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Antitumor activities of a novel indolin-2-ketone compound, Z24: more potent inhibition on bFGF-induced angiogenesis and bcl-2 over-expressing cancer cells

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Abstract

The present study was designed to select the effective dosage range of Z24 [3*Z*-3-[(1*H*-pyrrol-2-yl)-methylidene]-1-(1-piperidinylmethyl)-1,3-2*H*-indol-2-one], a novel synthetic indolin-2-ketone small-molecule compound, against tumorigenesis and angiogenesis in vitro and in vivo and to investigate the primary action mechanism of Z24 on the angiogenesis by comparing with SU5416 [3-[(2,4-dimethylpyrrol-5-yl)methyllidenyl]-indolin-2-one] in the selective effects on vascular endothelial growth factor (VEGF)/basic fibroblast growth factor (bFGF) signaling and Bcl-2-related cell vitality because Z24 is a potential inhibitor of the Bcl-2 that inhibits growth of multiple tumor types in vivo in our previous study. Per os Z24 inhibited dose-dependently the mouse S180 xenograft tumor growth and angiogenesis in mouse subcutaneous (s.c.) Matrigel plugs in vivo. The maximum growth inhibitory rate was 56.1% by 80 mg/kg/day on S180 mouse sarcoma cells; however, the maximum inhibitory potency on angiogenesis in C57BL/6 mouse subcutaneous Matrigel plug model was 50 mg/kg/day. Z24 inhibited angiogenesis in chicken chorioallantoic membrane (CAM) and invasion and inhibited tube formation of endothelial cells in a dose-dependent manner. Compared with SU5416, the IC_{50} (50% inhibition concentration) of Z24 on the proliferation of ECV-304 carcinoma cells induced by VEGF or bFGF was 24.4 and 17.99 μM, respectively, which is higher or lower, respectively, than that of SU5416 (14.2 μM for VEGF and 22.7 μM for bFGF). Furthermore, the IC_{50} of Z24 on the proliferation of Bcl-2 over-expressing HeLa cells and non-Bcl-2-expressing (wild-type) HeLa cells are 11.9 and 24.8 μM, respectively. SU5416 did not exert such a selective inhibiting effect on Bcl-2 over-expressing HeLa cells. These results suggest that Z24 per os has dose-dependent antitumor and antiangiogenesis pharmacological activity. The higher selectivity of Z24 on Bcl-2 protein and on bFGF other than VEGF signaling path may contribute to its efficiency against tumor and tumor-associated angiogenesis.

Keywords: Z24; Bcl-2; Antitumor; Antiangiogenesis; VEGF; bFGF

1. Introduction

Angiogenesis is the process of sprouting of capillaries from preexisting blood vessels, which is essential for the sustained growth and metastasis of primary solid tumors (Folkman, 1972; Holash et al., 1999). Antiangiogenic therapy has been believed to be an ideal strategy in the current cancer biological therapy. Until 2002, at least 10,000 cancer patients worldwide have received some form of experimental antiangiogenic therapy, more than 300 angiogenesis inhibitors have been discovered, of which 80 are in clinical trials (Madhusudan and Harris, 2002). However, several promising compounds, including 3-[(2,4-dimethylpyrrol-5-yl)methyllidenyl]-indolin-2-one, SU5416, a novel, potential inhibitor of angiogenesis, failed in Phase III

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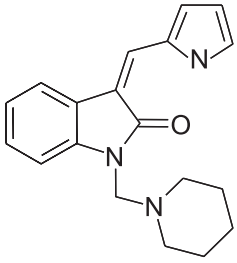

Fig. 1. Chemical structure of Z24.

clinical trial, although they exhibited good efficiency in preclinical tests (Gerber, 2003). To date, no antiangiogenic small-molecule compound has been approved as a therapeutic agent.

SU5416, the first synthetic indolin small-molecule compound, is a specific vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) signaling blocker that inhibits VEGF-stimulated Flk-1 phosphorylation (Fong et al., 1999). Because it is a specific angiogenesis antagonist, SU5416 blocks the angiogenic switch and prevents the growth of premalignant tumors; however, it does not directly inhibit tumor cells in vitro or induce regression of late-stage, well-vascularized tumors (analogous to those of typical Phase III clinical trial participants; Gerber, 2003). This may be the cause of the failure of SU5416 at Phase III clinical trial. Bikfalvi and Bicknell (2002) have shown that the makeup of the tumor vasculature varies at different stages of tumor development, so inhibitor efficacy might depend on its application during a specific phase of tumorigenesis. Novel approaches and targeting one or multiple angiogenic pathways might turn out to be more successful in the clinic (Gerber, 2003).

3*Z*-3-[(1*H*-pyrrol-2-yl)-methylidene]-1-(1-piperidinylmethyl)-1,3-2*H*-indol-2-one (Z24; Fig. 1), a novel small-molecule compound designed and synthesized by our lab, is a potential Bcl-2 inhibitor suggested by computer screening based on the Bcl-2 protein 3D structure. It is interesting that Z24 is a structural analog of SU5416 (Fig. 2), which belongs to indolin-2-ketones. It implies that the mechanism of bioactivity of Z24 may be related to SU5416 and Bcl-2 inhibitors. Preliminary biological screening tests have demonstrated that Z24 at a dose of 100 mg/kg body weight could inhibit the growth of tumors in various mice, including nude mouse subcutaneous xenograft tumor models, and could suppress the angiogenesis in chicken chorioallantoic membrane (CAM; Lu et al., 2003). Furthermore, Z24 could selectively inhibit the growth of human umbilical vein endothelial cells (HUVEC) and induce HUVEC and some tumor cells apoptosis in vitro (unpublished data). The aim of this study was to investigate the effective inhibitory dosage range of Z24 on tumorigenesis and the effect of Z24 on the angiogenesis in vitro and in vivo and to investigate the primary mechanism of the effect of Z24 on the angiogenesis by comparing with SU5416.

