

# Oral Antisense that Targets Protein Kinase A Cooperates with Taxol and Inhibits Tumor Growth, Angiogenesis, and Growth Factor Production<sup>1</sup>

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## ABSTRACT

Protein kinase A type I (PKAI) transduces mitogenic signals from different growth factors and oncogenes and is overexpressed in the majority of human cancers. We and other investigators previously have reported that different PKAI inhibitors, including antisense oligonucleotides, have antitumor activity. In this study, we used a novel hybrid DNA/RNA mixed-backbone oligonucleotide (MBO) targeting the PKAI subunit RI $\alpha$ . We demonstrated that after oral administration, the MBO antisense RI $\alpha$  inhibited the growth of human colon cancer xenografts in nude mice and showed a cooperative antitumor effect with Taxol, which outlasted treatment withdrawal and significantly prolonged survival of mice compared with untreated controls or to single-agent-treated mice. Immunohistochemical analysis of tumor specimens showed inhibition of target protein RI $\alpha$  and of growth factor expression along with a marked inhibition of angiogenesis and an increase in p27 expression. In conclusion, a novel MBO that targets PKAI, administered p.o., is effective and cooperates with the anticancer drug Taxol on both tumor growth and expression of factors involved in the control of cell proliferation, cell cycle, and angiogenesis. Because the MBO described has completed a phase I trial involving i.v. injection in cancer patients, these results provide the biological rationale of its activity after

oral administration and may be translated into a therapeutic strategy in a clinical setting.

## INTRODUCTION

PKA<sup>3</sup> plays a key role in the control of cell growth and differentiation of mammalian cells with two distinct isoforms, type I (PKAI) and type II (PKAII), which differ only in their regulatory subunits, defined RI and RII, respectively (1). It has been shown that PKAI is involved in cell proliferation and neoplastic transformation, is required for the G<sub>1</sub>→S transition in the cell cycle, and mediates mitogenic signals from different growth factors, including TGF $\alpha$  and EGF (1–3). PKAI overexpression is detected in human cancers, correlating with worse clinicopathological features in several tumor types, including colon, breast, and ovarian cancer (4–6). Conversely, PKAII is preferentially expressed in normal tissues and seems to be involved in cell growth arrest and differentiation (1). Growth factors binding to the EGFR, such as EGF, TGF $\alpha$ , and AR, play an important role in cancer pathogenesis and progression (7, 8). We have demonstrated that PKAI, through its RI $\alpha$  subunit, has a structural interaction with the ligand-activated EGFR, cooperating in the propagation to the mitogen-activated protein kinases of mitogenic signals originated and/or involving this tyrosine kinase receptor (9). For all of these reasons, PKAI is recognized as a relevant target for therapeutic intervention, and different PKAI inhibitors are now under clinical development.

Down-regulation of PKAI by different unmodified or phosphorothioate antisense oligodeoxynucleotides targeting its RI $\alpha$  subunit causes cell growth arrest and differentiation in a variety of cancer cell lines (10, 11), and has antitumor activity in nude mice (12). Modified oligodeoxynucleotides of a novel class, defined as MBOs, have been synthesized and have shown a significant improvement of pharmacokinetic and toxicological properties *in vivo* compared with phosphorothioate-oligodeoxynucleotides (13, 14). Moreover, it has been shown that MBOs administered p.o. are absorbed in the gastrointestinal tract and distributed to major organs of mice and rats (15, 16). We previously have shown that a first-series antisense RI $\alpha$  MBO, containing methylphosphonate linkages, given by an i.p. route, exerts a synergistic inhibitory effect on the growth of several human cancer cell lines when added to different cytotoxic drugs (17) and that it is able to synergize with the chimeric monoclonal antibody anti-EGFR MAb C225, which inhibits the growth of human renal cancer cells *in vitro* and *in vivo* (18).

Most recently, a novel antisense RI $\alpha$  MBO with a hybrid

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<sup>3</sup> The abbreviations used are: PKA, protein kinase A; TGF $\alpha$ , transforming growth factor  $\alpha$ ; EGF, epidermal growth factor; EGFR, EGF receptor; AR, amphiregulin; MBO, mixed-backbone oligonucleotide.

