Oral Administration of a Novel Taxane, an Antisense Oligonucleotide Targeting Protein Kinase A, and the Epidermal Growth Factor Receptor Inhibitor Iressa Causes Cooperative Antitumor and Antiangiogenic Activity

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ABSTRACT

Purpose: Protein kinase A type I (PKAI) and the epidermal growth factor receptor (EGFR) play a role in neoplastic transformation and interact with each other in transmitting mitogenic signals. We developed different PKAI and EGFR inhibitors, demonstrating their cooperation with cytotoxic drugs and the therapeutic potential of the combined blockade of PKAI and EGFR. In this study, we investigated the effect of orally active PKAI and EGFR inhibitors in combination with a novel taxane.

Experimental Design: We combined a hybrid PKAI antisense oligonucleotide sequence (AS-PKAI), the EGFR inhibitor ZD1839 (Iressa), and the taxane IDN5109, studying their effect on human cancer growth, apoptosis, and angiogenesis and measuring vascular endothelial growth factor (VEGF) expression and vessel formation in vitro and after oral administration in nude mice.

Results: We demonstrated cooperative growth inhibitory and proapoptotic effects and inhibition of VEGF expression with any combination of two drugs and a marked synergistic effect when all three agents were combined. Oral administration of AS-PKAI, ZD1839, and IDN5109 in combination to nude mice caused a remarkable antitumor effect with no histological evidence of tumors in 50% of mice 5 weeks after treatment withdrawal, accompanied by complete suppression of vessel formation and VEGF expression.

Conclusion: This is the first demonstration of the cooperative antitumor and antiangiogenic activity of three novel agents that block multiple signaling pathways after oral administration. Because all agents are under clinical evaluation in cancer patients, our results provide a rationale to translate this feasible therapeutic strategy in a clinical setting.

INTRODUCTION

A novel approach to cancer therapy is the integration of new cytotoxic agents with multiple selective inhibitors of relevant signaling pathways. Such a strategy indicates that it may be possible to use agents that cooperate at low doses and/or through simple administration modalities to increase tumor targeting and patient compliance and reduce toxicity. Novel inhibitors of cAMP-dependent protein kinase (PKA) and EGFR may satisfy these requirements.

PKA, which plays a key role in the control of cell growth and differentiation of mammalian cells, exists in two distinct isoforms, type I (PKAI) and type II (PKAII), which differ only in their regulatory subunits (RI and RII, respectively; Ref. 1). PKAI is mechanistically involved in cell proliferation and neoplastic transformation; is required for G1-S cell cycle transition and for the transduction of mitogenic signals from different growth factors, including TGFα and EGF; and is overexpressed in most human cancers, correlating with worse clinicopathological features in several tumor types (reviewed in Ref. 2). We have demonstrated that PKAI, through its RII subunit, structural interacts with the ligand-activated EGFR and cooperates in the propagation of the mitogenic signals originated by different growth factors and hormones (2, 3). For these reasons, PKAI is considered a relevant target for therapeutic intervention (2). Down-regu-

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1 This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), the Consiglio Nazionale delle Ricerche (CNR) Target Project on Biotechnologies, and the Regione Campania-La Ricerca sul Cancro (A.I.R.C.), the Consiglio Nazionale delle Ricerche.

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3 The abbreviations used are: PKA, protein kinase A; EGFR, epidermal growth factor receptor; PKAI and PKAII, PKA type I and II, respectively; RI and RII, regulatory subunits of PKAI and PKAII, respectively; TGFα, transforming growth factor α; AS-PKAI, antisense oligodeoxynucleotides targeting the PKAI RII subunit; MBO, mixed-backbone oligodeoxynucleotide; MAB, monoclonal antibody; VEGF, vascular endothelial growth factor; CM, conditioned medium.
lation of PKAI by different pharmacological tools, including antisense oligodeoxynucleotides targeting its RI\textsubscript{a} subunit (AS-PKAI) causes growth arrest and differentiation in a variety of cancer cell lines \textit{in vitro} and in nude mice (1, 2, 4, 5). An improvement of these effects has recently been obtained with a novel class of MBOs, which exhibit better pharmacokinetic and toxicological properties \textit{in vivo} (6). We have shown that the AS-PKAI MBOs have antitumor effects in different human cancer xenografts, synergizing with different classes of cytotoxic drugs (2, 7). A Phase I clinical trial in cancer patients with negligible toxicity has recently been completed with a hybrid DNA/RNA MBO AS-PKAI containing 2-O-methyl modifications (GEM 231; Ref. 8). We and others have recently shown that this novel AS-PKAI also exhibits a good pharmacokinetic profile and inhibits tumor growth after oral administration, potentiating Taxol activity (9, 10).

