Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 477 (2003) 17–21
www.elsevier.com/locate/ejphar

Short communication

Selective anti-endothelial effects of protracted low-dose BAL-9504, a novel geranylgeranyl-transferase inhibitor

Guido Bocci[a,b,*], Romano Danesi[b], Mario Del Tacca[b], Robert S. Kerbel[a]

[a] *Molecular and Cell Biology Research, Sunnybrook and Women's College Health Sciences Centre, Department of Medical Biophysics, University of Toronto, S-217, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5*

[b] *Division of Pharmacology and Chemotherapy, Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, Via Roma, 55, I-56126 Pisa, Italy*

Received 12 June 2003; received in revised form 28 July 2003; accepted 5 August 2003

Abstract

Because the anti-endothelial effects of agents such as interferon alpha, endostatin, and various chemotherapeutic drugs appear to be optimal after protracted, frequent dosing, both in vitro and in vivo, using low concentrations/doses of drugs, we tested the geranylgeranyl transferase inhibitor BAL-9504 ((*E*,*E*,*E*) [2-oxo-2-[[(3,7,11,15-tetramethyl-2,6,10,14 hexadecatetraenyl)-oxy]amino]ethyl] phosphonic acid) in this manner on different human cell types, including endothelial, breast cancer cells and fibroblasts. We found that human endothelial cells were preferentially affected with respect to inhibition of proliferation, induction of apoptosis, suppression of adhesion to fibronectin, and blockade of cell migration, by daily exposure to low concentrations of BAL-9504 for 6 days. These results may have implications in terms of both increasing anti-tumor efficacy, mediated by antiangiogenic mechanisms, and reducing toxic side effects.

Keywords: Geranylgeranyl transferase inhibitor; Endothelial cell, human; Breast cancer cell, human; Metronomic therapy

1. Introduction

A problem which has seriously impeded progress in the clinical application of various cytostatic therapies, including antiangiogenic agents, is determining the optimum biologic or therapeutic dose. Many of these drugs do not have dose-limiting toxicities precluding the determination of an acute and cumulative maximum tolerated dose (MTD), and moreover, even if an MTD can be defined, the optimal therapeutic effect is often found at doses well below this value (Eisenhauer, 1998). This paradigm is frequently illustrated by drugs developed as specific angiogenesis inhibitors such as endostatin (Kisker et al., 2001) as well as drugs that were not developed originally as angiogenesis inhibitors, but which are now known to have antiangiogenic properties, such as interferon alpha (Slaton et al., 1999) and conventional cytotoxic chemotherapeutic drugs (Browder et al., 2000; Klement et al., 2000). In these cases, it has been found that frequent, even daily, administration of such drugs at doses well below the MTD (if an MTD is known) induces more potent antiangiogenic effects, and sometimes greater anti-tumor efficacy, compared to less frequently administered regimens (e.g. once every week to once every 3 weeks) (Kisker et al., 2001; Slaton et al., 1999; Browder et al., 2000).

In addition to drugs such as interferon alpha, conventional cytotoxic agents, thalidomide, celecoxib, and many others, "accidental" antiangiogenic drugs may include oncogene targeted signalling inhibitors such as epidermal growth factor receptor (EGFR) inhibitors, erbB2/Her-2/neu inhibitors (Viloria-Petit et al., 2001).

It is conceivable that a direct inhibition of angiogenesis may be facilitated by more frequent administration of lower doses of signalling inhibitor drug, in a protracted manner, similar to the drugs discussed above. We decided to test this possibility with respect to a novel geranylgeranyl transferase inhibitor called BAL-9504 ((*E*,*E*,*E*) [2-oxo-2-[[(3,7,11,15-tetramethyl-2,6,10,14 hexadecatetraenyl)-oxy]amino]ethyl] phosphonic acid), a stable analog of geranylgeranyl diphosphate (Fig. 1A), which blocks prenylation of proteins,

* Corresponding author. Division of Pharmacology and Chemotherapy, Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, Via Roma, 55, I-56126 Pisa, Italy. Tel.: +39-050-830148; fax: +39-050-562020.
E-mail address: guido_bocci@libero.it (G. Bocci).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.08.001

