Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12586–12591, November 1997
Medical Sciences

Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotide targeting protein kinase A

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Communicated by Paul C. Zamecnik, Worcester Foundation for Biomedical Research, Shrewsbury, MA, September 15, 1997 (received for review July 1, 1997)

ABSTRACT **Protein kinase A type I plays a key role in neoplastic transformation, conveying mitogenic signals of different growth factors and oncogenes. Inhibition of protein kinase A type I by antisense oligonucleotides targeting its RIα regulatory subunit results in cancer cell growth inhibition *in vitro* and *in vivo*. A novel mixed backbone oligonucleotide HYB 190 and its mismatched control HYB 239 were tested on soft agar growth of several human cancer cell types. HYB 190 demonstrated a dose-dependent inhibition of colony formation in all cell lines whereas the HYB 239 at the same doses caused a modest or no growth inhibition. A noninhibitory dose of each mixed backbone oligonucleotide was used in OVCAR-3 ovarian and GEO colon cancer cells to study whether any cooperative effect may occur between the antisense and a series of cytotoxic drugs acting by different mechanisms. Treatment with HYB 190 resulted in an additive growth inhibitory effect with several cytotoxic drugs when measured by soft agar colony formation. A synergistic growth inhibition, which correlated with increased apoptosis, was observed when HYB 190 was added to cancer cells treated with taxanes, platinum-based compounds, and topoisomerase II selective drugs. This synergistic effect was also observed in breast cancer cells and was obtained with other related drugs such as docetaxel and carboplatin. Combination of HYB 190 and paclitaxel resulted in an accumulation of cells in late S-G_2 phases of cell cycle and marked induction of apoptosis. A cooperative effect of HYB 190 and paclitaxel was also obtained *in vivo* in nude mice bearing human GEO colon cancer xenografts. These results are the first report of a cooperative growth inhibitory effect obtained in a variety of human cancer cell lines by antisense mixed backbone oligonucleotide targeting protein kinase A type I-mediated mitogenic signals and specific cytotoxic drugs.**

Protein kinase A (PKA), a signal-transducing protein playing a key role in the control of cell growth and differentiation, is present in mammalian cells in two distinct isoforms, type I (PKAI) and type II (PKAII) (1, 2). PKAI is directly involved in cell proliferation and neoplastic transformation (1, 3), is required for the $G_1 > S$ transition of the cell cycle (3), mediates the mitogenic signals of different growth factors including epidermal growth factor and transforming growth factor type α (4–6), and is overexpressed in the majority of human cancers, correlating with worse clinicopathological features and prognosis in ovarian and breast cancer patients (7, 8). Conversely, PKAII is preferentially expressed in normal tissues and seems to be involved in cell growth arrest and differentiation (1, 9, 10). It has been shown that the selective down-regulation of PKAI by the site-selective cAMP analog 8-Cl-cAMP leads to inhibition of cancer cell growth in a wide variety of cancer cell types *in vitro* and *in vivo* (1, 4, 5, 11, 12) and is accompanied by inhibition of expression of different oncogenes and growth factors (1, 4, 5, 11). Several studies have also demonstrated that different antisense oligodeoxynucleotides targeting the RIα subunit of PKAI expression cause cell growth arrest and differentiation in a wide variety of cancer cell lines (13, 14). A recent study has shown that an RIα antisense phosphorothioate oligodeoxynucleotide (PS-oligo) is able to inhibit the growth of human colon cancer xenografts in nude mice (15).

A large number of *in vitro* and *in vivo* studies have demonstrated that PS-oligos complementary to the mRNA of proteins involved in the process of neoplastic transformation and progression are effective in inhibiting cancer cell growth (15–21). However, toxicity studies conducted with PS-oligos in animal models and humans have shown dose-dependent side effects, which may be due to the polyanionic structure of PS-oligos and to mitogenic immune response (22–24). Presently, PS-oligos are being tested for their therapeutic potential in human clinical trials. Although PS-oligos have shown promising results as the first generation of oligonucleotides, to further improve their therapeutic potential we have studied mixed-backbone oligonucleotides (MBOs). MBOs have appropriately placed segments of PS-oligo and segments of modified oligodeoxy- or oligoribonucleotides (24). The MBO that we have used in the present study contains five methylphosphonate linkages in the middle of the PS-oligo. These centrally modified oligonucleotides have shown a significant reduction of side-effects *in vivo* compared with PS-oligos (24).