EGFR is a main transducer of mitogenic signals involved in cancer pathogenesis and progression and is considered an important target for anticancer therapy (11). Several compounds that block ligand-induced EGFR activation have been developed. ZD1839 (Iressa) is a p.o. active quinazoline derivative that selectively inhibits the EGFR tyrosine kinase and is under clinical development in cancer patients. We and others have shown that ZD1839 inhibits the growth of a variety of human cancer cell lines \textit{in vitro}, achieving a supra-additive effect when combined with different cytotoxic drugs and inducing apoptotic cell death (12, 13). Moreover, ZD1839 shows antitumor activity and prolongs survival \textit{in vivo} in nude mice when given in combination with different drugs (12, 13).

On the basis of the molecular interactions between PKAI and EGFR, we have developed a therapeutic strategy that provides a combined blockade of these two signaling pathways by different selective inhibitors; we have demonstrated the ability of these inhibitors to target the respective kinases both \textit{in vitro} and \textit{in vivo} in nude mice models (2, 10, 14). Moreover, we have shown the ability of both PKAI and EGFR inhibitors to potentiate the cytotoxic activity of drugs (2, 13). In particular, we have reported a marked cooperative effect of different PKAI inhibitors with taxanes (2, 7, 10). Additional evidence of the possible interaction among PKAI, EGFR, and targets affected by taxanes is provided by our recent demonstration that a combination of AS-PKAI with the anti-EGFR MAb C225 and docetaxel cooperatively inhibited the \textit{in vitro} growth of a human breast cancer cell line (15), providing a potential basis for the development of a novel therapeutic strategy.

On this basis, in the present study we evaluated whether a cooperative effect could be obtained by combining the hybrid MBO AS-PKAI and ZD1839, which are both p.o. active, with IDN5109 [13-(N-boc-\textbeta-iso-butylisoserinyl)-14-hydroxy-baccatin-1,14-carbonate], a novel paclitaxel analogue from a new series of 14-OH-DA\textsubscript{b} derivatives, which has shown potent antitumor activity against a variety of human cancer types, including multidrug-resistant cells (16, 17), as well as after oral administration (18, 19).

We describe the effects of these drugs on tumor growth, angiogenesis, and apoptosis of human cancer, \textit{in vitro} and \textit{in vivo}, following oral administration.

**MATERIALS AND METHODS**

**Materials.** Clinical-grade ZD1839 and IDN5109 were provided by AstraZeneca (Macclesfield, United Kingdom) and Indena S.p.A. (Milan, Italy), respectively. MBOs were kindly provided by Dr. Sudhir Agrawal (Hybridon, Inc., Cambridge, MA). The AS-PKAI is a hybrid oligonucleotide, containing 2’-O-methyl-ribonucleosides, targeted against NH\textsubscript{2}-terminal codons 8–13 of the RI\textsubscript{a} regulatory subunit of PKA (5). The control was a scrambled MBO obtained by mixing all four nucleosides at each position. The sequence, structure, and purification methods were as published previously (10, 20).

**Cell Lines.** GEO colon, OVCAR-3 ovarian, and ZR-75-1 breast human cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). GEO, OVCAR-3 and ZR-75-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 g/ml streptomycin, and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified 95% air-5% CO\textsubscript{2} atmosphere at 37°C.

**Growth in Soft Agar.** On day 0, cells (10\textsuperscript{4} cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-well cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated with the following concentrations of drugs: 0.1 and 0.5 \textmu M AS-PKAI (on days 0, 1, and 2); 0.5, 1, and 5 \textmu g/ml IDN5109 (on day 0); and 0.05, 0.1 and 0.5 \textmu M ZD1839 (on days 1, 2, and 3). After 10–14 days, cells were stained with nitroblue tetrazolium (Sigma), and colonies larger than 0.05 mm were counted as described previously (4).