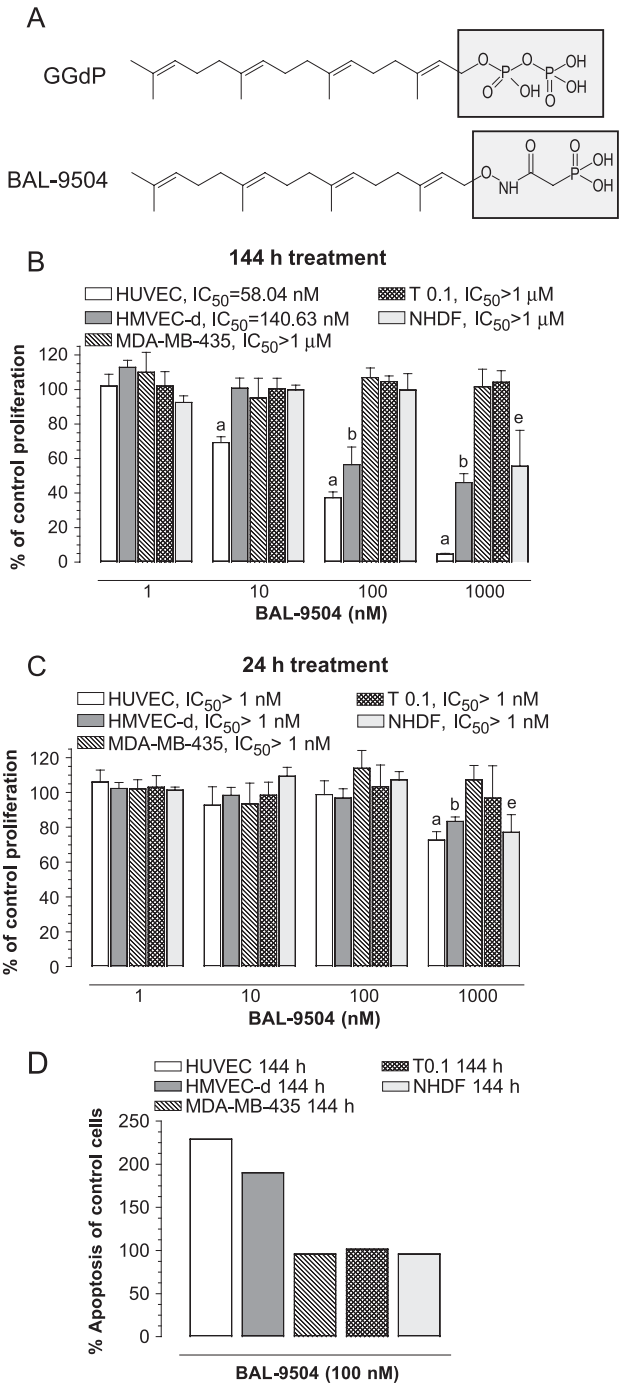

Fig. 1. (A) Chemical structure of geranylgeranyl diphosphate (GGdP) and its stable analog BAL-9504. Effect of low-dose BAL-9504 on in vitro cell proliferation using both prolonged continuous (144 h; B) and short-term exposures (24 h; C) on HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell lines; (D) induction of cell apoptosis after prolonged (144 h) low-dose treatment with BAL-9504 100 nM on the same cell lines. Columns and bars, mean values ± S.E.M., respectively. [a]$P<0.05$ vs. HUVEC controls; [b]$P<0.05$ vs. HMVEC-d controls; [c]$P<0.05$ vs. NHDF controls; IC_{50}, inhibitory concentration of 50% vs. controls.

including members of the *ras* superfamily of small GTP binding proteins, e.g. p21rho (Macchia et al., 1996). Treatment of endothelial cells by peptidomimetic geranylgeranyl transferase inhibitors has been shown to induce apoptosis (Li et al., 2002) and inhibition of geranylgeranylation of RhoA mediated by other drugs can suppress angiogenesis (Park et al., 2002).

The purpose of the present study was to evaluate the possibility of selective effects BAL-9504 on endothelial cells after a more protracted (6 day) exposure of various human cell types (e.g. fibroblasts, tumor cells, endothelial cells) to very low concentrations of the compound. The results suggest a highly selective effect for endothelial cells, within the limits of the study. As such, they may have implications for ways of reducing the doses of such type of drugs in vivo, without necessarily reducing treatment efficacy, and for "salvage" methods of administering the drug after tumor cells have become resistant to its effects using higher doses of the same drug.

