YM-231146, a Novel Orally Bioavailable Inhibitor of Vascular Endothelial Growth Factor Receptor-2, Is Effective against Paclitaxel Resistant Tumors

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Chemotherapy using anticancer drugs induces serious the problem of multidrug resistance (MDR) in the cancer cells. In contrast, endothelial cells so rarely acquire MDR that antiangiogenesis therapy has recently been considered as an effective means for cancer chemotherapy. We screened compounds in the chemical library to find a novel and orally active antitumor agent with vascular endothelial growth factor receptor-2 tyrosine kinase (VEGF-R2 TK) inhibition. The result was YM-231146 (IC50=0.080 μM). YM-231146 inhibited VEGF-stimulated proliferation, VEGF-R2 autophosphorylation, and vessel sprout formation of human vascular endothelial cells at concentrations between 0.15—0.30 μM. However, YM-231146 did not inhibit cancer cell proliferation at these concentrations (IC50>5 μM). In the in vivo studies, once-daily oral dosing of YM-231146 to human cancer xenografts elicited antitumor activity at doses of 3—100 mg/kg. Moreover, YM-231146 completely inhibited tumor growth of paclitaxel-resistant cancer cells without decreasing body weight at a dose of 100 mg/kg. These results suggest that YM-231146 is a novel orally bioavailable inhibitor of VEGF-R2 that would be useful for the treatment of multidrug resistant tumors.

Key words YM-231146; angiogenesis; endothelial cell; vascular endothelial growth factor (VEGF); multidrug resistance (MDR); paclitaxel

The prevalence of cancer has increased conspicuously in the past ten years. The diagnostic methods have progressed so rapidly that we can find cancer in the early stages. Chemotherapy using anticancer agents has been done for more than 50 years, and the number of anticancer agents has risen considerably. Unfortunately, though genes in cancer cells become unstable and mutated cells resistant to antitumor agents are frequently selected, bringing about a serious situation, multidrug resistance (MDR). Patients with this condition cannot be treated satisfactorily. Some of this suggested mechanisms of action for MDR include the following: the exclusion of drug from the cell by the overexpression of P-glycoprotein or a member of the MDR protein family; alteration in the levels or properties of drug targets; increased detoxification by activation of glutathione S-transferase; failure to change the drug into its active form; or increased resistance to a recombinant humanized monoclonal antibody to VEGF with efficacy against colorectal cancer and other malignancies, has already been approved for patients. The effects of VEGF are mediated by three endothelial cell-receptor tyrosine kinases, VEGF-R1 (Flt-1), VEGF-R2 (KDR/Flk-1), and VEGF-R3 (Flt-4). VEGF-R2 seems to mediate the major growth and permeability actions of VEGF. Mice engineered to lack VEGF-R2 fail to develop a vascular and have very few endothelial cells that abnormally coalesce into disorganized vessels. Although targeting VEGF-R2 would be the most suitable way to block the VEGF signaling cascade in endothelial cells, no orally active or small molecular antitumor agents that selectively inhibit vascular endothelial cell proliferation has yet been approved. For this reason, we screened compounds in the chemical library to find a novel and orally active antitumor agent with VEGF-R2 TK inhibition. This search yielded (3Z)-3-quinolin-2(1H)-ylidene-1,3-dihydro-2H-indol-2-one derivative, YM-231146. In this study, we evaluated the in vitro and in vivo efficacy of YM-231146, and suggested the possibility of its application as an antitumor agent based on its inhibition of endothelial cell proliferation. In addition, we examined its antitumor action against cancer cells resistant to the cytotoxic agent paclitaxel.
MATERIALS AND METHODS

Agents and Cell Lines  YM-231146, a (3Z)-3-[6-(2-morpholin-4-ylmethoxy)quinolin-2(1H)-ylidene]-1,3-dihydro-2H-indol-2-one (Fig. 1) was synthesized at Yamanouchi Pharmaceutical Co., Ltd. Paclitaxel was purchased from Sigma (St. Louis, MO, U.S.A.). Normal human endothelial cells, HUVECs (human umbilical vein endothelial cells), were obtained from Clonetics (San Diego, CA, U.S.A.) and cultured on gelatin-coated plates with endothelial growth medium-2 (EGM Bullet kit). Human colon cancer Colo205 and HCT-15 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, U.S.A.) and 1% antibiotic–antimycotic (Gibco BRL). Multidrug resistant MCF-7/ADR cells were selected for by growing MCF-7/ADR cells in 100% RPMI-1640 supplemented with 5% FBS (Gibco BRL) and 1% antibiotic–antimycotic (Gibco BRL). Treatment with 0.1% FBS. After 24 h, the cells were dosed with YM-231146 for 2 h and stimulated with human recombinant VEGF (50 ng/ml, R&D Systems) for 5 min. Cells were lysed in TNE buffer containing 10 mM Tris–HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM Na3VO4. Cell lysates were detected using western blot with anti-phosphotyrosine (4G10) and anti-VEGF-R2 antibody.