We have used a MBO targeting the RIα subunit of PKAI, alone or in combination with a series of cytotoxic drugs, to determine its antiproliferative effect *in vitro* and *in vivo* on a variety of human cancer cell lines. We have demonstrated that the RIα antisense MBO inhibits the growth of different human cancer cell lines at submicromolar concentrations and has a synergistic growth inhibitory activity with various classes of cytotoxic drugs, including taxanes, platinum-derived agents, and topoisomerase II-selective drugs. Finally, we have observed in absence of toxicity a cooperative antitumor effect of the antisense with paclitaxel in nude mice.

MATERIALS AND METHODS

Cell Lines. LS 174T and GEO human colon cancer, MDA-MB-231 and MDA-MB-468 human breast cancer and

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Abbreviations: PS-oligo, phosphorothioate oligodeoxynucleotide; MBO, mixed-backbone oligonucleotides; PKA, protein kinase A.

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OVCAR-3 human ovarian cancer cells were purchased from American Type Culture Collection. Docetaxel was a kind gift of Rhone-Poulenc Rorer (Antony Cedex, France). 5-Fluorouracil, methotrexate, cisplatin, camptothecin, doxorubicin, etoposide, paclitaxel, carboplatin, and vincristine were purchased from Sigma. All drugs were diluted in appropriate solvents and used as ×100 concentrated stock.

MBOs. The two oligonucleotides used in the study are HYB 190, GCGTGC***CTCCT***CACTGGC; [targeted against the N-terminal 8–13 codons of the RIα regulatory subunit of PKA (15)] and HYB 239, GCATGC***ATCCG***CACAGGC. HYB 190 and HYB 239 contain phosphorothioate and methylphosphonate internucleotide linkages. These linkages are identified by normal (phosphorothioate) and bold (methylphosphonate) face type for the nucleosides flanking each position. HYB 239 is a control oligonucleotide and contains four mismatched nucleosides as underlined. The two oligonucleotides have been synthesized by the protocol descibed earlier (24). The identity and purity of the oligonucleotides was confirmed by [^{31}P]NMR, capillary gel electrophoresis, hybridization melting temperature, and A_{269}/mass ratio.

Cell Growth Experiments and Antisense Treatment. LS 174T, MDA-MB-231, and MDA-MB-468 cell lines were maintained in DMEM. OVCAR-3 cells were grown in a 1:1 mixture of DMEM and Ham's-F12 medium. GEO cells were grown in McCoy medium. All media, purchased from Flow Laboratories, were supplemented with 10% heat inactivated fetal bovine serum/20 mM Hepes, pH 7.4/5 mM glutamine/100 units ml penicillin/100 μg/ml streptomycin (Flow Laboratories). Cells were maintained in a humidified atmosphere of 95% air/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 (4) and treated with different concentrations of the indicated cytotoxic drug (day 0). The HYB 190 or HYB 239 antisense RIα MBO were added after 12 h (day 1) and on days 2, 3, and 4. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Flow Cytometric Analysis of Cell Cycle by Propidium Iodide Staining. Cells seeded in monolayer in 6-well dish clusters were treated with the indicated cytotoxic drug. After 24 hrs (day 1) either HYB 190 or HYB 239 antisense RIα was added and the treatment was repeated on days 2, 3, and 4. At the indicated time points cells were harvested, fixed in 70% ethanol, stained with a propidium iodide solution (Sigma), and their DNA content was analyzed in duplicate by a FACScan flow-cytometer (Becton Dickinson) coupled with a Hewlett–Packard computer, as described (4). Cell cycle data analysis was performed by the CELL-FIT program (Becton Dickinson) (4).

Apoptosis. Flow cytometric analysis of apoptotic cell death was performed on cell pellet fixed in ethanol 70%, washed in PBS, and mixed with RNase (Sigma) and propidium iodide solution following the method reported (25). DNA content was analyzed by a FACScan flow-cytometer (Becton Dickinson) coupled with a Hewlett–Packard computer, and the percent of apoptotic cells was calculated using the LYSYS software (Becton Dickinson).

***In Vivo* Studies with Antisense in Nude Mice.** Five- to 6-week-old female BALB/c athymic (nu^+/nu^+) mice were purchased from Charles River Breeding Laboratories. The research protocol was approved and 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 being injected with cancer cells. Mice were injected s.c. with 10^7 GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 10 days, when well established tumors of approximately 0.4 cm^3 in diameter were detected, seven mice/group were treated i.p. either with paclitaxel (20 mg/kg) (26) (once, on day 1), or with the MBOs HYB 190 (10 mg/kg) or HYB 239 (10 mg/kg) (for 5 days, from day 2 to 6), or with paclitaxel in combination with either HYB 190 or HYB 239 in a sequential schedule; that is, the mice received the cytotoxic drug first (day 1), then, the oligonucleotides were administered for 5 consecutive days (day 2–6). Tumor size was measured twice weekly up to 67 days from tumor cell injection. Tumor size was measured using the formula $\pi/6 \times$ larger diameter × (smaller diameter)^2, as reported (27).

