Angiogenesis Inhibitor Z24 Induces Endothelial Cell Apoptosis and Suppresses Tumor Growth and Metastasis

Haiyan Lu¹*, Chen Lin¹, Zhibing Zheng², Song Li², Shunxing Guo³, Xueyan Zhang¹, Ming Fu¹, Xiao Liang¹, and Min Wu¹

¹National Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100021, People’s Republic of China
²Beijing Institute of Pharmacology and Toxicology, Beijing 100850, People’s Republic of China
³Institute of Medical Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100094, People’s Republic of China

Received October 14, 2004; Accepted February 28, 2005

Abstract. Z24, a small molecular compound with similar chemical structure to SU5416 designed and synthesized by our lab, has been proved to be an angiogenesis inhibitor. In this study, Z24 was shown to induce human umbilical venous endothelial cell (HUVEC) apoptosis confirmed by morphologic changes including the presence of apoptotic bodies, significant apoptotic sub-G1 peak upon flow-cytometric analysis, formation of DNA ladders upon agarose gel electrophoresis, and TUNEL (TdT mediated X-dUTP nick-end labeling) results. Systemic administration of Z24 at non-toxic dose in nude mice resulted in inhibition of subcutaneous tumor growth of human colon cancer HCT-8, while it did not inhibit this cell line in vitro, with 100-fold more potent growth-inhibition against endothelial cells. The immunohistochemical results showed that the microvessel density of tumor tissue of the Z24 group was significantly lower than that of the control groups ($P<0.05$), which supported its anti-angiogenic property. We further found that Z24 inhibited the pulmonary metastasis of mouse lung adenocarcinoma LA795, with fewer surface lung metastases (89.6%, $P<0.0001$) and decreased lung weights (38.5%, $P<0.01$) compared to the vehicle group. All these findings support that Z24 is a promising angiogenesis inhibitor for limiting tumor growth and metastasis.

Keywords: Z24, angiogenesis inhibitor, tumor growth and metastasis, microvessel density, endothelial cell apoptosis

Introduction

Angiogenesis, the process of sprouting of capillaries from preexisting blood vessels, is fundamental to reproduction and development. This process is typically short in duration and tightly regulated through a balance between angiogenesis stimulators and inhibitors. However, when this balance is disrupted, unchecked angiogenic factors can be released from hypoxic cells, migrate to the nearby blood vessel endothelia, and signal the activation of the angiogenic response. Angiogenesis takes part in many pathologic processes, including cancer, cardiovascular diseases, chronic inflammation, diabetes, endometriosis psoriasis, and adiposity (1, 2). Angiogenesis plays a very important role in the process of tumor growth and metastasis. The progressive growth and metastasis of tumor cells is dependent on the formation of new blood vessels stimulated by the factors released by tumors, to supply tumors with oxygen and essential nutrients. Without neovascularization, tumors rarely grow beyond 2 – 3 mm (3), will become necrotic and/or apoptotic (4 – 6), and most persist in a relatively benign or dormant state and are often clinically undetectable (3, 5). The importance of angiogenesis in human tumor growth and metastasis is reflected by many studies that demonstrated that the angiogenic phenotype as measured by vessel density is prognostic of survival in people with various types of cancer (7 – 11).
There are many advantages of antiangiogenic approach in comparison to traditional tumor therapy methods. Many clinical protocols rely on very aggressive approaches based on high-dose irradiation or chemotherapy. Both methods show only partial efficacy on certain tumor types, generally with severe side effects. However, the endothelial cell represents a preferential target for therapy, as it is a cell type common to all solid tumors. Even though every cancer is virtually a unique disease, the endothelial tumor is generally a relatively uniform, normal cell type. Stimuli are constantly present so that the differentiation of the tumor endothelium into a mature vessels show an abnormal morphology. These patterns suggest that it may be possible to specifically target tumor angiogenesis by inhibiting endothelial cell recruitment by the tumor and its proliferation. Another advantage of the antiangiogenic approach is the apparent inability by the endothelial cell to counteract therapy through development of multi-drug resistance mechanisms, due to the low mutagenesis rate of this normal cell type (12, 13).

