Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Inhibition of expression of vascular endothelial growth factor and its receptors in pulmonary adenocarcinoma cell by TNP-470 in combination with gemcitabine

WANG Xue-fen[†1], TU Ling-fang[1], WANG Li-hong[2], ZHOU Jian-ying[1]

([1]*Department of Respirology, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

([2]*Department of Cardiology, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

[†]E-mail: wxfzy636@hotmail.com

Received Dec. 23, 2005; revision accepted Apr. 17, 2006

Abstract: Angiogenesis is required for solid tumor growth and facilitates tumor progression and metastasis. The inhibition effects of O-(chloroacetyl-carbamoyl) fumagillol (TNP-470), an angiogenesis inhibitor, and gemcitabine, a chemotherapeutic agent, on expression of growth factors were investigated using human pulmonary adenocarcinoma cell line, A549. The A549 cells were divided into four groups: control group, 10^{-6} mg/ml gemcitabine treated group, 10^{-4} mg/ml TNP-470 treated group and gemcitabine+TNP-470 treated group. The mRNA and protein expression of vascular endothelial growth factor (VEGF) and its receptors, FMS-like tyrosine kinase-1 (FLT-1) and kinase insert domain-containing receptor (KDR), in different groups were measured. The growth of A549 cell cultured with gemcitabine or TNP-470 was inhibited in an almost dose-dependent manner. Although gemcitabine (10^{-6} mg/ml) alone and TNP-470 (10^{-4} mg/ml) alone had no effect on the mRNA and protein expression of VEGF and its receptors (FLT-1, KDR) in A549 cells compared to the control (P>0.05), 10^{-6} mg/ml gemcitabine in combination with 10^{-4} mg/ml TNP-470 had significant effect (P<0.01). Moreover, combination of the two drugs significantly inhibited the mRNA expression of VEGF, FLT-1 and KDR compared to either drug alone (P<0.05). This study suggests that combined treatment with TNP-470 plus gemcitabine may augment the antiangiogenic and antineoplastic effects in lung cancer cells in vitro.

Key words: TNP-470, Gemcitabine, VEGF, FLT-1, KDR
doi:10.1631/jzus.2006.B0837 **Document code:** A **CLC number:** R734

INTRODUCTION

Angiogenesis is required for solid tumor growth and facilitates tumor progression and metastasis (Folkman, 1990; Weidner *et al*., 1991; Weinstat-Saslow and Steeg, 1994). Moreover, angiogenesis decreased overall survival in patients with various tumors, such as lung cancer (Sauter *et al*., 1999; Johnson *et al*., 2005). Angiogenesis is thought to be regulated by a number of growth factors, including basic fibroblast growth factor (bFGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) (Rifkin *et al*., 1993). VEGF is a disulfide-linked dimeric glycoprotein that increases blood vessel permeability, endothelial cell growth, proliferation, migration, and differentiation (Senger *et al*., 1993). It is seemingly produced by human tumors solely for the stimulation of tumor vascularization. VEGF mediates angiogenic signals to the vascular endothelium via the receptors of FMS-like tyrosine kinase-1 (FLT-1) and kinase insert domain-containing receptor (KDR) (Miura *et al*., 2004). O-(chloroacetyl-carbamoyl) fumagillol (TNP-470), an analog of fumagillin derived from *Aspergillus fumigatus*, inhibits angiogenesis both in vivo and in vitro, regardless of the presence of angiogenesis factors. It is also less toxic than fumagillin (Kusaka *et al*., 1994). In addition, it was reported that TNP-470 has an inhibition effect on the growth and metastasis of human cell lines and rodent tumors (Yanase *et al*., 1993; Yamamoto *et al*., 1994; Shishido *et al*., 1996). In this study, we investigated the effects of TNP-470 and

gemcitabine, a chemotherapeutic agent, against human pulmonary adenocarcinoma cell line A549, which has high metastatic potential among the lung cancers.