2. Materials and methods

2.1. Reagents and materials

Z24, 3*Z*-3-[(1*H*-pyrrol-2-yl)-methylidene]-1-(1-piperidinylmethyl)-1,3-2*H*-indol-2-one, whose structure (Fig. 1) was identified by nuclear magnetic resonance spectroscopy–mass spectroscopy and elemental analysis, and SU5416, 3*Z*-3-[(3,5-dimethyl-1*H*-pyrrol-2-yl)-methylidene]-1,3-2*H*-indol-2-one was synthesized at Beijing Institute of Pharmacology and Toxicology, as described previously (Li et al., 2002). Bcl-2 plasmid DNA was a gift provided by professor Zhang Xue-Min (Beijing Biomedical Analysis Center, China); recombinant human vascular endothelial growth factor (VEGF), 3-(4,5-dimethylthiazoyl-2-yl)-2,5 diphenyltetrazolium bromide (MTT), penicillin, streptomycin were purchased from Sigma-Aldrich (St. Louis, MO); recombinant human basic fibroblast growth factor (bFGF or FGF-2) were purchased from Calbiochem–Novabiochem International (San Diego, California); Matrigel, 24-well plate transwell with 8-μm pores were provided by Becton Dickinson (Bedford, Massachusetts); 60-mm petri dish, 24-well plate, 13-mm coverslip were from NUNC (Naperville, Illinois). Dulbecco's modified Eagle's medium (DMEM), medium 199 (M199). PRMI 1640 medium, new calf serum were all purchased from Invitrogen (San Diego, CA).

2.2. Cell lines and cell culture

Cells were cultured at 37 °C in a 5% CO_2 atmosphere. ECV-304 human carcinoma cell line (TCCCAS, Type Culture Collection of Chinese Academy of Sciences), mouse vein endothelial cell (SVEC, a generous gift from Qian Huang, the first Shanghai People's Hospital, Shanghai, P.R. China), S180 mouse sarcoma cell line (kept by our lab) and ECV-304 were grown in DMEM supplemented with 10% new calf serum. Wild-type HeLa cells and Bcl-2 overexpressing HeLa cells (established through transfected with Bcl-2 plasmid and condition-selected in our lab) were maintained in RPMI 1640 medium with 10% new calf serum. Those cells were used at passages between the fourth and the sixth.

2.3. Subcutaneous xenograft model in mice

The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Exper-

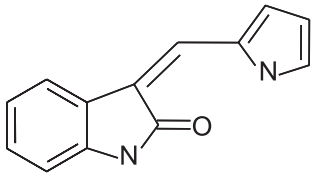

Fig. 2. Chemical structure of SU5416.

imentation. The sterilely harvested S180 mouse sarcoma cells with ascites, diluted with sterilized physiological saline at a ratio of 1:4 (v/v, cell concentration was adjusted to 1×10^7 /ml), were implanted subcutaneously in the front flank region of female 5- to 6-week-old Kunming mice, as described by others (Lu et al., 2003). After the mice were administered with the tumor cell suspension (0.2 ml per mouse), they were randomly divided into four groups (at least 10 mice per group) and were treated immediately with vehicle or test compound; 20 mice were treated with vehicle as the negative group; Z24 doses of 20, 40 and 80 mg/kg body weight were used for the treatment groups. The mice were dosed per os—continuously with the test compounds for 10 days—and were then sacrificed, and the tumor growth was quantified by weighting the tumor tissues (Table 1).

The rate of antitumor activity was calculated as: tumor growth inhibitory rate (%)=(1−tumor weight of test group/tumor weight of negative control group)×100.

2.4. Assay of selective inhibition on cell proliferation

ECV-304 was plated in 96-well plates at a density of 3×10^3 cells per well in 100 μl starvation medium composed of containing 1% new calf serum. After overnight culture, the medium was replaced with starvation medium supplemented with bFGF (10 ng /ml) or VEGF (10 ng /ml) containing fivefold serial dilutions of Z24 or SU5416. After 72 h of incubation with vehicle and compounds at 37 °C, MTT assays were performed with Bio-Rad model 550 plate reader (Bio-Rad), which was used to determine the number of living cells, and the absorbance was measured at 540 nm in triplicate wells.

The selective inhibition of Z24 and SU5416 on the proliferation of HeLa cells or Bcl-2 over-expressing HeLa cells were determined using the same protocol as ECV-304. The density of plated cells was also 3,000 cells per well and the medium was RPMI 1640 medium supplemented with 10% new calf serum and did not change. The inhibition of test compounds on cell proliferation was expressed as inhibition rate and the IC_{50} values.

The selective inhibition of Z24 and SU5416 on bFGF- or VEGF-stimulated cell proliferation and on Bcl-2-related cell proliferation were determined by comparing the inhibited potency of Z24 and SU5416 on different inducer and different cells.

Table 1
The inhibition rate of Z24 per os on the rumor growth at different dosages in mouse sarcoma 180 xenograft mice model

Dose (mg/kg/day)	*n*	Tumor weight (g)	Inhibition rate (%)
Vehicle	19	2.51±0.878	
20	10	2.30±1.044	8.37
40	10	1.67±0.642[a]	33.4
80	10	1.10±0.550[b]	56.1

Data represent as means±S.D.
[a] $p<0.05$.
[b] $p<0.01$ vs. vehicle group.

2.5. In vitro invasion assay

The effect of Z24 on invasive activity was detected as we described previously (Li et al., 2001). Briefly, 5×10^4 mouse vascular endothelial cells (SVEC) were suspended in 100 μl serum-free medium and inoculated into a transwell (cell culture inserts; 8-μm pore) layer pretreated with 10 μl of Matrigel on upper bottom and 10 μl collagen type I on lower bottom. The inserts were then set on 24-well plates filled with 600 μl of primary medium supplemented with positive stimulus bFGF (as chemoattractive motile activity) and test compound at various concentrations or vehicle, respectively. After being incubated at 37 °C in 5% CO_2 atmosphere for 24 h, the cells on the upper surface of the insert were removed with a cotton swab, while those on its lower surface were fixed in methanol and stained with Giemsa solution. The number of cells invading the Matigel-coated membranes to the outside was determined by counting the number of cells in each insert membrane, under a microscope at 200× magnification. Each treatment was repeated at least three times.

