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Letters to the Editor

## Comments on “Blockade of Angiogenesis by Small Molecule Antagonists to Protease-Activated Receptor-1: Association with Endothelial Cell Growth Suppression and Induction of Apoptosis”

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This letter is in response to the report by Zania et al. (2006). The authors used *in vitro* (Matrigel) and *in vivo* (chick chorioallantoic membrane) models of angiogenesis to demonstrate that small molecule PAR1 antagonists, SCH79797 and RWJ56110, block formation of new blood vessels through their ability to inhibit PAR1-mediated signal transduction. They also showed that SCH79797 and RWJ56110 inhibit HUVEC proliferation and induce apoptosis when the cells are grown in the presence of 4% serum (FBS).

We would like to point out that SCH79797 is able to inhibit cell proliferation and induce apoptosis *in vitro* in a PAR1-independent manner. Di Serio et al. (2007) demonstrated that SCH79797 (starting from a concentration of 100 nM) interferes with the growth of several human and mouse cell lines, including PAR1 null fibroblasts, when the cells are stimulated to grow with 10% serum, FGF-2 (25 ng/ml), or PDGF (20 ng/ml), whereas hirudin has no effect on the ability of these cells to grow in culture. Because neither FGF-2 nor PDGF depends on PAR1 receptor activation for its mitogenic effect, the ability of SCH79797 to inhibit cell proliferation under these experimental conditions is not likely to depend on its PAR1-blocking activity. Moreover, the ability of this drug to inhibit the growth of PAR1 null cells validates the hypothesis of a PAR1-independent biological effect of this molecule. We now have tested the growth inhibitory effect of SCH79797 on HUVEC, stimulated in culture by 10% serum (FBS) or FGF-2 (25 ng/ml), and found a significant inhibition of cell growth in both experimental conditions (see Fig. 1). Zania et al. (2006) reported that, in HUVEC treated with PAR1 inhibitors, phosphorylation of extracellular signal-regulated kinase 1/2 stimulated by thrombin and 4% serum is reduced. However, they found no effects on FGF-2, vascular endothelial growth factor (VEGF), and epidermal growth fac-

tor-mediated extracellular signal-regulated kinase 1/2 activation. They presented this finding to indicate a specific effect of the inhibitors on PAR1-mediated mitogen-activated protein kinase activation. However, when we characterized the growth inhibitory effect of SCH79797, we found that the inhibitor was able to reduce phosphorylation of mitogen-activated protein kinase induced by 10% serum, but not FGF-2 or PDGF, although it was still able to block the ability of all growth factors to induce cell proliferation (data not shown). It will be of interest to test the ability of PAR1 small inhibitors to block FGF-2- or VEGF-stimulated angiogenesis *in vitro* and *in vivo*.

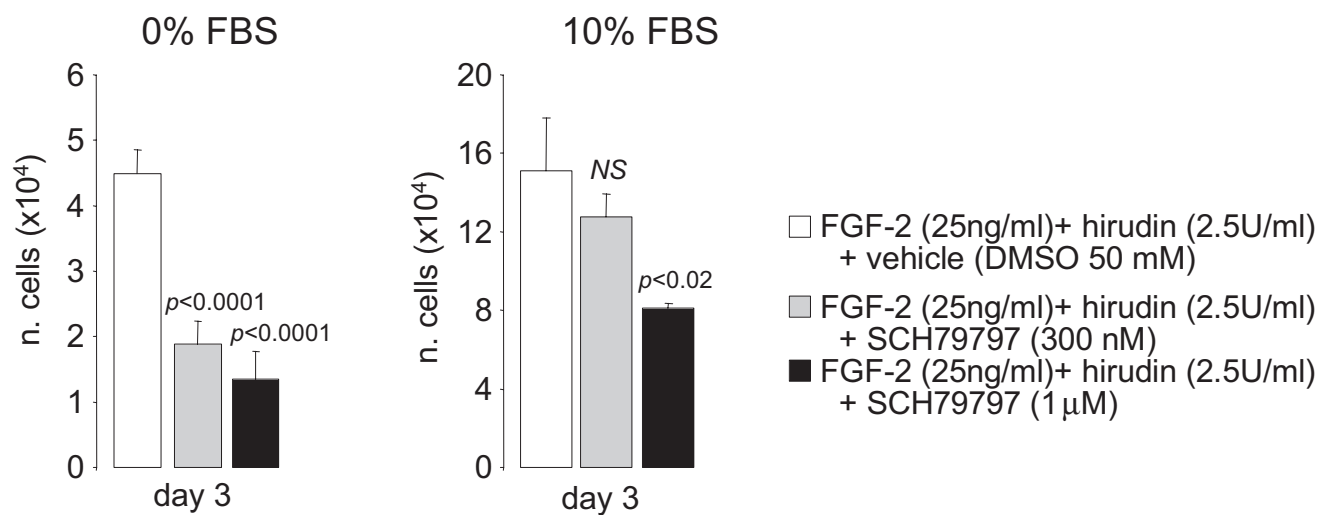
We have demonstrated that higher concentrations of the inhibitor (200 nM) induce apoptosis in all of the cell lines tested, including PAR1 null fibroblasts (Di Serio et al., 2007). Zania et al. (2006) reported that the antiangiogenic effect of SCH79797 is evident in fast-growing cultures of HUVEC (50–60% confluency) but less pronounced in quiescent cells (100% confluency), which they interpreted to indicate an effect of the inhibitor only on “activated” endothelial cells involved in angiogenesis. However, we demonstrated that the growth inhibitory effect of SCH79797 depends on cell density. Cells seeded at higher density still respond to increasing concentrations of the inhibitor (Di Serio et al., 2007).