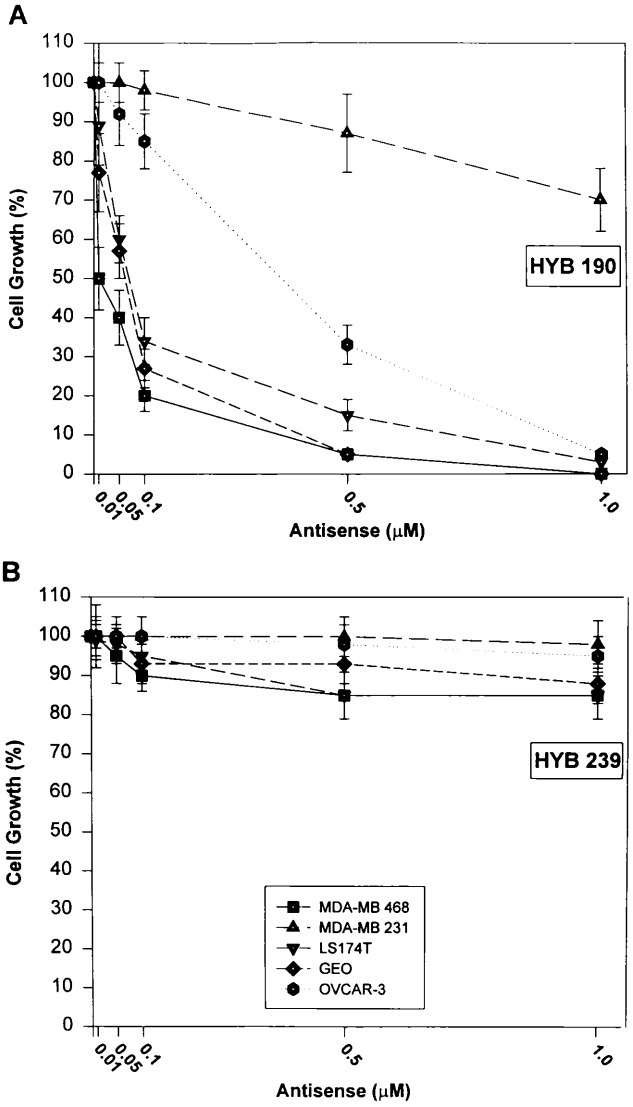

FIG. 1. Dose-dependent effect of the RIα antisense MBO HYB 190 and its control sequence HYB 239 in different cancer cell lines. (*A*) HYB 190. (*B*) HYB 239. Data represent means and standard errors of three different experiments with each performed in triplicate.

Statistical Analysis. The Student's *t* test (28) was used to evaluate the statistical significance of the results. All *P* values represent two-sided tests of statistical significance. All analyses were performed with the BMDP NEW SYSTEM statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles).

RESULTS

Effect of the RIα Antisense MBO in Cancer Cells. HYB 190, an 18-mer MBO antisense to the N-terminal 8–13 codons of the RIα subunit of PKAI, and the control HYB 239, containing four mismatched nucleotide bases, were tested to study