2.6. In vitro tube formation assay

Endothelial tube-structure formation on Matrigel was also conducted as we described previously (Li et al., 2001). Briefly, 300 μl Matrigel at 4 °C was added to a 24-well plates and then allowed to polymerize at 37 °C, 5% CO_2 for about 30 min. SVEC was suspended at the concentration of 5×10^5/ml in primary medium, and plated onto the plates preloaded with Matrigel basement membrane matrix and incubated for 6 h in the presence of the VEGF of 50 ng/ml and test compound at various concentrations, or vehicle, at 37 °C in a humidified 5% CO_2 chamber. After incubation, photographs of the formation of tube-like structures by endothelial cells on the Matrigel were taken under a stereomicroscope by a CoolSNAP cf CCD camera (Titan Electro-Optics) at 40× magnification. The number of tubular structures per unit area were counted, and the arrangement, form and integrity of tubular structures were evaluated qualitatively. Each treatment was carried out at least in triplicate.

2.7. Chicken chorioallantoic membrane assay

The chicken chorioallantoic membrane assay was conducted as we described previously (Li et al., 2001). Briefly, after incubation at 37 °C for 5 days in a humidified atmosphere, fertilized eggs were opened at the air cell and the shell membrane was taken off in order to expose chorioallantoic membrane (CAM). Then, the test compounds dissolved in 5 μl vehicle (1% dimethyl sulfoxide, DMSO in saline) were added on sterilized 1%

methylcellulose (MC) membrane of 35-mm diameter and placed on the CAM. Finally, the window was re-covered with glass-adhesive plaster and the fertilized egg was incubated continuously for another 96 h. At the end of the experiment, the photographs were taken off the CAM and the inhibition of angiogenesis was evaluated. Eggs treated by vehicle or 2 ng bFGF were used as the negative or positive controls, respectively. The results were expressed as the percentage of eggs showing inhibition of angiogenesis.

2.8. In vivo mouse Matrigel plug assay

The Matrigel plug assay was also performed as we described previously (Li et al., 2001). Briefly, female, 5- to 6-week-old clean C57BL/6 mice were randomly divided into 4 groups (at least 6 mice each group), which were cared for and handled according to accepted ethical practices. Angiogenesis was evaluated according to the growth of blood vessels from host mouse subcutaneous tissue into a solid Matigel containing the angiogenesis stimulus bFGF (25 ng/mouse) as a model. Vehicle, or Z24 of 25, 50 and 100 mg/kg/day was given as normal manner intraperitoneally (i.p.) for 5 days. Matrigel, in liquid form at 4 °C, was mixed with the bFGF and injected (0.5 ml) into the abdominal subcutaneous tissue of mice along the peritoneal midline. Matrigel rapidly forms a solid gel plug at body temperature, thereby trapping the factors and allowing their slow release and prolonged exposure to surrounding tissues. The mice were sacrificed by being injected i.p. with sodium pentobarbital (100 mg/kg body weight, i.p.) at day 5 of treatment, and the gels were removed and fixed in 10% neutral phosphate-buffered formalin and routinely embedded in paraffin. Sections cut at 5 μm and stained with hematoxylin and eosin were observed by light microscopy and photographed with a CoolSNAP cf CCD camera at 40× magnification. The number of vessels possessing a patent lumen and structure containing erythrocytes were evaluated qualitatively.

2.9. Statistics analysis

All results are expressed as mean±S.D., n representing the number of experiments or the number of animals per group. Statistical analysis was performed by one-way ANOVA analysis. Qualitative data was performed by X^2 assay with SPSS. $P<0.05$ was accepted as significant. IC_{50} value and its 95% confidence interval were calculated by Logit method.

3. Results

3.1. Inhibition of tumor growth in vivo

The broad-spectrum antitumor activity of Z24 of 100 mg/kg/day, i.p. in vivo has been shown in various of xenograft models including mouse sarcoma S180, mouse hepatoma 22 and nude mouse human hepatocellular carcinoma BEL-7402, and the inhibitory rate is 52% in sarcoma 180 (Lu et al., 2003). In this experiment, per os Z24 significantly inhibited the growth of subcutaneous xenograft of S180 sarcoma in dose-dependent manner (Fig. 3 and Table 1). At dosage of 80 mg/kg/day, the tumor inhibitory rate is 56.1%. It indicated that per os Z24 had better efficiency.

3.2. Inhibition of cell invasion and tube formation in vitro

The effect of Z24 on the angiogenic function of endothelial cells was determined by examining its effect on invasion and tube formation by SVEC in vitro.

In the invasion experiment, as a chemoattractive factor, bFGF of 100 ng/ml could induce the mobility of SVEC from the inner of inserts through the pores (8 μm) on bottom of insert cup. Z24 from 0.1 to 10 μM shows significant and concentration-dependent inhibition on invasion with SVEC (Fig. 4A and B). SU5416 at 10 μM also had significant inhibition on the invasion, but the potency is lower than Z24 at such concentration ($P<0.01$).