DNA/RNA structure containing 2'-*O*-methyl-ribonucleosides at the 5' and 3' ends has been synthesized. This MBO (GEM 231) has completed a phase I clinical trial in cancer patients and showed negligible toxicity (19). Moreover, it has shown good bioavailability and consistent concentrations in tumor xenografts after oral administration in nude mice (16).

In this study, we investigated the effect of the novel 2'-*O*-methyl hybrid antisense RI $\alpha$  given p.o., alone and in combination with Taxol, on the growth of human cancer xenografts and on the expression of a variety of factors involved in the control of proliferation, cell cycle, and angiogenesis.

## MATERIALS AND METHODS

**MBOs.** MBOs were kindly provided by Dr. Sudhir Agrawal (Hybridon, Inc, Milford, MA). The antisense RI $\alpha$  MBO is a hybrid oligonucleotide targeted against the NH<sub>2</sub>-terminal 8–13 codons of the RI $\alpha$  regulatory subunit of PKA (12) with the following sequence *GCGUGCCTCCTCA-CUGGC*; the control is a scramble MBO obtained by mixing all 4 nucleosides at each position. The two oligodeoxynucleotides contain phosphorothioate internucleotide linkages (regular font indicates the nucleosides flanking each position) and 2'-*O*-methyl-ribonucleoside modifications (in italics). MBOs were synthesized, identified, and purified by <sup>31</sup>P nuclear magnetic resonance, capillary gel electrophoresis, hybridization melting temperatures, and the ratio of the absorbance at 269 nm to the mass according to the protocol described previously (20).

**GEO Xenografts in Nude Mice.** Five- to 6-week-old female Balb/cAnNCrIBR athymic (nu+/nu+) mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and the mice were maintained in accordance to 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 prior to receiving injections of cancer cells. GEO human colon cancer cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, as reported previously (21). Cells (10<sup>7</sup>) were then resuspended in 200  $\mu$ l of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. into mice (21). After 7 days, when well-established tumors of  $\sim$ 0.2 cm<sup>3</sup> in diameter were detected, mice were randomized to receive different treatments. Ten mice per group were treated i.p. with Taxol and/or either antisense RI $\alpha$  or scramble MBOs as described in the "Results." In the experiments in which the MBOs were administered p.o., the daily dose of oligonucleotides was dissolved in 5% dextrose solution, and untreated mice received the 5% dextrose solution alone. Tumor volume was measured using the formula  $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$ , as reported previously (21).

**Immunohistochemical Analysis.** Formalin-fixed, paraffin-embedded tissue sections (5  $\mu$ m) were processed as reported previously (22). Reactions with the 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 hematoxylin were as described previously (22).

The following antibodies were used in this study. An anti-RI $\alpha$  monoclonal antibody (Transduction Laboratories, Lexington, KY) was used at 1:100 dilution, and an anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) was used at 1:100 dilution. An anti-TGF $\alpha$  mouse monoclonal antibody (Ab-2, 1:100 dilution; Oncogene Science, Manhasset, NY) and an anti-AR rabbit antiserum (AR56, 1:200 dilution) were used according to the method described previously (8). Each antibody was specific for TGF $\alpha$  and AR and did not cross-react with the other EGF-related peptides (8). An anti-p27 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:100 dilution.

All analyses were performed in a blind fashion. To determine the percentage of positive cells, at least 1000 cancer cells per slide were counted and scored at  $\times$ 40 magnification with a microscope.

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

**Statistical Analysis.** The Student's *t* test (24) and the Mantel-Cox log-rank test (25) were used to evaluate the statistical significance of the results. All reported *P* values 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).

