Combined Targeted Inhibition of *bcl*-2, *bcl*-XL, Epidermal Growth Factor Receptor, and Protein Kinase A Type I Causes Potent Antitumor, Apoptotic, and Antiangiogenic Activity

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ABSTRACT

Purpose: **This study investigated whether the functional and structural interactions between epidermal growth factor receptor (EGFR), protein kinase AI (PKAI), and *bcl*-2/*bcl*-xL could be exploited to obtain cooperative antitumor effects against models of human colon and breast cancer.**

Experimental design: **Antisense *bcl*-2/*bcl*-xL (4625), antisense PKAI (AS-PKAI), and ZD1839 ("Iressa"), a selective EGFR tyrosine kinase inhibitor, were administered as single agents and in combination against GEO colon and ZR-75-1 breast cancer cell lines *in vitro* and to mice bearing s.c. GEO human tumor xenografts *in vivo*. Effects on growth inhibition, vascular endothelial growth factor secretion, and induction of apoptosis were assessed.**

Results: **Antisense *bcl*-2/*bcl*-xL inhibited the growth of GEO and ZR-75-1 cells *in vitro*, reducing *bcl*-2 and *bcl*-xL expression and vascular endothelial growth factor secretion. Supra-additive growth inhibition and apoptosis induction were observed when 4625 was combined with ZD1839 or AS-PKAI. Combining all three agents resulted in a complete growth inhibitory effect *in vitro*. Antisense *bcl*-2/*bcl*-xL, AS-PKAI, and ZD1839 administered *in vivo* as single agents caused growth inhibition of GEO xenografts. Combining all three agents caused a marked and sustained effect, with 50% growth inhibition and 50% of mice tumor free 5 weeks after treatment withdrawal. The combination was well tolerated.**

Conclusions: **The combination of 4625, AS-PKAI, and ZD1839 resulted in a strong antiproliferative, proapoptotic, and antiangiogenic response, suggestive of a functional interaction between EGFR, PKAI, and *bcl*-2/*bcl*-xL and providing a rationale for the selection of specific molecular treatments for the development of therapeutic strategies.**

INTRODUCTION

Bcl-2 and *bcl*-xL are important members of a family of proteins responsible for dysregulation of apoptosis and resistance to chemotherapy and radiotherapy (1, 2). An AS[2] oligonucleotide targeting human *bcl*-2 (oblimersen, G3139) has completed early clinical studies in different malignancies, demonstrating target inhibition specificity and antitumor activity, alone and in combination with cytotoxic drugs, and is currently undergoing Phase III trials in combination with chemotherapy (3, 4). *Bcl*-xL results from the alternative splicing of the *bcl*-x pre-mRNA (5) and shares with *bcl*-2 high sequence homology regions; however, *bcl*-2 and *bcl*-xL have distinct biological roles and are coexpressed by many tumor types (6). A 20-mer MOE gapmer *bcl*-2/*bcl*-xL bispecific AS oligonucleotide, targeting the mRNA homology region of the two antiapoptotic effectors, has shown the ability to simultaneously down-regulate *bcl*-2 and *bcl*-xL expression, induce apoptosis, and inhibit growth of different tumor types *in vitro* and *in vivo* (7, 8).

Overexpression of PKA isoform type I (PKAI) is found in the great majority of human tumors and is associated with $G_1 \rightarrow S$ cell cycle transition, transduction of mitogenic signals from different growth factors, including transforming growth factor-α and epidermal growth factor, and multidrug-resistant phenotype (reviewed in Refs. 9 and 10). We have demonstrated that PKAI has a structural interaction with the ligand-activated EGFR and cooperates in the propagation of mitogenic signals originated by different growth factors and hormones (10–12). For these reasons, PKAI has been considered a relevant target for therapeutic intervention, and different pharmacological PKAI inhibitors have been developed (9, 10, 13). A mixed backbone oligodeoxynucleotide (MBO) AS targeting its RIα subunit with a DNA/RNA hybrid structure (AS-PKAI) has shown antitumor, proapoptotic, and antiangiogenic activity in a variety of cancer types *in vitro* and in nude mice, synergizing

Received 6/5/02; revised 9/12/02; accepted 9/13/02.
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[2] The abbreviations used are: AS, antisense; MOE, methoxy-ethyl; EGFR, epidermal growth factor receptor; CM, conditioned medium; VEGF, vascular endothelial growth factor; PKA, protein kinase A.

with different classes of cytotoxic drugs, and was also active after p.o. administration (14–16). Phase II clinical trials with this MBO AS-PKAI (defined GEM 231) are now ongoing in cancer patients.

