Combination of a Selective Cyclooxygenase-2 Inhibitor with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 and Protein Kinase A Antisense Causes Cooperative Antitumor and Antiangiogenic Effect[1]

Giampaolo Tortora,[2] Rosa Caputo, Vincenzo Damiano, Davide Melisi, Roberto Bianco, Gabriella Fontanini, Bianca Maria Veneziani, Sabino De Placido, A. Raffaele Bianco, and Fortunato Ciardiello

Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica [G. T., R. C., V. D., D. M., Ro. Bianco, S. D. P., Ra. Bianco, F. C.] e Dipartimento di Biologia e Patologia Cellulare e Molecolare [B. M. V.], Università di Napoli Federico II, 80131 Napoli, and Istituto di Anatomia Patologica, Università di Pisa, 56100 Pisa [G. F.], Italy

ABSTRACT

Purpose: **Epidermal growth factor receptor (EGFR) and protein kinase A type I (PKAI) play an important role in the control of cancer cell growth and angiogenesis. Inhibitors of EGFR and PKAI have antitumor activity *in vitro* and *in vivo* in a variety of tumor types, and some of these agents are active after oral administration. Increasing evidence shows that cyclooxygenase (COX)-2 also plays a role in promoting cancer cell proliferation and angiogenesis. COX-2 expression can be induced by EGFR activation and is regulated by cAMP and PKA. Combination of an EGFR inhibitor with a nonselective COX-1/COX-2 inhibitor prevents the development of intestinal cancer in nude mice. Therefore, we investigated whether any cooperative antitumor effect can be obtained by the combined blockade of COX-2, EGFR, and PKAI.**

Experimental Design: **The COX-2 inhibitor SC-236 was combined with the selective EGFR tyrosine kinase inhibitor ZD1839 (Iressa) and the DNA/RNA-mixed backbone oligonucleotide AS-PKAI to study their effect on human cancer growth and angiogenesis, measuring vascular endothelial growth factor (VEGF) and basic fibroblast growth factor expression and vessel formation, *in vitro* and after oral administration of these agents in mice.**

Results: **A cooperative effect was observed with SC-236 in combination with either ZD1839 or AS-PKAI, as well as with all three agents together, on the proliferation of human colon and breast cancer cells in soft agar at doses that were ineffective for each agent alone. The antiproliferative effect was accompanied by inhibition of COX-2 expression. Moreover, combination of SC-236 with either agent or the triple combination markedly reduced VEGF secretion in the conditioned medium and completely suppressed VEGF and basic fibroblast growth factor expression. In nude mice bearing human colon cancer xenografts, a low, noninhibitory dose of SC-236 with ZD1839 and AS-PKAI, all given p.o., caused a dramatic cooperative antitumor effect, with no histological evidence of tumor in 60% of mice 5 weeks after treatment withdrawal, at which time all mice were alive. Moreover, analysis of tumor specimens revealed inhibition of vessel formation and expression of COX-2 and VEGF.**

Conclusions: **This is the first demonstration that three novel agents blocking multiple signaling pathways, in absence of cytotoxic drugs, may have a potent antitumor and antiangiogenic activity after oral administration. Because all agents are under clinical evaluation, our results provide a rationale to translate this feasible therapeutic strategy into a clinical setting.**

INTRODUCTION

The blockade of signaling molecules playing a key role in cell proliferation, angiogenesis, and apoptosis may be a major part of future strategies for cancer therapy. In this respect, the EGFR[3] and the cAMP-dependent PKAI have recently been recognized as among the potentially relevant therapeutic targets (reviewed in Refs. 1, 2).

EGFR is a major transducer of mitogenic signals involved in cancer pathogenesis and progression and is considered an important target for anticancer therapy (1). ZD1839 (Iressa, AstraZeneca) is a p.o. active, selective EGFR tyrosine kinase

Received 5/21/02; revised 9/17/02; accepted 9/17/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro and the CNR-MIUR 449/97-99.
[2] To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Dipartimento Endocrinologia e Oncologia Molecolare e Clinica, Università di Napoli Federico II Via S. Pansini 5 80131 Napoli, Italy. Phone: 39-081-7462061; Fax: 39-081-7462066; E-mail: gtortora@unina.it.

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; PKAI, protein kinase A type I; VEGF, vascular endothelial growth factor; TGF-α, transforming growth factor α; MBO, mixed backbone oligodeoxynucleotide; COX, cyclooxygenase; bFGF, basic fibroblast growth factor; CRE, cAMP-responsive element; CM, conditioned medium.

inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells, and other host-dependent processes promoting cancer growth and is under clinical development in cancer patients (1, 3). ZD1839 inhibits the growth of a variety of human cancer types, and additive/cooperative effects have been observed when combined with a variety of cytotoxic drugs *in vitro* and *in vivo* in nude mice (4). EGFR activation has been directly related to induction of angiogenic growth factor expression and secretion. We and others have shown that ZD1839 is able to inhibit VEGF expression and neoangiogenesis *in vitro* and in nude mice xenografts (5).

