Novel Toll-Like Receptor 9 Agonist Induces Epidermal Growth Factor Receptor (EGFR) Inhibition and Synergistic Antitumor Activity with EGFR Inhibitors

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Abstract

Purpose: Immunostimulating Toll-like receptor 9 (TLR9) agonists cause antitumor activity interfering also with cancer proliferation and angiogenesis by mechanisms still incompletely understood. We hypothesized that modified TLR9 agonists could impair epidermal growth factor receptor (EGFR) signaling and, by this means, greatly enhance EGFR inhibitors effect, acting on both the receptor targeting and the immunologic arm.

Experimental Design: We used a novel second-generation, modified, immunomodulatory TLR9 agonist (IMO), alone and in combination with the anti-EGFR monoclonal antibody cetuximab or tyrosine kinase inhibitor gefitinib, on the growth of GEO and cetuximab-resistant derivatives GEO-CR colon cancer xenografts. We have also evaluated the expression of several proteins critical for cell proliferation, apoptosis, and angiogenesis, including EGFR, mitogen-activated protein kinase, Akt, bcl-2, cyclooxygenase-2, vascular endothelial growth factor, and nuclear factor-κB.

Results: IMO inhibited GEO growth and signaling by EGFR and the other proteins critical for cell proliferation and angiogenesis. IMO plus the anti-EGFR antibody cetuximab synergistically inhibited tumor growth, signaling proteins, and microvessel formation. EGFR signaling inhibition by IMO is relevant because IMO cooperated also with EGFR tyrosine kinase inhibitor gefitinib in GEO tumors, while it was inactive against GEO-CR xenografts. On the other hand, IMO boosted the non-EGFR-dependent cetuximab activity, causing a cooperative antitumor effect in GEO-CR cells. Finally, combination of IMO, cetuximab and chemotherapeutic irinotecan eradicated the tumors in 90% of mice.

Conclusion: IMO interferes with EGFR-related signaling and angiogenesis and has a synergistic antitumor effect with EGFR inhibitors, especially with cetuximab, boosting both the EGFR dependent and independent activity of this agent. Moreover, this therapeutic strategy could be translated in patients affected by colorectal cancer.

Unmethylated CpG dinucleotides exhibit a wide range of immune-related effects, including activation of macrophages, dendritic cells, and natural killer cell lytic activity (1–3), and potent induction of (*a*) the secretion of cytokines, such as interleukin-12, interleukin-6, tumor necrosis factor-α, and type 1 IFNs, and (*b*) the up-regulation of costimulatory molecules (1–4). These events occur mainly through Toll-like receptor 9 (TLR9), a transmembrane protein of the TLR family that recognizes CpG DNA and triggers a cascade of intracellular signaling events (5). Phosphorothioate-modified CpG oligonucleotides may have advantages in the activation of immune cells and escape rapid degradation by ubiquitous nucleases present in cells (6).

CpG DNA has shown antitumor activity (7–9) and ability to enhance the topoisomerase I–selective drug topotecan and radiotherapy (10, 11) and the antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 12). For these reasons, several early clinical studies are ongoing in patients affected by different types of cancer and are evaluating the potential cooperative activity of CpG DNA in combination with anti-CD20 antibody rituximab in non-Hodgkin's lymphoma and with anti-erbB-2 antibody trastuzumab in breast cancer patients (13).

Second-generation immunomodulatory oligonucleotides (IMO) containing 3′-3′-attached novel structures (immunomers)