## RESULTS

We investigated the antitumor activity of the antisense RI $\alpha$  MBO in nude mice bearing GEO colon cancer xenografts, using either the i.p. or the oral route of administration. When established GEO tumors of  $\sim$ 0.2 cm<sup>3</sup> were detectable, groups of 10 mice were treated i.p. with either the antisense RI $\alpha$  or the scramble MBO, at doses of 1, 5, or 10 mg/kg daily on days 7–11 and 14–18. Fig. 1 shows that antisense RI $\alpha$  i.p. caused a dose-dependent inhibition of growth up to 50% at the dose of 10 mg/kg, whereas the scramble MBO was unable to do so even at a dose of 10 mg/kg. It has been shown that the MBOs, including the antisense RI $\alpha$  used in the present study, are absorbed in the gastrointestinal tract and distributed to major organs (15, 16). Therefore, in parallel groups of 10 mice, we performed the same experiment but administered the MBOs p.o. As shown in Fig. 2, oral antisense RI $\alpha$  caused a dose-dependent inhibition of growth, which achieved  $\sim$ 60% inhibition at the dose of 10 mg/kg after two cycles of treatment compared with untreated mice, whereas the tumor volume of the mice treated with the scramble MBO was only moderately inhibited.

We reported previously that a methylphosphonate MBO antisense RI $\alpha$  administered i.p. in combination with Taxol causes a cooperative growth inhibition of GEO tumor xenografts (17). We investigated whether such cooperative effect

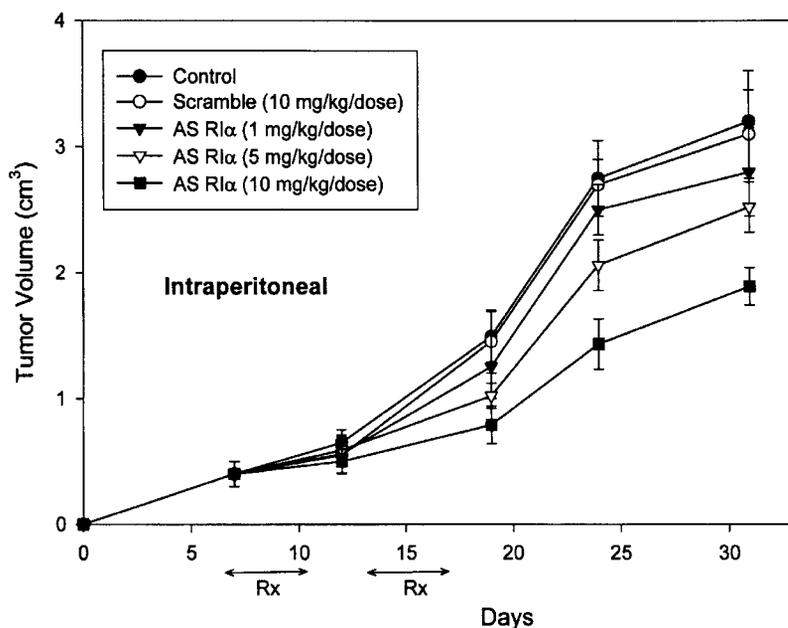
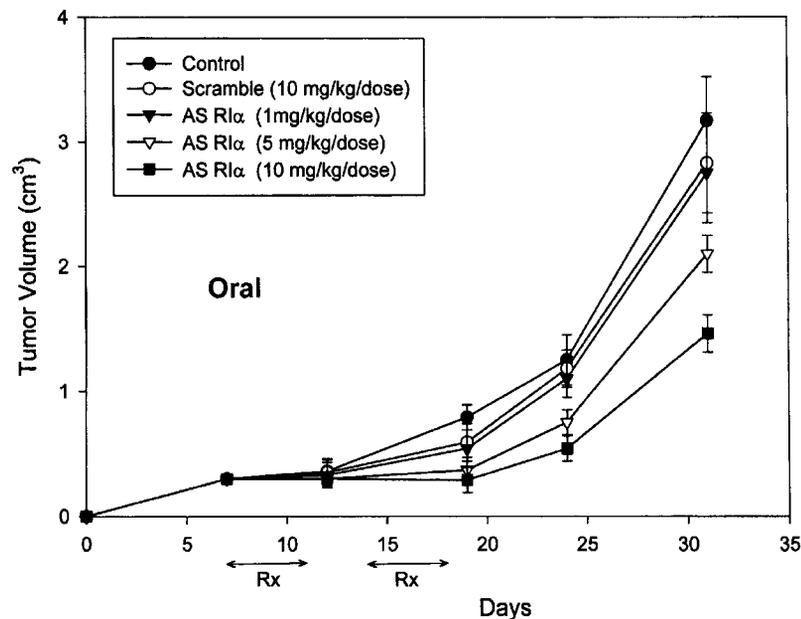


