properties and NMR spectral data with those reported in the literature (Okamoto *et al.*, 1986a; Kaouadji *et al.*, 1990).

Compound **1** was obtained as white needle crystals. The UV spectrum of **1** have λ_{max} at 210 and 266 nm. It exhibited negative ESI-MS ion at m/z 325 indicating a molecular weight of 326. The $^1\text{H-NMR}$ spectrum of **1** showed two sets of quartets between δ 4.22 ($J=12.8, 5.7$ Hz) and δ 4.33 ($J=12.8, 5.7$ Hz) which was assigned to the methylene protons of the primary alcohol fragment. Further, the doublets at δ 3.12 ($J=17.7$ Hz) and δ 3.64 ($J=17.7$ Hz) were assigned to the endocyclic methylene protons at C-10. The allylic protons of the hydrated benzene ring appear as a doublet of doublets at δ 4.54 and δ 4.85 with a coupling constant of 13.0 Hz. The coupling between these protons in compound **1** is due to the trans diaxial orientation (Waring *et al.*, 1986). The

remaining signals was assigned to the N-methyl signal at δ 3.10 and the characteristic 1,2,3-substituted aromatic ring signals at δ 5.64 (d, $J=9.3$ Hz), δ 5.97 (dd, $J=9.6, 3.0$ Hz) and δ 6.00 (br d, $J=5.7$ Hz). The $^{13}\text{C-NMR}$ and DEPT spectra of **1** showed 13 carbon signals including two sets of methylenes at δ 36.2 and δ 59.0, one N-methyl at δ 28.0 and two carbonyl carbons at δ 164.6 and δ 166.0. In the HMBC spectrum of **1**, the methylene protons (δ 4.22 and δ 4.33) of the primary alcohol were correlated with δ 78.6 and δ 164.6, which were assigned to the signals of C-3 and C-4, respectively. Another methylene protons at δ 3.12 and δ 3.64 were correlated with δ 76.2 and δ 166.0, which were assigned to the C-10a and C-1, respectively. The doublet signal at δ 4.85 was assigned to the H-5a due to the correlation with δ 73.0 (C-6) and δ 132.9 (C-9a). Another methine proton of H-6 was correlated with δ 69.6 (C-5a) and δ 129.6 (C-7). Accordingly, the structure of compound **1** was identified as gliotoxin, an epidithiodioxopiperazine class of antibiotic, in comparison with spectral data reported previously (Avent *et al.*, 1993; Haraguchi *et al.*, 1992; Kaouadji *et al.*; 1990, Waring *et al.*, 1986).

Compound **2** was obtained as white oil. The UV spectrum of **2** showed similar pattern of λ_{max} to those of **1**. Its positive ESI-MS showed the $[\text{M}+\text{Na}]^+$ ion peak at m/z 379, which was 30 mass units larger than that of **1**. This difference corresponded to the mass of additional two S-methyl moieties. The $^1\text{H-}$ and $^{13}\text{C-}$ NMR spectra of **2** were closely related to those of **1** except for the appearance of two sets of additional S-methyl signals at δ 2.19 and δ 2.20, indicating that compound **2** would be the form of reductive methylation of disulfide linkage of compound **1**. Accordingly, compound **2** was thus identified as methylthiogliotoxin, a dethiobisdimethylether derivative of gliotoxin by the comparison of spectral data of **2** with those reported previously (Avent *et al.*, 1993; Kirby *et al.*, 1988; Okamoto *et al.*, 1986a; Waring *et al.*, 1986).

These compounds significantly inhibited angiogenesis assessed by tube formation assay *in vitro*. The control HUVECs began to start the differentiation onto Matrigel in 6 hr after seeding, and formed the complete tubular structure in 16h. However, compounds **1** and **2** inhibited the tube formation of HUVECs in a dose dependent manner. The effects of the compounds on tube formation were quantified by measuring total tube length (Fig. 2). Compound **1** exhibited at least 10 times more potent inhibitory effect on tube formation of HUVEC than that of its derivative, compound **2**.