Overexpression of PKA isoform type I (PKAI), as compared with the type II isoform (PKAII), is a hallmark of the great majority of human tumors, correlating with worse clinicopathological features in several tumor types (reviewed in Ref. 2). PKAI overexpression is associated with cell proliferation and neoplastic transformation, with $G_1 \rightarrow S$ cell cycle transition and with transduction of mitogenic signals from different growth factors, including TGF-α and epidermal growth factor. We have demonstrated that PKAI, through its RIα subunit, has a structural interaction with the ligand-activated EGFR and cooperates in the propagation of mitogenic signals originated by different growth factors and hormones (6, 7). For these reasons, PKAI is considered a relevant target for therapeutic intervention and different pharmacological tools able to inhibit PKAI expression and function have been developed (2, 6, 8). The most recent one is a DNA/RNA hybrid MBO targeting its RIα subunit (AS-PKAI), which causes growth arrest and differentiation in a variety of cancer cell lines *in vitro* and in nude mice, synergizing with several class of cytotoxic drugs, and is active after oral administration (9–12). Phase II clinical trials in cancer patients are now ongoing with this MBO AS-PKAI (defined GEM 231) in combination with different cytotoxic agents.

On the basis of the molecular interactions between PKAI and EGFR, we have developed a therapeutic strategy achieving the combined blockade of these two signaling pathways by different selective inhibitors and demonstrating their ability to target the respective kinases. This results in a cooperative antitumor and antiangiogenic activity both *in vitro* and *in vivo* in nude mice models (6, 13).

COX-2 is an enzyme known to be involved in inflammation but increasing evidence now supports its role in promoting tumor cell growth and angiogenesis, probably through the activity of COX-2-derived prostaglandins (14). COX-2 overexpression has been shown in the biopsies of a variety of human cancers, including colon, cervix, lung, prostate, and breast, and has been associated with poor prognosis in patients affected by some of these cancers (14–17). It has been reported that newly formed blood vessels in tumors express COX-2, whereas quiescent vasculature expresses only the COX-1 enzyme (14, 18). Moreover, COX-2-dependent promotion of neoangiogenesis has been associated with induction of bFGF and VEGF (14, 18). Recent studies have demonstrated that activation of receptor tyrosine kinases such as EGFR may induce COX-2 expression and prostaglandin production (14, 16) and that cAMP may regulate COX-2 transcription through the CRE present in its promoter (14, 19).

An earlier study has shown that sulindac, a nonsteroid anti-inflammatory drug that inhibits both COX-1 and COX-2 in combination with an irreversible inhibitor of EGFR, is able to confer protection from development of intestinal neoplasia in a murine model of familial adenomatous polyposis (20).

On the basis of these functional interactions between EGFR, PKAI, and COX-2, we have evaluated whether a combined blockade of these signaling pathways by the respective inhibitors may result in significant antitumor effects. For this purpose, we have used ZD1839, AS-PKAI, and SC-236, a COX-2 inhibitor that has shown antiproliferative and antiangiogenic activity after oral administration (21). We have studied the effect of these inhibitors on growth and angiogenesis of human cancer cells *in vitro* and in nude mice after oral administration.

MATERIALS AND METHODS

Materials. Clinical grade ZD1839 was provided by AstraZeneca (Macclesfield, United Kingdom), and SC-236 was provided by Pharmacia-Upjohn and Searle Monsanto Company (St. Louis, MO), respectively. MBOs were kindly provided by Dr. Sudhir Agrawal (Hybridon, Inc., Cambridge, MA). The AS-PKAI is a hybrid oligonucleotide, containing 2′-*O*-methyl-ribonucleosides, targeted against the NH_2-terminal 8–13 codons of the RIα regulatory subunit of PKA (9). The control is a scramble MBO obtained by mixing all four nucleosides at each position. Sequence, structure, and purification methods were as published previously (12, 22).

Cell Lines. GEO colon and ZR-75-1 breast human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). GEO and ZR-75-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), penicillin (100 UI/ml), streptomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Growth in Soft Agar. On day 0, cells (10^4 cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated on days 0, 2, and 4 with the following concentrations of drugs: SC-236, from 0.01 to 2.5 μM; ZD1839 (0.1 μM); and AS-PKAI (0.1 μM). After 10–14 days, cells were stained with nitro blue tetrazolium (Sigma), and colonies > 0.05 mm were counted.

Western Blot Analysis. Total cell lysates were obtained from either cells cultured *in vitro* or from homogenized tumor specimens. The protein extracts were resolved by 4–15% SDS-PAGE and probed with antihuman polyclonal COX-2, monoclonal VEGF, and monoclonal bFGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, London, United Kingdom), as described previously (7).