**Apoptosis.** The induction of apoptosis was determined by the Cell Death Detection ELISA Plus Kit (Roche Molecular Biochemicals, Mannheim, Germany), which detects cytosolic histone-associated DNA fragments. Briefly, GEO, OVCAR-3 or ZR-75-1 cells (5 \times 10\textsuperscript{4} cells/dish) were seeded into 35-mm dishes. After treatment with 0.1 \textmu M AS-PKAI (days 0, 1, and 2)
Antitumor Effect of p.o. Taxane and PKA and EGFR Inhibitors

Inhibition if drugs have an additive effect. The height of these stacked columns also represents the expected total least two experiments.

bars data represent means and SE (bars) of triplicate determinations from at supra-additive effect was obtained and the magnitude of such effect. The column and that of the second column of each pair shows whether a combination. Therefore, the comparison between the height of the first column of each pair shows the effect obtained when the drugs were actually used in percentage of colony formation inhibition compared with untreated /H9262

and/or 5 ng/ml IDN5109 (day 0) or 0.05 μM ZD1839 (days 1, 2, and 3), on day 5 cells were plated in 60-mm dishes (Becton Dickinson) and treated for 4 days with 0.1 μM AS-PKAI, 5 ng/ml IDN5109, and 0.1 μM ZD1839 alone and in combination. Assays were performed using 24-h collected serum-free CM.

**GEO Xenografts in Nude Mice.** Female Balb/cAnNcrlBR athymic (nu/nu) mice (5–6 weeks old) were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized to the University of Naples Medical School Animal Facility for 1 week prior to receiving injections of cancer cells. GEO human colon cancer cells (10^5) were resuspended in 200 liters of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice. After 7 days, when well-established tumors of ∼0.2 cm^3 were detected, mice were randomized to receive different treatments. Groups of 10 mice were treated with p.o. AS-PKAI and/or ZD1839 daily on days 7, 11, 14, 18. Tumor volume was measured using the formula π/6 × (smaller diameter)^2, as reported previously (14). Two mice were sacrificed at day 21 for biochemical and immunohistochemical analyses.

**Immunohistochemical Analysis.** New blood vessels were detected as described previously (21, 22), with a MAb against the human factor VIII-related antigen (Dako, Milan, Italy) at 1:50 dilution and stained with a standard immunoperoxidase method (Vectastain ABC kit; Vector). Each slide was first scanned at low power (magnification, ×10–100), and the area with the highest number of new vessels was identified (hot spot) and then scanned at ×250 magnification (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 counts for the five fields. All analyses were performed in a blinded fashion.

**RESULTS**

**Dose-Response Effect of IDN5109 on Different Cancer Cell Lines.** We evaluated the antitumor activity in vitro of the novel taxane IDN5109 on the soft agar growth of a variety of human cell lines, including GEO colon, OVCAR-3 ovarian, and ZR-75-1 breast cancer cells. As shown in Fig. 1, IDN5109 caused dose-dependent growth inhibition of all tested cells, with
an IC₅₀ demonstrating higher sensitivity for ZR-75-1 and OVCAR-3 compared with GEO colon cells.

Cooperative Effect of the Different Agents in Combination. When we combined the AS-PKAI with different doses of IDN5109, a supra-additive effect was observed in all cell lines, particularly with lower doses of AS-PKAI (Fig. 2). In contrast, no cooperative effect was observed when the IDN5109 was combined with an oligonucleotide with a scrambled sequence.

We previously demonstrated the growth-inhibitory activities of AS-PKAI and ZD1839 as single agents on GEO, OVCAR-3, and ZR-75-1 cells (7, 13). We evaluated the effect of combinations of different doses of the two agents on the soft agar growth of these human cancer cells. Fig. 3 shows a clearly supra-additive inhibitory effect on GEO, whereas a cooperative effect with low doses and a mostly additive effect with higher doses were observed for OVCAR-3 and ZR-75-1 cells, probably because ZD1839 is extremely effective as a single agent in ovarian and breast cells. Unlike the AS-PKAI, the combination of a scrambled-sequence oligonucleotide with ZD1839 caused, at most, an additive effect in all cell lines. We then combined different doses of the taxane IDN5109 with ZD1839. A clearly cooperative growth-inhibitory effect was observed at all tested doses in the three cell lines (Fig. 4).

On the basis of our former hypothesis of potential cooperativity between the three drugs (18), we combined the three agents together, obtaining a dramatic synergistic inhibitory effect on the soft agar growth of GEO, OVCAR-3, and ZR-75-1 cancer cells. In fact, low doses that alone induced up to 10–15% inhibition of colony formation caused almost complete suppression of growth (Fig. 5).

Cooperative Effect of the Combinations on Apoptosis. We studied the effect of the combination of these agents on the induction of apoptosis in vitro. We used suboptimal doses of AS-PKAI and ZD1839 (unable to induce apoptosis) and a dose of IDN5109 that caused up to 3-fold induction of apoptosis compared with untreated cells. When any two drugs were com-

Fig. 3 Effect of the combination of AS-PKAI or scrambled MBO with ZD1839 on the soft agar growth of GEO, OVCAR-3, and ZR-75-1 cells. Column pairs: a–c, 0.1 μM AS-PKAI; d–f, 0.5 μM AS-PKAI; g–i, 0.5 μM scrambled MBO; a, d, and g, 0.05 μM ZD1839; b, e, and h, 0.1 μM ZD1839; c, f, and i, 0.5 μM ZD1839. Graphic representation as in Fig. 2. The data represent means and SE (bars) of triplicate determinations from three experiments.