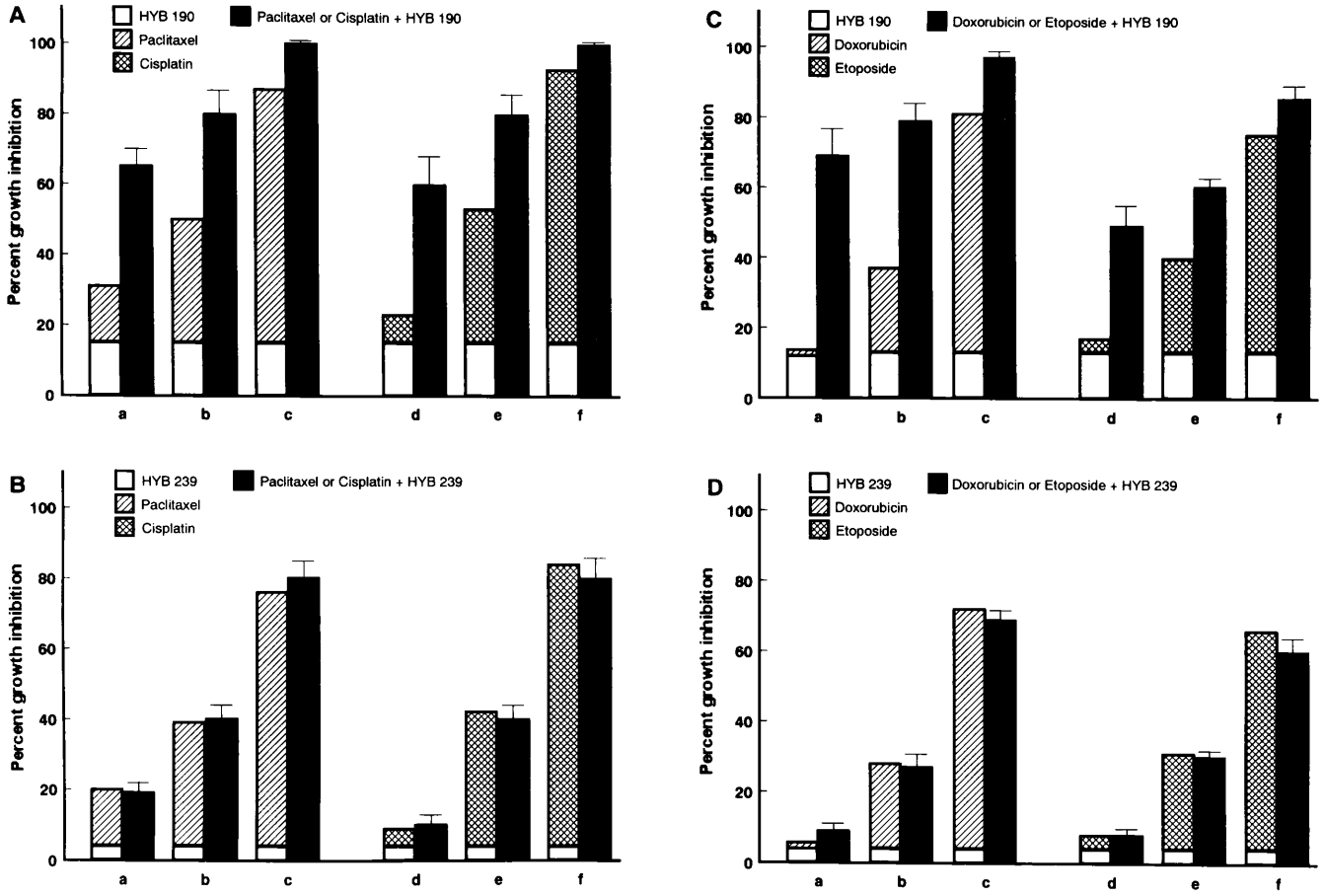

FIG. 2. Effect of different cytotoxic drugs and antisense RIα MBO on the growth of OVCAR-3 cancer cells. (*A*) HYB 190 (0.1 μM) in combination with paclitaxel or cisplatin. (*B*) HYB 239 (1 μM) in combination with paclitaxel or cisplatin. (*C*) HYB 190 (0.1 μM) in combination with doxorubicin or etoposide. (*D*) HYB 239 (1 μM) in combination with doxorubicin or etoposide. The drugs were used at the following doses: a–c, 1, 2.5, and 5 nM paclitaxel; d–f, 5, 10, and 50 ng/ml cisplatin; a–c, 0.01, 0.05, and 0.1 μg/ml doxorubicin; d–f, 0.05, 0.1, and 1 μg/ml etoposide. Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The open portion of the bars represents the percentage growth inhibition values for HYB 190 (*A* and *C*) or HYB 239 (*B* and *D*). The striped or squared portion of the bars represents the percentage growth inhibition values for the cytotoxic drugs as indicated in the respective legends. The height of the bars on the left represents the sum of the individual agents' effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs 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 standard errors of triplicate determination of at least two experiments.

their effect on the growth of OVCAR-3, GEO, LS 174T, MDA-MB-231, and MDA-MB-468 human cancer cells in soft agar. Although HYB 190 showed a dose-dependent inhibition of colony formation at doses ranging between 0.01 and 1 μM in all cell lines (Fig. 1*A*), the control MBO HYB 239 at the same doses showed a modest or no growth inhibitory effect (Fig. 1*B*). Western blot analysis after treatment with the antisense RIα MBO in OVCAR-3 ovarian and GEO colon cancer cells confirmed a dose-dependent inhibition of RIα expression in HYB 190- but not in HYB 239-treated cells (data not shown).