2. Materials and methods

2.1. Drug and cell lines

BAL-9504 (Laboratori Baldacci) was dissolved in distilled water and diluted in culture medium immediately before its use. Cell culture media and supplies were obtained from Gibco. As previously described (Bocci et al., 2002), human umbilical vein endothelial cells (HUVEC) and the human dermal microvascular endothelial cells (HMVEC-d; Clonetics; doubling time ~ 48–72 h) were maintained in MCDB131 culture medium (JRH Biosciences); normal human dermal fibroblasts (NHDF; Clonetics; doubling time ~ 48–72 h) were cultured in 5% fetal bovine serum (FBS) RPMI medium. The human tumor cell line MDA-MB-435 (Dr. J. Lemontt, Genzyme; doubling time ~ 26 h) and its *P*-glycoprotein-positive multidrug resistant variant T0.1 (Dr. D. Cohen, Novartis), obtained by in vitro exposure to increasing concentrations of paclitaxel, were maintained in 10% FBS DMEM medium.

2.2. Cell proliferation assay and apoptosis measurements

HUVEC, HMVEC-d, NHDF, MDA-MB-435 and T0.1 cells were plated in 96-well sterile plastic plates (1% gelatin-coated for the endothelial cells) and were treated for 24 h (3×10^3 cells/well in 200 μl of medium) or continuously for 144 h (1×10^3 and 0.5×10^3 cells/well of normal and cancer cells, respectively, in 200 μl of medium) with BAL-9504 (1–1000 nM) adding fresh solutions with new medium every 24 h. At the end of the experiment, cells were pulsed for 6 h with 2 μCi/well of methyl-[^{3}H]thymidine (Amersham Life Science). The concentration of drug that reduced cell proliferation by 50% (IC_{50}) vs. controls was calculated by nonlinear regression fit of the mean values of the data obtained in triplicate experiments (10 wells for each concentration). In order to quantify the apoptosis induced by the treatment, HUVEC, HMVEC-d, MDA-MB-435, T0.1

and NHDF cells were continuously treated for 144 h with BAL-9504 100 nM, a concentration around the experimental IC_{50} of endothelial cell proliferation, and vehicle alone. At the end of the experiment, cells were collected and the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) Plus kit (Roche) was used. All the absorbance values were plotted as a percentage of apoptosis relative to control cells (vehicle only), which were labeled as 100%. All experiments were repeated two times with at least two replicates per sample.

2.3. Cell adhesion assay

HUVEC, HMVEC-d, NHDF, MDA-MB-435 and T0.1 cells were treated for 144 h with BAL-9504 100 nM and vehicle alone. At the end of the sixth day of treatment, cells from each treatment group were detached with 0.5 mM EDTA, washed in serum-free media and resuspended to a final concentration of 4×10^5 cells/ml. A viable cell suspension (100 μl) was plated in 96-well plates that were previously coated with fibronectin, 10 μg/ml for 1 h at 37 °C, and then blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin; after 1 h, the wells were washed with PBS to remove the non-adherent cells. The remaining bound cells were quantified by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay using the CellTiter 96™ AQ_{ueous} kit (Promega). The data were presented as the percentage of the adhesion of vehicle-treated cells.

2.4. Migration assay

HUVEC and HMVEC-d cells were seeded at a density of 8.4×10^4 cells in 500 μl of serum-free MCDB131 in the upper chamber of 8 μm pore-modified Boyden chamber (Becton Dickinson). Cells were treated with BAL-9504 100 nM and vehicle alone and allowed to migrate in 250 μl of medium without or with vascular endothelial growth factor (VEGF) 100 ng/ml as chemoattractant; after 4 h, the membranes were fixed, stained with haematoxylin and mounted on slides. The pictures of the migrated cells were acquired as 512×1024 pixel images at 400× magnification with an Axioplan2 microscope (Carl Zeiss) and processed by image analysis software Northern Eclipse v.6.0 (Empix Imaging). Twenty different regions were analyzed in each slide to calculate the number of migrated cells and the experiments were performed in triplicates.

2.5. Statistical analysis

The results (mean values ± S.E.M.) of cell proliferation, adhesion and migration assays were subjected to analysis of variance between groups (ANOVA), followed by the Student–Neumann–Keuls test. The level of significance was set at $P < 0.05$.

