Combined Blockade of Protein Kinase A and Bcl-2 by Antisense Strategy Induces Apoptosis and Inhibits Tumor Growth and Angiogenesis[1]

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ABSTRACT

Protein kinase A type I (PKAI) plays a key role in neoplastic transformation, conveys mitogenic signals from different sources, and is overexpressed in the majority of human tumors. Inhibition of PKAI by different tools results in cancer-cell growth inhibition *in vitro* and *in vivo*. We and others have recently shown that a novel class of mixed-backbone oligonucleotides targeting the PKAI subunit RIα exhibits improved pharmacokinetic properties and antitumor activity accompanied by increased apoptosis in several human cancer types *in vitro* and *in vivo*. The role of bcl-2 in the control of apoptosis has been widely documented, and the inhibition of bcl-2 expression and function may have important therapeutic implications. In fact, oligonucleotides antisense bcl-2 have shown antitumor activity in animal models and have successfully completed early clinical trials. Recent studies have demonstrated a direct role of PKA in the regulation of the bcl-2-dependent apoptotic pathway. Therefore, we have investigated the combined blockade of PKA and bcl-2 by antisense strategy as a potential therapeutic approach. The novel hybrid DNA/RNA mixed-backbone oligonucleotide antisense RIα (AS RIα) in combination with the antisense bcl-2 (AS bcl-2), cooperatively inhibited bcl-2 expression and soft agar growth and induced apoptosis in different human cancer cell lines. p.o. administration of AS RIα in combination with i.p. AS bcl-2 caused a marked antitumor effect and a significant prolongation of survival in nude mice bearing human colon cancer xenografts. Moreover, histochemical analysis of tumor specimens showed inhibition of RIα and Ki67 expression, inhibition of angiogenesis, and parallel induction of apoptosis *in vivo*. The results of our study imply an interaction between the PKA and bcl-2 signaling pathways and, because both antisenses have now entered Phase II trials, provide the rationale to translate this novel therapeutic strategy in a clinical setting.

INTRODUCTION

PKA[3] plays a key role in the control of cell growth and differentiation of mammalian cells. PKA is composed of two distinct isoforms, PKAI and PKAII, which differ only in their regulatory subunits, defined RI and RII, respectively (1). PKAI is involved in cell proliferation and neoplastic transformation, is required for the G_1-S transition of cell cycle, and mediates mitogenic signals from different growth factors, including transforming growth factor α and epidermal growth factor (1–3). PKAI overexpression is detected in the majority of human cancers, correlating with worse clinicopathological features in colon, breast, and ovarian cancer (2). Conversely, PKAII is preferentially expressed in normal tissues and is involved in growth arrest, differentiation, and induction of apoptosis (1). PKAI subunit RIα has a structural interaction with the ligand-activated epidermal growth factor receptor as well with other receptors, cooperating in the propagation of mitogenic signals originated by different growth factors and hormones (2). For the above reasons, PKAI represents a relevant target for therapeutic intervention.

Down-regulation of PKAI by different pharmacological tools, including AS oligodeoxynucleotides targeting its RIα subunit, causes cell growth arrest and differentiation in a variety of cancer cell lines (2, 4) and shows antitumor activity in nude mice (5). Recently, these effects have been obtained also with a novel class of oligonucleotides containing MBOs. MBOs exhibit improved pharmacokinetic and toxicological properties *in vivo* as compared with PS oligonucleotides (6). We and others have shown the antitumor effect of a MBO AS RIα and its synergism with different classes of cytotoxic drugs in several human cancer xenografts in nude mice (2, 7). Recently, a hybrid DNA/RNA MBO AS RIα (defined as GEM 231) has completed a Phase I clinical trial in cancer patients with negligible toxicity

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[3] The abbreviations used are: PKA, protein kinase A; PKAI, PKA type I; PKAII, PKA type II; AS, antisense; MBO, mixed-backbone oligonucleotide; PS, phosphorothioate; JNK, c-Jun NH_2-terminal kinase; TdT, terminal deoxynucleotidyl transferase; MAb, monoclonal antibody.

(8). We and others have recently shown that the novel AS RIα exhibits a good pharmacokinetic profile and inhibits tumor growth in cooperation with several anticancer drugs with p.o. administration (9, 10).

Bcl-2 is the prominent member of a family of proteins responsible for dysregulation of apoptosis, prevention of cancer-cell death, and resistance to chemotherapy and radiotherapy (11, 12). Antiapoptotic bcl-2 family members counteract proapoptotic proteins, such as BAD and BAX, in the control of the pathways leading to the release of cytochrome *c* from the mitochondrial membrane, to the activation of the caspases cascade, and, finally, to the execution of apoptosis (11–13). Paclitaxel and other microtubule-damaging agents cause apoptosis, inducing bcl-2 phosphorylation and inactivation (12, 14). These effects may be obtained also by noncytotoxic tools, including AS bcl-2 oligonucleotides. A 18-mer PS- oligonucleotide targeting the human bcl-2 (G3139; GENTA) is able to block bcl-2 expression, inhibit the growth of human melanoma and prostate cancer in animal models, and synergize with cytotoxic drugs (15, 16). Moreover, it is able to delay the progression to androgen-independence (16). This same effect was obtained in a Shionogi murine tumor by an oligonucleotide targeting the murine bcl-2 (17). The AS human bcl-2 has completed early clinical studies in different malignancies including melanoma, non-Hodgkin's lymphoma, and prostate cancer, demonstrating antitumor activity alone and in combination with cytotoxic drugs (18–20).