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and synthetic immunomodulatory CpR (R = 2′-deoxy-7-dezaguanosine) dinucleotides have shown a remarkable antitumor activity in a broad spectrum of tumor models through induction of strong Th1 immune responses (6, 14, 15). The rationale behind using 3′-3′-attached DNA structure is that TLR9 reads DNA sequence from the 5′-end and that an accessible 5′-end is required for its recognition (16). IMOs contain two accessible 5′-ends and have shown to induce higher levels of immune responses compared with conventional CpG DNAs (14, 16–18). Previous studies have shown that IMOs induce immune responses through activation of TLR9 but not of other TLRs, including TLR3, TLR7, or TLR8, in HEK293 cells transfected with TLR9 (18). Moreover, removal of CpG sequence does not allow the activation in HEK293 cells expressing TLR9 (18). The IMO used in the present study contains a mouse-specific immunostimulatory sequence cross-reacting with human TLR9 and has shown enhanced antitumor activity in mice bearing melanoma and colon cancer xenografts and ability to cooperate with conventional chemotherapeutics, such as doxorubicin and taxol (14, 15). A phase I study with a human analogue of this IMO, IMOxine, is completed in healthy volunteers and cancer patients and a phase II study is now ongoing.

Epidermal growth factor receptor (EGFR) plays a pivotal role in the control of cell growth, apoptosis, and angiogenesis; is overexpressed in the majority of human carcinomas; is associated with resistance to cytotoxic drugs and hormone therapy; and is generally an indicator of poor prognosis (19, 20). For these reasons, the blockade of the EGFR-driven autocrine pathway has been successfully proposed as a target for anticancer therapy (19–21). The chimeric anti-EGFR monoclonal antibody (mAb) cetuximab has been proven very active, alone and in combination with chemotherapy and radiotherapy, in several types of cancer, particularly colorectal cancer (22, 23).

Little information is available on the ability of CpG DNA to affect signaling proteins involved in tumor growth and angiogenesis, especially those related to EGFR pathway. We have hypothesized that the novel TLR9 agonist IMO may interfere with EGFR-dependent signaling. If this is the case, IMO could greatly enhance the activity of EGFR antagonists, including cetuximab, with a EGFR-dependent mechanism, additional to the possible boost of ADCC, which has been shown previously that be an important component of the antitumor activity of cetuximab (24, 25).

To this aim, we have investigated the effect of IMO, cetuximab and another EGFR antagonist, on EGFR and a set of proteins directly involved in the control of cell proliferation, apoptosis, and angiogenesis and on the growth of GEO human colon cancer xenografts and an established derivative resistant to cetuximab (GEO-CR; ref. 26). Finally, we have evaluated the antitumor activity of IMO in combination with cetuximab and the topoisomerase I–selective drug irinotecan, reproducing the regimen that has shown recently a relevant activity in colorectal cancer patients (22, 23).

Materials and Methods

Drugs. IMO, 5′-TCTGACRTTCT-X-TCTTRCAGTCT-5′ (X and R are glycerol linker and 2′-deoxy-7-deazaguanosine, respectively), was synthesized with phosphorothioate backbone, purified, and analyzed as described previously (14, 16). The mAb anti-EGFR cetuximab was kindly provided by Dr. H. Waksal (ImClone Systems, New York, NY). Irinotecan was kindly provided by Pharmacia (St. Louis, MO).

Cell cultures. GEO and GEO cetuximab-resistant (GEO-CR; ref. 26) colon cancer cells were maintained DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mmol/L HEPES (pH 7.4), penicillin (100 IU/mL), streptomycin (100 μg/mL), and 4 mmol/L glutamine (ICN, Irvine, CA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell growth assay. On day 0, 10^4 cells per well were plated in 24-multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated on days 0 to 2 with doses of IMO ranging from 0.1 to 5 μmol/L. Cells were counted on days 3 and 5 by a hemocytometer.

Western blot analysis. Total cell lysates were obtained from cell line lysates or homogenized tumor specimens removed on day 25. The protein extracts were resolved by 4% to 15% SDS-PAGE and probed with anti-human, polyclonal Akt, monoclonal phosphorylated Akt (pAkt), monoclonal phosphorylated mitogen-activated protein kinase (pMAPK), and polyclonal cyclooxygenase-2 (Cell Signaling Technologies, Beverly, MA); monoclonal actin (Sigma-Aldrich, Milan, Italy), monoclonal EGFR (Lab Vision, Fremont, CA); monoclonal vascular endothelial growth factor (VEGF), monoclonal MAPK, monoclonal transforming growth factor-α, and monoclonal bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal phosphorylated EGFR and polyclonal KDR (Upstate, Lake Placid, NY); and monoclonal TLR9 (Imgenex, San Diego, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) as described previously (26).