To further study how compound **1** and **2** exerted the inhibitory activity, we investigated the effect of these compound on the migration of HUVECs. Both compounds dose-dependently inhibited the migration of HUVECs assessed by wounding migration assay (Fig. 3). Consistent with tube formation assay, compound **1** showed more potent inhibitory activity on the migration

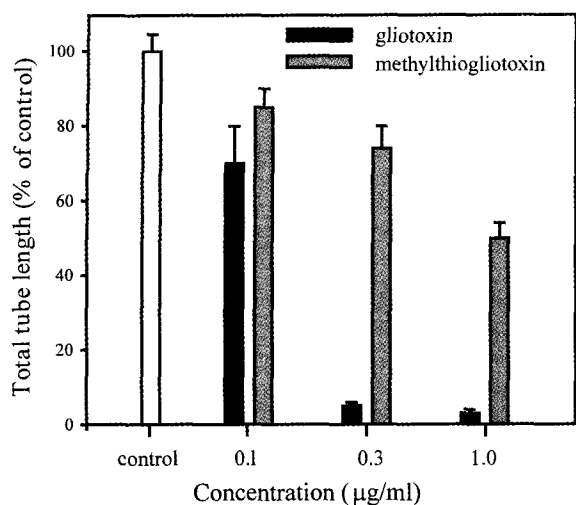


Fig. 2. Effect of gliotoxin and methylthiogliotoxin on the tube formation of HUVECs on matrigel

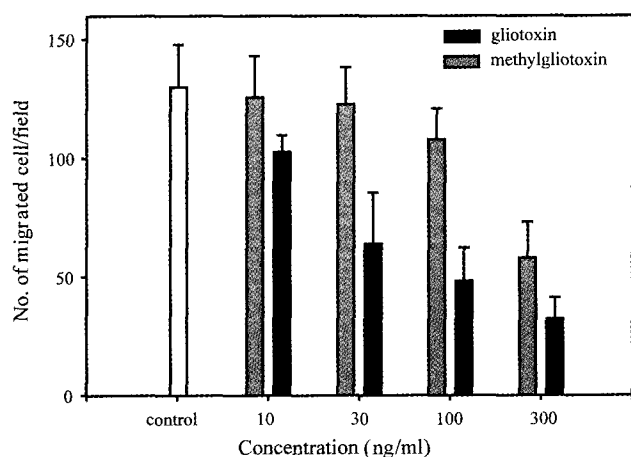


Fig. 3. Effect of gliotoxin and methylthiogliotoxin on the migration of HUVECs

of HUVECs. In addition, the effects of compounds **1** and **2** on the proliferation of HUVECs were investigated by MTT assay. As a control, we also measured the effect of these compounds on the proliferation of other cancer cell lines such as MCF-7, HeLa, and KB-3-1 (Fig. 4, 5). Compounds **1** and **2** significantly inhibited the proliferation of HUVECs in a dose dependent manner with IC_{50} values of 40 and 400 ng/ml, respectively. However, both compounds did not significantly inhibit the proliferation of HeLa, MCF-7, and KB-3-1 cell lines at concentrations that inhibited the proliferation of HUVECs. The difference of IC_{50} values of these compounds between HUVECs and other three cancer cell lines was at least 10-fold (Fig. 4, 5).

Both migration and proliferation of endothelial cells are critical and essential events in the initiation of new blood vessel formation. Compound **1** and its derivative compound **2** dose dependently inhibited the migration and

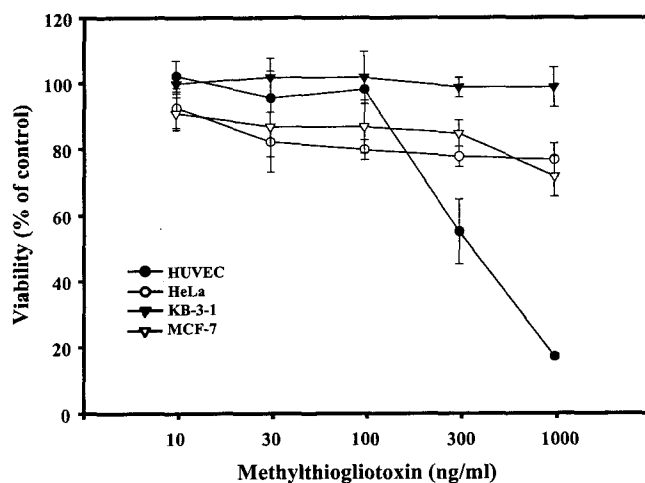


Fig. 4. Effect of gliotoxin on the proliferation of HUVECs, HeLa, KB-3-1 and MCF-7 cell lines