In past years, a large number of studies elucidated the role of different signaling proteins in the control of bcl-2-dependent apoptotic events. In addition to JNK and Akt proteins (11, 12, 21, 22), PKA seems to play a prominent role. In fact, after treatment with paclitaxel and other microtubule-damaging agents, PKA as well as JNK cause specific phosphorylation of bcl-2 and activate the cascade that leads to apoptosis (23, 24). Agents that inhibit PKAI and induce PKAII, such as AS RIα or DBcAMP, are able to inhibit bcl-2 expression and function, to induce cleavage of PARP and caspase 3 activation, causing apoptosis (25, 26). Taken together, these data suggest that the combined blockade of PKAI and bcl-2 may have a therapeutic potential. Therefore, in the present study, we have used two AS oligonucleotides as selective tools. We have investigated whether the hybrid MBO AS RIα and the PS-AS bcl-2, used in combination, have any cooperative effect on tumor growth and apoptosis of different human cancer cells *in vitro* and *in vivo*. Moreover, we have performed immunohistochemical analysis of tumor specimens to evaluate their *in vivo* effect on cell proliferation, angiogenesis, and apoptosis.

MATERIALS AND METHODS

Oligodeoxynucleotides. All oligonucleotides were synthesized by Dr. Sudhir Agrawal at Hybridon, Inc. (Milford, MA). The PS-AS bcl-2, corresponding to the first six codons of bcl-2 mRNA site and its mismatch control oligonucleotide have the following sequences: TCTCCCAGCGTGCGCCAT and TCTCCCAGCATGTGCCAT, respectively, as published previously (15). The AS RIα MBO is a hybrid oligonucleotide targeted against the NH_2-terminal 8–13 codons of the RIα regulatory subunit of PKA (5) with the following sequence: *GCGU*GCCTCCTCAC*UGGC*; the RIα control oligonucleotide is a scramble MBO obtained by mixing all four nucleosides at each position (10). The two MBO oligonucleotides contain PS internucleotide linkages, identified by roman type for the nucleosides flanking each position, and 2′-*O*-methyl-ribonucleosides modifications, identified by italic type. Oligonucleotides were synthesized, identified, and purified according to the protocol described earlier (27).

Cell Growth Experiments and AS Treatment. GEO human colon cancer cells were grown in McCoy medium, OVCAR-3 human ovarian and ZR-75-1 human breast cancer cells were maintained in DMEM (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, (pH 7.4), penicillin (100 IU/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. For cell growth experiments in soft agar, 10^4 cells/well were seeded in 24 multiwell cluster dishes as described previously (28) and treated with different concentrations of the indicated oligonucleotides. In all of the combination experiments shown, the two oligonucleotides were administered simultaneously, (AS bcl-2 on days 1–3, AS RI MBO or Scramble MBO on days 1 and 3). We also performed experiments by administering the compounds in a sequential fashion, using the AS bcl-2 on days 1, 2, and 3 with the MBOs on days 4 and 6 or, alternatively, the MBOs on days 1 and 3 and the AS bcl-2 on days 4, 5, and 6. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma Chemical Co., Milan, Italy), and colonies >0.05 mm were counted.

Western Blot Analysis. Total cell lysates (50 μg) were fractionated through 7.5% or 12% SDS-polyacrylamide gels, transferred to nitrocellulose filters, incubated with specific MAbs and then horseradish-peroxidase antiserum (Bio-Rad Laboratories, Milano, Italy). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, United Kingdom), as described previously (3). Antihuman RIα mouse MAb (Transduction Laboratories, Lexington, KY), antihuman bcl-2 mouse MAb (Santa Cruz Biotechnology, Santa Cruz, CA), and antihuman actin mouse MAb (Sigma Chemical Co.) were used following the methods described previously (3).

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 OVCAR-3 cells (5×10^4 cells/dish) were seeded into 35-mm dishes. After treatment with different concentrations of either AS RI (days 1 and 3), Scramble MBO (days 1 and 3, or AS bcl-2 (days 1, 2, and 3), alone and in combination, on day 4 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. Total number of cells was measured with an hemocytometer in additional plates receiving an identical treatment. The values resulting from the readings of optical density at 405 nm ($A_{280\ nm}$) were normalized for cell number, and the ratio of optical density-treated cells:optical density-untreated cells was defined as the apoptotic index.