MATERIALS AND METHODS

Cell proliferation assay

A549 cells (5×10^3 $well^{-1}$, obtained from Shanghai Cell Resources Center, China) were cultured in a 96-well plate. Culture supernatants were replaced the next day (day 1) with fresh medium, containing TNP-470 (Takeda Pharmaceutical Company, Japan) or gemcitabine (Eli Lilly and Company, USA) at concentrations of 0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml. On day 3, to count viable cells, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay was performed. The MTT assay was previously described. Briefly, 20 μl of 5 mg/ml MTT was added and the cells were incubated for 4 h. This product was solubilized by the addition of dimethyl sulfoxide (DMSO). The absorbance of each well was quantified with a NovaPath microplate reader using a test wavelength of 570 nm. The assay was repeated at least four times.

Cell culture and anti-angiogenic treatment

A549 cells were cultured in 9 cm Petri dish at 2×10^5 cells/ml for 10 ml in RPMI 1640 medium with 10% FCS. Culture media were changed to RPMI 1640 containing no FCS but concentrations of gemcitabine (0, 10^{-6} mg/ml) with/without TNP-470 (10^{-4} mg/ml). After 2 d, the cells were collected and cultured in three groups.

Reverse transcription (RT) and polymerase chain reaction (PCR)

RNA was isolated from cell lines using TRIzol reagent (Life Technologies, Inc.). RT was carried out using a kit (Fermentas) according to the manufacturer's instructions. RT products were stored at −70 °C. The amount of RNA was determined by measuring the specific absorption at 260 nm. Oligonucleotide primers used were (written in the 5' to 3' direction): (1) VEGF sense primer, TCGGGCCTCC GAAACCATGA, and antisense primer, CCTGGT GAGAGATCTGGTTC; (2) FLT-1 outer sense primer, ATTTGTGATTTTGGCCTTGC, and outer antisense primer, CAGGCTCATGAACTTGAAA GC; FLT-1 inner sense primer, CACCAAGAGCGA CGTGTG, and inner antisense primer, TTTTGG GTCTCTGTGCCAG; (3) KDR sense primer, GTCAAGGGAAAGACTACGTTGG, and antisense primer, AGCAGTCCAGCATGGTCTG; (4) β-actin sense primer, CGCTGCGCTGGTCGACA, and antisense primer, GTCACGCACGATTTCCCGCT. The VEGF primers correspond to sequences in the untranslated 5' and 3' region resulting in amplication of three different splice variants of size 516, 648 and 720 bp. Primers specific for KDR, FLT-1 and β-actin recognize coding sequences. PCR product sizes are, for FLT-1 outer primer pair, 555 bp; for FLT-1 inner primer pair, 196 bp; for KDR, 591 bp; and for β-actin, 619 bp, respectively.

VEGF, FLT-1 and KDR expression were determined by semi-quantitative PCR using β-actin as an internal standard. The samples were subjected to 35 cycles of amplification as follows: the samples were initially heated to 94 °C for 3 min to ensure complete denaturation of DNA (30 s for subsequent cycles) followed by 30 s at 60 °C to anneal the primers, and then 1-min at 72 °C for extension of the annealed primer. The PCR reaction was concluded by a 30 s elongation phase, again at 72 °C. The PCR products (5 μl) were visualized on 1.5% agarose gel. Electronic images were captured by using a solid state black-and-white video camera (Cohu Electronic), and the intensity of the bands was determined using Kodak Digital imaging software (EDAS290). Every sample was repeated three times.

Protein isolation and Western blot analysis

Cell cultures grown to 70%~80% confluence were lysed with an SDS-based single lysis buffer. The lysate was centrifuged at 12000 r/min, and the supernatant was collected. Total protein concentration of the soluble cell extract was determined based on absorbance. Total protein (40 μg) of each sample was labelled in the presence of SDS before the addition of 2-mercaptoethanol and applied to a discontinuous 10% polyacrylamide gel for electrophoretic separation. Proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane, which was placed in blocking solution (5% nonfat dry milk), probed with VEGF, or FLT-1, or KDR, or

β-actin-specific antibody (1:400, Santa Cruz, Inc.) followed by a phosphatase-conjugated second antibody (1:5000, Santa Cruz, Inc.), and washed. Substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were added for signal detection. Every sample was repeated three times.