Strategies for exploiting tumor angiogenesis for novel cancer drug discovery include the following: i) inhibition of proteolytic enzymes that breakdown the extracellular matrix surrounding existing capillaries; ii) inhibition of endothelial cell migration; iii) inhibition of endothelial cell proliferation; iv) enhancement of tumor endothelial cell apoptosis. Identification of potential mediators of angiogenesis is an active area of research, and angiogenesis inhibitors have shown some potential in the treatment of cancer in both preclinical and clinical studies (14 – 17). SU5416, a selective inhibitor of the VEGF receptor (Flk-1/KDR), is proved to be an effective angiogenesis inhibitor and inhibits the growth and metastasis of multiple types of tumors in vivo (18 – 20). In our previous work and L.L. Wang’s unpublished work, it has been proved that Z24, a small molecular compound with similar chemical structure to SU5416, selectively inhibited EC proliferation, migration, and differentiation (21). In this study, we want to determine its effects on EC apoptosis and further to test its inhibitory activity on the growth and metastasis of different tumor models in vivo.

Materials and Methods

Synthesis of Z24

The chemical name of Z24 is (3Z)-3-[(1H-pyrrol-2-y1)-methylidene]-1-[(piperidylmethyl)-1,3-dihydro-2H-indol-2-one mesylate. The structural formula is shown in Fig. 1. The chemical identity of Z24 is supported by nuclear magnetic resonance, mass spectroscopy, and elemental analysis.

Cell line and animals

Human umbilical venous endothelial cells (HUVEC) were isolated from umbilical cords as previously described (21). Culture medium consisted of RPMI-1640 supplemented with 20% (v/v) fetal bovine serum, 10 ng/ml bFGF (Invitrogen, Beijing, China); 2 mM l-glutamine, 5 U/ml penicillin G, 5 μg/ml streptomycin sulfate. HUVECs were passaged with 0.05% trypsin / 0.02% EDTA. Confluent HUVEC monolayers (passage 2 – 4) were used in the experiments described below. The 7 – 8-week-old BALB/c male nude mice and 6 – 7-week-old T-739 mice were bred at the Cancer Institute, PUMC. The mice were housed five mice per box in wire-topped plastic boxes maintained on a 12-h light / 12-h dark cycle under the specific pathogen-free condition. The temperature and humidity in the room were controlled at 24 – 26°C and 30 – 50%, respectively.

Flow cytometric analysis

HUVECs were plated in 25 cm² plastic flasks (Costar, Cambridge, MA, USA). After 24 h, cells were treated with 1, 10, or 100 μM Z24 for 72 h. Cells were then trypsinized, washed twice with PBS, and fixed in 1 ml ice-cold 70% ethanol overnight at 4°C. Fixed cells were pelleted, resuspended in 0.5 ml PBS containing 75 U/ml RNase A (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 30 min, and stained with propidium iodide (PI) (Sigma, Beijing, China). The DNA content of cells was analyzed by a FACSCalibur flow cytometer (Becton-Dickinson, Beijing, China). Data were analyzed by the ModFit LT™ V2.0 software.

DNA ladder assay

DNA fragmentation analysis by gel electrophoresis was performed according to Grimm et al. (22). HUVECs
in the log phase of growth were treated with different concentrations of Z24 or vehicle for 72 h. Cells were harvested and washed twice with PBS and then pelleted and lysed in 400 μl cell lysis buffer [1% NP40, 20 mM EDTA, 50 mM Tris-HCl (pH 7.5)] for 10 s. After centrifugation, the supernatant was digested with 42 μl 10% SDS and 15 μl RNase A (10 mg/ml) for 2 h at 37°C and then with 10 μl proteinase K (20 mg/ml, Sigma) for 2 h at 56°C. Then DNA was isolated by phenol/chloroform extraction and ethanol precipitation, finally dissolved in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.6)] and analyzed on a 1.0% agarose gel containing ethidium bromide (0.5 μg/ml). DNA was visualized and photographed under UV light.

**TUNEL stain for the apoptotic cells**

The apoptotic HUVECs were also detected by the TUNEL method. HUVECs were harvested after being treated for 72 h with different concentrations of Z24 or vehicle. Cells were distributed onto glass slides and dried at room temperature. Slides were fixed in 4% paraformaldehyde for 30 min and then treated with permeabilizing buffer (0.1% Triton X-100, 0.1% potassium citrate) at 4°C for 2 min. A 20-μl aliquot of TUNEL reaction buffer from the In Situ Cell Death Detection kit (R&D Systems, Beijing, China) was added to the slides, which were then incubated at 37°C for 1 h. Slides were washed in PBS 3 times and then examined by a fluorescent microscope (Olympus, Beijing, China) and photographed.

