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Biological evaluation of a multi-targeted small molecule inhibitor of tumor-induced angiogenesis

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Abstract—RO4396686 is a small molecule KDR, FGFR, and PDGFR inhibitor with good pharmacokinetic properties in rodents. In a mouse corneal neovascularization assay, this compound inhibited VEGF-induced angiogenesis. Tested in a H460a xenograft tumor model this agent effected significant tumor growth inhibition at doses as low as 50 mg/kg.

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature of a host, is a process absolutely necessary for the growth and metastasis of most cancerous tumors.[1] As such, a great part of contemporary anticancer research has been devoted to the elucidation of the drivers of tumor angiogenesis and the discovery of agents that inhibit this process.[2,3] Particular effort has been placed on the discovery of small orally administered molecules that can block the activity of receptor tyrosine kinases (RTKs) involved in the various angiogenic signaling cascades by the displacement of ATP from their catalytic sites.[4,5] In this paper, we report on one such agent, RO4396686 (Fig. 1); a racemic pyrimidopyrimidine that selectively inhibits three key pro-angiogenic RTKs KDR, FGFR, and PDGFR. The general synthesis, SAR, and receptor binding mode for the series RO4396686 belongs to has been presented elsewhere.[6] As such, herein we will focus solely on the in vitro and in vivo results we obtained with this compound.

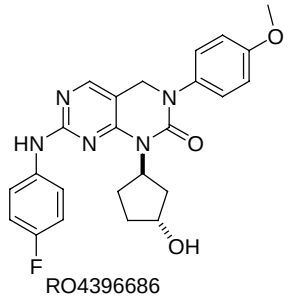

Figure 1. Chemical structure of RO4396686.

In vitro, in a panel of 13 kinases, RO4396686 selectively inhibited KDR, FGFR, and PDGFR with IC_{50} values of 50, 92, and 83 nM, respectively, while it was markedly less potent against the other kinases of that panel (Table 1).[7] This profile of inhibition of multiple key pro-angiogenic receptors is of particular interest, especially since recent evidence suggests that the targeting of multiple pro-angiogenic receptors may afford better therapeutic outcomes than the selective targeting of only one receptor.[8]

In cellular assays, agents that target pro-angiogenic RTKs should inhibit the proliferation of endothelial cells stimulated by the agonists of these RTKs. As shown in Table 2, RO4396686 potently inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) induced by either VEGF (KDR agonist) or bFGF (FGFR agonist).[9] To demonstrate that this inhibition of cellular proliferation is indeed mechanism-based and not due to general cytotoxicity, we tested

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Table 1. In vitro kinase selectivity of RO4396686

Kinase	IC_{50} in nM[a]
KDR	50
FGFR	92
PDGFR	83
Fyn	466
EGFR	545
EphB3	1,250
PKA	9,010
Erk2	12,600
PKCα	>125,000
PKCδ	>125,000
CDK1/cyclin B	>125,000
SGK	>125,000
AKT	>125,000

[a] IC_{50} values were determined by a single experiment run in duplicate.

Table 2. Antiproliferative activity of RO4396686

Cell line	Growth factor	IC_{50} in nM[a]
HUVEC	VEGF	35
HUVEC	bFGF	149
HCT116	—	3,000
H460a	—	2,500

[a] IC_{50} values were determined by a single experiment run in duplicate.

RO4396686 against the HCT116 and H460a cancer cell lines in a general cytotoxicity assessment assay (MTT).[10] In that test, RO4396686 was practically inactive (Table 2).

In vivo, in Wistar rats and in athymic nude mice, RO4396686 had very favorable clearance and volume of distribution. When formulated as a suspension in 2% Klucel LF, 0.1% Tween 80 in water, it also had very high oral absorption and plasma exposure (Tables 3 and 4).[11] However, the plasma exposure did not increase in a dose proportional manner with increasing dose (Table 4).

The next step in the in vivo evaluation of RO4396686 was the assessment of its anti-angiogenic properties.