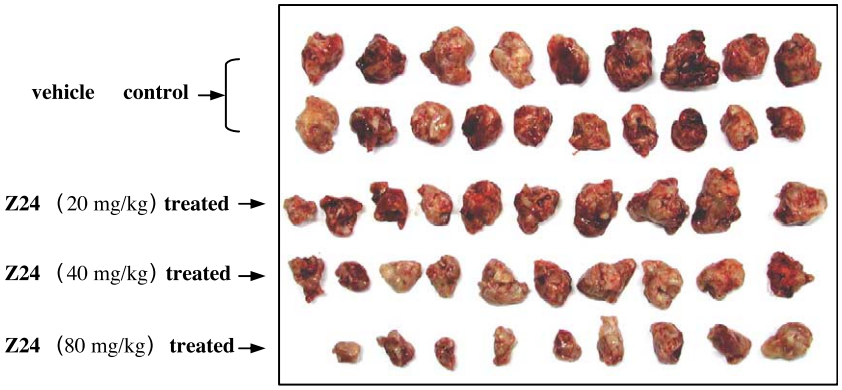

Fig. 3. Effect of Z24 at different dosages on the tumor growth in mouse sarcoma 180 xenograft mice model. The S180 mouse sarcoma cells with ascites which were implanted (2×10^6/mouse) subcutaneously in the front flank region of female Kunming mice. One day after implantation, animals were treated per os once daily with a 0.5 ml either Z24 at 20, 40 or 80 mg/kg/day in 5% DMSO in saline or 5% DMSO in saline only for 10 days. On day 10 after implantation, animals bearing tumor were euthanized, and their tumors were isolated and weighted.

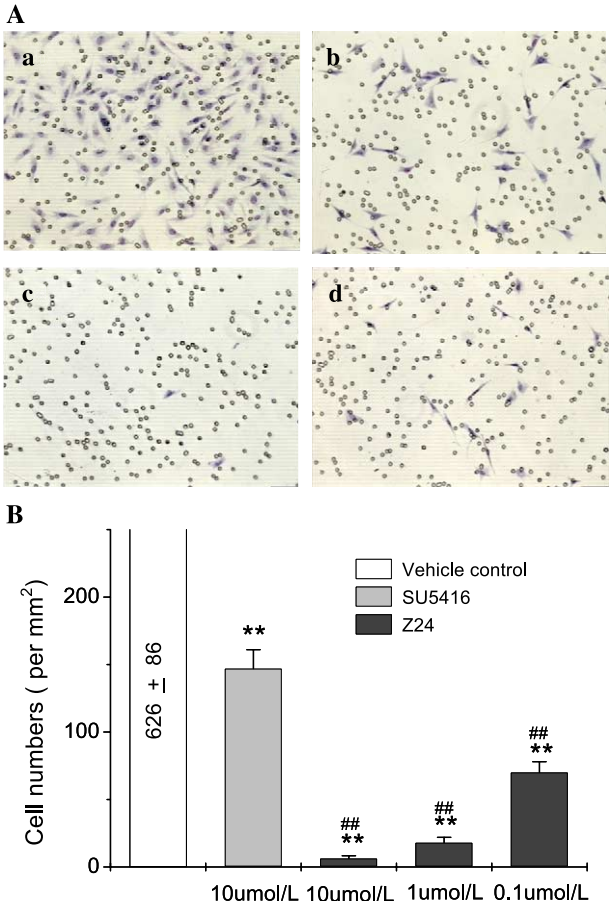

Fig. 4. Effect of Z24 at different dosages on invasion of mouse vein endothelial cell SVEC. Inhibition of SU5146 and Z24 at concentrations from 0.1 to 10 μM on SVEC invasion through Matrigel be present of 100 ng/ml bFGF. Mouse vein endothelial cell SVECs (5×10^4) suspended in serum-free medium were inoculated into cell culture inserts (with pores of 8-μm diameter) layer pretreated with Matrigel on upper bottom and collagen type I on lower bottom. The inserts were then set on 24-well plates filled with primary medium supplemented with positive stimuli bFGF and test compound or vehicle. After incubated for 24 h, invaded cells through the Matigel-coated membranes to the outside were stained with Giemsa and were determined by counting the number of cells in each insert membrane under a microscope at 200× magnification. (A) Image of invasion endothelial cells of different treatment groups: (a) bFGF (100 ng/ml), (b) bFGF (100 ng/ml)+SU5416 (10 μM), (c) bFGF (100 ng/ml)+Z24 (10 μM) and (d) bFGF (100 ng/ml)+Z24 (0.1 μM). (B) Effect of SU5416 and Z24 on SVEC invasion. Columns represent the cell number of SVEC invasion at per mm^2 area of insert membrane. Each value represents means±S.D. of at least three repeats. Statistical significance of differences calculated by one-way ANOVA analysis. $^{**}p<0.01$ vs. control; $^{\#\#}p<0.01$ vs. SU5416.

VEGF of 100 ng/ml could induce tube formation on the Matrigel layer in the well for about 6 h (Fig. 5). SU5416 at 10 μM significantly inhibited tube formation and exhibited broken tube connection structures formed by SVEC. Z24 at 0.1–10 μM showed significant inhibition of tube formation by SVEC and the concentration-dependent range is between 0.1 and 1 μM. The maximum inhibition of invasion by Z24 at 10 μM was obviously weaker than SU5416 at 10 μM (Fig. 4). It suggested that Z24 could inhibit the tube formation in Matrigel induced by VEGF and its blocking potency is weaker than SU5416 at the same concentration.

3.3. Antiangiogenesis in chicken chorioallantoic membrane

Chicken chorioallantoic membrane was formed from day 4 when fertilized egg was incubated at 37 °C. The significant neovascularization in chicken chorioallantoic membrane was observed at about day 5. Then, the bFGF or testing compounds were added on chicken chorioallantoic membrane for another 96 h. In the presence of bFGF, angiogenesis on the chicken chorioallantoic membrane was significantly enhanced. Z24 at dosages from 100 to 1000 ng/egg strongly inhibited the angiogenesis on the chicken chorioallantoic membrane in a dosage-dependent manner. At the dosage of 300 ng/egg, the inhibition rate of Z24 almost resembled that of SU5416 (Fig. 6 and Table 2).