Effects of RIα Antisense MBO in Combination with Cytotoxic Drugs. HYB 190 at a dose of 0.1 μM showed an inhibition of OVCAR-3 ovarian cancer cell growth of approximately 15% (Fig. 1*A*), whereas HYB 239 at the same concentration caused less than 10% inhibition (Fig. 1*B*). Therefore, we selected this low dose to study whether any cooperative antiproliferative effect may occur between HYB 190 or HYB 239 and a series of cytotoxic drugs acting by different mechanisms of action in OVCAR-3 cells. When HYB 190 or HYB 239 were added to OVCAR-3 cells treated with different doses of fluorouracil (0.05–0.1 μg/ml), methotrexate (0.05–0.1 μM), vincristine (5–10 ng/ml), or camptothecin (0.05–0.1 ng/ml), which, when used as single agent, showed a growth inhibition between 20% and 60%, an additive growth inhibitory effect was observed on soft agar colony formation (data not shown). A marked synergistic effect was observed when HYB 190 was added to OVCAR-3 treated with paclitaxel, cisplatin, doxorubicin, or etoposide (Fig. 2 *A* and *C*). Conversely, HYB 239 at doses ranging between 0.1 and 1 μM had no such effect when combined with the same cytotoxic agents (Fig. 2 *B* and *D*). The cooperative antiproliferative effect was not restricted to ovarian cancer cells. In fact, when 0.01 μM HYB 190 , which caused 22% growth inhibition in GEO colon cancer, or at 0.1 μM, which inhibits 5% of the growth of MDA-MB-231 breast cancer cells, were added to paclitaxel, cisplatin, or doxorubicin, a synergistic growth inhibitory effect was observed. For example, in GEO cells, treatment with 0.01 μM HYB 190 in combination with 1 nM paclitaxel, 0.05 μg/ml cisplatin, or 0.05 μg/ml doxorubicin, which used alone showed 7%, 2%, and 25% growth inhibition, respectively, caused a 60%, 62%, and 78% inhibition of colony formation, respectively.

To study whether this cooperative effect occurs also with other drugs of the same classes, we tested the effect of HYB190 when added to OVCAR-3 cells treated with either the taxane docetaxel or the platinum derivative carboplatin. With each drug, HYB 190 caused a synergistic inhibition of growth. In fact, in cells treated with 0.5 or 1 nM docetaxel, which alone