Evaluation of VEGF Secretion. The concentration of VEGF in the CM obtained from cultured cells was measured using commercially available sandwich ELISA kits and according to manufacturers' instructions. The ELISA kit for VEGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Cells were plated in 60-mm dishes (Becton Dickinson) and

treated for 4 days with SC-236 (0.1 μM), ZD1839 (0.1 μM), and AS-PKAI (0.1 μM), alone and in combination. Assays were performed using 24-h collected serum-free CM.

GEO Xenografts in Nude Mice. Five to 6-week-old female Balb/cAnNCrlBR athymic (nu+/nu+) mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized to the University of Naples Medical School Animal Facility for 1 week before they received injections of cancer cells: 10^7 GEO human colon cancer cells were resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice. After 7 days, when well-established tumors of ~0.2 cm^3 were detected, mice were randomized to receive different treatments. Groups of 10 mice were treated with oral administration of SC-236 (6 mg/kg) and/or AS-PKAI (10 mg/kg) and/or ZD1839 (150 mg/kg) daily on days 7–11, 14–18, 21–25, and 28–32. Tumor volume was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (13). Two mice were sacrificed at day 32 to perform biochemical and immunohistochemical analysis.

Immunohistochemical Analysis. New blood vessels were detected as previously described (23), using a monoclonal antibody against the human factor VIII-related antigen (Dako, Milan, Italy) at 1:50 dilution and stained with a standard immunoperoxidase method (Vectastain ABC kit; Vector). Each slide was firstly scanned at low power (×10–100 magnification), and the area with the higher number of new vessels was identified (hot spot) and then scanned at ×250 microscope magnification (0.37 mm^2). Stained blood vessels were counted in each of five different fields. For individual tumors, the microvessel count was scored by averaging the counts from five fields. All analyses were performed in a blind fashion.

RESULTS

Dose-Response Effect of SC-236 on Cancer Cell Growth. We have evaluated the antiproliferative effect *in vitro* of the COX-2 inhibitor SC-236 on the soft agar growth of GEO colon and ZR-75-1 breast cancer cells. SC-236 caused a dose-dependent growth inhibition with an IC_{50} of 0.5 μM for GEO and 0.8 μM for ZR-75-1 cells (data not shown).

Cooperative Effect of the Different Agents in Combination. Combination of either AS-PKAI or ZD1839 with different doses of SC-236 caused a supra-additive effect in both cell lines, particularly with lower doses (Fig. 1, *A* and *B*). A similar supra-additive effect was also obtained with the combination of AS-PKAI and ZD1839 (Fig. 1, *A* and *B*). When we combined the three agents together, a clear cooperative inhibitory effect was observed on the soft agar growth of GEO and ZR-75-1 cancer cells. In fact, low doses that alone induce up to 15–20% inhibition of colony formation caused an almost complete suppression of growth (Fig. 1, *A* and *B*). No cooperative effect was obtained when either SC-236 or ZD1839 were combined with the scramble sequence oligo (data not shown).

We performed a Western blot analysis of COX-2 expression in ZR-75-1 cells treated with the different agents. Fig. 1*C* shows that SC-236 and ZD1839 inhibit COX-2, whereas AS-PKAI is unable to do so. Combination of any two agents markedly reduced COX-2 expression, which was completely suppressed by the three agents in combination. Protein expression was compared with that of actin (Fig. 2). Interestingly, Western blot analysis of RIα, EGFR, and phosphorylated EGFR tyrosine expression showed that SC-236 is unable to affect their expression (data not shown).