3.4. Antiangiogenesis in mouse Matrigel plug in vivo

At day 5 of treatment by Z24 and SU5416, Matrigel plugs of every group were isolated, routinely paraffin-

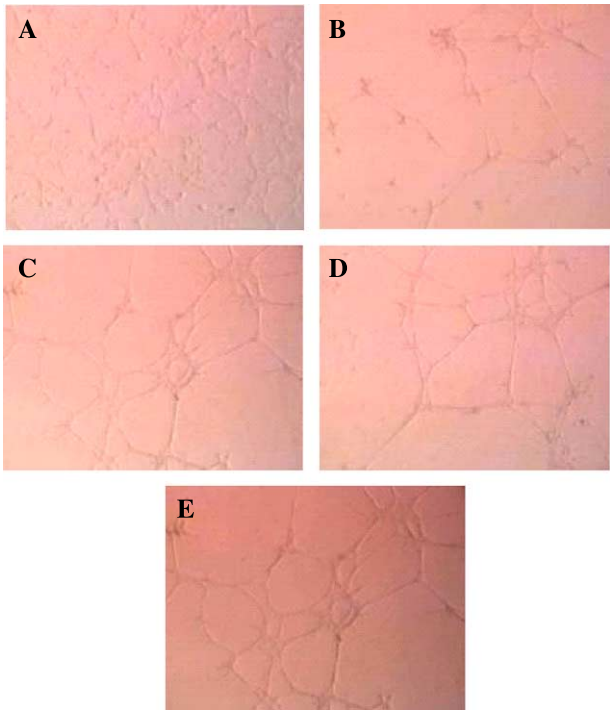

Fig. 5. In vitro tube formation assay. Inhibition of SU5146 and Z24 at concentration from 0.1 to 10 μM on tube formation by SVEC in Matrigel incubated for 6 h be present of 50 ng/ml VEGF. Mouse vein endothelial cell (SVEC, 5×10^5/ml) were suspended in primary medium and were plated onto the plates preloaded with Matrigel basement membrane matrix. After incubating for 6 h at the presence of the VEGF of 50 ng/ml and test compound, the photography of the formation of tube-like structures by endothelial cells on the Matrigel was taken under Zeiss Stemi 2000 stereomicroscopy by CoolSNAP cf CCD camera at ×40 magnification. (A) VEGF (50 ng/ml)+vehicle control; (B)VEGF (50 ng/ml)+SU5416 (10 μM); (C) VEGF (50 ng/ml)+Z24 (0.1 μM); (D) VEGF (50 ng/ml)+Z24 (10 μM); and (E) VEGF (50 ng/ml)+Z24 (10 μM).

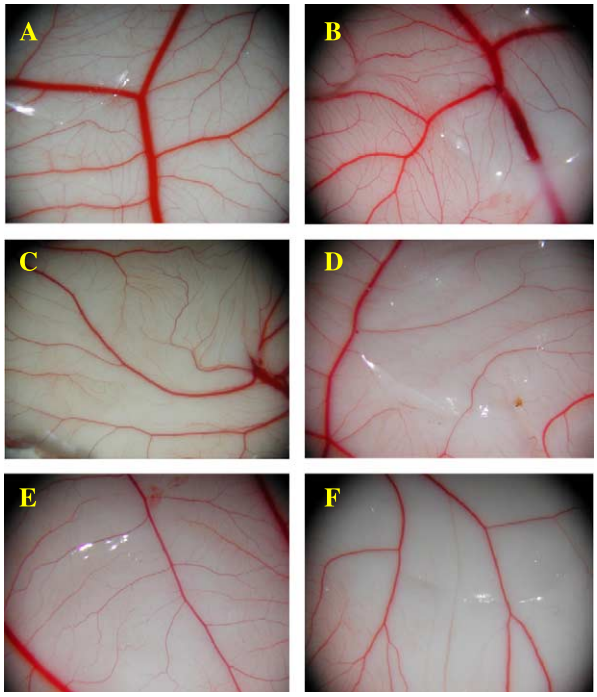

Fig. 6. Chicken chorioallantoic membrane assay. A group of typical pictures showing effect of Z24 on angiogenesis in chick chorioallantoic membrane of day 9 fertilized egg. The testing compounds dissolved in 5-μl vehicle were added on sterilized 1% methylcellulose membrane and placed on the chick chorioallantoic membrane of 5-days fertilized egg. After incubation continuously for another 96 h, the chick chorioallantoic membrane was taken pictures and the inhibition of angiogenesis was evaluated. (A) Vehicle control (3 μl 5% DMSO saline); (B) bFGF (4 ng/egg) as positive control; (C) SU5416 300 ng/egg as positive drug treatment; (D) Z24 100 ng/egg treatment; (E) Z24 300 ng/egg treatment ; and (F) Z24 1000 ng/egg treatment.

sectioned, hematoxylin and eosin stained. As shown in Fig. 7, mice subcutaneously injected with Matrigel containing bFGF (control group) showed significantly enhanced endothelial cell emigration and vessels formation. Z24 at dosage of 25, 50 and 100 mg/kg i.p. significantly inhibited the number of endothelial cells and the formation of canalized vessels in the Matrigel plug. The inhibitory potency of Z24 at dosage of 25 mg/kg is similar with that of SU5416 at the same dose.

Table 2
Inhibition rate of Z24 on angiogenesis in chick embryonic chorioallantoic membrane (CAM)

Compound (ng/egg)	Positive number/ total number	Positive rate (%)	Inhibition rate (%)
Control	4/15	26.6	–
SU5416			
300	8/13	61.5	47.6
Z24			
100	8/15	53.3	36.4
300	9/13	69.2[a]	58.0
1000	10/16	62.5	48.9

[a] $p<0.05$ vs. control group.