Several studies have established a link between PKA, apoptosis, and *bcl*-2, demonstrating a specific PKA phosphorylation site on *bcl*-2 protein, a structural interaction between PKAI and the cytochrome *c* oxidase, and the ability of AS-PKAI to induce *bcl*-2 phosphorylation, cleavage of poly(ADP-ribose) polymerase, caspase 3 activation, and apoptosis (17–20). More recently, it has been shown that the MBO AS-PKAI can induce phosphorylation of *bcl*-2 and hypophosphorylation of BAD, thus causing *bcl*-2 inactivation and induction of apoptosis in androgen-independent human prostate cancer cells (21).

The EGFR, a major transducer of mitogenic signals leading to cell proliferation and angiogenesis, is involved in cancer pathogenesis and progression and is associated with poor prognosis in human epithelial cancers (22). For such reasons, EGFR is now considered an important target for anticancer therapy, and compounds that block ligand-induced EGFR activation have been developed (23, 24). ZD1839 ("Iressa") is a p.o. active, selective EGFR-tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth (23, 24). We *et al.* (24, 25) have shown that ZD1839 inhibits the growth of a variety of human cancer cell lines and potentiates cytotoxic drug activity *in vitro* and *in vivo* in nude mice. Moreover, antiproliferative effects of ZD1839 have been associated with induction of apoptosis and inhibition of *bcl*-2 expression (25, 26). ZD1839 is now undergoing Phase III trials in cancer patients.

We have demonstrated previously that the combined blockade of EGFR and PKAI or of PKAI and *bcl*-2 by their respective inhibitors can result in a cooperative antitumor and antiangiogenic effect (10, 27, 28).

Therefore, based on the above findings, we have investigated whether the functional and structural interactions among EGFR, PKAI, and *bcl*-2/*bcl*-xL could be exploited to obtain a cooperative antitumor effect, taking advantage of the respective selective inhibitors ZD1839, AS-PKAI, and AS *bcl*-2/*bcl*-xL.

MATERIALS AND METHODS

Materials. AS-PKAI MBO was kindly provided by Dr. Sudhir Agrawal (Hybridon, Inc., Cambridge, MA); clinical grade ZD1839 was provided by AstraZeneca (Macclesfield, United Kingdom). The AS *bcl*-2/*bcl*-xL, 4625, is a 20-mer 2′-*O*-(2-methoxy)ethyl (2′-MOE) gapmer targeting a high homology region shared by the *bcl*-2 and the *bcl*-xL mRNAs, whereas 4626 is a control 20-mer 2′-MOE oligonucleotide; their sequence, structure, and purification methods were as described previously (7). The AS-PKAI is a hybrid oligonucleotide, containing phosphorothioate DNA sequences and 2′-*O*-methyl-ribonucleosides, targeted against the NH_2-terminal 8–13 codons of the RIα regulatory subunit of PKA (13). Sequence, structure, and purification methods were as published previously (7, 29).

Cell Lines. GEO colon and ZR-75-1 breast human cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). GEO and ZR-75-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum; HEPES (20 mM, pH 7.4), penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (4 mM; ICN, Irvine, United Kingdom) were maintained 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: (*a*) 0.1–2.5 μM 4625 or 4626; (*b*) 0.1 μM ZD1839; and (*c*) 0.1 μM AS-PKAI. Lipofectin (Life Technologies, Inc., Glasgow, United Kingdom) was mixed with oligonucleotides as recommended by the manufacturer. After 10–14 days, cells were stained with nitro blue tetrazolium (Sigma Chemicals, Milan, Italy), and colonies > 0.05 mm were counted.