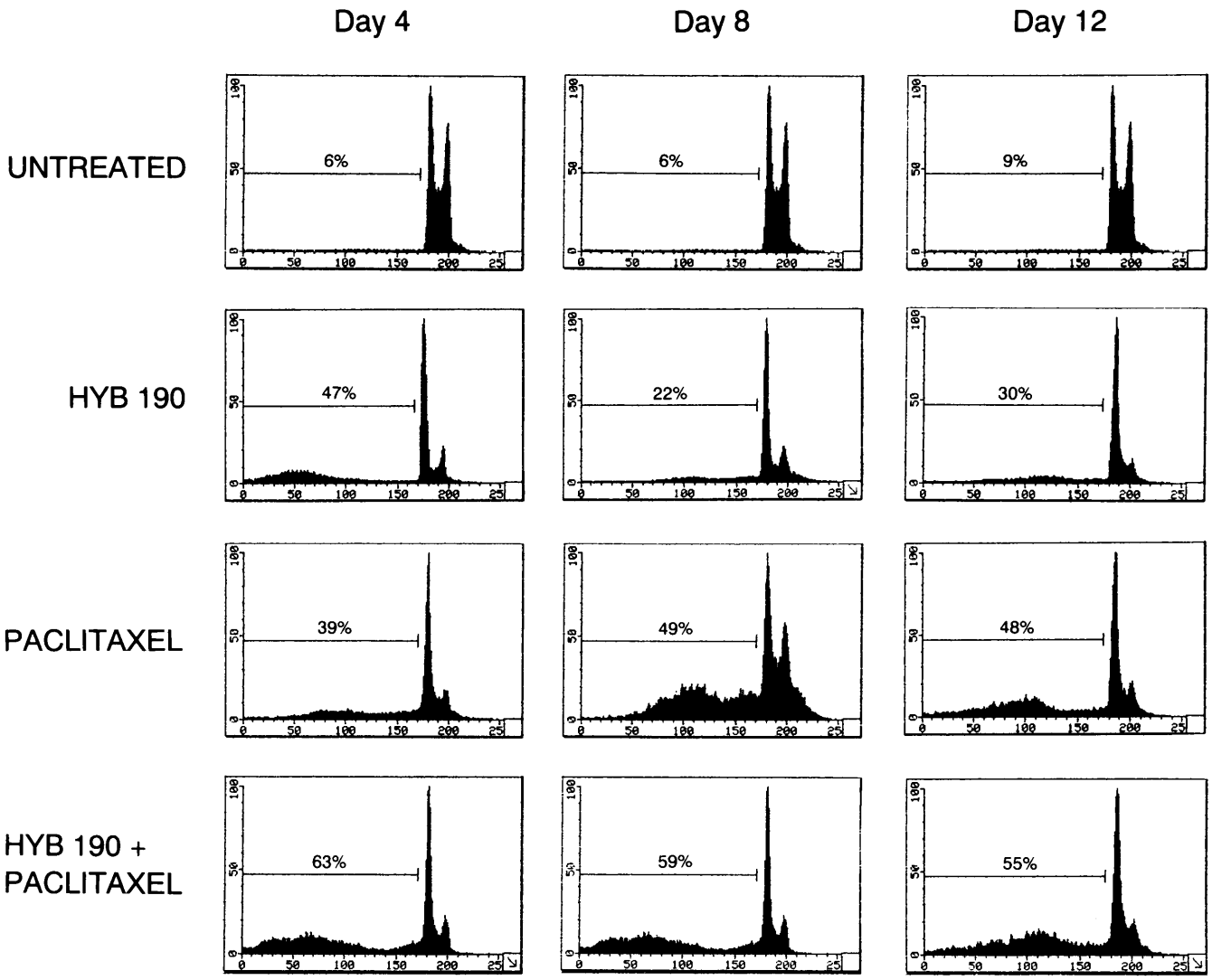

FIG. 3. Flow-cytometric analysis of the effect of 1 nM paclitaxel and/or 0.1 μM HYB 190 on the induction of apoptosis in OVCAR-3 cells. Apoptotic cells are present in the area indicated by a bar on the left side of each histogram. The numbers in each panel represent the percent of apoptotic cells calculated by flowcytometric analysis (25). Data represents one of three different experiments showing similar results.

cause 20% and 45% growth inhibition, respectively, addition of 0.1 μM HYB 190 caused a growth inhibition of 60% and 85%, respectively. An even more dramatic effect was obtained with carboplatin, where the mild growth inhibitory effect produced by this drug at 0.5 μg/ml (6%) or 1 μg/ml (11%) increased to 70% and 90%, respectively, after the addition of 0.1 μM HYB 190 to OVCAR-3 cells.

Cell Cycle Analysis and Evaluation of Apoptosis. We have analyzed the effect of the treatment with the MBOs and/or the cytotoxic drugs on the cell cycle distribution of the cancer cells. After 4 days of treatment with HYB 190, an increase of the cells in G_0/G_1 was observed at all doses between 0.05 and 0.5 μM, whereas 1 nM paclitaxel or 0.05 μM cisplatin alone moderately increased the percent of cells in G_2-M phases. Addition of HYB 190 to the cytotoxic drugs caused accumulation of cells between middle S and G_2-M phases, preventing their progression into M phase. For example, the percent of cells in S and early G_2-M phases was 41% in untreated cells, 32% in cells treated with 0.1 μM HYB 190, 76% in those treated with 1 nM paclitaxel, and 91% in cells treated with both agents. A similar cell cycle distribution was observed at 8 days and 12 days after treatment, except for the fact that the cells treated with the HYB 190 alone began to accumulate in middle S/early G_2-M phases. In the same cell population, we conducted a time-dependent analysis of apoptosis by flow-cytometry (Fig. 3). Although apoptosis was present only in 6–9% of untreated cells, a marked percent of apoptotic cells (46%) was present after 4 days of treatment with 0.1 μM HYB 190 alone and remained over 20% in the cell population examined at 8 and 12 days after treatment. Paclitaxel (1 nM) showed a high percent (about 40% or more) of apoptotic cell death throughout the time course, whereas treatment with both agents in combination always showed, at all time points examined, a percent of apoptosis higher than that obtained with each agent alone.

Effect of RIα Antisense in Nude Mice. To evaluate whether the cooperative effect observed *in vitro* could be obtained also *in vivo*, we used nude mice bearing GEO cell xenografts (27). When large GEO tumors of approximately 0.4 cm^3 were detected, seven mice/group were treated i.p. with paclitaxel (20 mg/kg, the maximum tolerated dose in mice) (26), HYB 190 (10 mg/kg/dose), and HYB 239 (10 mg/kg/dose) used as single agent, or with paclitaxel in combination with either HYB 190 or HYB 239 in a sequential schedule (Fig. 4). As compared with untreated animals, a significant delay of tumor growth was observed in mice either treated with paclitaxel (two-sided $P = 0.05$) or with antisense HYB 190 (two-sided $P = 0.05$), whereas the control oligomer HYB 239 exhibited only a mild growth inhibitory effect. A marked tumor growth inhibition was obtained in mice treated with paclitaxel plus