Fig. 4 Effect of the combination of ZD1839 and/or IDN5109 on the soft agar growth of GEO, OVCAR-3, and ZR-75-1 cells. Column pairs: a–c, 0.05 μM ZD1839; d–f, 0.1 μM ZD1839; a and d, 0.5 ng/ml IDN5109; b and e, 1 ng/ml IDN5109; c and f, 5 ng/ml IDN5109. Graphic representation as in Fig. 2. The data represent means and SE (bars) of triplicate determinations from three experiments.
combined, a mostly additive effect was observed (Fig. 6). A clearly supra-additive effect was achieved in all cell lines tested when the three drugs were combined (Fig. 6), in agreement with the marked antiproliferative effect observed with the same combination.

**Effect of the Combinations on VEGF Production.** The endogenous levels of the angiogenic growth factor VEGF were measured in the CM of GEO cells. Untreated GEO cells secreted \( \sim 150 \) pg VEGF/10\(^6\) cells. Treatment with either AS-PKAI or ZD1839 alone caused \( \sim 25\% \) inhibition of VEGF secretion, whereas IDN5109 was ineffective. Combination of AS-PKAI with either ZD1839 or IDN5109 caused \( \sim 50 \) and \( \sim 30 \)\% inhibition of VEGF secretion, respectively, whereas IDN5109 and ZD1839 together caused \( \sim 40\% \) inhibition. A cooperative inhibitory effect of almost 75\% was observed when the three agents were used together, reducing the VEGF secretion to 36 pg/10\(^6\) cells.

**Effect on the Growth of Tumor Xenografts.** We investigated the antitumor activity of AS-PKAI, ZD1839, and IDN5109 administered p.o. alone and in combination in nude mice bearing GEO colon cancer xenografts. When established GEO tumors of \( \sim 0.2 \) cm\(^3\) were detectable, groups of 10 mice were treated with AS-PKAI, IDN5109, and ZD1839 alone and in combination. Two mice were sacrificed on day 21 for biochemical and histochemical analyses; tumor growth studies were therefore performed on the remaining eight mice. As shown in Fig. 7, within \( \sim 5 \) weeks, GEO tumors reached a size not compatible with normal life in all untreated mice, and treatment with the IDN5109 had only a small inhibitory effect. On the other hand, AS-PKAI and ZD1839 caused a similar inhibition and delay of tumor growth. However, shortly after the end of treatment, these tumors resumed a growth rate similar to that in untreated mice. When we treated the animals with two agents in combination, a remarkably similar effect was obtained with any two drugs tested, causing a significant inhibition of growth. In fact, treated tumors grew very slowly for almost 3 weeks after withdrawal of treatment; they then resumed a faster growth rate (Fig. 7). As shown in Table 1, at this time point, no more than 25\% of animals were tumor free. At the end of the experiment, 10 weeks after tumor cell injection, all animals were still alive in all groups treated with two drugs in combination, but only one of the eight mice in each of the groups treated with the AS-PKAI in combination with either IDN5109 or ZD1839 was tumor free after pathology examination. A dramatic and sustained inhibitory effect was obtained by the combination of three drugs together. In fact, no tumor growth was observed for 3 weeks after treatment withdrawal, and a modest increase in tumor size was recorded at the end of the experiment, 10 weeks after tumor cell injection (Fig. 7). Moreover, at this time point, pathological evaluation showed that five of eight mice were still tumor free (Table 1). The combined treatment with any two drugs or with all three drugs together was well tolerated; no weight loss or other signs of acute or delayed toxicity were observed.

**Effect of Treatment on Angiogenesis in Vivo.** In the present study, 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 (21). At day 21, two tumor samples were analyzed for each group of mice. As reported in Fig. 7, their volumes were similar to that measured at the same time point in
DISCUSSION

In recent years, several molecular targets relevant for cancer cell growth have been identified, and consequently, different selective inhibitors have been developed. The tyrosine kinase growth factors receptors, such as EGFR, and the protein kinases that transduce intracellular signaling, such as PKA, play an important and complex role in the control of neoplastic growth, apoptosis, and angiogenesis (2). We have previously demonstrated that PKAI and EGFR have structural and functional interaction and that a combined blockade of these two pathways have therapeutic implications (2, 3, 14).