Western Blot Analysis. Total cell lysates were obtained from either cells cultured *in vitro* or from homogenated tumor specimens. Protein extracts were resolved by 4–15% SDS-PAGE and probed with an antihuman *bcl*-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an antihuman *bcl*-xL polyclonal antibody (Transduction Laboratories, Lexington, KY), an antihuman VEGF monoclonal antibody (Santa Cruz Biotechnology), or an antiactin monoclonal antibody (ICN Biomedicals, Inc., Aurora, OH). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, United Kingdom), as described previously (11).

Apoptosis in Cultured Cells. The induction of apoptosis was determined by the Cell Death Detection ELISA Plus Kit, which detects cytosolic histone-associated DNA fragments (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, GEO and ZR-75-1 cells (5×10^4 cells/dish) were seeded into 35-mm dishes. On day 4, after treatment on days 1–3 with different concentrations of 4625, 4626, AS-PKAI, or ZD1839, alone and in combination, cells were washed once with PBS; then 0.5 ml of lysis buffer was added. After a 30-min incubation, the supernatant was recovered and assayed for DNA fragments as recommended by the manufacturer. Each treatment was performed in quadruplicate. The total number of cells was measured with a hemocytometer in additional plates receiving an identical treatment. The values resulting from readings of absorbance at $A_{405\ nm}$ were normalized for cell number, and the ratio of absorbance-treated cells:absorbance untreated cells was defined according to apoptotic index.

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 manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN). Cells were plated in 60-mm dishes (Becton Dickinson) and treated for 4 days with either 0.5 μM 4625 or 1 μM 4626 oligos. Assays were performed using 24-h collected, serum-free CM.

GEO Xenografts in Nude Mice. Female BALB/cAnNCrlBR athymic (*nu*+/*nu*+) mice (5–6 weeks old) 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 for 1 week before being injected with cancer cells. Then, 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 20 mg/kg 4625 or 4626 i.p., 150 mg/kg ZD1839 i.p., and/or 10 mg/kg AS-PKAI p.o. daily on days 7–11, 14–18, and 21–25. Tumor volume was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (27). Two mice were sacrificed at day 25 to perform biochemical and immunohistochemical analysis.

RESULTS

Effect of Oligonucleotides on Cell Growth, Protein Expression, and VEGF Secretion. We have evaluated the antitumor activity *in vitro* of AS *bcl*-2/*bcl*-xL 4625 and control sequence 4626 on the soft agar growth of GEO colon and ZR-75-1 breast human cancer cells. Fig. 1*A* demonstrates that 4625 caused a dose-dependent growth inhibition in both cell lines, with an IC_{50} demonstrating higher sensitivity in ZR-75-1 than GEO cells; 4626 caused only a mild growth inhibitory effect, even at the higher dose, confirming the observations reported previously in other cell types (7, 8).

We evaluated the effect of 4625 and 4626 on target proteins *bcl*-2 and *bcl*-xL by Western blot analysis. Fig. 1*B* shows that 4625 is able to markedly down-regulate the expression of *bcl*-2, as well as that of *bcl*-xL in both GEO and ZR-75-1 cells as compared with untreated cells. Conversely, treatment with control sequence 4626 did not affect either *bcl*-2 or *bcl*-xL levels in the two cell lines tested.

Treatment of ZR-75-1 cells with 4625 caused >40% inhibition of the VEGF secretion in the CM, whereas 4626 was unable to affect angiogenic factor secretion compared with untreated control cells (Fig. 1*C*).

Cooperative Growth Inhibitory Effect of Different Agents in Combination. The combination of either AS-PKAI or ZD1839 with different doses of 4625 caused a supra-additive effect on the soft agar growth of both GEO and ZR-75-1 cell lines (Fig. 2*A*). The cooperative inhibitory effect was even more evident when we combined the three agents in both cell lines. When AS-PKAI and ZD1839 were combined with the control sequence 4626, no additive growth inhibitory effect was observed in the two cell lines (Fig. 2*B*).