We have little information regarding the cellular effects of RWJ56110; however, we now have tested this molecule against the ability of PAR1 null fibroblasts to grow in culture and found that, like SCH79797, RWJ56110 significantly impairs the proliferative potential of these cells (Fig. 2).

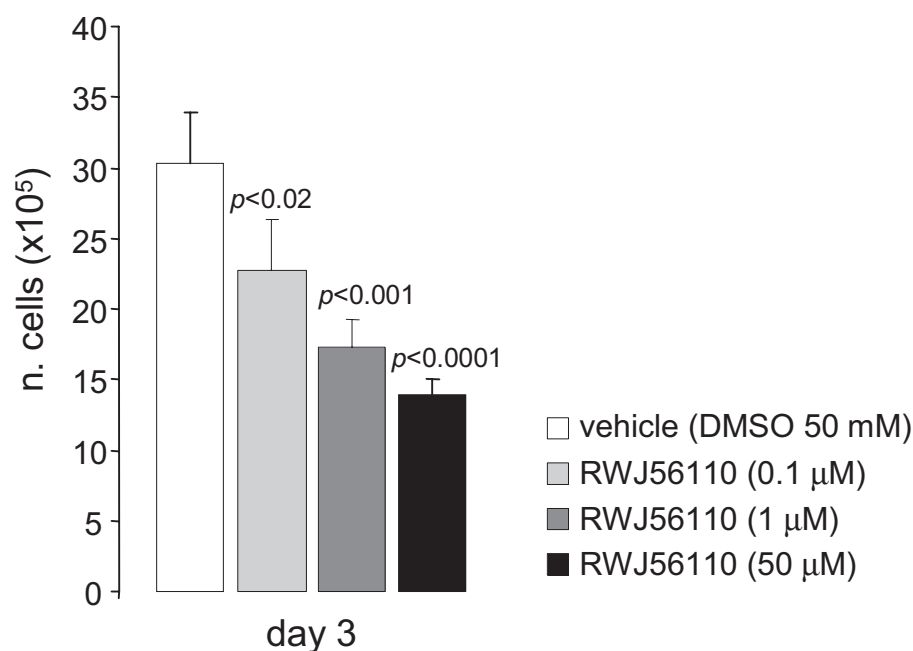
In conclusion, we suggest that the results reported by Zania et al. (2006) are not conclusive for an antiangiogenic effect of SCH79797 solely dependent on its ability to inhibit PAR1-mediated intracellular signal. Although we agree that SCH79797 and RWJ56110 may be useful as antiangiogenic agents and should be further developed for

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**ABBREVIATIONS:** PAR1, proteinase-activated receptor 1; SCH79797, *N*-3-cyclopropyl-7-[[4-(1-methylethyl)phenyl]-methyl]-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine); RWJ56110, [(*S*)-*N*-[(1*S*)-3-amino-1-[[[phenylmethyl]amino]carbonyl]propyl]-[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1*H*-indol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide]; FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell(s); PDGF, platelet-derived growth factor.



**Fig. 1.** HUVEC cells ( $2 \times 10^4$  cells/well) were plated in triplicates and grown in M199 containing 10% FBS or no serum in the presence of 25 ng/ml FGF-2, 2.5 U/ml hirudin, and SCH79797 (300 nM and 1  $\mu$ M). Cells were counted at day 3 using an hemocytometer. Data are presented as mean  $\pm$  S.D. DMSO, dimethyl sulfoxide.



**Fig. 2.** PAR1 null fibroblasts ( $2 \times 10^4$  cells/well) were plated in triplicates and grown in Dulbecco's modified Eagle's medium containing 10% FBS, in the presence of RWJ56110 (0.1, 1, and 50  $\mu$ M). Cells were counted at day 3 using an hemocytometer. Data are presented as mean  $\pm$  S.D. DMSO, dimethyl sulfoxide.

therapeutic applications, there is data to support that their biological effect is, at least in part, independent from PAR1 inhibition.

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