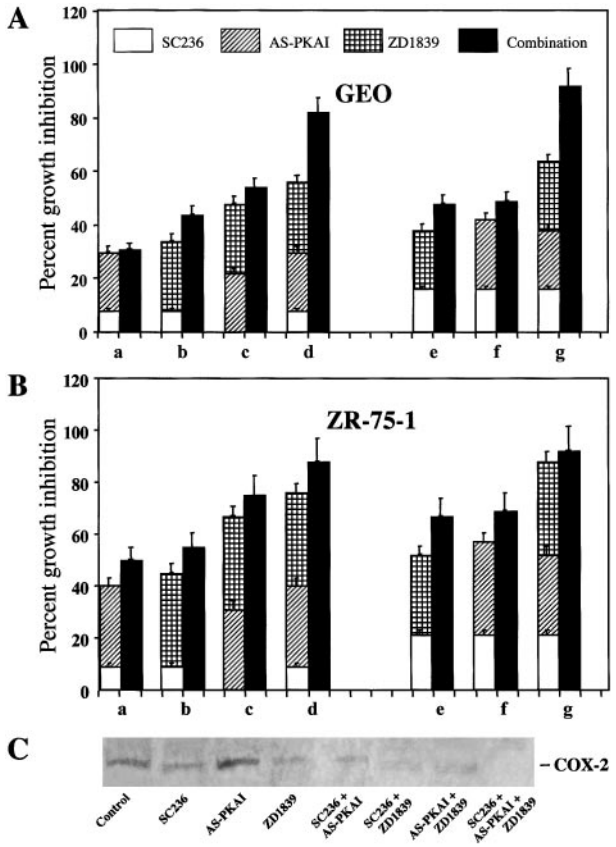

Fig. 1 Effect of SC-236, AS-PKAI, and/or ZD1839 on the soft agar growth of (*A*) GEO and (*B*) ZR-75-1 cells and on (*C*) COX-2 expression. *A* and *B*, SC-236 (0.1 μM; *a*, *b*, *d*), 0.5 μM (*e–g*); AS-PKAI (0.1 μM; *a*, *c*, *d*, *f*, *g*) and ZD1839 (0.1 μM; *b*, *c*, *d*, *e*, *g*). Data are expressed as percentage of colony formation inhibition compared with untreated control cells. The first of each couple of bars shows, as stacked bars, the individual effects of each drug when used alone. Thus, the total height of these stacked bars represents also the expected total inhibition, if drugs have an additive effect. The second bar (■) of each couple shows the effect obtained when the drugs were actually used in combination. Therefore, the comparison between the height of the first bar and that of the second bar of each couple shows whether a supra-additive effect is obtained and the magnitude of such effect. The data represent means and SEs of triplicate determination of at least two experiments. *C*, Western blotting analysis of COX-2 expression in ZR-75-1 cells. Cells were plated in 60-mm dishes (Becton Dickinson) and treated for 4 days with SC-236 (1 μM), AS-PKAI (0.5 μM), and ZD1839 (0.5 μM), alone and in combination. Cell lysates were processed as described in "Materials and Methods."

Effect of the Different Agents on Angiogenic Factors. The endogenous levels of the angiogenic growth factor VEGF were measured in the CM of ZR-75-1 cells. Untreated ZR-75-1 secreted ~18 ng of VEGF for 10^6 cells in 24 h. Treatment with

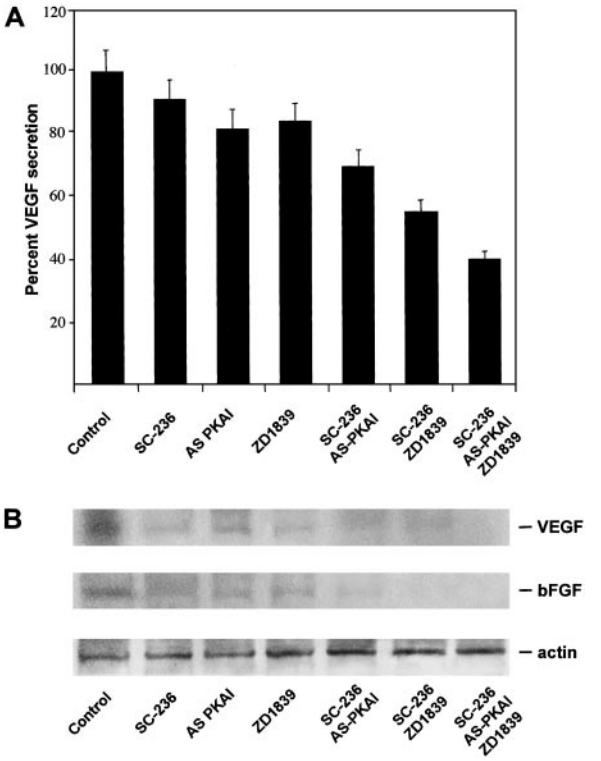

Fig. 2 Effect of the different treatments on (*A*) VEGF secretion and (*B*) VEGF and bFGF expression in ZR-75-1 cells. *A*, inhibition of VEGF secretion in the CM collected from the indicated human cancer cell lines. Cancer cells were plated in 60-mm dishes and treated for 4 days with SC-236 (1 μM), AS-PKAI (0.5 μM), and ZD1839 (0.5 μM), alone and in combination. Data represent the average (±SD) of two different experiments each performed in triplicate. *B*, Western blot analysis of the expression of VEGF, bFGF, and actin in ZR-75 cell lysates described in Fig. 1.