VEGF-R2 Kinase Domain Expression and Purification  The VEGF-R2 cDNA-encoding amino acids 790—1168 (catalytic domain) were obtained by performing RT-PCR using total RNA isolated from HUVECs. VEGF-R2, nucleotide sequences encoding a FLAG epitope recognized by the M2 monoclonal antibody, were incorporated into the forward PCR primer. The cDNA was subeloned into the pFASTBAC1 vector (Gibco BRL). SF-9 cells expressed recombinant KDR kinase domain using the Bac-To-Bac expression system were sonicated and centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was then collected. The VEGF-R2 kinase domain was bound to M2-agarose (Sigma) and extracted with a FLAG epitope.

VEGF-R2 Kinase Assay  We used a homogeneous time-resolved fluorescent (HTRF) assay format.26 The kinase reaction solution (25 μl) consisted of 100 ng VEGF-R2 kinase domain in assay buffer (50 mM Hepes, pH 7.5, 1 mM MgCl2, 4 mM MnCl2, and 0.1% BSA). The kinase reaction was initiated by adding 25 μl of 2 μl ATP in a black 96-well Opti-plate (Perkin-Elmer). After a 20-min incubation at room temperature, 10 μl of 0.5 mM EDTA was added to terminate the reaction. Fifty microliters of HTRF reagent mixture [6.5 ng cryptate-conjugated anti-phosphotyrosine antibody (PT66, Cis Bio International) and 100 ng XL665-conjugated FLAG (M2) antibody (Cis Bio International) in quench buffer (50 mM Hepes pH 7.5, 0.1% BSA, and 0.5 M KF)] was added to the reaction mixture. The quenched reaction was incubated for 2 h at room temperature and then read using Discovery (Perkin-Elmer), a time-resolved fluorescence detector.

VEGF-Stimulated VEGF-R2 Autophosphorylation Assay  HUVECs were plated in gelatin-coated 12-well plates (1×104 cells/well) for 24 h, and then transferred into Medium 199 supplemented with 0.1% FBS. After 24 h, the cells were dosed with YM-231146 for 2 h and stimulated with human recombinant VEGF (50 ng/ml, R&D Systems) for 5 min. Cells were lysed in TNE buffer containing 10 mM Tris–HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM Na3VO4. Cell lysates were detected using western blot with anti-phosphotyrosine (4G10) and anti-VEGF-R2 antibody.

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In Vivo Tumor Xenograft Models  Male nude mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). For MCF-7/ADR cells only, female nude mice were used. A single-cell suspension of each tumor cell line (2—5×104 cells) was inoculated into the flank of the nude mice by subcutaneous injection. When the tumors reached volumes of 50—150 mm3, the tumor-bearing mice were ran-
domized into treatment groups (n=5—6/group), and then either YM-231146 or 0.5% methyl cellulose (MC) as the vehicle control was given p.o. daily for more than 2 weeks. Paclitaxel (20 mg/kg) was i.v. injected into the tail vein 5 times a week for more than 2 weeks. Tumor size and body weight were measured twice a week. The tumor volume was calculated using the following formula: $1/2 \times (\text{length}) \times (\text{width})^2$. The percent inhibition was also calculated as follows: $\frac{1-(V_i-V_x)}{V_i} \times 100$, where $V_x$ represents the tumor volume on day $x$ and $V_i$ represents the initial tumor volume.

**Statistical Treatment** Data are expressed as the mean ± S.E.M. The significance of differences between the two groups was determined using the unpaired Student’s $t$-test, and the comparison of more than two groups was determined using Dunnett’s multiple range test. $p$ values were calculated. Differences of $p<0.05$ versus control were considered statistically significant.

**RESULTS**

**Effect of YM-231146 on VEGF-R2 Kinase Activity**
The primary assay for identifying inhibitors of VEGF-R2 kinase activity uses an ELISA-based assay with a time-resolved fluorescence readout. The effects of YM-231146, (3Z)-3-[6-(2-morpholin-4-ylethoxy)quinolin-2(1H)-ylidene]-1,3-dihydro-2H-indol-2-one (Fig. 1), was also evaluated in enzyme-based assays for the inhibition of VEGF-R2 kinase activity. The slope for YM-231146 inhibition of human VEGF-R2 kinase is shown in Fig. 2A, and the IC$_{50}$ value was 0.080 μM.