Statistical analysis

Data are presented as mean±*SD*. Datasets containing the four different groups were analyzed by ANOVA. Mean values between two different groups were compared by a least-significant difference (*LSD*) test, after an *F* test for homogeneity of variances had been performed. If data failed to meet the requirements for equal variance, a Tamhane's T2 test was used. Differences were considered statistically significant at a value of $P<0.05$.

RESULTS

Effects of gemcitabine and TNP-470 on cell proliferation

As shown in Fig.1a, the growth of A549 cell cultured with gemcitabine was inhibited in an almost dose-dependent manner. Half-maximum inhibition of gemcitabine was 10^{-4} mg/ml approximately. The dose of gemcitabine that we chose to study was 10^{-6} mg/ml in order to reduce the possible severe side effects induced by high dose drug.

The growth of A549 cell cultured with TNP-470 was also inhibited in an almost dose-dependent manner (Fig.1b). Half-maximum inhibition of TNP-470 occurred at approximately 10^{-3} mg/ml. The dose of TNP-470 that we chose to study was 10^{-4} mg/ml.

Effects of gemcitabine or/and TNP-470 on mRNA expression of VEGF, FLT-1, KDR

As shown in Fig.2, gemcitabine (10^{-6} mg/ml) alone and TNP-470 (10^{-4} mg/ml) alone had no effect on the mRNA expression of VEGF and its receptors (FLT-1, KDR) in A549 cells compared to the control ($P>0.05$). However, 10^{-6} mg/ml gemcitabine in combination with 10^{-4} mg/ml TNP-470 significantly inhibited the mRNA expression of VEGF, FLT-1 and KDR compared to the control ($P<0.01$). Moreover, the combination of two drugs had significant effect on the mRNA expression of VEGF, FLT-1 and KDR compared to either drug alone ($P<0.05$).

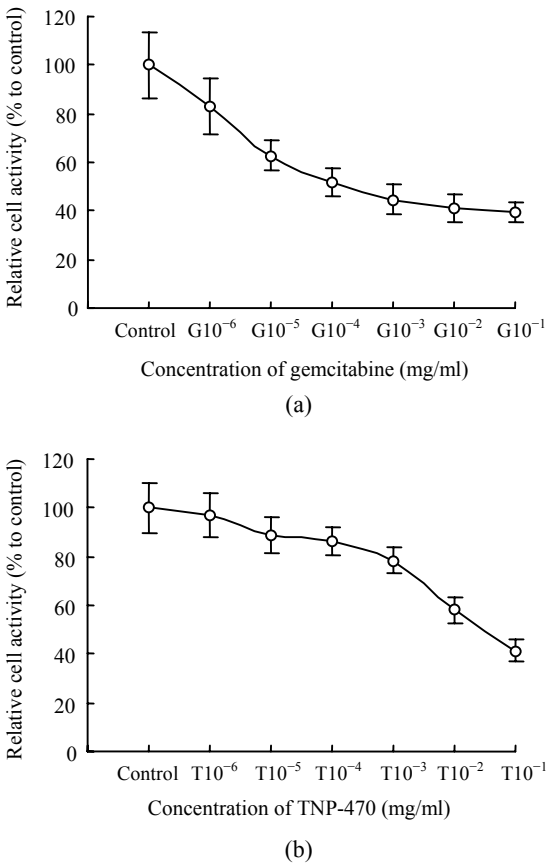
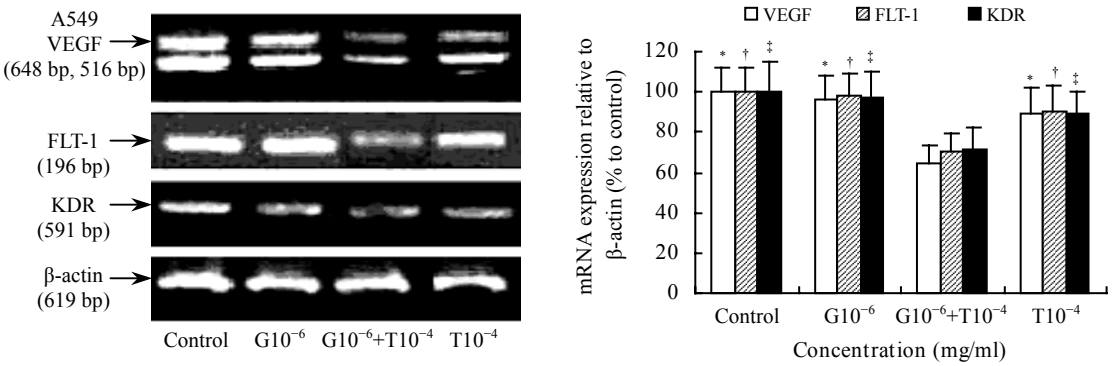