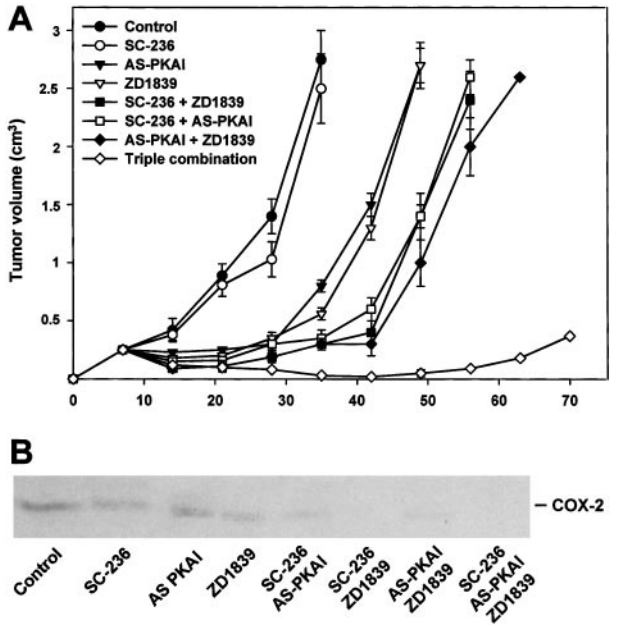

Fig. 3 Effect of the treatment with SC-236, AS-PKAI, and ZD1839 on the growth (*A*) and COX-2 expression (*B*) of GEO tumor xenografts. The schedule of oral administration of each single agent, alone or in combination, is described in "Materials and Methods." The doses of each drug, used alone or in combination, were: SC-236 (6 mg/kg); AS-PKAI (10 mg/kg); and ZD1839 (150 mg/kg). Western blot analysis was performed on total lysates from tumor specimens of two mice sacrificed at day 32.

either AS-PKAI or ZD1839 alone caused ~20% inhibition of VEGF secretion, respectively, whereas SC-236 was almost ineffective (Fig. 2*A*). When SC-236 was combined with AS-PKAI, an additive inhibitory effect was observed, whereas combination of SC-236 with ZD1839 caused a supra-additive effect with ~50% inhibition of VEGF secretion. An additional cooperative inhibitory effect was achieved by the three agents used together, reducing the VEGF secretion to 7 ng/10^6 cells (Fig. 2*A*).

Western blot analysis of VEGF and bFGF expression on the same cells demonstrated that each single agent is able to reduce the amount of both angiogenic factors, as compared with the expression of the control protein actin (Fig. 2*B*). Combination of SC-236 with either AS-PKAI or ZD1839 caused >80% inhibition of both VEGF and bFGF expression, whereas the three agents together completely suppressed the expression of these two angiogenic factors (Fig. 2*B*).

Effect on the Growth of Tumor Xenografts. We investigated the antitumor activity of SC-236, AS-PKAI, and ZD1839 administered p.o., alone and in combination, in nude mice bearing GEO colon cancer xenografts. When established GEO tumors of ~0.2 cm^3 were detectable, groups of 10 mice were treated with SC-236, AS-PKAI, and ZD1839, alone and in combination. Two mice were sacrificed on day 32 to perform biochemical and histochemical analysis; therefore, tumor growth studies were performed on the remaining 8 mice. As shown in Fig. 3*A*, within ~5 weeks, GEO tumors reached a size not compatible with normal life. Treatment with the SC-236 alone at the low dose of 6 mg/kg was almost ineffective. AS-PKAI and ZD1839 alone each caused a similar significant inhibition of tumor growth, delaying the death of mice by ~2 weeks. When SC-236 was combined with either AS-PKAI or ZD1839, an increase in the growth inhibitory effect and a delay of ~1 week in the death of mice were observed, as compared with PKAI or EGFR inhibitors alone. An additional inhibitory effect was obtained when AS-PKAI and ZD1839 were used in combination. A dramatic and sustained inhibitory effect was obtained by the combination of three drugs altogether. In fact, no tumor growth was observed for ~5 weeks after treatment withdrawal, and a modest increase in tumor size was recorded at the end of the experiment, 10 weeks after tumor cell injection (Fig. 3*A*). Moreover, at this time point, pathologic evaluation showed that 5 of 8 mice were still tumor free. The combined treatment with two drugs or with three drugs altogether was well tolerated; no weight loss or other signs of acute or delayed toxicity were observed.

Western blot analysis of tumor specimens removed at the end of treatment, on day 32, demonstrated a marked inhibition of COX-2 expression in animals treated with SC-236 or ZD1839 and a moderate inhibition produced by AS-PKAI (Fig. 3*B*).