Fig. 1 Effect of i.p. administration of antisense RI $\alpha$  and scramble MBOs on GEO xenografts. Different doses of antisense RI $\alpha$  (AS RI $\alpha$ ) or scramble MBOs (Scramble) were administered daily on days 7–11 and 14–18. Each group was composed of 10 mice. Double-headed arrows represent the periods of treatment. Bars, SD.

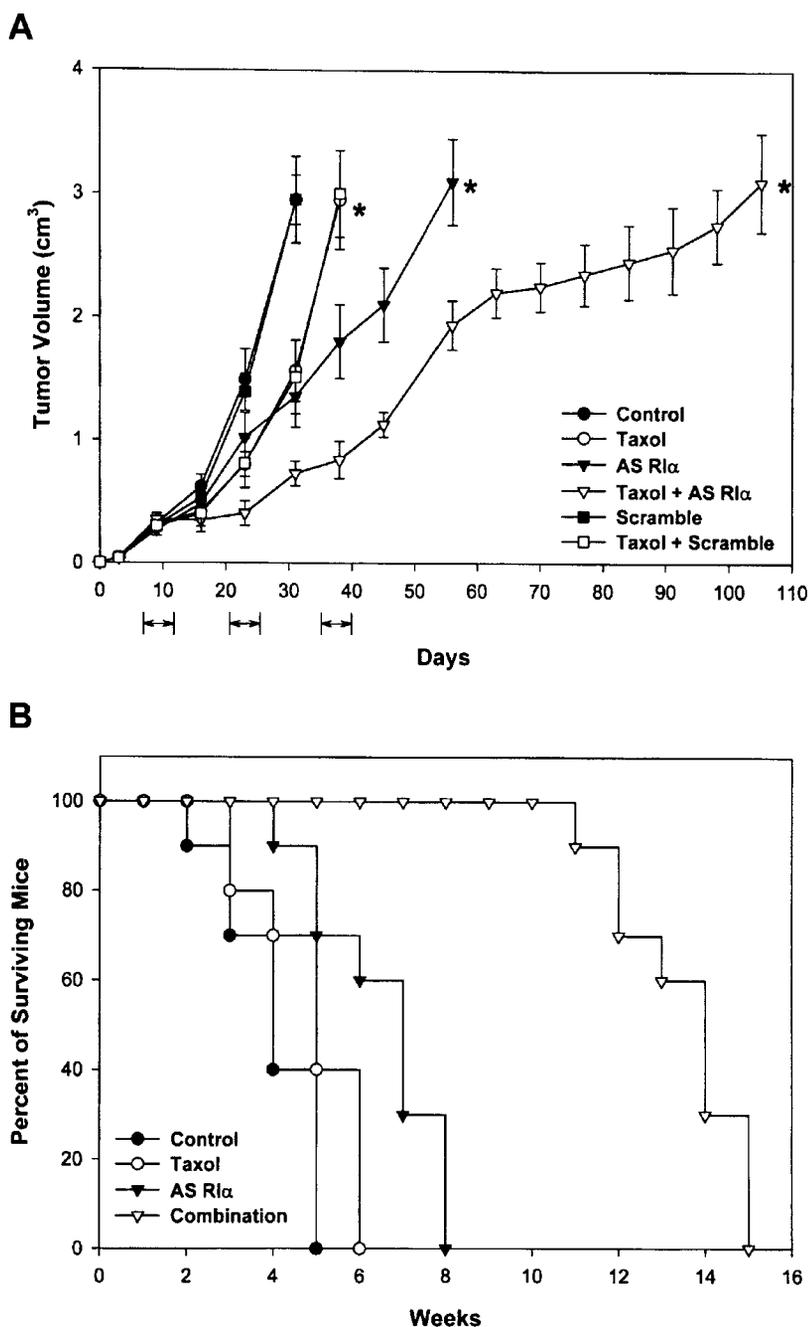
Fig. 2 Effect of oral administration of antisense RI $\alpha$  and scramble MBOs on GEO xenografts. Different doses of antisense RI $\alpha$  (AS RI $\alpha$ ) or scramble MBOs (Scramble) were administered daily on days 7–11 and 14–18. Each group was composed of 10 mice. Double-headed arrows represent the periods of treatment. Bars, SD.