Cooperative Effect of the Combinations on Apoptosis. We studied the effect of the combination of 4625, AS-PKAI, and ZD1839 on the induction of apoptosis *in vitro*, using only mildly effective doses of each agent. A moderately supra-additive proapoptotic effect was observed with any two drugs or with the three drugs together in GEO cells (Fig. 3*A*). A clearly supra-additive effect was achieved in ZR-75-1 cells treated with two drugs in combination and, more evidently, with three drugs together, resulting in an apoptotic index 4-fold higher than the sum of each individual agent (Fig. 3*A*). Conversely, the combination of AS-PKAI and ZD1839 with the control sequence 4626 did not significantly affect the apoptotic index of either GEO or ZR-75-1 cells (Fig. 3*B*).

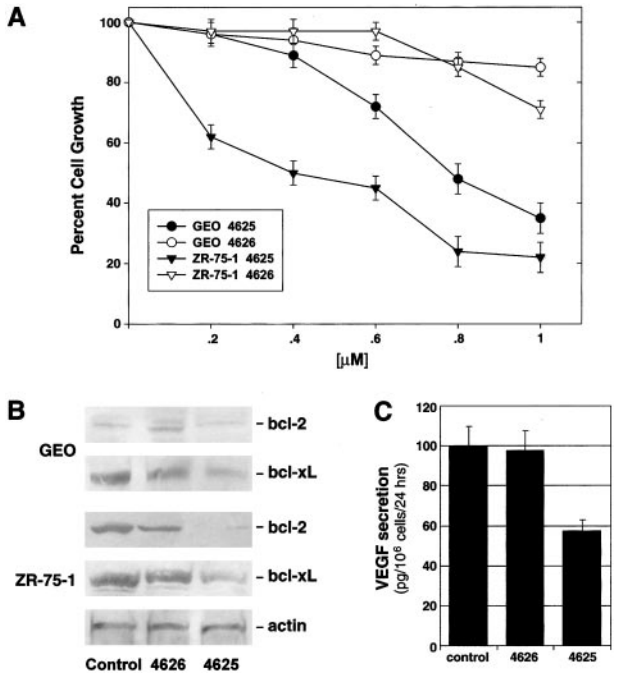

Fig. 1 The effect of AS 4625 and control 4626 on cancer cell growth (*A*), protein expression (*B*), and VEGF secretion (*C*). *A*, dose-response effect of 4625 and 4626 on the soft agar growth of GEO colon and ZR-75-1 breast cancer cells. Data represent means and SE of three different experiments, each performed in triplicate. Cells were counted as described in "Materials and Methods." *B*, effect of 4625 and 4626 on *bcl*-2, *bcl*-xL, and actin protein expression. Western blotting analysis of protein expression in GEO and ZR-75-1 cells grown in monolayer, untreated or treated for 4 days with 1 μM 4625 or 4626. Cell lysates were processed as described in "Materials and Methods." *C*, VEGF secretion in the CM collected from ZR-75-1 cells, untreated or treated for 4 days with either 0.5 μM 4625 or 1 μM 4626, as described in "Materials and Methods." Data represent the average (±SD) of two different experiments each performed in triplicate.

Effect on the Growth and Protein Expression of Tumor Xenografts. We investigated the antitumor activity of 4625, 4626, AS-PKAI, and ZD1839, 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 either agent alone or in combination. As shown in Fig. 4*A*, within ~5 weeks, untreated GEO tumors or tumors treated with 4626 alone reached a size not compatible with normal life; 4625, AS-PKAI, and ZD1839 each caused a similar tumor growth inhibitory effect, delaying the death of mice by ~1 week. When AS 4625 was combined with either AS-PKAI or ZD1839, we observed an additional increase of the growth inhibitory effect, with a delay of death of ~1 week, as compared with the PKAI or EGFR inhibitors alone. A marked and sustained inhibitory effect was obtained by the combination of three drugs. In fact, >50% tumor growth inhibition was still observed 5 weeks after treatment withdrawal and 10 weeks after tumor cell injection (Fig. 4*A*). Moreover, at this time point, pathologic evaluation showed that 50% of mice were still tumor