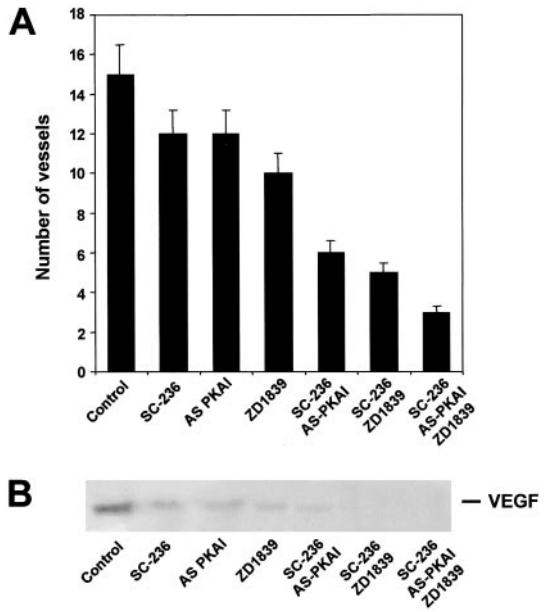

Fig. 4 Number of vessels (*A*) and VEGF expression (*B*) in tumor specimens from mice treated with the different agents. Evaluation of new blood vessels, determined by a monoclonal antibody against the human factor VIII-related antigen, and Western blotting analysis of VEGF expression were carried out as described in "Materials and Methods."

Combination of SC-236 with AS-PKAI caused ~90% inhibition of COX-2 expression, whereas protein was no longer detectable in the specimens from animals treated with SC-236 in combination with ZD1839 or with the three agents together (Fig. 3*B*).

Effect of Treatment on Angiogenesis *in Vivo*. We have quantified by immunohistochemistry the tumor-induced vascularization as microvessel count in the most intense areas of neovascularization, using an antifactor VIII-related antigen monoclonal antibody. At day 32, two tumor samples were analyzed for each group of mice. SC-236 or AS-PKAI used as single drug caused ~20% inhibition of vessel formation, whereas ZD1839 caused ~30% inhibition, as compared with samples from untreated mice (Fig. 4*A*). A supra-additive effect was achieved by treatment with SC-236 combined with ZD1839 or AS-PKAI or with the three agents together, which inhibited vessel formation by ~50–60 and 80%, respectively (Fig. 4*A*).

Western blot analysis of VEGF expression in the same tumor samples revealed an inhibition by SC-236, which was even more evident with AS-PKAI or with ZD1839. VEGF protein levels were reduced ~70% by combination of SC-236 and AS-PKAI and completely suppressed in the animals treated with SC-236 and ZD1839 in combination or with the three agents altogether (Fig. 4*B*).

DISCUSSION

In recent years, the tyrosine kinase growth factor receptors such as EGFR and the protein kinases transducing the intracellular signaling such as PKA have been recognized as key players in the control of neoplastic growth, apoptosis, and angiogenesis. Therefore, they have been considered as potential targets for anticancer therapy, prompting the development of different selective inhibitors (1, 2, 8). We have previously demonstrated that PKAI and EGFR have a structural and functional interaction and that a combined blockade of these two pathways by PKAI and EGFR inhibitors may have a cooperative antitumor effect, enhancing the activity of chemotherapy and radiotherapy (6, 7, 13).

Several recent studies have correlated EGFR ligands such as TGF-α and EGFR activation with the ability to stimulate normal endothelial cell growth through paracrine mechanisms and to up-regulate VEGF production in human cancer cells at the transcriptional level (24, 25). In this regard, we have demonstrated that ZD1839 inhibits the expression of TGF-α, bFGF, and VEGF in a large variety of human cancer cell lines (5). We have also shown that PKAI is involved in the angiogenesis and that PKAI-selective inhibitors, including oral AS-PKAI, are able to down-regulate the production of angiogenic growth factors (12, 26).

Many studies have shown that COX-2 is involved in cancer cell proliferation, induction of neoangiogenesis, and increased invasiveness (14, 27–29). These events may be responsible for the worse prognosis observed in patients with different types of cancer overexpressing COX-2 (14–17). For such reasons, different selective inhibitors have been developed and proposed as novel agents for cancer treatment and for chemoprevention (14). It has been demonstrated that COX-2 expression is transcriptionally induced by the signaling cascade triggered by EGFR activation and is controlled by the cAMP-dependent pathway through a CRE-regulated AP-1 transcription factor (14, 19).

In this study, we have evaluated the possibility of obtaining a control of tumor growth without using cytotoxic drugs, by the combined blockade of EGFR, PKAI, and COX-2, three molecules that interact in nodal points of distinct yet related signaling pathways. To translate this hypothesis in an experimental setting, we have used three novel agents with specific properties, including oral activity: the selective EGFR tyrosine kinase inhibitor ZD1839; a hybrid DNA/RNA MBO AS-PKAI; and the COX-2 inhibitor SC-236. All these agents have demonstrated antiproliferative and antiangiogenic properties in different tumor models, alone and in combination with cytotoxic drugs.

We have demonstrated, in human colon and breast cancer cell types, that these agents in combination have a cooperative growth inhibitory effect, achieving maximal activity when the three agents are used together, even at very low doses. The antitumor effect is accompanied by down-regulation of the expression of COX-2 as well as of VEGF and bFGF angiogenic proteins.

Moreover, secretion of VEGF in the CM was inhibited by combined treatments. In all cases, the combination of SC-236 and ZD1839 was the most effective.