GEO Xenografts in Nude Mice. Female Balb/cAnNCrlBR athymic (nu+/nu+) mice 5–6 weeks of age 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 acclimated to the University of Naples Medical School Animal Facility for 1 week before being injected with cancer cells. GEO cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, as described above. Then, 10^7 cells were resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice (7, 29). After 7 days, when well established tumors of approximately 0.2 cm^3 were detected, mice were randomized to receive different treatments. Ten mice for each group were treated with either p.o. AS RIα (10 mg/kg/dose) in a 5% dextrose solution, i.p. AS bcl-2 (10 mg/kg/dose), or i.p. mismatch control bcl-2 (10 mg/kg/dose) alone or in combination (AS RIα + AS bcl-2 or AS RIα + mismatch bcl-2) for 5 days a week for a total of 3 weeks. Tumor volume was measured using the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as previously reported (29).

Immunohistochemical Analysis. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were processed as reported previously (30). Reactions with appropriate primary antibody, secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit; Vector Laboratory, Burlingame, CA), avidin-biotinylated horseradish peroxidase H complex, diaminobenzidine, and hydrogen peroxide and counterstaining with hematoxilin were as described previously (30).

The following antibodies were used in this study: an anti-RIα MAb (Transduction Laboratories, Lexington, KY), 1:100 dilution; and an anti-Ki67 MAb (clone MIB1; DBA, Milan, Italy), 1:100 dilution. All analyses were performed in a blind fashion. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored at ×40.

New blood vessels were detected as described previously (31) using a MAb against the human factor VIII-related antigen (Dako, Milan) at a dilution of 1:50 and stained using a standard immunoperoxidase method (Vectastain ABC kit; Vector). Each slide was first scanned at low power (×10–100), and the area with the higher number of new vessels was identified (the "hot spot"). This region was then scanned at ×250 (0.37 mm^2). Stained blood vessels were counted in each of five different fields. For individual tumors, microvessel count was scored by averaging the five fields counts.

Determination of apoptosis was performed by a modified TdT-mediated nick end labeling assay (32) with the ApopTag Kit (Intergen, DBA, Milan, Italy). The processing of paraffin-embedded tissues, washings, the addition of working-strength TdT enzyme, antidigoxigenin peroxidase conjugate, and diaminobenzidine and counterstaining with hematoxilin were carried out according to the ApopTag Kit manual and as described previously (32). The counting of apoptotic nuclei was performed at ×40, evaluating at least 100 cells and an average of 10 fields.

Statistical Analysis. The Mantel-Cox log-rank test (33) was used to evaluate the statistical significance of the results. All reported *P*s were two-sided. All analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

Table 1 IC_{50} (μM) of different oligonucleotides

The effect of the different oligonucleotides was measured on the soft agar growth of GEO, ZR-75-1, and OVCAR-3 human cancer cells (described in "Materials and Methods"). The data represent the means of triplicate determination of at least two experiments with SDs < 15%.

	AS RI MBO	Scramble MBO	AS bcl-2	Bcl-2 control
GEO	0.8	>25	4.6	>15
ZR-75-1	1.2	>25	3	>15
OVCAR-3	0.7	>25	7	>15

RESULTS

Effect of Different Oligonucleotides on the Growth of Human Cancer Cells. We have evaluated the antitumor activity of the MBO AS RIα, its control scramble MBO, the PS-AS bcl-2, and its mismatch control on the soft agar growth of a variety of human cancer cells, including GEO colon, OVCAR-3 ovarian, and ZR-75-1 breast cells. As shown in Table 1, the AS RIα caused a dose-dependent growth inhibition on all tested cells, achieving the IC_{50} at doses ranging between 0.7 and 1.2 μM. Conversely, an IC_{50} could not be reached with the control scramble MBO, even with doses as high as 25 μM. A dose-dependent growth inhibitory effect was also observed in all cell lines with the AS bcl-2, with an IC_{50} ranging between 3 and 7 μM, whereas the same degree of inhibition was reached only by higher concentrations of mismatch bcl-2 oligonucleotide (Table 1).

Cooperative Effect of AS RIα and AS Bcl-2. We evaluated whether the AS RIα and AS bcl-2 in combination were able to cooperate in inhibiting human cancer cell growth. We selected different doses of AS RIα (0.1–1 μM), Scramble MBO (0.5 and 1 μM), AS bcl-2 (0.5–5 μM), and mismatch bcl-2 (1–5 μM) and used these oligonucleotides alone or in combination. Different schedules of treatment were studied, administering the two oligonucleotides simultaneously and, in a sequential order, giving either the AS bcl-2 first and then the AS RIα or *vice versa*. Remarkably, the antiproliferative effect obtained was similar, regardless of the schedule used (data not shown). For this reason, thereafter we performed all of the experiments using a simultaneous administration, as described in "Materials and Methods." Fig. 1 shows that AS RIα and AS bcl-2, when used in combination, have a dose-dependent cooperative effect, which is particularly evident with lower doses, on GEO, ZR-75-1, and OVCAR-3 soft agar colony formation. For instance, in ZR-75-1 breast cancer cells, AS RIα (0.1 μM) and AS bcl-2 (2 μM), which alone cause 10% and <40% inhibition, respectively, completely suppressed colony formation in soft agar (Fig. 1, *middle*). Conversely, the combination of Scramble MBO (0.5 μM) with increasing doses of AS bcl-2 caused, at most, an additive effect (Fig. 1). In the same fashion, the addition of mismatch bcl-2 oligonucleotide to AS RIα MBO did not modify the inhibitory effect obtained with the latter alone in GEO cells (data not shown).