Table 3. Rodent pharmacokinetic profile of RO4396686 after iv dosing

Rodent species	Cl (mL/min/kg)	AUC (ng h/mL)	V_{dss} (L/kg)	$t_{1/2}$ (h)
Nude mice[a]	13.8	12,058	1.9	2.0
Wistar rats[b]	32.5	2770	3.7	4.3

[a] Single dose, 10 mg/kg.
[b] Single dose, 5 mg/kg.

Table 4. Mean pharmacokinetic parameters of RO4396686 after single oral dose of 50 and 200 mg/kg in rodents

Rodent species	Dose (mg/kg)	AUC (ng h/mL)	C_{max} (ng/mL)	$t_{1/2}$ (h)
Nude mice	50	96,465	29,100	1.9
	200	260,244	82,600	1.7
Wistar rats	50	40,901	3,163	5.0
	200	284,844	10,267	7.8

For that purpose a mouse corneal pocket assay (CPA) was employed.[12] The assay involved the implantation of VEGF-containing pellets in the cornea of C57/BL6 mice followed by oral administration of RO4396686. The mice used in this study were separated in three dose cohorts and each cohort received orally twice a day (bid) 12.5, 50, and 200 mg/kg of RO4396686 for a period of 7 days. At the end of this period the area of corneal angiogenesis was quantified and showed that 50 mg/kg bid administration of RO4396686 led to a 67% ($p = 0.004$) inhibition of VEGF-induced corneal angiogenesis. At 200 mg/kg bid the inhibition achieved was 91% ($p < 0.001$) (Fig. 2).

During the CPA study, the mice in all dose groups were monitored for changes in their average weight as a gross indicator of toxicity. As shown in Figure 3 no appreciable changes in the weight of test animals were observed in that study suggesting no overt toxicity.

These excellent results warranted the further in vivo evaluation of RO4396686 in a tumor model.

Athymic nude mice were implanted with H460a non-small cell lung carcinoma (NSCLC) cells. Ten days post implantation the mice were treated orally with RO4396686 at doses of 12.5, 25, 50, 100, and 200 mg/kg bid for a period of 28 days. The average tumor size

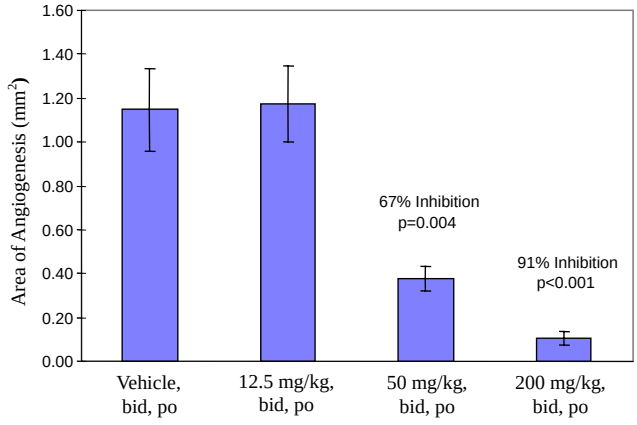

Figure 2. Inhibition of VEGF-induced corneal angiogenesis in C57/BL6 mice by oral bid dosing of RO4396686.

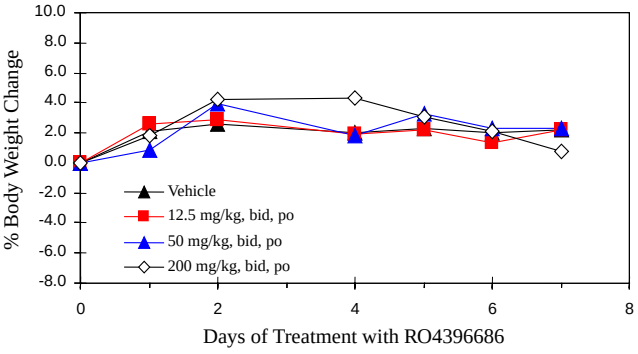

Figure 3. Average percentage of body weight change in C57/BL6 mice treated with three different doses of RO4396686 for a period of 7 days.

for each dose group prior to treatment was between 128 and 137 mm^3. Tumor size measurements were taken throughout the 28-day study period and showed that there was a statistically significant tumor growth inhibition at the 50, 100, and 200 mg/kg dose groups. Specifically, the 50 mg/kg dose group had a 78% ($p < 0.001$) tumor growth inhibition. The 100 and 200 mg/kg groups showed an 87% ($p < 0.001$) and a 95% ($p < 0.001$) tumor growth inhibition, respectively (Fig. 4).