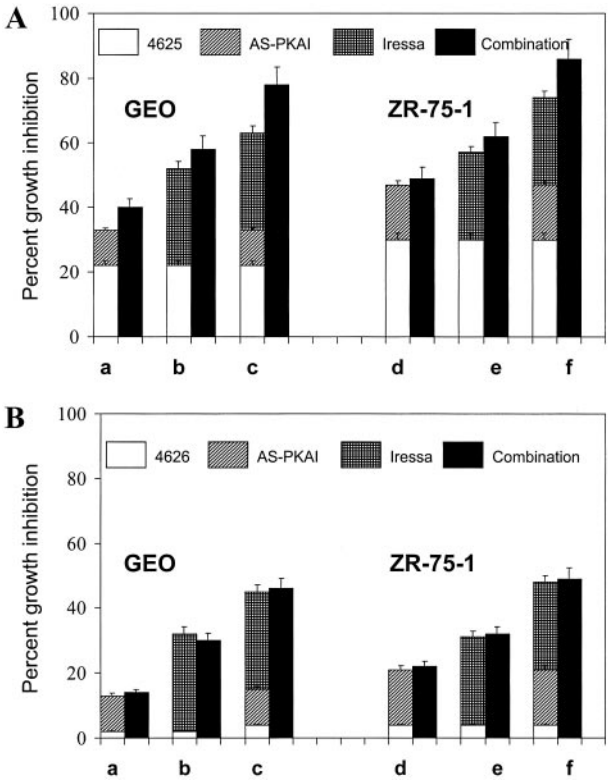

Fig. 2 The effect of either 4625 (*A*) or 4626 (*B*) in combination with AS-PKAI and ZD1839 on the soft agar growth of GEO and ZR-75-1 cells. *A*, 0.5 μM 4625 (*a–c*), 0.25 μM 4625 (*d–f*), 0.1 μM AS-PKAI (*a, c, d,* and *f*), and 0.1 μM ZD1839 (*b, c, e,* and *f*). *B*, 0.5 μM 4626 (*a–f*), 0.1 μM AS-PKAI (*a, c, d,* and *f*), and 0.1 μM ZD1839 (*b, c, e,* and *f*). Data are expressed as a percentage of colony formation inhibition compared with untreated control cells. The first bar of each couple shows the individual effects of each drug when used alone (represented as *stacked bars*). Thus, the total height of these stacked bars also represents the expected total inhibition, if drugs have an additive effect. The second bar of each couple (*black bar*) 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 SE of triplicate determination of at least two experiments.