with Taxol could be obtained with the novel hybrid MBO administered p.o. On day 7 after tumor cell injection, one group of 10 mice was treated with Taxol at a dose of 20 mg/kg i.p.; the treatment was then repeated every 2 weeks (days 21 and 35) for a total of three cycles. Two other groups of 10 mice were treated with either the antisense RI $\alpha$  or the scramble MBO, at a dose of 10 mg/kg p.o., daily for 5 days (days 8–12). Treatment was repeated every 2 weeks (days 22–26 and 36–40) for a total of three cycles. Two more groups of 10 mice were treated with

both Taxol and either MBO, with the Taxol administered at a dose of 20 mg/kg i.p. on day 7, followed by either antisense RI $\alpha$  or scramble oligonucleotide, administered p.o. for 5 days (days 8–12). The sequential treatment was repeated with the same schedule every 2 weeks for a total of three cycles. As illustrated in Fig. 3A, treatment with either Taxol or antisense RI $\alpha$  alone inhibited tumor growth compared with control untreated mice or mice treated with the scramble MBO. Student's *t* test was used to compare tumor volumes among different treatment groups at

**Fig. 3** Effect of Taxol and/or oral MBOs on GEO tumor growth and on mice survival. **A**, effect of Taxol, oral antisense RI $\alpha$  (AS RI $\alpha$ ), or oral scramble (Scramble) alone and in combination. Each group of 10 mice received three cycles of treatment as described in the "Results," and tumor volumes were compared among the different treatment groups at day 35 after GEO cell injection. *Double-headed arrows* represent the cycles of treatment. \*,  $P < 0.05$ . Taxol *versus* control,  $P = 0.05$ ; AS RI $\alpha$  *versus* control,  $P = 0.02$ ; Taxol plus AS RI $\alpha$  *versus* control,  $P = 0.01$ . **B**, effect on mouse 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 Taxol plus AS RI $\alpha$  ( $\nabla$ ) and control untreated mice ( $\bullet$ ;  $P < 0.0001$ ), Taxol-treated mice ( $\circ$ ;  $P < 0.0001$ ), or AS RI $\alpha$ -treated mice ( $\blacktriangledown$ ;  $P < 0.0001$ ) is significant.



day 35 after GEO cell injection. The antisense RI $\alpha$  was an effective treatment (Taxol *versus* control,  $P = 0.05$ ; antisense RI $\alpha$  *versus* control,  $P = 0.02$ ; Taxol *versus* antisense RI $\alpha$ ,  $P = 0.06$ ), causing  $>50\%$  reduction of tumor volume at the completion of the three cycles of treatment. However, shortly after the end of the treatment, GEO tumors resumed the growth rate of those in untreated mice or in mice treated with the scramble MBO. When the cytotoxic drug and the MBOs were used in combination, we observed a marked and sustained inhibition of tumor growth in mice receiving the oral antisense RI $\alpha$  in combination with Taxol (Taxol plus antisense RI $\alpha$  *versus* control,

$P = 0.01$ ; Taxol plus antisense RI $\alpha$  *versus* antisense RI $\alpha$ ,  $P = 0.03$ ), whereas administration of the scramble MBO after Taxol showed an effect similar to that of the cytotoxic agent alone (Taxol plus scramble MBO *versus* control,  $P = 0.05$ ). In fact, tumors in mice treated with Taxol and antisense RI $\alpha$  grew very slowly for  $\sim 60$  days after the end of the treatment, and then resumed a faster growth rate (Fig. 3A).