**Effect of YM-231146 on VEGF-R2 Autophosphorylation Induced by VEGF**
The VEGF-R2 kinase inhibitory activity of YM-231146 was evaluated for its dose-related inhibition of VEGF-R2 autophosphorylation induced by VEGF in cell-based assays. The inhibitory concentration of YM-231146 was 0.3 μM with HUVECs (Fig. 2B), which is comparable with its observed inhibitory concentration in enzyme-based assays. At concentrations of YM-231146 of less than 1 μM, there was no decrease in total VEGF-R2 protein when human endothelial cells were exposed for 2 h in vitro.

**Effect of YM-231146 on Cell Proliferation**
YM-231146 potently inhibited VEGF-stimulated HUVEC proliferation (IC$_{50}$=0.19 μM). The IC$_{50}$ values for Colo205 and MCF-7/MDR cell proliferation were higher than 5 μM, approximately 30 times more than that for HUVEC proliferation. (Table 1).

**Effect of YM-231146 on Angiogenesis in Vitro**
The vessel sprout formation assay has been widely used in vitro systems that effectively model the distinct temporal and spatial events underlying in vitro angiogenesis, which is sensitive to the antiangiogenic effects of inhibitors. YM-231146 displayed statistically significant and dose-related inhibition of vessel sprout formation as well as anti-VEGF antibody (Fig. 3). The IC$_{50}$ of capillary-tube formation by YM-231146 was 0.15 μM.

**Plasma Concentrations of YM-231146 after Oral Administration to Mice** Pharmacokinetic studies in mice demonstrated that YM-231146 was detected in the plasma
after p.o. administration. Dose-proportional plasma levels of YM-231146 were observed at levels of 3 mg/kg when orally administered. Peak concentrations of YM-231146 reached about 0.36 μM at 1 h after oral administration. At 8 h after administration, the concentrations were still more than 0.1 μM. The p.o. bioavailability of YM-231146 was 48%. Oral administration of YM-231146 produced good overall systemic exposure (Fig. 4) in i.v. administration of 1 mg/kg, total body clearance was 1026 ml/h/kg, approximately one fifth of the hepatic blood flow, 5000 ml/h/kg. Plasma elimination half-life was 8.75 h (data not shown).

Effect of YM-231146 on Antitumor Activity in Xenograft Models in Vivo

The antitumor activity of YM-231146 was examined in human colon cancer Colo205 xenograft model. Chronic oral dosing of YM-231146 produced a significant and dose-dependent inhibition of tumor xenograft growth. Treatment with 3, 10, 30, and 100 mg/kg of YM-231146 for 14 d resulted in inhibition of tumor growth of 56, 63, 83, and 97%, respectively (Fig. 5). In human lung cancer NCI-H358, human prostate cancer PC-3, and human kidney cancer Caki-1 xenograft models, treatment with 100 mg/kg of YM-231146 resulted in the almost complete inhibition of tumor growth (data not shown).

Effect of YM-231146 on Antitumor Activity in Paclitaxel-Resistant Xenograft Model in Vivo

The multidrug resistant human breast cancer MCF-7/ADR cell line was first created by another group in 1986 after exposing an MCF-7 cell line to increasing concentrations of the drug Adriamycin. The cells that survived drug treatment were isolated and found to be 200 times more resistant to Adriamycin than the parental cell line. MCF-7/ADR cells were also found to be cross-resistant to a range of other anti-neoplastic agents from the Vinca alkaloid and anthracycline groups.25) In a preliminary study using MCF-7/ADR cells obtained by a similar method, we observed that the cells were also resistant to paclitaxel in its xenograft model. In this study, therefore, the antitumor activity of YM-231146 and paclitaxel in the xenograft model was examined for 14 d. While treatment...
with 20 mg/kg of paclitaxel did not significantly inhibit tumor growth, treatment with 100 mg/kg of YM-231146 resulted in an inhibition of tumor growth of more than 100% (Fig. 6A). Additionally, treatment with 20 mg/kg of paclitaxel significantly decreased body weight compared with the day that administration of YM-231146 was started, but treatment with 100 mg/kg of YM-231146 did not cause body weight to decrease (Fig. 6B). YM-231146 also had a tendency toward antitumor efficacy against paclitaxel-resistant human colon cancer HCT-15 xenografts (Figs. 6C, D).