The mice in all dose groups were again monitored for signs of toxicity through the measurement of changes in their average weight (Fig. 5). Again, as in the CPA experiment, all cohorts of mice showed no significant changes in their average weight or any signs of gross toxicity.

In conclusion, RO4396686 is a novel inhibitor of the three key pro-angiogenic receptors KDR, FGFR, and PDGFR. This agent has good clearance and volume of distribution as well as very high oral absorption in rodents. The absorption of this agent in the species tested appears to be non-linear. In vivo, the anti-angiogenic properties of RO4396686 were demonstrated in a CPA study that showed inhibition of VEGF-induced corneal angiogenesis at doses as low as 50 mg/kg. Finally, at doses between 50 and 200 mg/kg bid, this agent effected a statistically significant, dose-dependent, tumor growth inhibition in a H460a NSCLC tumor model in nude mice.

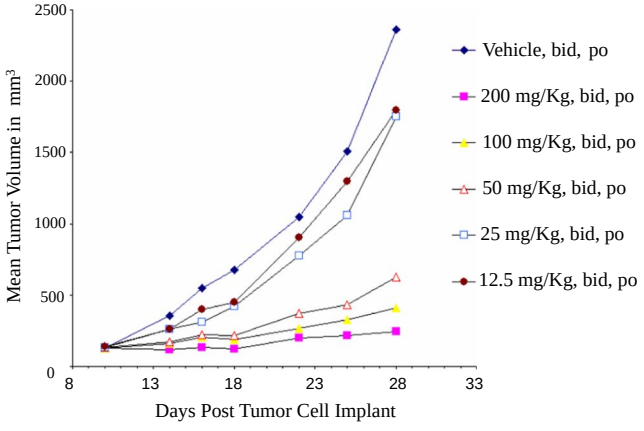

Figure 4. Tumor growth inhibition by oral bid dosing of RO4396686 in nude mice implanted with H460a (NSCLC) tumors.

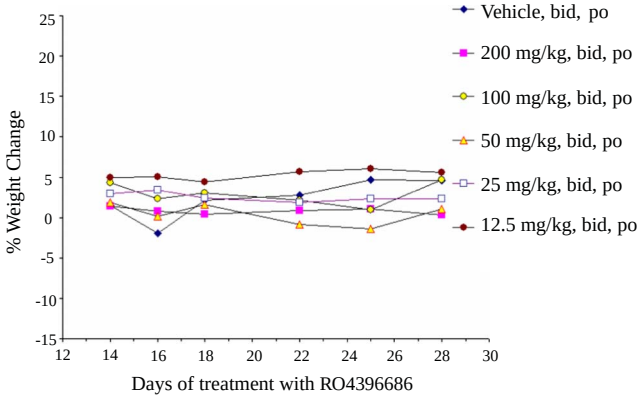

Figure 5. Percentage of change in average body weight of nu/nu mice implanted with H460a (NSCLC) tumors after treatment with RO4396686 for a period of 28 days.