Taken together, these results support the evidence of a functional interaction among EGFR, PKAI, and COX-2 in promoting cell proliferation and angiogenesis.

We translated these results *in vivo* in nude mice bearing GEO xenografts using an oral administration for all of the agents tested. We used a suboptimal dose of SC-236 and effective doses of AS-PKAI and ZD1839 to determine any potential for cooperative effect among the drugs used in combination. Addition of SC-236 to either ZD1839 or AS-PKAI clearly

increased the effect of the latter two agents alone. An effective cooperative activity was also obtained when AS-PKAI and ZD1839 were used in combination, as demonstrated previously (30). A dramatic cooperative effect was obtained when the three agents were used together, resulting in no tumor growth at the end of the experiment on day 70 after tumor injection and ~6 weeks after treatment withdrawal. At this time point, 60% of mice were still tumor free. Analysis of tumor samples removed at the end of treatment demonstrated a clear inhibition of COX-2 expression by two drugs in combination and a complete suppression when the three agents were used together. Moreover, we have demonstrated that two drugs in combination are able to reduce the VEGF expression in the specimen from treated nude mice, whereas a complete suppression was caused by the three drugs together. An inhibitory effect was also obtained on the vessels count, suggesting a direct interference of the respective drugs on the angiogenic signaling pathways.

The dramatic *in vivo* activity of the three agents together, as compared with even two agents in combination, may be explained only by a potent cooperativity. Because each pathway blocked by the respective inhibitors has different downstream branches, involving other signaling molecules, it may be hypothesized that alternative signaling pathways may lead to proliferation. In this context, combination of the three agents together would provide an effective multisignaling blockade. The previously reported ability of SC-236 to prevent the DNA synthesis and proliferative response to other growth factors such as platelet-derived growth factor (31) and the prostaglandin-dependent activation of CREB, which stimulates cell proliferation in certain murine models (19), may greatly enhance the cooperative effect previously demonstrated for AS-PKAI and ZD1839 in combination.

There is growing consensus that the blockade of key mitogenic and angiogenic molecules may be a successful strategy for both chemoprevention and long-term control of cancer. Agents suitable for these purposes should have mild and differing toxicity patterns and simple administration routes. In agreement with this approach, we have shown that using oral administration of different noncytotoxic selective agents targeting molecules involved in mitogenic signaling and angiogenesis, it is possible to achieve an antitumor and antiangiogenic effect. Because all of the agents used in our study are under clinical development, we believe that this study provides a strong rationale to translate this feasible therapeutic strategy into a clinical setting.

ACKNOWLEDGMENTS

We thank Gaetano Borriello for his excellent technical assistance.

REFERENCES

1. Ciardiello, F., and Tortora, G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin. Cancer Res., *7:* 2958–2970, 2001.

2. Cho-Chung, Y. S., Pepe, S., Clair, T., Budillon, A., and Nesterova, M. cAMP-dependent protein kinase: role in normal and malignant growth. Crit. Rev. Oncol. Hematol., *21:* 33–61, 1995.

3. Woodburn, J. R. The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol. Ther., *82:* 241–250, 1999.

4. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A. R., and Tortora, G. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an EGFR-selective tyrosine kinase inhibitor. Clin. Cancer Res., *6:* 2053–2063, 2000.

5. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R., and Tortora, G. Inhibition of growth factors production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin. Cancer Res., *7:* 1459–1465, 2001.

6. Tortora, G., and Ciardiello, F. Targeting of epidermal growth factor receptor and protein kinase A: molecular basis and therapeutic applications. Ann. Oncol., *11:* 777–783, 2000.

7. Tortora, G., Damiano, V., Bianco, C., Baldassarre, G., Bianco, A. R., Lanfrancone, L., Pelicci, P. G., and Ciardiello, F. The RIα subunit of protein kinase A (PKA) binds to Grb2 and allows PKA interaction with the activated EGF-receptor. Oncogene, *14:* 923–928, 1997.

8. Tortora, G., and Ciardiello, F. Protein kinase a type I: a target for cancer therapy. Clin. Cancer Res., *8:* 303–304, 2002.

9. Nesterova, M., and Cho Chung, Y. S. A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth. Nat. Med., *1:* 528–533, 1997.

10. Tortora, G., Caputo, R., Damiano, V., Bianco, R., Pepe, S., Bianco, A. R., Jiang, Z., Agrawal, S., and Ciardiello, F. Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotide targeting protein kinase A. Proc. Natl. Acad. Sci. USA, *94:* 12586–12591, 1997.

11. Wang, H., Cai, Q., Zeng, X., Yu, D., Agrawal, S., and Zhang, R. Antitumor activity and pharmacokinetics of a mixed-backbone oligonucleotide targeted to the RIα subunit of protein kinase A following oral administration. Proc. Natl. Acad. Sci. USA, *96:* 13989–13994, 1999.