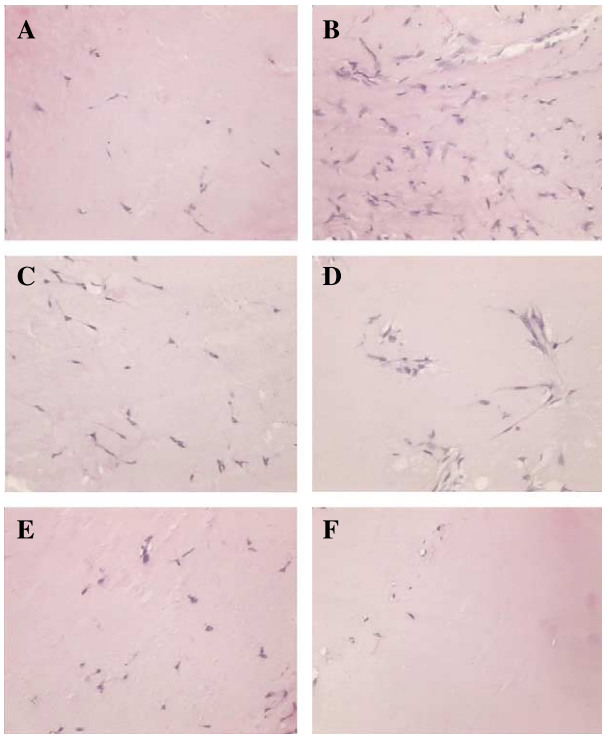

Fig. 7. In vivo mouse Matrigel plug assay. Histological analysis of Matrigel plug sections stained with hematoxylin–eosin (HE). Effect of Z24 at different dosages on angiogenesis in Matrigel plug, in C57BL/6 mice. Angiogenesis was assayed as growth of blood vessels from subcutaneous tissue into a solid Matigel containing the angiogenesis stimuli bFGF. Matrigel, in liquid form, was mixed with the bFGF (25 ng per mouse) and injected into the abdominal subcutaneous tissue of C57BL/6 mice along the peritoneal midline and rapidly formed a solid gel plug at body temperature. Vehicle and different concentrations testing compounds was given as normal manner, i.p. The mice were sacrificed at the day 5 of treatment, and the gels were removed and fixed in 10% phosphate-buffered formalin and routinely embedded in paraffin. Sections cut at 5 μm and stained with hematoxylin and eosin were observed and analyzed by light microscopy. (A) Vehicle control; (B) bFGF (25 ng/mice) as positive control; (C) SU5416 (25 mg/kg) as positive drug treatment; (D) Z24 25 mg/kg treatment; (E) Z24 50 mg/kg treatment; and (F) Z24 100 mg/kg treatment.

3.5. Selectivity and potency of Z24 on VEGF- or bFGF-stimulated proliferation of ECV-304 cell

ECV-304 cells (3×10^3/well) in starvation medium composed of containing 1% new calf serum were incubated at 37 °C in a 5% CO_2 atmosphere for about 3 days. The proliferation of the cells was very slow and almost kept in resting state. This is shown by the OD value by MTT that is almost same as the day after cells seeded. About 10 ng/μl of VEGF or bFGF markedly enhanced the proliferation of ECV-304 in starvation medium (data not shown), Z24 and SU5416 of 1–100 μM blocked the proliferation induced by VEGF or bFGF in a concentration-dependent manner but with a different concentration–inhibition rate curve (Fig. 8) and IC_{50} value (Table 3). The IC_{50} values of SU5416 on the proliferation induced by VEGF is lower than that induced by bFGF, especially when SU5416 is below 30 μM; the inhibition rate on VEGF-induced proliferation is obviously

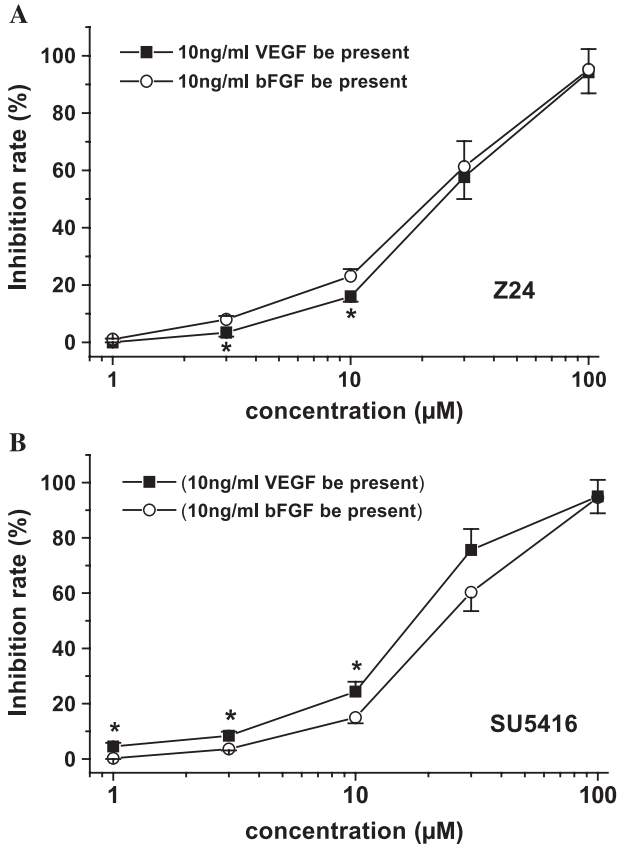

Fig. 8. The concentration–inhibition rate curves of Z24 (A) and SU5416 (B) on the proliferation of ECV-304 cell induced by VEGF or bFGF. ECV-304 of 3×10^3 cells per well in 100 μl starvation medium was plated in 96-well plates. After overnight culture, the starvation medium was supplemented with bFGF (10 ng /ml) or VEGF (10 ng /ml) containing Z24 or SU5416. After 72 h incubation with vehicle and compounds at 37 °C, MTT assays were performed. All data were expressed as means±S.D.; *p<0.05, **p<0.01 vs. bFGF-treated group.