Fig.1 Effects of gemcitabine (a) and TNP-470 (b) on in vitro proliferation of A549 cells

Effects of gemcitabine or/and TNP-470 on protein expression of VEGF, FLT-1, KDR

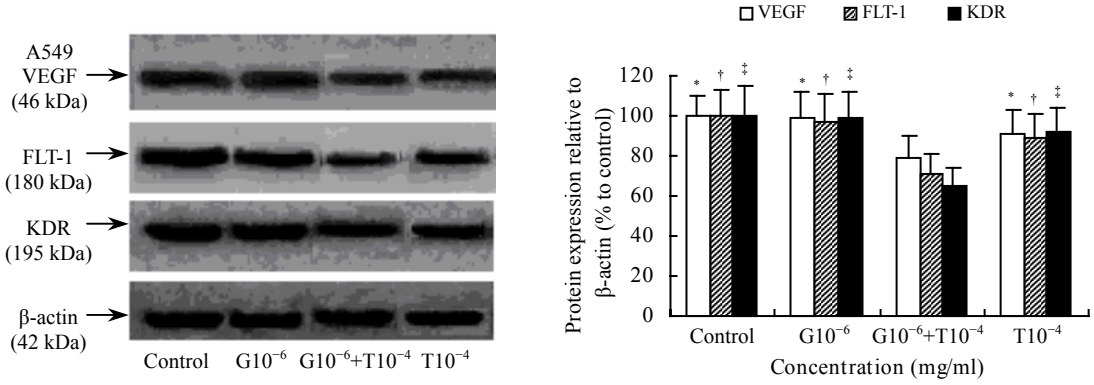
The protein expression of VEGF, FLT-1 and KDR in A549 cells was also examined, which provided a very sensitive approach for the results of relative expression of mRNA (Fig.3). Gemcitabine (10^{-6} mg/ml) alone and TNP-470 (10^{-4} mg/ml) alone also had no effect on the protein expression of VEGF, FLT-1 and KDR in A549 cells compared to the control ($P>0.05$). However, there was significant difference in inhibition of protein expression of VEGF and its receptors (FLT-1, KDR) between the group cultivated using 10^{-6} mg/ml gemcitabine combined with 10^{-4} mg/ml TNP-470 and control group ($P<0.01$). Furthermore, the combination of gemcitabine and TNP-470 enhanced the inhibition of VEGF and its receptors compared to gemcitabine/TNP-470 alone ($P<0.05$).



(a) (b)

Fig.2 Effects of gemcitabine or/and TNP-470 on mRNA expression of VEGF, FLT-1 and KDR in A549 cells. (a) The representative RT-PCR products from normal control, 10^{-6} mg/ml gemcitabine treatment, 10^{-6} mg/ml gemcitabine+10^{-4} mg/ml TNP-470 treatment, and 10^{-4} mg/ml TNP-470 treatment; (b) The data expressed by mean±*SD* for VEGF, FLT-1 and KDR mRNA levels. All the data were showed by the ratio of the expression of aim mRNA and that of β-actin

$^{*}P$<0.05 vs VEGF expression in gemcitabine+TNP-470 treatment group; $^{\dagger}P$<0.05 vs FLT-1 expression in gemcitabine+TNP-470 treatment group; $^{\ddagger}P$<0.05 vs KDR expression in gemcitabine+TNP-470 treatment group



(a) (b)

Fig.3 Effects of gemcitabine or/and TNP-470 on protein expression of VEGF, FLT-1 and KDR in A549 cells. (a) The representative Western blot products from normal control, 10^{-6} mg/ml gemcitabine treatment, 10^{-6} mg/ml gemcitabine+10^{-4} mg/ml TNP-470 treatment, and 10^{-4} mg/ml TNP-470 treatment; (b) The data expressed by mean±*SD* for VEGF, FLT-1 and KDR protein levels. All the data was showed by the ratio of the expression of aim protein and that of β-actin