DISCUSSION

Lack of specificity and the consequent generation of toxic side effects when high doses are administered often limit cytotoxic drug use. Low drug doses are not very effective and their use generally results in patient relapse and development of drug resistance. The ability of tumor cells to tolerate these low levels of toxic stress and become drug resistant must be also kept to a minimum. Strategies targeting not cancer cells, but vascular endothelial cells could prove to be very useful in cancer therapy, particularly with regard to the development of drug resistance. Recently, the usefulness of VEGF-signaling blockage has attracted attention in the clinical field because VEGF signaling is an important part of vascular endothelial cell proliferation. Several orally active and low molecular compounds that use VEGF-R2 TK inhibition as their mechanism of action, similar to bevacizumab, an anti-VEGF antibody currently on the market, are being aggressively developed as remedies for cancer.25—33

YM-231146 is a (3Z)-3-quino1in-2(1H)-ylidene-1,3-dihydro-2H-indol-2-one derivative that was found through screening for the inhibition of VEGF-R2 TK activity. The IC50 value of VEGF-R2 TK activity was 0.080 μM in an enzyme assay (Fig. 2A). YM-231146 is not a nonspecific kinase inhibitor, because the IC50 values of protein kinase C-β2 (PKCβ2), PKCα, cyclin-dependent kinase-2 (CDK2), CDK4, and CDK5 were higher than 1 μM (data not shown). YM-231146 inhibited VEGF-stimulated autophosphorylation of VEGF-R2 at a concentration of 0.3 μM (Fig. 2B). In the present study, we immunoblotted cell lysates with anti-phosphotyrosine antibody to detect the phosphorylation of VEGF-R2. Relating to the inhibition of VEGF-R2 enzymatic activity by YM-231146, there would be really very little possibility that the positive band represents the phosphorylation of another kinase substrate protein in Fig. 2B. YM-231146 has a high rate cell permeability because the IC50 value of HUVEC proliferation induced by VEGF was 0.19 μM (Table 1), as well as vessel sprout formation (IC50=0.15 μM) at the inhibitory concentrations of VEGF-R2 enzymatic activity.

Since YM-231146 resulted in significant inhibition of tumor growth of 56—97% in colon cancer Colo205 xenografted model after repeated administration at 3—100 mg/kg, it would be effective enough to exert antitumor activities in the clinical stage (Fig. 5). Compared to the total dose per day in tumor-bearing xenografted mice of these VEGF-R2 TK inhibitors: 50—100 mg/kg (PTK-787), 33—37 25—100 mg/kg (ZD6474), 38,39 50 mg/kg (CP-547,632), 40 and 40 mg/kg (SU11248).41 YM-231146 would have similar potency. Moreover, YM-231146 also showed antitumor efficacy in paclitaxel-resistant tumor xenografts without decreasing body weight (Fig. 6). MCF-7/ADR and HCT-15 cells have been reported to overexpress P-glycoprotein, which is in high correlation with MDR progress when displacing intracellular drugs to the outside of the cell using two ATPs. Especially, MCF-7/ADR showed approximately 200 times more resistance to paclitaxel than the parent MCF-7 cells.25

In this pharmacokinetics study of YM-231146 in mice, the bioavailability was 48%, and peak concentrations of YM-231146 reached about 0.36 μM at 1 h after oral administration of 3 mg/kg. At 8 h after administration, the concentrations were still more than 0.1 μM, which indicated that ADME values were also satisfactory. The plasma concentrations of YM-231146 in mice were consistently lower than 1 μM (Fig. 4). YM-231146 did not inhibit cancer cell proliferation, but vessel sprout formation at lower than 1 μM in media (Table 1, Fig. 3). Therefore, the compound would be expected to inhibit angiogenesis without direct effects on cancer cells in the xenografts. In addition, the IC50 value of paclitaxel against MCF-7/MDR cell proliferation was 3.6 μM (data not shown). The reason why YM-231146 was effective against MCF-7/MDR tumor growth in vivo although it did not inhibit MCF-7/MDR cell proliferation at a concentration of 10 μM in vitro may be that the compound potently suppressed vascular endothelial cell proliferation and angiogenesis in tumor.

In this study, we showed that YM-231146 was a potent and orally active antitumor agent based on VEGF-R2 TK inhibition. Since angiogenesis is a physiological phenomenon observed in almost all solid tumors, YM-231146, which potently inhibits vascular endothelial cell proliferation, can be expected to exert antitumor efficacy without severe toxic side effects, such as decreased body weight, in clinical patients bearing various types of tumors, including multidi drug resistant tumors.

REFERENCES