Inhibition of Bcl-2 Expression. We measured the effect of the AS oligonucleotides on the expression of the target gene

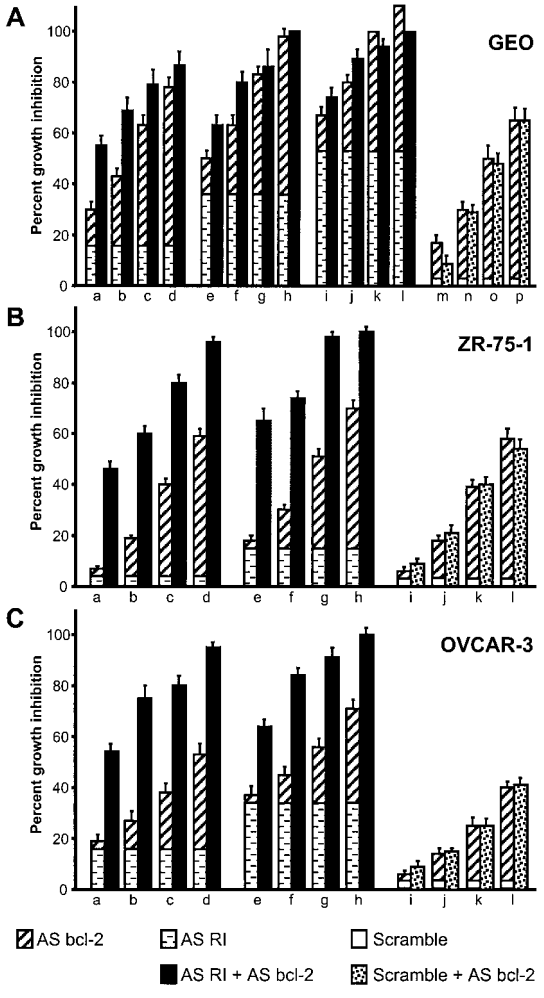

Fig. 1 Effect of the AS RIα MBO, scramble MBO and AS bcl-2 on GEO, ZR-75-1, and OVCAR-3 cell growth in soft agar. Concentrations of AS bcl-2 were 0.5, 1, 2, and 5 μM in all cell lines. *A*, in GEO cells, AS RIα doses are: 0.1 μM (*a–d*); 0.5 μM (*e–h*); and 1 μM (*i–l*). AS bcl-2 doses are 0.5 μM (*a, e, i,* and *m*); 1 μM (*b, f, j,* and *n*); 2 μM (*c, g, k,* and *o*); and 5 μM (*d, h, l,* and *p*) in all cell lines. In ZR-75-1 (*B*) cells and OVCAR-3 (*C*) cells, AS RIα doses are 0.1 μM (*a–d*) and 0.5 μM (*e–h*); AS bcl-2 doses are 0.5 μM (*a, e,* and *i*), 1 μM (b, f, j), 2 μM (*c, g,* and *k*), and 5 μM (*d, h,* and *l*). Scramble MBO dose is 1 μM in all three cell lines. Data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. Each *bar,* the percentage of growth inhibition, as indicated in the respective legends. *Bars* on the *left,* the sum of the effects of the individual agents and the expected percentage of growth inhibition if oligonucleotides are additive when used in combination. Total height of the *solid bar* on the *right,* the actual observed growth inhibition when oligonucleotides were used in combination. Therefore, the differences between the heights of the *paired bars* reflect the magnitude of synergism of growth inhibition. The data represent means and SEs of triplicate determination of at least two experiments.

products RIα and bcl-2 by Western blotting analysis. RIα protein expression was inhibited in GEO cells by AS RIα, as shown previously (34), whereas it was unaffected by AS bcl-2 (data not shown). Fig. 2 illustrates the effect on bcl-2 expression of the different oligonucleotides at concentrations close to the IC_{50}. AS bcl-2 (5 μM) inhibited the total bcl-2 protein expression,

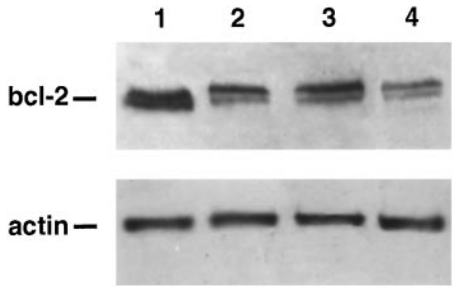

Fig. 2 Western blot analysis of bcl-2 and actin protein levels. Treatments were as follows: *Lane 1,* untreated; *Lane 2,* AS bcl-2, 5 μM; *Lane 3,* AS RIα, 1 μM; and *Lane 4,* AS bcl-2 μM plus AS RIα μM.

reducing >70% its nonphosphorylated form, whereas the AS RIα (1 μM) prevalently increased the relative amount of phosphorylated bcl-2 without affecting the total amount of protein. Interestingly, the combined treatment with the two oligonucleotides caused an additive effect, markedly decreasing the total amount of bcl-2 protein and inducing a prevalently phosphorylated bcl-2 (Fig. 2). Combination of either AS with an equal dose of the relative control sequence did not affect the results obtained with each AS alone (data not shown).