$^{*}P$<0.05 vs VEGF expression in gemcitabine+TNP-470 treatment group; $^{\dagger}P$<0.05 vs FLT-1 expression in gemcitabine+TNP-470 treatment group; $^{\ddagger}P$<0.05 vs KDR expression in gemcitabine+TNP-470 treatment group

DISCUSSION

Tumor angiogenesis plays an important role in tumor growth, maintenance and metastatic potential. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and a potent inducer of vessel permeability and angiogenesis in vivo (Senger *et al*., 1993) and is a marker of tumor invasion and metastasis (Sauter *et al*., 1999). Non-small cell lung cancer (NSCLC) tissue produces numerous growth factors, which are multifunctional and considered predictive of patient survival. The previous studies showed that a high expression of VEGF (≥25% of cells) was observed in squamous cell carcinomas and adenocarcinomas, and in all cases of large cell carcinomas (Iwasaki *et al*., 2004; Stefanou *et al*., 2003). The level of VEGF was independent prognostic factor in cases of NSCLC involving patients who had undergone curative resection (Iwasaki *et al*., 2004). Furthermore, the VEGF levels showed

differences with respect to histological type. The levels of VEGF in adenocarcinoma were higher than for squamous cell carcinoma (Iwasaki *et al*., 2004). Therefore, VEGF may be important in the growth of lung adenocarcinoma. A number of observations have spurred extensive investigation of VEGF inhibitors as possible therapies for cancer. Inhibitors in development include monoclonal antibody to VEGF and inhibitors of VEGFR activation following ligand binding (Fong *et al*., 1999; Yukita *et al*., 2000; Yilmaz *et al*., 2003).

The VEGF mediate angiogenic signals to the vascular endothelium via high-affinity receptor tyrosine kinase (RTK). To date, three receptors for the VEGFs have been identified, FLT-1, KDR/FLK-1 (fetal liver kinase-1), and FLT-4 (Shibuya *et al*., 1999; Neufeld *et al*., 1999; Aprelikova *et al*., 1992). All three are relatively specific for endothelial cells and have seven immunoglobulin homology domains in their extracellular part and an intracellular tyrosine kinase signaling domain split by a kinase insert. In adults, FLT-1 and KDR/FLK-1 are expressed mainly in the blood vascular endothelium, whereas FLT-4 is restricted largely to the lymphatic endothelium (Veikkola *et al*., 2000). Current reports show that a number of tumor cell lines, including melanoma, ovarian carcinoma, pancreatic carcinoma, and Kaposi sarcoma, produce VEGF and its cognate receptors and show impaired viability in response to VEGF ablation by either VEGF ASODN (antisense oligodeoxynucleotides) or neutralizing VEGFR antibodies (Lacal *et al*., 2005; Vieira *et al*., 2005). We further demonstrate that the human lung adenocarcinoma cell line also expresses VEGF and its receptors, FLT-1 and KDR. The presence of autocrine growth pathways in some tumors implies that VEGF antisense therapy acts on 2 levels: antiangiogenic effects on the tumor vasculature and antineoplastic effects on the tumor cell population (Masood *et al*., 2001).