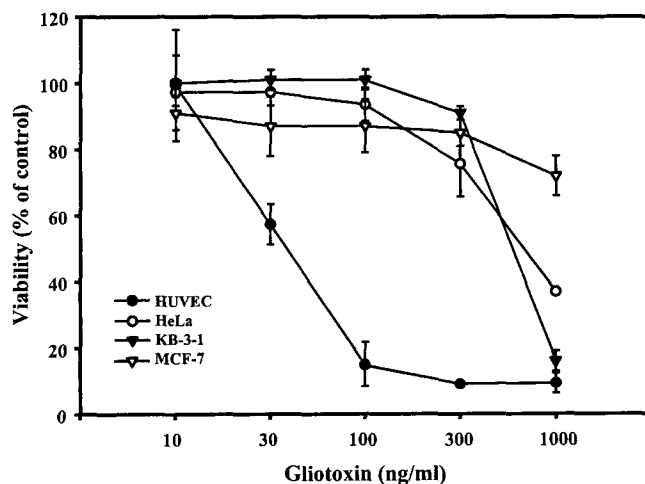


Fig. 5. Effect of methylthiogliotoxin on the proliferation of HUVECs, HeLa, KB-3-1 and MCF-7 cell lines

proliferation of HUVECs. Moreover, these compounds did not significantly inhibit the proliferation of three cancer cell lines. Therefore, it is possible that anti-angiogenic activity of gliotoxin and bisdethiobis (methylthio) gliotoxin may be partially explained by the inhibition of migration and the selective inhibition of proliferation of endothelial cells among several angiogenesis steps.

Compound **1** and its derivatives, **2** have known to be a kind of common epipolythiopiperazine-3,6-dione fungal metabolites. Compound **1** was isolated because of its potent antibiotic properties against bacteria, fungi, and RNA viruses. It has diverse biological activities. Compound **1** has been reported to have profound immunosuppressive activity *in vivo*. It induces apoptosis in thymocytes, splenocytes and mesenteric lymph node cells, and can selectively deplete bone marrow of mature lymphocytes (Pahl *et al.*, 1996). Compound **1** also accelerated the rate of constitutive apoptosis in human

neutrophils and eosinophils by inhibition of transcription factor nuclear factor κ B (Ward *et al.*, 1999). Compound **1** and its derivatives also showed inhibitory activities on platelet activating factor-induced platelet aggregation and farnesyl-protein transferase (Okamoto *et al.*, 1986b; Pyl *et al.*, 1992). But, compounds **1** and **2** have not been reported to have anti-angiogenic activity yet.

Compound **1** exhibited much more potent inhibitory activities on tube formation, proliferation, and migration of HUVECs than those of compound **2** *in vitro*. It appears that a disulfide group in compound **1** could be a critical functional group to exert its various biological activities. Consistent with this suggestion, it was reported that compound **1** has more potent activity than compound **2** on the inhibition of NF- κ B and platelet aggregation (Ward *et al.*, 1999; Sakai and Watanuki, 1987).

In conclusion, our results suggest that compound **1** and **2** are novel angiogenic inhibitors. Inhibition of migration and proliferation of endothelial cells might be directly associated with the anti-angiogenic activities of these compounds. However, more detailed evaluation on anti-angiogenic mechanisms of these compounds remains to be investigated.