Cooperative Effect of the Two Oligonucleotides on Apoptosis. We measured the effect of different AS treatments on the induction of apoptosis *in vitro*. Previously, we have shown by flow cytometry that the apoptotic cells are ~4% in untreated GEO cells and ~6% in untreated OVCAR-3 cells (35). In GEO, we have observed that the AS RIα (1 μM) is able to double the rate of cells undergoing apoptosis as compared with untreated control cells, whereas the AS bcl-2 causes a 1.5-fold increase in the apoptotic index at 1 μM and a 4-fold increase at 2 μM, respectively (Fig. 3*A*). When the MBO AS RIα was combined with the PS-AS bcl-2, either 1 μM or 2 μM, they determined a supraadditive apoptotic effect, causing an 8-fold and an ~13-fold increase in apoptotic index, respectively. Conversely, the addition of scramble MBO to either dose of AS bcl-2 did not modify the effect observed with the latter oligonucleotides alone. A supraadditive effect, although less pronounced, was also observed in ovarian OVCAR-3 cells (Fig. 3*B*). In fact, AS RIα (1 μM) caused a 1.8-fold increase in the apoptotic index and AS bcl-2 (1 μM and 2 μM), caused a 1.5-fold and a 2.4-fold increase, respectively. The AS RIα combined with the AS bcl-2 (1 μM and 2 μM) induced a 4.3-fold and a 6.1-fold increase in apoptosis, respectively. The scramble MBO was not effective, causing a less-than-additive effect when added to either dose of AS bcl-2 (Fig. 3*B*).

Cooperative Antitumor Activity *in Vivo*. We evaluated whether the cooperative antitumor effect observed *in vitro* could be reproduced *in vivo* using the model of nude mice bearing GEO xenografts. Seven days after tumor injection, groups of 10 mice were randomly selected to receive different treatments, including p.o. AS RIα, i.p. AS bcl-2, i.p. mismatch bcl-2 oligonucleotide, or the combination of either p.o. AS RIα plus i.p. AS bcl-2 or p.o. AS RI plus i.p. mismatch bcl-2 oligonucleotide (described in "Materials and Methods"). Treatment was performed for 5 days a week, for 3 consecutive weeks, up to day 25.