Within  $\sim 5$  weeks, GEO tumors reached a size not compatible with normal life in all untreated mice (Fig. 3B) and in mice treated with the scramble oligonucleotide. A small increase in mice survival was observed in the group treated with Taxol

Table 1 Immunohistochemical analysis of GEO tumors following treatment with Taxol and/or oral MBOs

Analysis was performed at the end of the second cycle of treatment (day 27) on three different tumor specimens randomly selected in each group. Numbers represent the percentage of positive cell staining for each antigen (see "Materials and Methods"). Numbers in parentheses represent the SD (%) in the measurement of each biological parameter. The intensity of staining is represented by a score from + to +++.

	Ki67	RI $\alpha$	AR	TGF $\alpha$	p27	Vessels
Control	40% ( $\pm$ 5) ++	70% ( $\pm$ 4) +++	85% ( $\pm$ 7) ++	50% ( $\pm$ 4) ++	10% ( $\pm$ 2) +	15 ( $\pm$ 2) +++
Taxol	20% ( $\pm$ 2) +	60% ( $\pm$ 5) +++	70% ( $\pm$ 5) ++	50% ( $\pm$ 3) ++	10% ( $\pm$ 2) +	5 ( $\pm$ 2) +
AS RI $\alpha$ MBO	28% ( $\pm$ 5) +	35% ( $\pm$ 3) +	50% ( $\pm$ 3) ++	20% ( $\pm$ 3) +	15% ( $\pm$ 3) +	3 ( $\pm$ 1) ++
Scramble MBO	30% ( $\pm$ 4) ++	60% ( $\pm$ 5) +++	85% ( $\pm$ 8) ++	50% ( $\pm$ 3) ++	8% ( $\pm$ 2) +	14 ( $\pm$ 2) +++
AS RI $\alpha$ MBO + Taxol	6% ( $\pm$ 1) +	15% ( $\pm$ 3) +	25% ( $\pm$ 3) +	30% ( $\pm$ 3) +	25% ( $\pm$ 4) ++	0
Scramble MBO + Taxol	28% ( $\pm$ 3) +	60% ( $\pm$ 5) ++	70% ( $\pm$ 5) ++	50% ( $\pm$ 5) ++	8% ( $\pm$ 2) +	7 ( $\pm$ 2) +

alone, which was similar to that of mice treated with Taxol plus the scramble oligonucleotide (data not shown). Treatment with antisense RI $\alpha$  alone also resulted in a better survival compared with the control group. The delayed GEO tumor growth observed in the group treated with Taxol plus antisense RI $\alpha$  was accompanied by a prolonged life span in mice that, analyzed with the log-rank test, was significantly different from controls ( $P < 0.0001$ ), the Taxol-treated group ( $P < 0.0001$ ), or the group treated with scramble MBO plus Taxol ( $P < 0.0001$ ). In fact, all mice treated with Taxol plus antisense RI $\alpha$  MBO were alive 10 weeks after tumor injection, representing the only animals alive at this time point. Furthermore, ~50% of mice in this group were still alive after 15 weeks. The combined treatment with Taxol and antisense RI $\alpha$  was well tolerated: no weight loss or other signs of acute or delayed toxicity were observed.

Tumor specimens from the different groups of mice were examined by immunohistochemical analysis to evaluate the expression of a variety of biological parameters. Table 1 reports the results of the analyses performed on three tumor samples, randomly selected in each group after two cycles of treatment, on day 27. Treatment with antisense RI $\alpha$  inhibited the expression of the target protein RI $\alpha$  and of Ki67, a protein related to cell proliferation (Fig. 4). When the MBO antisense RI $\alpha$  was used in combination with Taxol, the combination markedly reduced RI $\alpha$  expression and almost completely suppressed cell proliferation, as demonstrated by Ki67 staining (Table 1 and Fig. 4). Interestingly, no other treatment was able to affect RI $\alpha$  expression, not even Taxol, although it showed antitumor activity and ~50% inhibition of expression of Ki67 (Table 1). These results suggest that inhibition of RI $\alpha$  expression can be selectively obtained only by treatment with the specific antisense RI $\alpha$  MBO and, therefore, is not a consequence of growth inhibition.