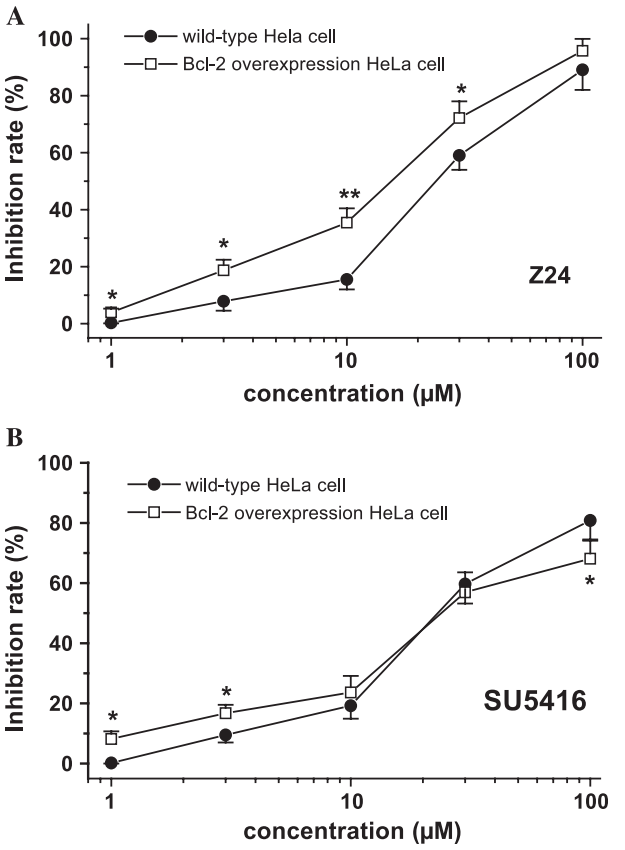

Fig. 9. The concentration–inhibition rate curves of Z24 (A) and SU5416 (B) on the proliferation of wild-type and Bcl-2 over-expressing HeLa cell. Wild-type (●) and Bcl-2 over-expressing (□) HeLa cell of 3×10^3 cells per well in 100 μl RPMI 1640 medium supplemented with 10% new calf serum was plated in 96-well plates. After overnight culture, vehicle or Z24 or SU5416 were added and incubation for 72h, then MTT assays were performed. All data were expressed as means±S.D.; *p<0.05 vs. wild-type HeLa cell.

enhanced (P<0.05, compared with bFGF-inducer; Fig. 8B). It suggested that SU5416 at 1–30 μM has more selectivity on VEGF-stimulating proliferation of ECV-304 cells than that of bFGF. However, the inhibition rates of Z24 at dosage of 3 and 10μM on the proliferation induced by bFGF is higher than that induced by VEGF (P<0.05, respectively) and the inhibition of Z24 seems to be more potent than that of SU5416 on the proliferation induced by bFGF. This showed that Z24 at low concentration has more selectivity on bFGF signaling pathway than that of VEGF. That was different from the SU5416 which has more selectivity on VEGF-stimulating proliferation of ECV-304 cells.

Table 3
IC_{50} values and its 95% confidence interval of Z24 and SU5416 on proliferation of ECV-304 at the presence of 10 ng/ml bFGF or VEGF

Compound	IC_{50} (95% confidence interval; μM)	
	10 ng/ml bFGF	10 ng/ml VEGF
Z24	18.0 (14.4–22.5)	24.4 (14.8–40.3)
SU5416	22.7 (18.6–27.6)	14.2 (9.8–20.9)

IC_{50}, and its 95% confidence interval, was calculated by Logit method.

3.6. Selectivity of Z24 on Bcl-2 over-expressing HeLa cell

We successfully established a stable Bcl-2-specific over-expressing HeLa cell line, which was validated by Western blot (data not shown); for wild-type HeLa, cell line almost does not express Bcl-2 protein. As shown in Fig. 9, Z24 and SU5416 from 1 to 100 μM inhibited the proliferation of both wild-type HeLa cell and Bcl-2 over-expressing HeLa cell in a concentration-dependent manner but with different concentration–inhibition rate curve (Fig. 9) and IC_{50} value (Table 4). The IC_{50} values of Z24 on the stable Bcl-2 over-

Table 4
IC_{50} values and its 95% confidence interval of Z24 and SU5416 on proliferation of wild-type and Bcl-2 over-expressing HeLa cell

Compound	IC_{50} (95% confidence interval; μM)	
	Wild-type HeLa cell	Bcl-2 over-expressing HeLa cell
Z24	24.8 (16.1–38.3)	11.8 (9.3–15.4)[a]
SU5416	24.4 (12.8–46.5)	30.4 (21.4–43.4)

IC_{50}, and its 95% confidence interval, was calculated by Logit method.
[a] p<0.05 vs. wild-type HeLa cell.

expressing HeLa cells are lower than that on wild-type HeLa cells ($P<0.05$; Table 4); however, SU5416 did not exert this effect. It suggests that Z24 has selective inhibitory activity on Bcl-2 over-expressing cells.

4. Discussion

Z24 was selected as a Bcl-2 inhibitor through structure-based computer screening. It is interesting that Z24 has a structure analogous to SU5416, a known selective inhibitor of VEGF receptor-2, having definite effect against tumor angiogenesis and has developed as an antitumor agent (Mendel et al., 2000). The present study shows that Z24 inhibits the mouse S180 xenograft tumor growth and angiogenesis in a dose-dependent manner in vivo and inhibited invasion and tube formation of endothelial cell in a concentration-dependent manner. These data indicate that the antitumor and antiangiogenesis effect of Z24 has a dose-dependent pharmacological property. In addition to this, the maximal inhibitory effect of Z24 on tumor growth and angiogenesis is almost equal to that of SU5416 in vivo. It is speculated that the antitumor activities of Z24 are related to its antiangiogenic activity, which is similar to that of SU5416 (Lu et al., 2003). However, relative less-biological evidences have been obtained about the mechanisms by which Z24 exerts antitumor and antiangiogenesis effects.