The angiogenesis inhibitor TNP-470 has been reported to inhibit neovascularization by preventing endothelial cell proliferation (Shishido *et al*., 1996; Kato *et al*., 1994). One possible mechanism for its effect is that it acts on endothelial cells to inhibit growth factor-induced DNA synthesis. TNP-470 has inhibitory activities against both tumor growth and metastasis (Yanase *et al*., 1993; Yamaoka *et al*., 1993a). Antitumor and antimetastatic activities of TNP-470 were evaluated in various human cell lines (Yamaoka *et al*., 1993b; Tanaka *et al*., 1995). In this study, we also found the inhibitory effect of TNP-470 on tumor cell proliferation by using a human lung adenocarcinoma cell line, A549. However, TNP-470 is rapidly cleared from the circulation with a short terminal half-life ((0.88±2.5) h) (Figg *et al*., 1997). Thus, for treatment of lung adenocarcinoma, which, because of its high biological malignancy, requires prolonged maintenance of effective blood concentrations of drugs, we should employ combination chemotherapy.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a novel nucleoside analogue that exerts its antitumor activity via multiple action mechanisms. These include inhibiting DNA replication and cell growth and masked DNA chain termination (Storniolo *et al*., 1997). It is widely used in lung adenocarcinoma as a first-line chemotherapy and has been shown to produce significant survival benefits (Ferrigno *et al*., 2004). However, single-agent systemic chemotherapy achieves objective response rates ranging from 10% to 25%. Systemic polychemotherapies achieve variable response rates, but with consistent toxicity (Andre *et al*., 2004). Therefore, it is important to find a combination chemotherapy that is safe and well tolerated.

To our knowledge, this is the first report describing the antiangiogenic activity of TNP-470 in combination with gemcitabine in a human lung adenocarcinoma cell line. In this study, we investigated the inhibition of expression of VEGF and its receptors, FLT-1 and KDR, in human lung adenocarcinoma cell line using the established model, A594. In order to reduce the toxicity of drugs, we choose the dose of TNP-470 and gemcitabine lower than the half-maximum inhibition occurring at approximately 10^{-3} mg/ml and 10^{-4} mg/ml respectively. Although the expression of VEGF and its receptors, FLT-1 and KDR, was not reduced in human lung adenocarcinoma cell line treated with either TNP-470 or gemcitabine. The inhibition effects of the combination of TNP-470 and gemcitabine were stronger than those of TNP-470 alone or gemcitabine alone. This study suggests that combined treatment with TNP-470 plus gemcitabine may augment the antiangiogenic and antineoplastic effects in lung cancer cells in vitro. The

present findings may be of importance for further exploration of the potential application of this combined approach in the treatment of lung cancer.

Although the mechanism of this combination therapy is not clear, it appeared to be due to a synergistic effect. In in vitro experiments treatment of KoTCC-1 cells with gemcitabine and TNP-470 decreased the IC_{50} of gemcitabine by greater than 50% and markedly enhanced gemcitabine induced apoptosis (Muramaki *et al*., 2004), then it may reduce the expression of VEGF and its receptor, FLT-1 and KDR. Moreover, gemcitabine enhanced the aggregation of TNP-470 and increased its cell concentration (Jia *et al*., 2005), which may strengthen the inhibition effects of TNP-470 on VEGF, FLT-1 and KDR.

In summary, by combining antiangiogenic agents with each other and/or with other modalities in the treatment of cancer, the limitations of each therapeutic approach could be overcome, leading to enhanced efficacy with diminished toxicity (O′Reilly, 2002). Antiangiogenic therapy (TNP-470 in combination with gemcitabine) shows significant anti-tumor and anti-metastatic effects and is helpful for reducing the dosage for cytotoxic drugs and the side effects (Lin *et al*., 2005). Our angiocytotoxic therapy (TNP-470 in combination with low dose gemcitabine) also showed excellent inhibitory effects of VEGF and its receptor on A549 model of human lung adenocarcinoma cell line. These results suggest that angiocytotoxic therapy may provide a new safe and effective strategy for treatment of lung adenocarcinoma. However, before these agents can be used in clinical practice, a better understanding of their action mechanism and regulation is needed.

LIMITATION

Limitations of our lab prevent our carrying out specific experiment in vivo and in vitro to show the anti-angiogenesis and anti-tumor effect of the treatment by gemcitabine and TNP-470. It should be done in our subsequent experiment.

References

Andre, T., Tournigand, C., Rosmorduc, O., Provent, S., Maindrault-Goebel, F., Avenin, D., Selle, F., Paye, F., Hannoun, L., Houry, S., *et al*., 2004. Gemcitabine combined with oxaliplatin (GEMOX) in advanced biliary tract adenocarcinoma: a GERCOR study. *Ann. Oncol.*, **15**(9):1339-1343. [doi:10.1093/annonc/mdh351]

Aprelikova, O., Pajusola, K., Partanen, J., Armstrong, E., Alitalo, R., Bailey, S.K., McMahon, J., Wasmuth, J., Huebner, K., Alitalo, K., 1992. FLT4, a novel class III receptor tyrosine kinase in chromosome 5q33-qter. *Cancer Res.*, **52**(3):746-748.