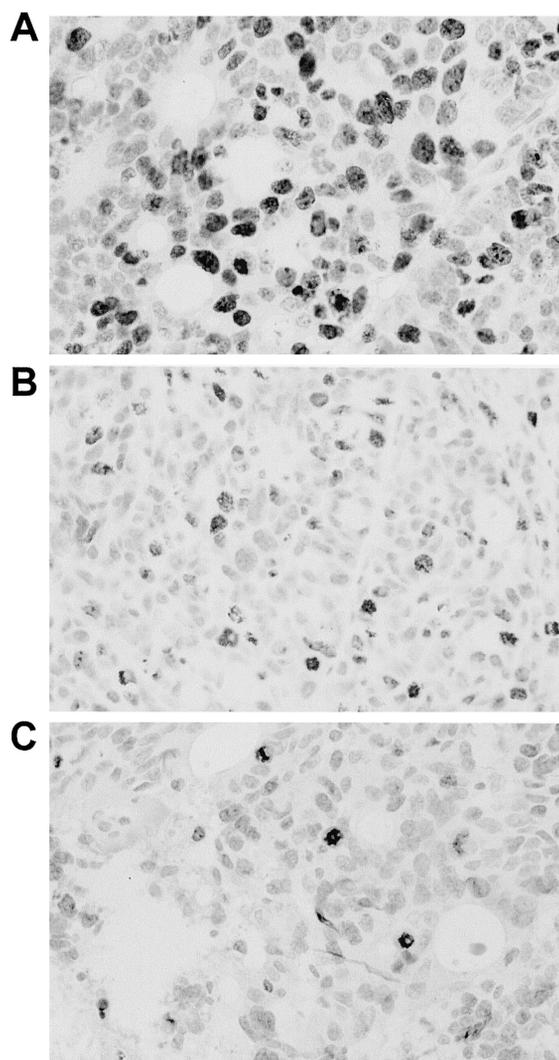
We then examined the expression of several factors that have been implicated in the growth and metastasis of colon cancer, such as TGF $\alpha$  and AR (7, 8). Unlike Taxol, treatment with antisense RI $\alpha$  inhibited the expression of TGF $\alpha$  and AR. Inhibition of AR was further enhanced when Taxol was used in combination with antisense RI $\alpha$ . Reduced expression of p27, a protein involved in cell cycle control, correlates with a worse

prognosis in colon cancer patients (26). We have demonstrated that, unlike Taxol, oral antisense RI $\alpha$  alone is able to increase p27 expression. Moreover, a 2.5-fold increase in cells staining intensely positive for p27 was observed in the tumor samples from mice treated with Taxol and antisense RI $\alpha$  (Table 1).

In recent years, the key role of tumor-induced neovascularization in neoplastic development, progression, and metastasis has been elucidated (27). In the present study, we quantified by immunohistochemistry the tumor-induced vascularization, as microvessel count, in the most intense areas of neovascularization using a monoclonal antibody for an anti-factor VIII-related antigen (23). As reported in Table 1, a significant inhibition of staining was obtained with antisense RI $\alpha$  (~80%) as well with Taxol (>60%) compared with samples from untreated mice or mice treated with the scramble MBO. Combined treatment with Taxol and antisense RI $\alpha$  completely suppressed vessel formation in GEO tumors, demonstrating that the cooperative anti-tumor effect was associated with marked inhibition of several factors that control cell cycle, proliferation, and angiogenesis of this human colon cancer model.