VEGF and FGFs are two key regulators in tumor-associated angiogenesis (Schmidt et al., 1999). VEGF is a critically important angiogenic growth factor and its signaling is very important during the angiogenic switch and initial tumor growth (Gerber, 2003). The FGFs and their receptors are expressed in a number of cancers (De Jong et al., 1998). FGF-directed signaling can induce proliferation of both vessels and tumor cells and can synergize with or induce VEGF-directed signaling (Seghezzi et al., 1998). It is hopeful that the efficacy agents against tumor-associated angiogenesis could be developed through targeting the receptor or signaling pathway of VEGF and FGFs. SU5416 is such an agent with more selectivity on VEGF receptor (Mendel et al., 2000). It is because of the ability of VEGF or bFGF to attract the endothelial cell migration and enhance vascular endothelial cells to form tube-like structures when cultured on extracellular matrix components like Matrigel, which is composed of basement membrane components (Kubora et al., 1988; Grant et al., 1989), that these two reactions the endothelial cell migration and tube-like structures forming induced by VEGF or bFGF are regarded as model of angiogenesis in vitro. In this experiment, when VEGF and bFGF were used to stimulate tube formation and invasion, Z24 and SU5416 exhibited different inhibitory potencies. In the invasion assay, Z24 exhibited more potency when bFGF was the stimulant, but in the tube formation test, SU5416 had more potency when VEGF was the stimulant. In order to validate the different selectivity of Z24 and SU5416 on VEGF and bFGF signaling, ECV-304, which was reported expressing the VEGF receptor (Genersch et al., 2003), was chosen in this experiment. About 10 ng/μl of VEGF or bFGF markedly enhanced the proliferation of ECV-304, formed an VEGF- or bFGF-predominated cell proliferation in starvation medium and Z24 and SU5416 blocked the proliferation induced by VEGF or bFGF in a concentration-dependent manner but with different concentration–inhibition rate curve and IC_{50} values. The IC_{50} values of SU5416 on the proliferation induced by VEGF is lower than that induced by bFGF, especially when SU5416 is at 1–30 μM; the inhibition rates on VEGF-induced proliferation is obviously enhanced. It indicated that SU5416 has more selectivity on VEGF-stimulating proliferation of ECV-304 cells than that of bFGF. This result verified the statement that SU5416 is a specific VEGF receptor-2 (VEGFR-2) signaling blocker (Fong et al., 1999). Moreover, the inhibition rates of Z24 at dosage of 3 and 10μM on the proliferation induced by bFGF is higher than that induced by VEGF and the inhibition of Z24 seems to be more potent than that of SU5416 on the proliferation induced by bFGF. This suggested that Z24 has more selectivity on bFGF signaling pathway than that of VEGF. As for which step in bFGF signaling pathway was targeted specially by Z24 in bFGF signaling pathway needs further investigating.

Bcl-2 is an important member of antiapoptosis protein family. It overexpresses in 70% of breast cancer, 30–60% of prostate cancer, 80% of B-cell lymphomas and 90% of colorectal adenocarcinoma and other cancers (Buolamwini, 1999). Moreover, the expression of Bcl-2 protein also correlates with resistance to a wide spectrum of chemotherapeutic drugs and γ-radiation therapy (Ziegler et al., 1997; Reed, 1997). Therefore, Bcl-2 is a new attractive anticancer target (Zhang, 2002). More recently, three independent groups reported their discovery of small molecule inhibitors of Bcl-2 or Bcl-xL (Wang et al., 2000; Degterev et al., 2001; Tzung et al., 2001). Those inhibitors were all discovered through structure-based computer screening and had the characteristic of inducing apoptosis in cancer cells with high Bcl-2 expression and potency correlated with the Bcl-2 expression level in cancer cells (Enyedy et al., 2001). In this experiment, the growth-inhibitory effect of Z24 and SU5416 on wild-type HeLa cells and Bcl-2 over-expressing HeLa cells were compared. The IC_{50} of Z24 on the growth of Bcl-2 over-expressing HeLa cells is obviously lower than that of wild-type HeLa cells. However, SU5416 did not exhibit this effect. The IC_{50} of SU5416 on the growth of Bcl-2 over-expressing HeLa cells is higher than that of primary HeLa cells but with no obvious difference. It indicated that Bcl-2 was not the work site of SU5416. These results confirmed that Z24 has better selectivity on Bcl-2 over-expressing cells and is a potential Bcl-2 inhibitor.

With advances in basic biological study of angiogenesis, more and more evidence has shown that Bcl-2 has a close

relationship with tumor-associated pathologic angiogenesis (tumor angiogenesis). At the beginning stage of tumor-angiogenesis, hypoxia and VEGF expression induced Bcl-2 over-expression in tumor tissues and endothelia (Gerber et al., 1998; Mazure et al., 1997). In human MCF-7 breast cancer cells, Bcl-2 over-expression induces matrix matelloproteinases-9 (MMP-9) transcription (Ricca et al., 2000). Bcl-2 up-regulation or over-expression enhances intratumoral angiogenesis and accelerates tumor growth (Iervolino et al., 2002; Nör et al., 2001). Therefore, Bcl-2 could potentially be used as a therapeutic target within pathologic vasculogenesis. Antitumor and antiangiogenesis effects should be related to Bcl-2 protein in tumor cells and endothelial cells. The directed relationships of Z24 and tumor angiogenesis need further investigation.

In conclusion, Z24, a novel small molecule compound, combines both antiangiogenic and antitumor properties. The action mechanism of Z24 is different from that of SU5416, but is related to the inhibition of Bcl-2 and has more selectivity on bFGF signaling pathway than VEGF signaling pathway.

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