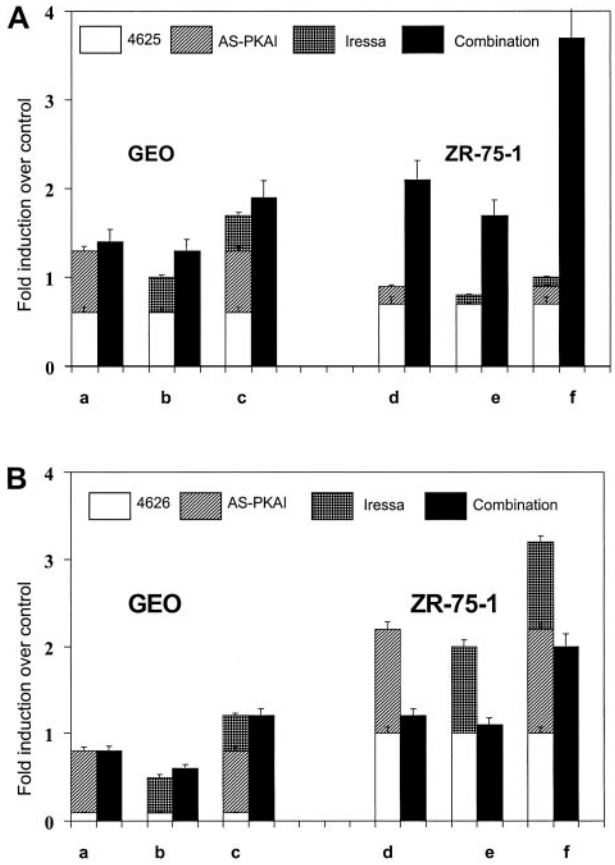

Fig. 3 The effect of either 4625 (*A*) or 4626 (*B*) in combination with AS-PKAI and ZD1839 on apoptosis of GEO and ZR-75-1 cells. *A*, 1 μM 4625 (*a–c*), 0.5 μM 4625 (*d–f*), 0.5 μM AS-PKAI (*a, c, d,* and *f*), and 0.5 μM ZD1839 (*b, c, e,* and *f*). *B*, 1 μM 4626 (*a–f*), 0.5 μM AS-PKAI (*a, c, d,* and *f*), and 0.5 μM ZD1839 (*b, c, e,* and *f*). Treatments were carried out as described in "Materials and Methods." Data are expressed as apoptotic index, which represents the ratio between the absorbance of treated cells and that of untreated cells, normalized for the same number of cells. Therefore, results for each treatment are presented relative to control untreated cells, referred to as 1. The percentage of apoptotic cells is ~4% in untreated GEO cells and ~6% in untreated ZR-75-1 cells, as determined by flow cytometry. The data represent means and SE of duplicate determination of at least two experiments.

free. The combined treatment was well tolerated; no weight loss or other signs of acute or delayed toxicity were observed.

Western blot analysis of protein lysates from tumor specimens removed at the end of treatment, on day 25, demonstrated an inhibition of *bcl*-xL and VEGF expression in animals treated with 4625 or ZD1839. A more evident inhibition was caused by the combination of 4625 with either AS-PKAI or ZD1839 (Fig. 4*B*). A complete suppression of both *bcl*-xL and VEGF proteins was obtained in the specimens from animals treated with the three agents together (Fig. 4*B*).

DISCUSSION

A growing body of evidence supports the role of key proteins in controlling cell proliferation, apoptosis, and angiogenesis in the pathogenesis and progression of cancer. Novel therapeutic strategies based on the integration of selective inhibitors of these proteins with conventional treatments are now being widely explored in preclinical and clinical studies. 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 potential therapeutic targets for their role in the control of cell proliferation, apoptosis, and angiogenesis and for the frequent correlation of their overexpression with more aggressive disease and worse prognosis (9, 10, 22). More recently, *bcl*-2 and *bcl*-xL have also been regarded as potential therapeutic targets on the basis of their ability to disrupt apoptosis and confer resistance to chemo and radiotherapy in cancer cells (1, 2). Recent studies have suggested that *bcl*-xL may have specific functions during tumor development, *e.g.*, in breast cancer patients, *bcl*-2 is associated with hormone-sensitive disease, whereas *bcl*-xL

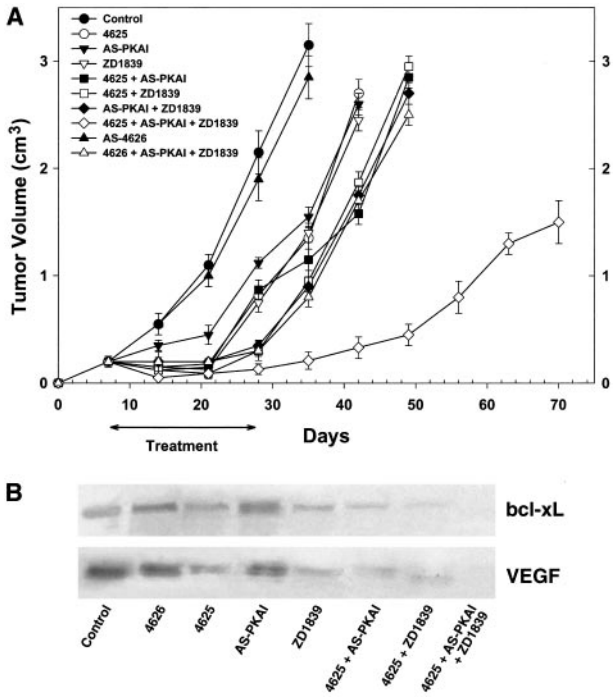

Fig. 4 The effect of the treatment on the growth (*A*) and protein expression (*B*) of GEO tumor xenografts in nude mice. *A*, the schedule of each single agent, alone or in combination, is described in "Materials and Methods." The doses of each drug, used alone or in combination, were: (*a*) 20 mg/kg 4625; (*b*) 20 mg/kg 4626; (*c*) 10 mg/kg AS-PKAI; and (*d*) 150 mg/kg ZD1839. *B*, Western blot analysis was performed on total lysates from tumor specimens of two mice sacrificed at day 25, as described in "Materials and Methods."

overexpression is associated with higher tumor grade and lymph node metastases (30).