Ferrigno, D., Buccheri, G., Cuneo Lung Cancer Study Group, 2004. Front-line weekly chemotherapy with gemcitabine for unfit patients with non-small cell lung cancer (NSCLC). *Lung Cancer*, **45**(3):373-380. [doi:10.1016/j.lungcan.2004.01.023]

Figg, W.D., Pluda, J.M., Lush, R.M., Saville, M.W., Wyvill, K., Reed, E., Yarchoan, R., 1997. The pharmacokinetics of TNP-470, a new angiogenesis inhibitor. *Pharmacotherapy*, **17**(1):91-97.

Folkman, J., 1990. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.*, **82**(1):4-6.

Fong, T.A., Shawver, L.K., Sun, L., Tang, C., App, H., Powell, T.J., Kim, Y.H., Schreck, R., Wang, X., Risau, W., *et al*., 1999. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res.*, **59**(1):99-106.

Iwasaki, A., Kuwahara, M., Yoshinaga, Y., Shirakusa, T., 2004. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) levels, as prognostic indicators in NSCLC. *Eur. J. Cardiothorac. Surg.*, **25**(3):443-448. [doi:10.1016/j.ejcts.2003.11.031]

Jia, L., Zhang, M.H., Yuan, S.Z., Huang, W.G., 2005. Antiangiogenic therapy for human pancreatic carcinoma xenografts in nude mice. *World J. Gastroenterol.*, **11**(3):447-450.

Johnson, F.M., Saigal, B., Talpaz, M., Donato, N.J., 2005. Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. *Clin. Cancer Res.*, **11**(19 Pt 1):6924-6932. [doi:10.1158/1078-0432.CCR-05-0757]

Kato, T., Sato, K., Kakinuma, H., Matsuda, Y., 1994. Enhanced suppression of tumor growth by combination of angiogenesis inhibitor O-(chloroacetyl-carbamoyl) fumagillol (TNP-470) and cytotoxic agents in mice. *Cancer Res.*, **54**(19):5143-5147.

Kusaka, M., Sudo, K., Matsutani, E., Kozai, Y., Marui, S., Fujita, T., Ingber, D., Folkman, J., 1994. Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). *Br. J. Cancer*, **69**(2):212-216.

Lacal, P.M., Ruffini, F., Pagani, E., D′Atri, S., 2005. An autocrine loop directed by the vascular endothelial growth factor promotes invasiveness of human melanoma cells. *Int. J. Oncol.*, **27**(6):1625-1632.

Lin, J., Mei, H.Z., Shi, Z.Y., Wen, G.H., 2005. Antiangiogenic therapy for human pancreatic carcinoma xenografts in

nude mice. *World J. Gastroenterol.*, **11**(3):447-450.

Masood, R., Cai, J., Zheng, T., Smith, D.L., Hinton, D.R., Gill, P.S., 2001. Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood*, **98**(6):1904-1913. [doi:10.1182/blood.V98.6.1904]

Miura, S., Emoto, M., Matsuo, Y., Kawarabayashi, T., Saku, K., 2004. Carcinosarcoma-induced endothelial cells tube formation through KDR/Flk-1 is blocked by TNP-470. *Cancer Lett.*, **203**(1):45-50. [doi:10.1016/j.canlet.2003.08.020]

Muramaki, M., Miyake, H., Hara, I., Kawabata, G., Kamidono, S., 2004. Synergistic inhibition of tumor growth and metastasis by combined treatment with TNP-470 and gemcitabine in a human bladder cancer KoTCC-1 model. *J. Urol.*, **172**(4 Pt 1):1485-1489. [doi:10.1097/01.ju.0000133653.74536.43]

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.*, **13**(1):9-22.

O'Reilly, M.S., 2002. The combination of antiangiogenic therapy with other modalities. *Cancer J.*, **8**(Suppl. 1):S89-99.