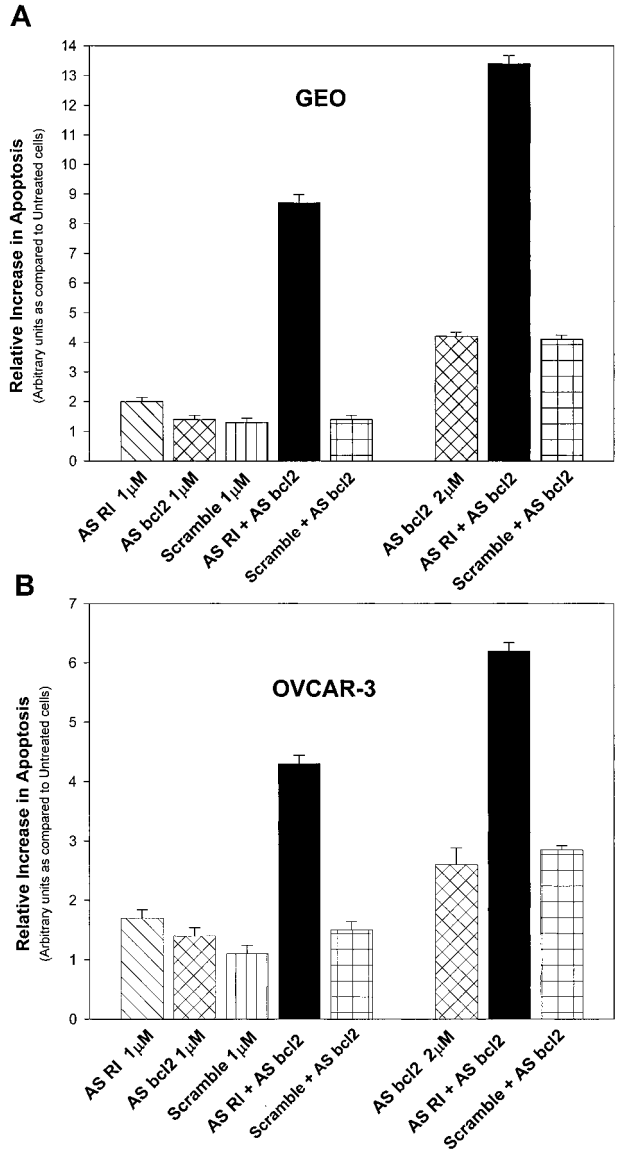

Fig. 3 Effect of the AS RIα MBO, scramble MBO, and AS bcl-2 on the induction of apoptosis in GEO (*A*) and OVCAR-3 (*B*) cells. Treatments were carried out as described in "Materials and Methods." Doses of each oligonucleotide alone and in combination are reported. Data are expressed as apoptotic index (*AI*), which represents the ratio between the absorbance in optical density of treated cells and that of untreated cells normalized for the same number of cells. Therefore, results for each treatment are intended as relative to control untreated cells, referred to as 1. The percentage of apoptotic cells is ~4% in untreated GEO cells and ~6% in untreated OVCAR-3 cells, as determined by flow cytometry (35). The data represent means and SEs of quadruplicate determination of at least two experiments.

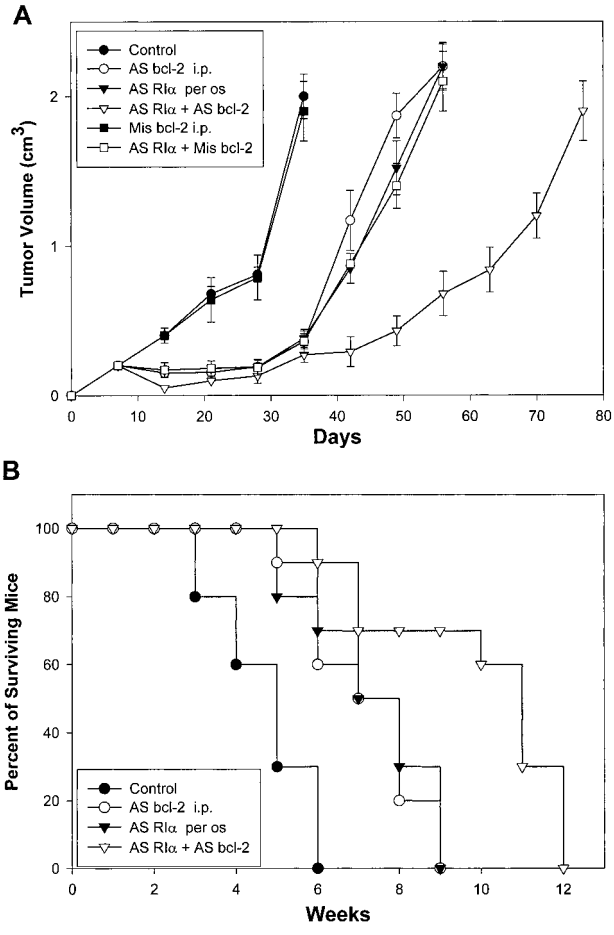

Fig. 4 Effect of different oligonucleotides on GEO tumor growth and on mice survival. *A*, effect of p.o. AS RIα MBO, i.p. AS bcl-2, i.p. mismatch bcl-2 (*mis bcl-2*) alone and in combination. Each group of 10 mice received three cycles of treatment as described in "Materials and Methods." *B*, effect on mice survival of the different treatments described in *A*. The Mantel-Cox log-rank test was used to evaluate the statistical significance of the results. The difference between the treatment with either AS RIα or AS bcl-2 is significant as compared with control untreated ($P < 0.001$). The difference between the treatment with AS RIα plus AS bcl-2 is significant as compared with control untreated or mismatch bcl-2-treated mice ($P < 0.0001$), with AS bcl-2-treated mice ($P < 0.001$), or with AS RIα-treated mice ($P < 0.001$).

As illustrated in Fig. 4*A*, treatment with either AS RIα or AS bcl-2 alone inhibited tumor growth as compared with untreated mice. In fact, by day 35, each AS oligonucleotide caused a 80–90% inhibition of tumor growth as compared with control untreated mice. However, shortly after the end of treatment, tumors resumed the growth rate observed in untreated mice, achieving their same size by day 56. We have shown previously that a scramble MBO oligonucleotide is ineffective in the same tumor model (10). Similarly, the mismatch control bcl-2 oligonucleotide was ineffective (Fig. 4*A*). When the p.o. AS RIα was used in combination with the AS bcl-2, we observed a marked and sustained inhibition of tumor growth. In fact, tumors achieved an average volume of about 2 cm^3 6 weeks later than mice that were untreated or treated with mismatch control and 3 weeks later than animals treated with each single AS alone. In contrast, treatment with AS RIα plus mismatch bcl-2 caused an antitumor effect similar to that obtained with the AS RIα alone. Within ~6 weeks, GEO tumors reached a size not compatible with normal life in all untreated mice and in mice treated with the control bcl-2 oligonucleotide (Fig. 4*B*). A significant increase in survival was observed in mice groups treated with

Table 2 Immunohistochemical analysis of GEO tumors subsequent to treatment with different oligonucleotides

Analysis was performed at the end of the second cycle of treatment (day 21) on three different tumor specimens randomly selected in each group. Numbers represent the percentage of positive cells staining for each antigen and for apoptotic nuclei or the number of microvessels/field (see "Materials and Methods"). Numbers in parenthesis represent the SD in the measurement of each biological parameter. The intensity of staining is represented by a score from + to +++.