**Tumor cell line and growth assays**

Human colon cancer cell line HCT-8 used in the in vitro growth and subcutaneous xenograft studies was maintained by our lab and was cultured in RPMI-1640 media at 37°C in 5% CO₂. The culture media was supplemented with 10% FBS and 2 mM l-glutamine. The growth-well cells were seeded in 96-well, flat-bottomed plates at 3 × 10³ cells/100 μl per well. Z24 and SU5416 were serially diluted in media containing DMSO (<0.5%) and added to cultures of the cells 1 day after the initiation of culture. Cell growth was measured after another 72 h using the MTT method (23). Plates were read on an ELISA plate reader (Bio-Kinetic Reader, Microplate EL312e; Bio-TEK Instruments, Winooski, VT, USA) at a wavelength of 490 nm. Results were compared to those from cells treated with media alone and expressed as a percentage relative to untreated cells to generate a dose-response curve.

**Subcutaneous xenograft model**

HCT-8 human colon cancer cells were implanted subcutaneously in the axillary region of BALB/c male nude mice. After the tumor tissue grew to 0.5 cm in diameter, it was separated from the mouse, cut into same-size masses, and implanted subcutaneously in the axillary region of the same old nude mice. Animals received i.p. injections once daily of 80 mg/kg Z24 dissolved in 0.05% CMC (in PBS) vehicle beginning 1 day after implantation. The animals of the positive control group were injected once daily with 25 mg/kg SU5416, which was also dissolved in 0.05% CMC (in PBS) vehicle. Tumor growth during the treatment period was monitored by measuring the tumor masses in the animals using venier calipers. Tumor volumes were calculated using the formula a²b/2, where a and b are the shorter and longer diameters of the tumor, respectively. After treatment for 23 days, animals were euthanized in dry ice, and tumor growth was quantitated by weighing the tumors.

**Metastasis tumor model**

LA-795 mouse lung adenocarcinoma tissues that grew well in T-739 mouse were cut into pieces of same-size masses. Then the tumor masses were implanted subcutaneously in the back foot pad of T-739 mice. Mice were randomized to one of three groups (10 mice/group). Treatment began 1 day after implantation, like in the subcutaneous xenograft model. After 11 days, the primary tumor was removed by excision of the foot pad. Therapy was then continued for 30 more days. Mice were euthanized in dry ice. Lungs were excised, weighted, and fixed in Bouin’s solution. The number of total surface lung metastases was counted.

**Microvessel staining**

This experiment was carried out according to Barnhill et al. (24). Tumors were fixed in 10% buffered formalin and embedded in paraffin and then cut into 3-μm-thick sections. Sections were deparaffinized. Endogenous peroxidase was blocked by covering the sections with 3% (v/v) H₂O₂ in absolute methanol for 30 min at room temperature (RT). Sections were heated at 95 – 97°C in 0.01 M sodium citrate pH 6 for 15 min. Then they were incubated with 5% nonfat dry milk in TBS-T (1 × TBS + 0.05% Tween-20) for 30 min at RT, followed by incubation with rabbit anti-factor-VIII serum (Zhongshan Biotechnology, Beijing, China) at 1:50. Antibody binding was detected by sequential incubation with HRP-labeled anti-rabbit IgG (Zhongshan Biotechnology) at 1:100. Immune complexes were visualized by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H₂O₂ in PBS for 5 min. The sections were counterstained with hematoxylin (Sigma).

Microvessel density counts were derived by averaging the number of factor VIII-positive vessels per four
“hot spots” fields at ×200 magnification. Microscope fields (using a ×400 magnification) were acquired and digitized. The results were expressed as the mean ± S.E.M.

Statistical analysis
The statistical analysis was performed by Student’s two-tail t-test. Unless otherwise indicated, average values were expressed as mean values with S.E.M. P<0.05 was considered as statistically significant.

Results

Morphological evaluation of apoptosis
Growth of HUVECs was obviously inhibited in the presence of Z24. The morphological changes of the apoptotic cells became obvious after incubation with Z24 (100 μM) for 24 h compared to the vehicle control as assessed by morphology (Fig. 2). The morphological changes of the apoptotic cells included membrane blebbing, shrunken cytoplasm, nuclear condensation, and loss of adhesion. The morphological changes were more obvious after treatment for 72 h, in which almost all cells began to detach from the substratum (Fig. 2).

Z24 induces HUVECs apoptosis
Apoptotic HUVECs induced by Z24 were first examined by flow-cytometric analysis of PI-stained cells. Treatment of HUVECs with various concentrations of Z24 for 72 h causes cell apoptosis. The 1 μM Z24 group showed a sub-G1 peak (being considered apoptotic cells), in which the apoptotic cells were about 19.8%. The rate of apoptotic cells became higher with the increase of Z24 concentration (Fig. 3).