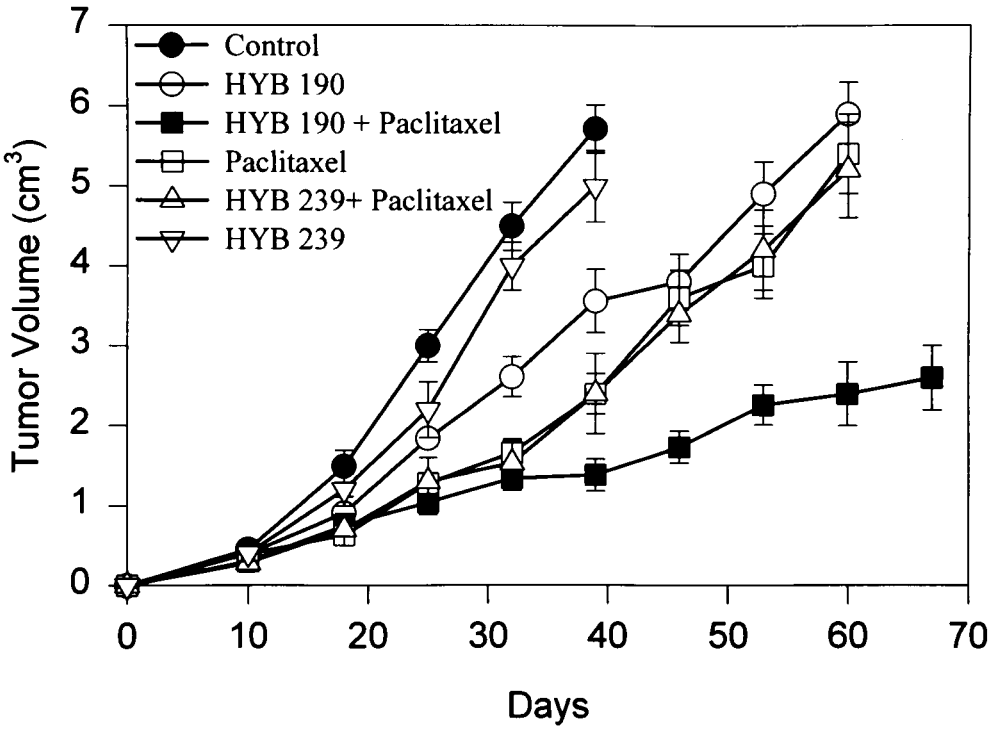

FIG. 4. Effect of the treatment with paclitaxel and/or HYB 190 on the growth of GEO human colon cancer xenografts in nude mice. The administration of each single agent alone or in sequential schedule is described in *Materials and Methods*. Paclitaxel at 400 μg/dose and HYB 190 at 200 μg/dose.

HYB 190, which was statistically significant as compared with control untreated mice (two-sided $P = 0.01$), or mice treated with either paclitaxel (two-sided $P = 0.04$) or HYB 190 (two-sided $P = 0.01$) alone. In contrast, in mice treated with paclitaxel plus the control oligomer HYB 239 the tumor growth inhibition was similar to that determined by the cytotoxic drug alone. Moreover, tumors grew to a size not compatible with normal life in all untreated mice or in those treated with HYB 239 within 40 days after tumor cell injection, and in all mice treated with either paclitaxel, HYB 190 alone, or paclitaxel plus HYB 239 within 60 days. In contrast, all mice treated with the sequential combination of paclitaxel and antisense RIα HYB 190 were still alive and tumor growth was still very slow up to 67 days after GEO cell injection. Finally, no weight loss or other signs of acute or delayed toxicity were observed in this group of mice.

DISCUSSION

Emerging novel strategies of cancer treatment are based on the selective down-regulation of specific targets involved in the process of neoplastic transformation and progression. PKAI seems to be a relevant target for such therapeutic intervention, and antisense oligonucleotides against its RIα subunit have shown promising results in inhibiting cancer cell growth *in vitro* and *in vivo* (13–15). In the present study, we have used a second generation modified oligonucleotide with mixed backbone structure (18, 24) designed to target PKAI. Such MBOs have shown better pharmacokinetic and toxicology profile *in vivo* (24). The 18-mer RIα antisense MBO HYB 190 showed a dose-dependent inhibitory effect on different human cancer cell types, including ovary, colon, and breast, at submicromolar concentrations ranging between 0.01 and 1 μM. The effect appeared to be sequence-specific, as HYB 239, a similar MBO with only four mismatched nucleotide bases, at the same concentrations was ineffective in inhibiting cancer cell growth. The HYB 190 is complementary to the same sequence of RIα targeted by an antisense oligonucleotide previously shown effective in inhibiting PKAI expression and function (15). Indeed, HYB 190, but not HYB 239, inhibited the expression of RIα protein in OVCAR-3 and GEO colon cells. To evaluate whether any cooperative effect may exist between cytotoxic drugs and a novel PKAI selective down-regulator such as the HYB 190 MBO, we tested the effect of HYB 190 or its control sequence HYB 239 in combination with cytotoxic drugs of different classes in several cancer cell lines. We have demonstrated that HYB 190 is additive when added to cells treated with the antimetabolites fluorouracil or methotrexate, the vinca alcaloid vincristine, or the topoisomerase I-selective drug camptothecin. A marked synergistic effect was observed when HYB 190 was used in combination with the taxanes paclitaxel and docetaxel, the platinum derivatives cisplatin and carboplatin, and the topoisomerase II-selective agents doxorubicin and etoposide. In contrast, no cooperative effect was obtained when the same drugs were added to the same or higher doses of control MBO HYB 239. It is likely that the inhibition of the PKAI-related mitogenic pathway plays a key role in the observed cooperative effect with the cytotoxic drugs. In fact, we have recently shown that 8-Cl-cAMP, another selective inhibitor of PKAI expression, has a synergistic growth inhibitory effect with taxanes and platinum-derived compounds in a variety of cancer cell types (29, 30).