Several critical links can be identified in the functional roles that EGFR, PKAI, and *bcl*-2/*bcl*-xL play in the control of apoptosis and angiogenesis, *e.g.*, EGFR activation can up-regulate VEGF production in human cancer cells and stimulate normal endothelial growth, but it can also induce apoptosis (22–26, 31). PKAI, which interacts with EGFR in the transduction of mitogenic signaling, is involved in the production of angiogenic factors and can control apoptosis by affecting *bcl*-2 and BAD phosphorylation (21, 28, 32). In this respect, inhibitors of EGFR and PKAI, such as ZD1839 and AS-PKAI, respectively, are able to inhibit production of angiogenic factors and neoangiogenesis and induce apoptosis *in vitro* and in nude mice (15, 33). In the same fashion, the combination of PKAI and *bcl*-2 selective inhibitors causes a cooperative antitumor, antiangiogenic, and proapoptotic effect (28), supporting the hypothesis that functional interactions may occur between these target proteins. More recently, a correlation has been shown between *bcl*-2, VEGF production, and angiogenesis. In fact, overexpression of *bcl*-2 may act synergistically with hypoxia to induce VEGF expression and angiogenesis in breast cancer (34) and may induce VEGF overexpression, favoring angiogenesis and tumor progression in a prostate cancer cell line (35).

Taking together the above information, we have hypothesized that EGFR, PKAI, and *bcl*-2 may be part of a loop of signaling proteins interplaying in the concerted control of cell proliferation, apoptosis, and angiogenesis. In such a situation, the combined inhibition of EGFR, PKAI, and *bcl*-2 might be able to inhibit tumor growth by interfering with angiogenesis and apoptosis.

To experimentally verify this hypothesis, we have evaluated the possibility of controlling human colon and breast cancer growth, without using cytotoxic drugs, by combining the EGFR-tyrosine kinase inhibitor ZD1839, the DNA/RNA hybrid oligonucleotide AS-PKAI, and the novel bispecific AS *bcl*-2/*bcl*-xL 4625. All these agents have demonstrated antiproliferative and proapoptotic properties in different tumor models, alone and in combination with cytotoxic drugs.

We have shown that AS *bcl*-2/*bcl*-xL 4625 is able to down-regulate the growth of human breast and colon cancer cells. The antiproliferative effect is mirrored by a selective inhibition of *bcl*-2 and *bcl*-xL target proteins and by inhibition of angiogenic factor VEGF secretion. In addition, we have demonstrated that 4625, AS-PKAI, and ZD1839 are able to cause a supra-additive antiproliferative and proapoptotic effect in GEO and ZR-75-1 cells when two agents are used in combination or, even more notably, as three drugs together.

We translated these results *in vivo* in nude mice bearing GEO xenografts combining 4625 or 4626 with AS-PKAI and ZD1839. Although control 4626 was ineffective, 4625, AS-PKAI, and ZD1839 alone caused a moderate growth inhibitory effect. The addition of 4625 to either AS-PKAI or ZD1839 resulted in an increased antitumor effect. A marked cooperative effect was obtained when the three agents were used together.

The marked antiproliferative, proapoptotic, and antiangiogenic activity of the three agents in combination, in the absence of cytotoxic drugs, strongly supports the hypothesis of a functional interaction among the tyrosine kinase receptor EGFR, transducing protein kinase PKAI, and antiapoptotic proteins *bcl*-2 and *bcl*-xL. In this context, the combination of the three respective inhibitors provides an effective multisignaling blockade, reducing the chances of tumor escape. We believe that this study also provides a robust biological rationale in the selection of specific molecular treatments for the development of future therapeutic strategies.

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Clin Cancer Res 2003;9:866-871.