References and notes

1. Folkman, J. *Sci. Am.* **1976**, *234*, 58.
2. For reviews about receptors and signaling pathways involved in tumor angiogenesis, see: (a) Bicknell, R.; Harris, A. L. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 219; (b) Lutsenko, S. V.; Kiselev, S. M.; Severin, S. E. *Biochemistry (Moscow)* **2003**, *68*, 286; (c) Yancopoulos, G. D.; Davis, S.; Gale, N. W.; Rudge, J. S.; Wiengand, S. J.; Holash, J. *Nature* **2000**, *407*, 242.
3. For reviews on angiogenesis inhibitors and the clinical aspects of anti-angiogenic therapy, see: (a) Rhee, J.; Hoff, P. M. *Expert Opin. Pharmacother.* **2005**, *6*, 1701; (b) Verheul, H. M. W.; Pinedo, H. M. *Expert Opin. Emerg. Drugs* **2005**, *10*, 403; (c) Scappaticci, F. A. *Expert Opin. Investig. Drugs* **2003**, *12*, 923.
4. For some reviews, see: (a) Mazitscheck, R.; Giannis, A. *Curr. Opin. Chem. Biol.* **2004**, *8*, 432; (b) Traxler, P. *Expert Opin. Ther. Targets* **2003**, *7*, 215; (c) Magnetti, F.; Botta, M. *Curr. Pharm. Des.* **2003**, *9*, 567; (d) Boyer, S. J. *Curr. Top. Med. Chem.* **2002**, *2*, 973.
5. Recently, the US FDA gave marketing approval for two such compounds, gefitinib (Iressa®) and erlotinib (Tarceva®). Both agents are selective inhibitors for the pro-angiogenic receptor EGFR.
6. (a) Rossman, P.; Luk, K.; Cai, J.; Chen, Y.; Daniewski, A. R.; Dermatakis, A.; Flynn, T.; Garofalo, L.; Gillespie, P.; Goodnow, R.; Graves, B.; Harris, W.; Huby, N.; Jackson, N.; Kabat, M.; Konzelmann, F.; Li, S.; Liu, J.-J.; Liu, W.; Lukacs, C.; Michoud, C.; Perrotta, A.; Portland, L.; Qiao, J.; Roberts, J.; Schutt, A.; Simcox, M.; So, S.-S.; Tamborini, B.; Toth, K.; Wen, Y.; Xiang, Q.; Yang, H.; Zhang, Z. *Abstracts of Papers*, 95th National Meeting of the American Association for Cancer Research, Orlando, FL, USA, 2004; Abst. 2478.; (b) Rossman, P.; Luk, K.; Chen, Y.; Garofalo, L.; Graves, B.; Jackson, N.; Kabat, M.; Konzelmann, F.; Liu, J.-J.; Lukacs, C.; McDermott, L. A.; Michoud, C.; Portland, L.; Roberts, J.; Schutt, A.; Simcox, M.; So, S.-S.; Tamborini, B.; Yang, H. *Abstracts of Papers*, 229th National Meeting of the American Chemical Society, San Diego, CA, USA, March 13–17, 2005; MEDI-124.
7. KDR, FGFR, PDGFR, and EGFR kinase assays were conducted using hTRF (Homogeneous Time-Resolved Fluorescence) assays. The KDR reactions contained 1 μM KDR substrate (Biotin-EEEEYFELVAKKKK), 1.0 nM activated KDR (recombinant human EE-tagged intracellular domain), and dilutions of test compound (final reaction [DMSO] 1%) in kinase buffer (100 mM HEPES, pH 7.4, 1 mM DTT, 0.1 mM Na_2VO_4, 25 mM $MgCl_2$, 0.02% BSA, and ATP at the K_m of 0.3 mM). FGFR reactions contained 1.0 nM activated human recombinant GST-tagged intracellular domain (ICD), 1 μM substrate (Biotin-EEEEYFELV), test compound in 100 mM HEPES, 1 mM DTT, 0.4 mM $MgCl_2$, 0.4 mM $MnCl_2$, 50 mM NaCl, 1% DMSO, 10 μM ATP (K_m = 8.5 μM for FGFR), 0.1 mM Na_2VO_4, and 0.02% BSA. PDGFR and EGFR assays included human recombinant His-tagged ICD of PDGFR and recombinant human ICD of EGFR. For PDGFR and EGFR the substrate peptide used was Biotin-EEEEYFELV. ATP

concentrations for these assays were at the K_m for each enzyme (2.3 μM for PDGFR and 0.5 μM for EGFR). EphB3, SGK, PKA, CDK1/cyclinB, Erk2, AKT, PKCα, PKCδ, and Fyn assays were conducted using an assay based on IMAP (Immobilized Metal Assay for Phosphochemicals). This is a homogeneous FP-based technology (Molecular Devices) that enables quantitation of kinase activity via preferential binding of phosphorylated fluorescent peptide substrates to immobilized metal beads. Reactions were carried out at ATP concentrations of three times the K_m.