In the attempt to understand the kinetics of these events, we analyzed the effect on cell cycle and apoptosis of the antisense compounds with or without the cytotoxic drugs. Whereas paclitaxel causes accumulation of cells in G_2-M phases, the antisense HYB 190 tends to first accumulate cells in G_0/G_1 and, later, in S/G_2-M phase. These results are consistent with our previous report that PKAI is important in the G_1-S transition (3) and with our most recent observation that PKAI, together with the surge at the G_1-S transition, has a second peak of expression at the late S/early G_2 phase (31). Along with these cell cycle perturbations, apoptosis seems to be present very early after treatment with either RIα antisense or the cytotoxic drug alone. When the antisense MBO is administered in combination with the cytotoxic drug in the sequen-

tial treatment, a marked increase in the S-G_2 phase is observed throughout the course of the experiment. Interestingly, a consistent amount of apoptotic cells, over 50%, is present for several days after treatment, suggesting that cells undergo several rounds of apoptosis. These data seem in agreement with a recent study suggesting that uncoupling between increase of S phase and inhibition of cell division is a major event occurring after treatment with certain cytotoxic drugs inducing apoptosis (32).

We next evaluated whether the cooperative effect obtained *in vitro* could also be reproduced *in vivo* in nude mice bearing GEO human colon cancer xenografts. We have shown that, unlike the control oligo HYB 239, which only mildly inhibited GEO tumor growth, HYB 190 significantly delayed tumor growth as compared with control mice. Moreover, whereas HYB 239 combined with paclitaxel had an effect similar to that of paclitaxel alone, combination of paclitaxel with HYB 190 determined a significantly increased tumor growth inhibition as compared with untreated mice or to mice treated with each single agent. In addition, all control mice or mice treated with HYB 239 died within 40 days whereas mice treated with HYB 190 alone, paclitaxel alone, or paclitaxel in combination with HYB 239 died within 60 days. In contrast, at the same time point, all animals treated with paclitaxel and HYB 190 (*i*) showed over 50% tumor growth inhibition as compared with mice treated with either agent alone, (*ii*) were alive at 67 days, and (*iii*) the tumor growth was still very slow although the combination treatment was completed 15 days after tumor cell injection. Remarkably, HYB 190 significantly increased the activity of paclitaxel but not the toxicity, considering that the taxane was used at the maximum tolerated dose in nude mice (26).

Our study reports that a relevant cooperative effect toward tumor growth inhibition can be achieved *in vitro* and *in vivo* by combining specific cytotoxic drugs with second generation oligodeoxynucleotides targeting PKAI. Moreover, we have provided evidence that the inhibitory effect correlates with a marked induction of apoptosis and does not result in increased toxicity. Experiments are ongoing to study whether the synergistic inhibitory effect correlate not only with suppression of RIα, but also with that of other relevant growth regulators such as growth factors and angiogenic factors. Taken together, these data suggest that selective down-regulation of PKAI, a key mitogenic signal transducer, is an important event able to markedly increase the antitumor efficacy of certain conventional cytotoxic drugs. As HYB 190 is a prototype of novel second generation oligodeoxynucleotides (the MBOs), which have shown an improved *in vivo* pharmacologic profile without toxicity (24), our study provides a rationale for translating this combination treatment strategy into a clinical setting.

This study was supported by the Associazione Italiana per la Ricerca sul Cancro and from the Progetto Finalizzato-Applicazioni Cliniche della Ricerca Oncologica (Consiglio Nazionale delle Ricerche).

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