During cell apoptosis, an early biochemical event is activation of an endogenous nuclear endonuclease, leading to degradation of genomic DNA, which can be shown by flow cytometry.
revealed as a characteristic DNA ladder pattern by gel electrophoresis. After HUVECs were treated with different concentrations of Z24 for 72 h, DNA gel electrophoresis revealed that a DNA ladder pattern was produced by treatment with 1 \mu M Z24 (Fig. 4), indicating degradation of nucleosomal DNA in cells.

The apoptotic cell death of HUVECs induced by Z24 was confirmed by TUNEL (TdT mediated X-dUTP nick-end labeling). After treatment of cells with serially diluted Z24 for 72 h, a considerable percentage of apoptotic cells was detected (data not shown).

**Effect of Z24 on HCT-8 human colon cancer cell line in vitro**

To rule out the possibility that Z24 had an effect on the growth of tumor cells directly, the in vitro growth inhibitory property of Z24 on the HCT-8 tumor cell line used in the in vivo study was tested by the MTT method. Z24 treatment had no effects on the in vitro growth of HCT-8 cells (IC_{50} > 40 \mu M), similar to that of the positive control SU5416. Given the 100-fold more potent growth-inhibitory properties on endothelial cells, we concluded that the inhibitory effect of Z24 may be mediated by a direct effect on the angiogenic process associated with tumor growth.

**Z24 inhibited HCT-8 colon tumor growth in vivo**

Nude mice were implanted s.c. with tissue masses of the HCT-8 tumor cell line and randomized into three experimental groups. The drug treatment with Z24 began the next day after implantation. Tumor growth of drug treatment groups was inhibited compared to that of the vehicle group (Fig. 5A). By day 23 of treatment, mice treated with Z24 (80 mg/kg, i.p.) had a significant decrease in tumor burden (51.4%, P<0.001) compared to vehicle-treated mice. The positive control SU5416-treated mice (25 mg/kg, i.p.) also showed significantly lower tumor burden (32.7%, P<0.05) than the vehicle-treated mice (Fig. 5B).
Z24 inhibited tumor metastasis in vivo

Fewer surface lung metastases were present in the Z24 (89.6%, P<0.0001) and positive control SU5416 (68.4%, P<0.01) groups than that in the vehicle control group. There was no significant difference between the SU5416 group and Z24 group (P>0.05) (Fig. 6A). Harvested lungs were weighted as a gross measure of metastatic tumor burden. Relative to control mice, lung weights were decreased in the Z24 (38.5%, P<0.01) and positive control SU5416 (24.9%, P>0.05) groups. The lung weights of the Z24 group were significantly less than those of the positive control SU5416 group (P<0.05) (Fig. 6B).

Fig. 6. Effects of Z24 on the development of LA795 lung metastases. The number of surface lung metastases (A) and lung weight (a gross measure of tumor burden: B) for the three treatment groups are shown. Bars, S.E.M. *P<0.01, **P<0.001 vs vehicle; †P<0.05 vs SU5416.

Fig. 7. Effects of Z24 on the microvessel density of HCT-8 colon tumors. HCT-8 tumors from the above in vivo experiment were stained for microvessels using rabbit anti-factor VIII serum. The microvessels were counted in four random fields at ×200 magnification. Bars, S.E.M. *P<0.05, **P<0.001 vs vehicle; †P<0.01 vs SU5416.

Fig. 8. Immunohistochemical staining of microvessels of HCT-8 colon tumors. HCT-8 tumors from the above in vivo experiment were stained for microvessels using rabbit anti-factor VIII serum. Magnification = ×400.
Effect of Z24 on tumor vessel counts

In addition to inhibition of tumor growth, treatment with Z24 led to decreased tumor vascularization. Immunohistochemical staining for factor VIII-related antigen to detect microvessels in HCT-8 tumors revealed a significant decrease in tumor vessel counts in the Z24 group (71.9%, \( P < 0.001 \)) and positive control SU5416 group (34.3%, \( P < 0.05 \)) compared to those in the vehicle group (Figs. 7 and 8). Additionally, the tumor vessel counts in the Z24 group were much less than those of the SU5416 group (\( P < 0.01 \)), and this correlated with their antitumor activity in vivo (\( r = 0.983 \)).