12. Tortora, G., Bianco, R., Damiano, V., Fontanini, G., De Placido, S., Bianco, A. R., and Ciardiello, F. Oral antisense targeting protein kinase A cooperates with taxol and inhibits tumor growth, angiogenesis and growth factors production. Clin. Cancer Res., *6:* 2506–2512, 2000.

13. Ciardiello, F., Damiano, V., Bianco, R., Bianco, C., Fontanini, G., De Laurentiis, M., De Placido, S., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. J. Natl. Cancer Inst. (Bethesda), *88:* 1770–1776, 1996.

14. Turini, M. E., and DuBois, R. N. Cyclooxygenase-2: a therapeutic target. Annu. Rev. Med., *53:* 35–57, 2002.

15. Masunaga, R., Kohno, H., Dhar, D. K., Ohno, S., Shibakita, M., Kinugasa, S., Yoshimura, H., Tachibana, M., Kubota, H., and Nagasue, N. Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin. Cancer Res., *6:* 4064–4068, 2000.

16. Kulkarni, S., Rader, J. S., Zhang, F., Liapis, H., Koki, A. T., Masferrer, J. L., Subbaramaiah, K., and Dannenberg, A. J. Cyclooxygenase-2 is overexpressed in human cervical cancer. Clin. Cancer Res., *7:* 429–434, 2001.

17. Khuri, F. R., Wu, H., Lee, J. J., Kemp, B. L., Lotan, R., Lippman, S. M., Feng, L., Hong, W. K., and Xu, X-C. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin. Cancer Res., *7:* 861–867, 2001.

18. Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, M., Edwards, D. A., Flickinger, A. G., Moore, R. J., and Seibert, K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res., *60:* 1306–1311, 2000.

19. Rudnick, D. A., Perlmutter, D. H., and Muglia, L. J. Prostaglandins are required for CREB activation and cellular proliferation during liver regeneration. Proc. Natl. Acad. Sci., USA, *98:* 8885–8890, 2001.

20. Torrance, C. J., Jackson, P. E., Montgomery, E., Kinzler, K. W., Vogelstein, B., Wissner, A., Nunes, M., Frost, P., and Discafani, C. M. Combinatorial chemoprevention of intestinal neoplasia. Nat. Med., *6:* 1024–1028, 2000.

21. Masferrer, J. L., Koki, A., and Seibert, K. COX-2 inhibitors. A new class of antiangiogenic agents. Ann. N. Y. Acad. Sci., *889:* 84–86, 1999.

22. Agrawal, S., and Zhao, Q. Antisense therapeutics. Curr. Opin. Chem. Biol., *2:* 519–528, 1998.

23. Weidner, N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res. Treat., *36:* 169–180, 1995.

24. Schmitt, F. C., and Soares, R. TGFα and angiogenesis. Am. J. Surg. Pathol., *23:* 358–359, 1999.

25. Gille, J., Swerlick, R. A., and Caughman, S. W. Transforming growth factor α-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP2-dependent DNA binding and transactivation. EMBO J., *16:* 750–759, 1997.

26. Bianco, C., Tortora, G., Baldassarre, G., Caputo, R., Fontanini, G., Chinè, S., Bianco, A. R., and Ciardiello, F. 8-chloro-cAMP inhibits autocrine and angiogenic growth factor production in human colorectal and breast cancer. Clin. Cancer Res., *3:* 439–448, 1997.

27. Tsujii, M., Kawano, S., Tsujii, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93:* 705–716, 1998.

28. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci., USA, *94:* 3326–3340, 1997.

29. Gately, S. The contributions of cyclooxygenase-2 to tumor angiogenesis. Cancer Metastasis Rev., *19:* 19–27, 2000.

30. Tortora, G., Caputo, R., Damiano, V., Fontanini, G., Melisi, D., Veneziani, B. M., Zunino, F., Bianco, A. R., and Ciardiello, F. Oral administration of a novel taxane, an antisense oligonucleotide targeting protein kinase A, and the epidermal growth factor receptor inhibitor Iressa causes cooperative antitumor and antiangiogenic activity. Clin. Cancer Res., *7:* 4156–4163, 2001.

31. Castano, E., Bartrons, R., and Gil, J. Inhibition of cyclooxygenase-2 decreases DNA synthesis induced by platelet-derived growth factor in Swiss 3T3 fibroblasts. J. Pharmacol. Exp. Ther., *293:* 509–513, 2000.

Clinical Cancer Research

Combination of a Selective Cyclooxygenase-2 Inhibitor with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 and Protein Kinase A Antisense Causes Cooperative Antitumor and Antiangiogenic Effect

Giampaolo Tortora, Rosa Caputo, Vincenzo Damiano, et al.

Clin Cancer Res 2003;9:1566-1572.