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REFERENCES

- Ausprunk, D. H. and Folkman, J., Migration and proliferation of endothelial cells in preformed and newly formed vessels during tumor angiogenesis. *Microvasc. Res.*, 14, 53-165 (1977).
- Avent, A. G., Hanson, J. R., and Truneh, A., Metabolites of *Gliocladium flavofusum*. *Phytochemistry*, 32, 197-198 (1993).
- DAmato, R. J., Loughnan, M. S., Flynn, E., and Folkman, J., Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. USA.*, 91, 4082-4085 (1994).
- Filder, I. J. and Ellis, L. M., The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell*, 79, 185-188 (1994).
- Folkman, J., Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Medicine*, 1, 27-31 (1995).
- Folkman, J., Merler, E., Abernathy, C. and Williams, G., Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.*, 133, 275-288 (1971).
- Gastl, G., Hermann, T., Steurer, M., Zmija, J., Gunsilins, E., Unger, C., and Kraft, A. Angiogenesis as a target for tumor treatment. *Oncology*, 54, 177-184 (1997).
- Haraguchi, H., Hamatani, Y., Shibata, K., and Hashimoto, K., An inhibitor of acetolactate synthase from a microbe. *Biosci. Biotech. Biochem.*, 56, 2085-2086 (1992).
- Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H., and Folkman, J., Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. *Nature*, 348, 555-557 (1990).
- Kaouadji, M., Steiman, R., Seigle-Muran, F., Krivobok, S. and Sage, L., Gliotoxin: uncommon ^1H couplings and revised ^1H - and ^{13}C -NMR assignments. *J. Nat. Prod.*, 53, 717-719 (1990).
- Kirby, G. W., Rao, G. V., and Robins, D. J., New co-metabolites of gliotoxin in *Gliocladium virens*. *J. Chem. Soc. Perkin Trans. I.*, 301-304 (1988).
- McAuslan, B. R. and Hoffman, H. Endothelium stimulating factor from Walker carcinoma cells. *Exp. Cell Res.*, 119, 181-190 (1979).
- Okamoto, M., Yoshida, K., Uchida, I., Nishikawa, M., Kohsaka, M., and Aoki, H., Studies of platelet activating factor (PAF) antagonists from microbial products. I. Bisdethiobis(methylthio)gliotoxin and its derivatives. *Chem. Pharm. Bull.*, 34, 340-344 (1986a).
- Okamoto, M., Yoshida, K., Uchida, I., Kohsaka, M., and Aoki, H., Studies of platelet activating factor (PAF) antagonists from microbial products. II. Pharmacological studies of FR-49175 in animal models. *Chem. Pharm. Bull.*, 34, 345-348 (1986b).
- Pahl, H. L., Krauss, B., Schulze-Osthof, Decker T., Traenckner E. B., Vogt, M., Myers, C., Parks, T., Warring, P., Muhlbacher, A., Czernilofsky, A. P., and Baeuerle, P. A., The immunosuppressive fungal β -bolite gliotoxin specifically inhibits transcription factor NF-kappa B. *J. EXP. Med.*, 183, 1829-1840 (1996).
- Pepper, M. S., Vassalli, J. D., Ovci, L., and Montesano, R., Biphasic effect of transforming growth factor-1 on *in vitro* angiogenesis. *Exp. Cell Res.*, 204, 356-363 (1993).
- Pyl, D. V., Inokoshi, J., Shimoi, K., Yang, H., Takeshima, H., and Omura, S., Inhibition of farnesyl-protein transferase by gliotoxin and acetylgliotoxin. *J. Antibiotics*, 45, 1802-1805 (1992).
- Sakai, M. and Watanuki, M., Inhibitory effect of gliotoxin on platelet aggregation-Importance of disulfide structure. *Agric. Biol. Chem.*, 51, 2167-2170 (1987).
- Shinji, Y., Peter, R. N., and Kent, K. C., Role of protein kinase C in attachment, spreading and migration of human endothelial cells. *J. Surg. Res.*, 63, 349-354 (1996).
- Tada, H., Shiho, O., Kuroshima, K. I., Koyama, M., and Tsukamoto, K., An improved colorimetric assay for interleukin 2. *J. Immunol. Methods*, 93, 157-165 (1986).
- Ward, C., Chilvers, E. R., Lawson, M. F., Pryde, J. G., Fujihara, S., Farrow, S. N., Haslett, C., and Rossi, A. G., NF- κ B activation is a critical regulator of human granulocyte apoptosis *in vitro*. *J. Biol. Chem.*, 274, 4309-4318 (1999).
- Waring, P., Eichner, R. D., Palni, U. T., and Mullbacher, A., The isolation and identification of a new metabolite from *Aspergillus fumigatus* related to gliotoxin. *Tetrahedron Lett.*, 27, 735-738 (1986).