	Ki67	RIα	Vessels	Apoptosis
Control	60% (±5) ++	60% (±4) +++	10 (±1) ++	3 (±1)
AS bcl-2	40% (±2) +	60% (±5) +++	7 (±1) +	7 (±1)
AS RIα	40% (±5) +	20% (±3) +	7 (±1) +	4 (±1)
AS bcl-2 + AS RIα	20% (±4) +	10% (±5) +	3 (±1) +	11 (±1)

either AS RIα or AS bcl-2 alone, as compared with control mice ($P < .001$). A remarkable result was obtained in mice treated with AS RIα plus AS bcl-2 in combination. In fact, the delayed GEO tumor growth observed in this group was accompanied by a prolonged life span in mice, significantly different when compared by log-rank test to controls ($P < .0001$), to either the AS alone-treated group ($P < .001$) or to the AS RIα plus mismatch bcl-2-treated group ($P < .001$). Approximately 60% of mice treated with AS RIα plus AS bcl-2 were still alive 11 weeks after tumor injection, representing the only animals alive at this time point. The combined treatment with AS RIα plus AS bcl-2 was well tolerated, inasmuch as no weight loss or other signs of acute or delayed toxicity were observed.

Effect on Angiogenesis, Proliferation, and Apoptosis *in Vivo*. Tumor specimens from the different groups of mice were examined by immunohistochemical analysis to evaluate the expression of a variety of biological parameters. Table 2 reports the results of the analyses performed on two tumor samples randomly selected in each group after the 2nd week of treatment on day 21. Treatment with AS RIα was able to inhibit the expression of both the target protein, RIα, and Ki67, a protein related to cell proliferation, confirming our previous results (10). Interestingly, AS bcl-2 inhibited Ki67 but did not affect RIα expression (Table 1). Additional inhibition of RIα and Ki67 expression was observed when the MBO AS RIα was used in combination with the AS bcl-2 (Table 2).

We quantified by immunohistochemistry the tumor-induced vascularization as microvessel count in the most intense areas of neovascularization using an anti-factor VIII related-antigen MAb (31). As reported in Table 2, the AS RIα as well the AS bcl-2 caused inhibition of specific vessel staining, as compared with samples from untreated mice. Combined treatment with AS RIα and AS bcl-2 inhibited by >70% the vessel formation in GEO tumors (Table 2).

Finally, we studied the effect of treatment on the induction of apoptosis *in vivo* by a modified TdT-mediated nick end labeling technique (32). We observed that the i.p. AS bcl-2 was very effective after only 2 weeks of treatment, doubling the percentage of cells undergoing apoptosis, whereas at the same time point, p.o. AS RIα only slightly increased apoptosis. Combination of two oligonucleotides caused an additive effect on apoptosis.

DISCUSSION

Increased tumor expression of bcl-2 and other members of the antiapoptotic bcl-2 family as well as reduced expression of proapoptotic proteins are considered among the major determinants of resistance to chemotherapeutic drugs and radiotherapy and the persistence of microscopic proliferating clones, indicating a poor prognosis in several types of cancer (11, 12). Blockade of bcl-2 expression and function may represent a relevant therapeutic strategy, and AS bcl-2 oligonucleotides have been successful in several preclinical and clinical studies (15–20). Several studies suggest that PKA may play a major role in the control of bcl-2-dependent apoptosis. The PKAI isoform is involved in the transduction and amplification of a variety of mitogenic signals from cell membrane to core cellular machineries (2). Recently, different studies have linked PKA to the apoptotic machinery. For instance, microtubule-damaging agents determine apoptosis by a serine kinase-dependent phosphorylation of bcl-2. Both PKA and JNK have shown the ability to phosphorylate the specific serine-70 residue of bcl-2, to induce the cleavage of PARP, to activate caspase 3, and, finally, to cause apoptosis (21, 23, 24). It has also been demonstrated that selective inhibitors of PKAI and inducers of PKAIIβ, such as AS RIα, cAMP analogues, or selective unhydrolizable cAMP analogues, can inactivate bcl-2 and induce activation of the caspases cascade, cleavage of PARP, and apoptosis (24–26). A recent study has shown that a PKAII isoform is targeted by an anchoring protein to phosphorylate and inactivate BAD and that a selective inhibitor of PKAI activation may prevent this effect (36), suggesting that PKAI may be involved also in BAD functions. In this regard, an even more direct link between the apoptotic machinery and PKAI has been unraveled by Yang *et al.* (37), showing that PKAI subunit RIα is directly bound to cytochrome *c* oxidase subunit Vb. Pharmacological inhibition of PKAI causes cytochrome *c* release and apoptosis, whereas overexpression of mutant PKAI, harboring a defective RIα, prevents these events (37).

This large body of experimental evidence would imply that the selective blockade of bcl-2, combined with a selective inhibition of the multifunctional signaling protein PKAI, could trigger the apoptosis and suppress the transduction of survival signals, thus leading to cancer cell growth inhibition.