We have also demonstrated a cooperative effect of taxanes with PKAI and EGFR inhibitors, implying the possibility that these cytotoxic drugs may affect related molecular targets (2, 7, 13, 15).

To translate this hypothesis in an experimental setting, in this study we used three novel agents with peculiar properties, including oral activity: a hybrid DNA/RNA MBO, AS-PKAI; the selective EGFR tyrosine kinase inhibitor ZD1839; and the novel taxane IDN5109. All of these agents are under clinical evaluation in cancer patients.

In this study we demonstrated, in different human cancer cell types, that the combined use of AS-PKAI, ZD1839, and IDN5109 has a cooperative growth-inhibitory effect, achieving maximal activity when the three agents are used together, even at very low doses. Moreover, the in vitro antitumor effect is paralleled by increased induction of apoptosis and by inhibition of VEGF secretion by these drugs in combination, supporting the evidence of a role for PKAI and EGFR in the apoptotic and angiogenic pathways.

We translated these results in vivo in nude mice bearing GEO xenografts, using oral administration for all of the agents tested. We used moderately effective doses of AS-PKAI and ZD1839 and a suboptimal dose of IDN5109 to determine any cooperative effect among the drugs used in combination. We showed that any two drugs used in combination have a similar additive effect, supporting the hypothesis of a functional interaction among all of the targets specifically blocked by these agents. A marked cooperative effect was obtained when the three agents were used together. In fact, no tumor growth was observed at the end of the experiment, on day 70 after tumor injection, and several weeks after treatment withdrawal. Moreover, >50% of mice were still tumor free at this time point.

In recent years, a large body of evidence has emphasized the key role of tumor-induced neovascularization in neoplastic development, progression, and metastasis (23). EGFR may provide a major contribution to the development of neoangiogenesis (24, 25). In fact, EGFR ligand TGFα has been identified as a positive regulator of angiogenesis because it is secreted by cancer cells to stimulate normal endothelial cell growth through paracrine mechanisms (26). Moreover, EGF and TGFα can up-regulate the production of VEGF in human cancer cells at the transcriptional level (27, 28). We have previously shown that the anti-EGFR MAb C225 is able to inhibit the expression of TGFα, VEGF, and basic fibroblast growth factor in human GEO colon cancer cells and that it has a marked cooperative effect with an antisense oligonucleotide targeted to human VEGF (14, 29). More recently, we have demonstrated that ZD1839 is able to inhibit the expression of TGFα, basic fibroblast growth factor, and VEGF in a large variety of human cancer cell lines, including GEO, OVCAR-3, and ZR-75-1, and that this effect is independent of the EGFR expression levels (30).

We have shown that PKAI is also involved in angiogenesis because its selective inhibitor 8-Ci-cAMP or oral AS-PKAI inhibits the production of angiogenic growth factors (31). The antiangiogenic properties of taxanes have also been demonstrated (32).

In this study we found a cooperative effect of AS-PKAI, ZD1839, and IDN5109 in combination on the in vitro secretion of VEGF. More importantly, we demonstrated in nude mice that any two drugs used at very low doses in combination reduce the VEGF secretion of GEO tumor xenografts, supporting the hypothesis of a direct interference of the respective drugs on the angiogenic signaling pathways.
The possibility of a direct interplay of the different drugs on common targets is further supported by an intriguing recent study showing the direct association of PKAI with microtubules and its role in the organization of the mitotic spindle (33), thus providing a potential explanation for the potent interaction of PKAI inhibitors and taxanes.

A recently proposed effective therapeutic strategy, defined metronomic dosing, is based on the administration of continuous low doses of cytotoxic drugs to target tumor angiogenesis and cell proliferation without toxicity (34, 35). Such a strategy implies that the blockade of mitogenic and angiogenic molecules may play a critical role in the treatment and long-term control of cancer. In agreement with this approach, we have here shown that the use of nontoxic doses of different selective agents targeting molecules involved in mitogenic signaling, angiogenesis, and apoptosis can inhibit tumor growth and angiogenesis, thereby inducing apoptosis.

Ryan and Chabner (36) recently proposed the receptor tyrosine kinases as the most rational and promising targets for drug development and that the ultimate value of their selective inhibitors is through integration with cytotoxic drugs and/or radiotherapy. Our results not only confirm the relevance of such a strategy, but also demonstrate that further development and benefit may be provided by agents also active by oral administration. Because all of the agents are under clinical development, we believe that this study provides a strong rationale to translate this feasible therapeutic strategy into a clinical setting.

**ACKNOWLEDGMENTS**

We acknowledge the excellent technical assistance of Gaetano Borriello.

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