3. Results

A strong and dose-dependent antiproliferative effect (including at the lower compound concentrations) was observed when endothelial cells were continuously exposed over 144 h (Fig. 1B). BAL-9504 showed marked differences in induction of antiproliferative responses between endothelial and cancer cells ($IC_{50} = 58.04$ and 140.63 nM for HUVEC and HMVEC-d, respectively; $IC_{50} > 1$ μM for cancer cells; Fig. 1B). Conversely, when endothelial cells were exposed for only 24 h, a significant inhibition was only observed at the highest compound concentration tested (Fig. 1C). Moreover, the proliferation of NHDFs was affected only at the highest concentrations used, with the IC_{50} always higher than 1 μM (Fig. 1B,C). As shown in Fig. 1D, the Cell Death ELISA assay also revealed a markedly higher levels of apoptosis in the BAL-9504-treated endothelial cells when compared to the cancer cell lines or fibroblasts, after 6 days of 100 nM treatment (+210% and +230% for HUVEC and HMVEC-d vs. controls, respectively).

The protracted low-dose exposure of BAL-9504 significantly and specifically inhibited viable endothelial cell adhesion to fibronectin after 1 h of plating with a maximum

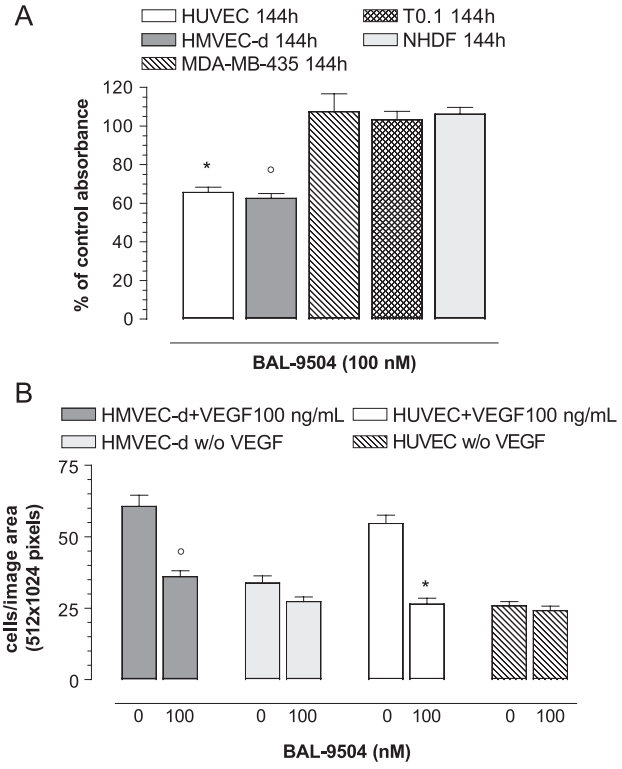

Fig. 2. (A) Effects of prolonged (144 h) low-dose treatment with BAL-9504 100 nM on HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell adhesion. Columns and bars, mean values ± S.E.M, respectively. *$P < 0.05$ vs. HUVEC controls; °$P < 0.05$ vs. HMVEC-d controls. (B) Effects of low-dose treatment with BAL-9504 100 nM on endothelial cell migration ± VEGF 100 ng/ml. Columns and bars, mean ± S.E.M., respectively. *$P < 0.05$ vs. HUVEC + VEGF controls; °$P < 0.05$ vs. HMVEC-d + VEGF controls.

inhibition observed in HMVEC-d cells (62.6 ± 2.4% vs. control adhesion, $P < 0.05$; Fig. 2A). Furthermore, the same low-concentration of BAL-9504 (100 nM) showed a maximum inhibition of VEGF-induced migration of HUVECs (26.4 ± 2.1 vs. 54.6 ± 2.9 control migrated cells/image area, $P < 0.05$; Fig. 2B). No significant differences were observed in the migration of the cells in the absence of the VEGF stimulus.