8. (a) Erber, R.; Thurnher, A.; Katsen, A. D.; Groth, G.; Kerger, H.; Hammes, H. P.; Menger, M. D.; Ullrich, A.; Vajkoczy, P. *FASEB* **2004**, *18*, 338; (b) Bergers, G.; Song, S.; Myer-Morse, N.; Bergsland, E.; Hanahan, D. *J. Clin. Invest.* **2003**, *111*, 1287; (c) Shaheen, R. M.; Tseng, W. W.; Davis, D. W.; Liu, W.; Reinmuth, N.; Vellagas, R.; Wieczoreck, A. A.; Ogura, Y.; McConkey, D. J.; Drazan, K. E.; Bucana, C. D.; McMahon, G.; Ellis, L. M. *Cancer Res.* **2001**, *61*, 1464.
9. Human umbilical vein endothelial cells (HUVEC, Clonetics cat. CC-2519) were cultured according to manufacturer's protocol. Cell passages 2–6 were used to determine VEGF- or bFGF-stimulated proliferation. Subconfluent cultures were serum starved for 24 h, followed by addition of test compound. After 2 h incubation with drug, 20 ng/mL VEGF (R&D Systems, cat.#293-VE) or 5 ng/mL bFGF (purified, recombinant) was added. DNA synthesis was evaluated using BrdU incorporation (Roche Biochemicals, cat. #1-647-229). After 20 h of incubation with compound, BrdU labeling reagent was added. Four hours later, incorporated label was quantitated using a peroxidase-conjugated BrdU antibody and colorimetric detection.
10. Exponentially growing tumor cells were plated in 96-well microtiter plates and incubated overnight at 37 °C prior to compound addition. Proliferation was assessed by measurement of formazan formation from MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) (Sigma) during a 2.5 h incubation 6 days after initial plating.
11. Single doses of the test compound were administered intravenously and orally to athymic nude mice and Wistar rats. In mice, the test agent was administered at a dose level of 10 mg/kg iv, and at doses of 50 and 200 mg/kg po. Blood samples were collected from the retro-orbital sinus or via terminal cardiac puncture at 1, 5, 15, 30 min, and 1, 2, 4, 8, 16, and 24 h postdose after iv dosing and 5, 15, 30 min, and 1, 2, 4, 8, 16, and 24 h postdose after oral dosing. In Wistar rat, a 5 mg/kg single dose of test compound was administered iv via a femoral arterial catheter. Again two oral doses of 50 and 200 mg/kg were used to determine oral absorption characteristics. Blood samples were collected via an implanted jugular catheter at 1, 7, 15, 30 min, and 1, 3, 6, 12, and 24 h postdose after iv dosing and at 5, 15, 30 min, and 1, 3, 6, 12, 24, and 48 h postdose after oral dosing. In all iv experiments, the test compound was formulated in 2% DMA, 10% PEG400, and 88% of 40% HPBCD in water. The oral formulation was 2% Klucel LF and 0.1% Tween 80 in water. Blood samples were collected in EDTA-containing tubes and centrifuged. The plasma was then removed and stored at −70 °C until analysis. For the analysis of samples a specific LC–MS/MS assay was developed. Fifty microliters of samples was extracted by precipitation of plasma proteins with acetonitrile. The supernatant was diluted with water before injecting onto a C18 (Zorbax® SB-C18 5 μm 2.1 × 50 mm) LC column connected to an LC–MS/MS system. Standard and QC samples were injected together with study samples. All concentrations were interpolated from calibration curves (ranged from 1 to 2000 ng/mL) and all PK parameters were calculated using a Watson laboratory information system (LIMS) software program (version 6.3.0.01a).
12. For an overview of the CPA and other assays in use for the assessment of angiogenesis in vivo and in vitro, see: Auerbach, R.; Lewis, R.; Shinners, B.; Kubai, L.; Akhtar, N. *Clin. Chem.* **2003**, *49*, 32.