To experimentally demonstrate this hypothesis, in the present study we have used two AS oligonucleotides that have shown their therapeutic potential in different preclinical and clinical settings. The PS-AS bcl-2 has shown antitumor activity in human melanoma and prostate cancer models in nude mice (15, 16). The same compound has shown antitumor and chemosensitizing activity in Phase I/II clinical trials in melanoma, non-Hodgkin's lymphomas, and prostate cancer subsequent to i.v. as well as s.c. administration (18–20). The AS RIα is a novel 2′-*O*-methyl-modified MBO that has shown antitumor activity *in vitro* and *in vivo* in a variety of tumor models in nude and severe combined immunodeficient mice (7, 8). We and others have also shown that this compound has good bioavailability and antitumor activity in the same tumor models after

p.o. administration (9, 10). Recently, the AS RIα MBO has completed a Phase I trial in cancer patients with negligible toxicity (8).

We have here demonstrated that both the AS bcl-2 and the MBO AS RIα are able to inhibit, in a dose-dependent fashion, the anchorage-independent growth of GEO colon, ZR-75-1 breast and OVCAR-3 ovarian cancer cells. When used in combination at doses causing only moderate inhibition as single agents, they show a marked cooperative growth-inhibitory effect, allowing the suppression of colony formation in agar. The fact that the cooperative antiproliferative effect can be obtained regardless of the schedule of administration used, further supports the hypothesis that the two pathways are distinct yet related to each other. Inhibition of bcl-2 expression as well as bcl-2 phosphorylation are critical events in the commitment to apoptosis. In this study we have demonstrated that AS RIα causes bcl-2 phosphorylation in GEO cells and that combination with AS bcl-2 markedly reduces the total expression of bcl-2, which then results mostly in a phosphorylated form. These data are in agreement with previous observations, showing that AS RIα affects bcl-2 expression and causes bcl-2 phosphorylation (23, 34). More importantly, the combination of AS oligonucleotides caused a remarkable cooperative effect on the induction of apoptosis *in vitro*. These data suggest that combined blockade of PKAI and bcl-2 imbalance antiapoptotic cellular mechanisms, causing cell death. We have then evaluated whether the effects *in vitro* could be obtained also *in vivo* in nude mice bearing GEO tumors. Previously, we have shown the inhibitory effect of p.o. MBO AS RIα in GEO tumor xenografts. In this study we have demonstrated the cooperative antitumor effect of p.o. AS RIα in combination with i.p. PS-AS bcl-2 after 3 weeks of treatment. The growth-inhibitory effect was sustained, lasting for several weeks after treatment withdrawal and causing a remarkable increase in the survival of treated mice. In fact, ~60% of mice treated with the two oligonucleotides in combination were still alive 11 weeks after tumor injection. We and others have shown previously that either the p.o. AS RIα or an AS bcl-2 cooperate with paclitaxel in inhibiting the growth of tumors in mice (10, 17). Interestingly, in the present study we have observed that the addition of paclitaxel to both ASs together minimally increases the antiproliferative and apoptotic effect obtained by the two oligonucleotides in combination.[4] We have demonstrated that the p.o. AS RIα selectively inhibits the expression of its target in the tumor. In fact, although bcl-2 also inhibits tumor growth and reduces expression of the proliferation-related protein Ki67, it does not affect RIα expression. The critical role of tumor-induced neovascularization in neoplastic development, progression, and metastasis has been emphasized in recent years (38). In the present study, we have shown that either oligonucleotide alone was active in inhibiting vessel staining after only 2 weeks of treatment and that combined treatment with the two oligonucleotides reduced >70% vessel formation in GEO tumors. Finally, we have observed that AS bcl-2 induced a more-than-double increase in apoptosis, whereas the AS RIα was modestly effective after 2 weeks of treatment. Interestingly, the two oligonucleotides in combination caused an additive apoptotic effect.

It has been proposed that novel therapeutic strategies should target the expression and function of mitogenic and antiapoptotic proteins as well as angiogenesis, causing apoptosis and/or inducing a status of tumor dormancy (39). In the same fashion it can be proposed that multiple selective inhibitors may be used to interfere with different growth-escape pathways. In this study, we have demonstrated that two AS oligonucleotides, a PS-oligonucleotide targeting bcl-2 and a hybrid MBO targeting PKAI, cooperatively inhibit the growth of and induce apoptosis in human cancer cells *in vitro*. The same compounds administered i.p. and p.o. for a short term caused a marked antitumor effect, accompanied by the inhibition of angiogenesis and the induction of apoptosis in nude mice bearing established GEO tumors.

This study represents the first demonstration that two oligonucleotides targeting two preeminent signaling pathways have a cooperative antitumor activity *in vivo* and further supports the hypothesis of a direct link between PKA and bcl-2. Because both AS RIα and AS bcl-2 have shown promising results in clinical trials and also can be used by p.o. and s.c. routes, respectively, our study provides the rationale to translate a feasible and novel treatment in cancer patients.

[4] Unpublished results.

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