4. Discussion

The proteins that undergo to geranylgeranylation, including members of the *ras* superfamily of small GTP binding proteins such as p21*ras*, p21*rap* 1, p21*rho*, p21*rac* and Cdc42, participate in important cell functions, involving signal transduction pathways and regulating cell adhesion, proliferation, and survival (Liang et al., 2002). Their mutations induce malignant transformation of cells and the angiogenic phenotype of tumors, upregulating VEGF expression and down-regulating angiogenesis inhibitors such as thrombospondin-1 (Rak et al., 2002). Prenylated proteins are modified by formation of cysteine thioethers with the isoprenoid lipids, farnesyl (C-15), or geranylgeranyl (C-20), at the carboxyl terminus. Geranylgeranylation and farnesylation are catalyzed by the enzymes geranylgeranyl transferase and farnesyl transferase, respectively (Liang et al., 2002). Recent research has focused on the synthesis of novel farnesyl transferase and geranylgeranyl transferase inhibitors (Lobell et al., 2001) to suppress the oncogenic potential of the transformed protein and to provide a specific approach to anti-cancer therapy. Among these, BAL-9504 has been designed and validated as a stable analog of geranylgeranyl diphosphate, an intermediate of the cholesterol biosynthetic pathway and an isoprenoid donor (Grunler et al., 1994), in which the biologically labile diphosphate moiety of geranylgeranyl diphosphate is replaced by a portion that can act as a stable isoster, i.e. the phosphonoacetamido oxy group (Fig. 1A), blocking the activity of the enzyme geranylgeranyl transferase (Macchia et al., 1996). Some previous reports have shown a decreased capacity of capillary structure (tube) formation of endothelial cells when the cells were treated with a peptidomimetic geranylgeranyl transferase inhibitor (Li et al., 2002; Park et al., 2002). Thus, our results show, for the first time, that vascular endothelial cells are preferentially affected for properties such as proliferation, induction of apoptosis, adhesion to a fibronectin matrix and migration by low concentrations of a similar compound, BAL-9504, when exposed continuously for protracted periods of time (e.g. 6 days).

The rationale behind the low-dose approach is as follows: (i) our results imply that tumor cells require higher doses of BAL-9504 to be affected; (ii) once tumor cell resistance becomes apparent, switching to a more frequent lower-dose metronomic schedule may result in resumption of responsiveness, by virtue of preferentially targeting the tumor's genetically stable host vasculature. There are some clear-cut precedents for success using this type of sequential dosing strategy. Our previous in vitro results (Bocci et al., 2002) are highly suggestive of an "antiangiogenic window" when low-dose chemotherapy is used, which becomes apparent after protracted exposure times (e.g., 144 h). Low concentrations of drug that had no or little effect on tumor cells or normal fibroblasts (e.g., for paclitaxel or 4-hydroperoxycyclophosphamide), greatly affected microvascular endothelial cells. However, not all antineoplastic drugs have this preferential activity against endothelial cells. This was the case of doxorubicin (Bocci et al., 2002), which even at low concentrations, affected equally cancer and endothelial cells. BAL-9504 results suggest the presence of such an "antiangiogenic window", especially if compared to non-antiangiogenic drugs such as doxorubicin.

The metronomic antiangiogenic schedules require protracted and continuous administrations of low-dose chemotherapeutic drugs. The oral intake has an undoubted experimental and clinical advantage if compared to other administration routes (e.g. continuous infusion or daily injections); with this in mind, future in vivo studies has been planned to investigate the possible per os use of compound BAL-9504.

Recently, the geranylgeranyl transferase inhibitor GGTI-298 was reported to inhibit tube formation, but not proliferation, of human dermal microvascular endothelial cells (Park et al., 2002) and to induce apoptosis of HUVECs (Li et al., 2002). In our study, using BAL-9504 in protracted continuous treatment ($IC_{50} = 58.04$ and 140.63 nM for HUVECs and HMVEC-d, respectively), we obtained striking results with respect to both antiproliferative and pro-apoptotic effects, with the calculated IC_{50}s being significantly lower when compared to the values previously obtained with the same drug when testing the human prostate cancer PC-3 and human pancreatic cancer MIA PaCa-2 cell lines ($IC_{50} = 18.5$ and 25 μM, respectively) (Macchia et al., 1996; Gesi et al., 1998).

In summary, our results illustrate the potential of protracted, low-dose metronomic dosing with a geranylgeranyl transferase inhibitor, similar to our previous results using a variety of chemotherapeutic drugs (Bocci et al., 2002), and thus raise the prospect of enhancing the direct acting antiangiogenic effects of such an agent, by using this type of dosing and administration regimen in future in vivo experiments, particularly when anti-tumor directed schedules of drug administration cease being effective. It will also be of interest to determine whether the antiangiogenic effects of protracted metronomic dosing using a drug such as BAL-9504 may be due, in part, to suppression of the levels and viability of circulating bone-marrow derived endothelial progenitor cells, as was shown recently (Bertolini et al., 2003) using low-dose metronomic chemotherapy.