Discussion

Tumor growth and metastasis are angiogenesis-dependent processes (25, 26). Endothelial cell activation, migration, proliferation, and differentiation are major cellular events in this process (27). In our previous work and Wang’s unpublished work, it was demonstrated that Z24 selectively inhibited endothelial cell proliferation without any influence on human normal tissue cell and tumor cells, inhibited endothelial cell migration, and inhibited the spontaneous formation of capillary-like structures by endothelial cells on Matrigel, which occurs as a result of endothelial cell differentiation (21). It has been reported that many angiogenesis inhibitors including SU5416 induce endothelial cells apoptosis (19). In this report, first we studied the apoptosis-induce activity of Z24 on HUVECs. Our data (morphology, flow-cytometric analysis, DNA ladder, and TUNEL test) showed that Z24 induced HUVECs apoptosis in a time- and dose-dependent manner. There were no obvious apoptotic cells when HUVECs were treated with 100 \( \mu \)M Z24 for 5 h up to 24 h. Upon 72-h treatment, HUVECs exhibited characteristic morphological and biochemical changes, including formation of apoptotic bodies, emergence of an apoptotic subpopulation in flow-cytometric analysis, and appearance of DNA ladders on gel electrophoresis and of apoptotic cells using the In Situ Cell Death Detection kit. The apoptotic cell rates increased from 19.8% to 49.6% as the treatment concentration of Z24 increased from 1 to 100 \( \mu \)M.

Angiogenesis in human solid tumors is a risk factor for metastasis and recurrence (25). The density of microvessels in primary carcinoma lesions significantly correlates with tumor growth and metastasis (25). Our data showed that Z24 reduced the microvessel density of HCT-8 human colon cancer tissue from nude mice in tumor growth-inhibition experiments in vivo. This result correlated with its antitumor activity in vivo (\( r = 0.983 \)). Our data also showed that the antitumor activity of Z24 in vivo should not be attributed to its direct cytotoxic activity on tumor cells. Z24 had no significant effect on the growth of tumor cells directly compared with its effects on endothelial cells, with 100-fold more potent growth-inhibitory properties on endothelial cells. All of these results proved that the antitumor activity of Z24 was the result of its inhibition of the formation of new vessels in tumors.

It has been mentioned before that angiogenesis is very important for tumor metastasis. We have proved Z24’s anti-angiogenesis activity. Following this, we determined its inhibitory activity on tumor metastasis using the mouse lung adenocarcinoma LA795 model. Our data suggested that Z24 has inhibitory activity on tumor metastasis in vivo, similar to but better than positive control SU5416. It inhibited mouse carcinoma LA795 lung metastasis when 80 mg/kg was injected i.p. once a day for 30 days, as demonstrated by fewer surface lung metastases (89.6%, \( P < 0.0001 \)) (Fig. 6A) and decreased lung weights (38.5%, \( P < 0.01 \)) (Fig. 6A) compared to those of the vehicle group.

The i.p. dose of Z24 used in the in vivo experiments was determined by a crude toxicity experiment that showed that the maximum dose of Z24 was 100 mg/kg, and because it was used for a long time, 80 mg/kg was used in the formal experiment. Fong et al. (18) reported the maximum i.p. dose of SU5416 was 25 mg/kg. From this study, we found that the maximum inhibition activity of Z24 was higher than that of SU5416 on HCT-8 growth and mouse carcinoma LA795 lung metastasis in vivo. What also deserved to be mentioned is that we did not observe toxic effects at the doses administered in mice tumor model experiments, as evidenced by body weight and grooming habits, which remained similar to those of the vehicle control mice during the treatment. It was because of operational injuries during excision of the primary tumor in the foot that several mice died in the three groups including the vehicle group.

We used different methods from traditional ones to prepare the mouse tumor models. The traditional method is to inject cultured cells to the experimental mice directly. First we injected the cells to several mice to get tumor tissue about 0.5 cm in diameter. Then the experimental mice were seeded subcutaneously with small same-sized pieces of these tumor tissues. This makes it easier to obtain tumors that grow to similar size, and it does not require preparation of a large number of cells.

In summary, in this study we have confirmed the apoptosis-induce activity of Z24 on HUVECs that contributes to its antiangiogenic activity besides its other actions on endothelial cells. It is also proved that Z24 inhibits tumor growth and metastasis in vivo, which is...
the result of its antiangiogenic activity. We can conclude that Z24 is a potential angiogenesis inhibitor that can be used in tumor therapy despite its unknown molecular target, which we are currently performing investigations to identify.

Acknowledgment

This research was supported by National 863 High Technology Foundation of China, Grant No.: 2001AA235091.

References