Rifkin, D.B., Kojima, S., Abe, M., Harpel, J.G., 1993. TGF-beta: structure, function, and formation. *Thromb. Haemost.*, **70**(1):177-179.

Sauter, E.R., Nesbit, M., Watson, J.C., Klein-Szanto, A., Litwin, S., Herlyn, M., 1999. Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck. *Clin. Cancer Res.*, **5**(4):775-782.

Senger, D.R., van de Water, L., Brown, L.F., Nagy, J.A., Yeo, K.T., Yeo, T.K., Berse, B., Jackman, R.W., Dvorak, A.M., Dvorak, H.F., 1993. Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev.*, **12**(3-4):303-324. [doi:10.1007/BF00665960]

Shibuya, M., Ito, N., Claesson-Welsh, L., 1999. Structure and function of vascular endothelial growth factor receptor-1 and -2. *Curr. Top Microbiol. Immunol.*, **237**:59-83.

Shishido, T., Yasoshima, T., Denno, R., Sato, N., Hirata, K., 1996. Inhibition of liver metastasis of human gastric carcinoma by angiogenesis inhibitor TNP-470. *Jpn. J. Cancer Res.*, **87**(9):958-962.

Stefanou, D., Goussia, A.C., Arkoumani, E., Agnantis, N.J., 2003. Expression of vascular endothelial growth factor and the adhesion molecule E-cadherin in non-small cell lung cancer. *Anticancer Res.*, **23**(6C):4715-4720.

Storniolo, A.M., Allerheiligen, S.R., Pearce, H.L., 1997. Preclinical, pharmacologic, and phase I studies of gemcitabine. *Semin. Oncol.*, **24**(2 Suppl. 7):S7-2-S7-7.

Tanaka, T., Konno, H., Matsuda, I., Nakamura, S., Baba, S., 1995. Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470. *Cancer Res.*, **55**(4):836-839.

Veikkola, T., Karkkainen, M., Claesson-Welsh, L., Alitalo, K., 2000. Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res.*, **60**(2):203-212.

Vieira, J.M., Santos, S.C., Espadinha, C., Correia, I., Vag, T., Casalou, C., Cavaco, B.M., Catarino, A.L., Dias, S., Leite, V., 2005. Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop. *Eur. J. Endocrinol.*, **153**(5):701-709. [doi:10.1530/eje.1.02009]

Weidner, N., Semple, J.P., Welch, W.R., Folkman, J., 1991. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N. Engl. J. Med.*, **324**(1):1-8.

Weinstat-Saslow, D., Steeg, P.S., 1994. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. *FASEB J.*, **8**(6):401-407.

Yamamoto, T., Sudo, K., Fujita, T., 1994. Significant inhibition of endothelial cell growth in tumor vasculature by an angiogenesis inhibitor, TNP-470 (AGM-1470). *Anticancer Res.*, **14**(1A):1-3.

Yamaoka, M., Yamamoto, T., Ikeyama, S., Sudo, K., Fujita, T., 1993a. Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. *Cancer Res.*, **53**(21):5233-5236.

Yamaoka, M., Yamamoto, T., Masaki, T., Ikeyama, S., Sudo, K., Fujita, T., 1993b. Inhibition of tumor growth and metastasis of rodent tumors by the angiogenesis inhibitor O-(chloroacetyl-carbamoyl) fumagillol (TNP-470; AGM-1470). *Cancer Res.*, **53**(18):4262-4267.

Yanase, T., Tamura, M., Fujita, K., Kodama, S., Tanaka, K., 1993. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines in vitro and in vivo. *Cancer Res.*, **53**(11):2566-2570.

Yilmaz, A., Kliche, S., Mayr-Beyrle, U., Fellbrich, G., Waltenberger, J., 2003. p38 MAPK inhibition is critically involved in VEGFR-2-mediated endothelial cell survival. *Biochem. Biophys. Res. Commun.*, **306**(3):730-736. [doi:10.1016/S0006-291X(03)01064-7]

Yukita, A., Asano, M., Okamoto, T., Mizutani, S., Suzuki, H., 2000. Suppression of ascites formation and re-accumulation associated with human ovarian cancer by an anti-VPF monoclonal antibody in vivo. *Anticancer Res.*, **20**(1A):155-160.