Acknowledgements

The authors thank Cassandra Cheng for her excellent secretarial assistance. Guido Bocci was financially supported by the Sunnybrook Trust for Medical Research. RSK is a recipient of a Canada Research Chair in Molecular Medicine. This work was supported by a grant (MT-5815) to RSK from the Canadian Institutes for Health Research (CIHR).

References

Bertolini, F., Paul, S., Mancuso, P., Monestiroli, S., Gobbi, A., Shaker, Y., Kerbel, R.S., 2003. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res. 63, 4342–4346.

Bocci, G., Nicolaou, K.C., Kerbel, R.S., 2002. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. Cancer Res. 62, 6938–6943.

Browder, T., Butterfield, C.E., Kraling, B.M., Shi, B., Marshall, B., O'Reilly, M.S., Folkman, J., 2000. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res. 60, 1878–1886.

Eisenhauer, E.A., 1998. Phase I and II trials of novel anti-cancer agents: endpoints, efficacy and existentialism. The Michel Clavel Lecture, held at the 10th NCI-EORTC Conference on New Drugs in Cancer Therapy, Amsterdam, 16-19 June 1998. Ann. Oncol. 9, 1047–1052.

Gesi, M., Pellegrini, A., Soldani, P., Lenzi, P., Paparelli, A., Danesi, R., Nardini, D., Macchia, M., 1998. Ultrastructural and biochemical evidence of apoptosis induced by a novel inhibitor of protein geranylgeranylation in human MIA PaCa-2 pancreatic cancer cells. Ultrastruct. Pathol. 22, 253–261.

Grunler, J., Ericsson, J., Dallner, G., 1994. Branch-point reactions in the biosynthesis of cholesterol, dolichol, ubiquinone and prenylated proteins. Biochim. Biophys. Acta 1212, 259–277.

Kisker, O., Becker, C.M., Prox, D., Fannon, M., D'Amato, R., Flynn, E., Fogler, W.E., Sim, B.K., Allred, E.N., Pirie-Shepherd, S.R., Folkman, J., 2001. Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. Cancer Res. 61, 7669–7674.

Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D.J., Bohlen, P., Kerbel, R.S., 2000. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Invest. 105, R15–R24.

Li, X., Liu, L., Tupper, J.C., Bannerman, D.D., Winn, R.K., Sebti, S.M., Hamilton, A.D., Harlan, J.M., 2002. Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. J. Biol. Chem. 277, 15309–15316.

Liang, P.H., Ko, T.P., Wang, A.H., 2002. Structure, mechanism and function of prenyltransferases. Eur. J. Biochem. 269, 3339–3354.

Lobell, R.B., Omer, C.A., Abrams, M.T., Bhimnathwala, H.G., Brucker, M.J., Buser, C.A., Davide, J.P., deSolms, S.J., Dinsmore, C.J., Ellis-Hutchings, M.S., Kral, A.M., Liu, D., Lumma, W.C., Machotka, S.V., Rands, E., Williams, T.M., Graham, S.L., Hartman, G.D., Oliff, A.I., Heimbrook, D.C., Kohl, N.E., 2001. Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. Cancer Res. 61, 8758–8768.

Macchia, M., Jannitti, N., Gervasi, G., Danesi, R., 1996. Geranylgeranyl diphosphate-based inhibitors of post-translational geranylgeranylation of cellular proteins. J. Med. Chem. 39, 1352–1356.

Park, H.J., Kong, D., Iruela-Arispe, L., Begley, U., Tang, D., Galper, J.B., 2002. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. Circ. Res. 91, 143–150.

Rak, J., Yu, J.L., Kerbel, R.S., Coomber, B.L., 2002. What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? Cancer Res. 62, 1931–1934.

Slaton, J.W., Perrotte, P., Inoue, K., Dinney, C.P., Fidler, I.J., 1999. Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. Clin. Cancer Res. 5, 2726–2734.

Viloria-Petit, A., Crombet, T., Jothy, S., Hicklin, D., Bohlen, P., Schlaeppi, J.M., Rak, J., Kerbel, R.S., 2001. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res. 61, 5090–5101.