## DISCUSSION

The central role of PKAI in mitogenic signaling and cell cycle progression in the process of neoplastic transformation renders this kinase a relevant target for cancer growth control. Several pharmacological tools devised in the past years to inhibit PKAI expression and function, including antisense oligonucleotides that target its RI $\alpha$  subunit, have shown antitumor activity *in vitro* and *in vivo*. A novel class of modified oligodeoxynucleotides with mixed backbones, the MBOs, has demonstrated improved pharmacokinetic properties and antitumor activity *in vivo*. The most advanced of this class of oligodeoxynucleotides, a hybrid RNA/DNA MBO antisense RI $\alpha$ , contains 2-*O*-methyl-ribonucleosides at the 5' and 3' end. This compound (GEM 231) given by an i.v. route, has successfully completed a phase I study in cancer patients, with minor toxicity (19). GEM 231 was administered i.v. in a 2-h infusion twice weekly. Cumulative toxicity after 4 weeks was seen, with a grade III increase in transaminases in three of six patients at 240 mg/m<sup>2</sup> and in three of three patients at 360 mg/m<sup>2</sup>. Other



**Fig. 4** Immunohistochemical analysis of Ki67 expression in GEO tumor specimens. Analysis was performed as described in "Materials and Methods." A, untreated; B, oral antisense RI $\alpha$ ; C, Taxol plus oral antisense RI $\alpha$ . Images were taken at  $\times 40$  hps.

toxicities were negligible. Therefore, 240 mg/m<sup>2</sup> for a 4-week treatment was considered the recommended dose for phase II studies (19).

In the present study, we have shown that this compound has antitumor activity in mice bearing GEO human colon carcinoma xenografts when administered p.o.

A most promising strategy in cancer treatment is the integration of conventional drugs with novel agents able to selectively target the pathways controlling mitogenic signaling and angiogenesis. It has been proposed that the success of this strategy relies on the possibility of affecting the expression and function of the mitogenic proteins, growth factors, and angiogenic factors responsible for tumor growth and relapse, inducing a status of tumor dormancy (27). In this study, we have demonstrated that MBO antisense RI $\alpha$  administered p.o. has a cooperative antitumor effect when used in combination with

Taxol. The growth inhibitory effect is sustained, lasting  $\sim 10$  weeks after suspension of treatment and causing a remarkable increase in the survival of treated mice. We have demonstrated that the oral antisense RI $\alpha$  inhibits the expression of its target RI $\alpha$  protein in the tumor in a selective fashion. In fact, although Taxol also inhibits tumor growth and reduces expression of the proliferation-related protein Ki67, it does not affect RI $\alpha$  expression. Moreover, oral antisense RI $\alpha$  alone, or even more dramatically when used in combination with Taxol, inhibits the expression of growth factors of the EGF family, such as TGF $\alpha$  and AR, and neovascularization. It has been demonstrated that the cyclin-dependent kinase inhibitor p27 is directly related to cell entry into S phase and proliferation and that reduction of its expression correlates with a poor prognosis in colon cancer patients (26). We have shown that the combined treatment with oral antisense and Taxol increases p27 expression.

A clinically relevant aspect of this report is provided by the tumor model studied and by the schedule of drug administration used. In fact, in GEO xenograft-bearing mice, treatment was started when well-established palpable tumors were detectable, mimicking a locally advanced tumor mass. Moreover, this schedule of treatment could be translated into a clinical setting because the cytotoxic agent is administered in repeated cycles in combination with the RI $\alpha$  antisense.

Our results represent the first demonstration of the antitumor activity of a combination of a second-generation hybrid oligonucleotide that targets a mitogenic protein after oral administration and a cytotoxic drug like Taxol. Moreover, the results show that this cooperative effect can produce sustained control of cancer growth by affecting the expression of mitogenic factors, neoangiogenesis, and cell cycle controllers. These results are in agreement with the most recent observation that this MBO has antitumor activity and elevated retention in tumor tissue in different human cancer xenografts after oral administration (28). Because the antisense RI $\alpha$  MBO has completed with negligible toxicity a phase I trial (19), these data collectively represent a rationale for transferring this therapeutic strategy into a clinical setting.

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