Electromobility shift assays. To analyze nuclear factor-κB DNA-binding activity, total cell extracts from tumors removed on day 25 were prepared according to the method published previously (27).

Xenografts in nude mice. Five-week-old BALB/c AnNCrlBR athymic (nu^+/nu^+) mice (Charles River Laboratories, Milan, Italy) were maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee and in accordance to the Declaration of Helsinki. Wild-type GEO or GEO-CR human colon cancer cells (10^7 per mice) were resuspended in 200 μL Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice. After 7 days, tumors were detected and groups of 10 mice were randomized to receive the following treatments (i.p. IMO 1 mg/kg thrice weekly for 4 weeks, i.p. cetuximab 10 mg/kg twice weekly for 3 weeks, oral gefitinib 125 mg/kg, i.p. irinotecan 50 mg/kg once weekly for 3 weeks, or the combination of these agents) on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Tumor volume was measured using the formula: $\pi / 6 \times \text{larger diameter} \times (\text{smaller diameter})^2$ as reported previously (28). Two mice were sacrificed on day 25 to perform biochemical analysis.

Immunohistochemical analysis. Immunocytochemistry was done on formalin-fixed, paraffin-embedded tissue sections (5 μm) of GEO xenografts. Sections were processed, reacted with avidin-conjugated horseradish peroxidase H complex, and stained as described previously (28). An anti-Ki-67 mAb (clone MIB1, DBA, Milan, Italy) was used at 1:100 dilution. To determine the percentage of positive cells, at least 1,000 cancer cells per slide were counted and scored. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. New blood vessels were detected using a mAb against the CD34 antigen (DAKO, Milan, Italy) at the dilution of 1:50 and stained with a standard immunoperoxidase method (Vectastain ABC kit). Each slide was scanned at low power (×10-×100 magnification) and the area with the higher number of new vessels was identified (hotspot). This region was then scanned at ×250 microscope magnification (0.37 mm^2). The number of microvessels per field was scored by averaging five field counts of two individual tumors for each group. Intraepithelial tumor lymphocytes were detected with an anti-CD8 mAb (DAKO). Two independent observers quantified the number of lymphocytes.

Statistical analysis. The Student's *t* test and the Mantel-Cox log-rank test were used to evaluate the statistical significance of the results. All

reported *P*s were two sided. All analyses were done with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

Results

IMO combined with cetuximab causes potent antitumor activity. BALB/c nude mice xenografted with GEO tumors were treated with IMO or cetuximab alone and in combination (Fig. 1). On day 56, 8 weeks after tumor injection, all untreated mice reached the maximum allowed tumor size of ~2 cm^3. Mice treated with IMO alone reached that size 12 weeks after tumor injection, gaining 4 weeks of survival compared with untreated animals. Treatment with cetuximab produced a more potent effect, as tumors reached the maximum size 15 weeks after tumor cell injection, 3 weeks longer than the group treated with IMO. When the IMO and cetuximab were used in combination, a potent cooperative antitumor activity was observed, because 11 weeks after treatment withdrawal a marked tumor growth inhibition was still evident and tumors just started to recover their growth (Fig. 1A). No treatment-related side effects were observed. Comparison of tumor sizes among different treatment groups as evaluated by the Student's *t* test were statistically significant (Fig. 1A). The median survival of mice treated with IMO or cetuximab was 9 and 13 weeks, respectively, compared with 6 weeks of control mice (Fig. 1B). Differences among the groups were calculated by log-rank test (Fig. 1B). IMO plus cetuximab did not reach a median survival, because at the end of the experiments 80% of mice were still alive.

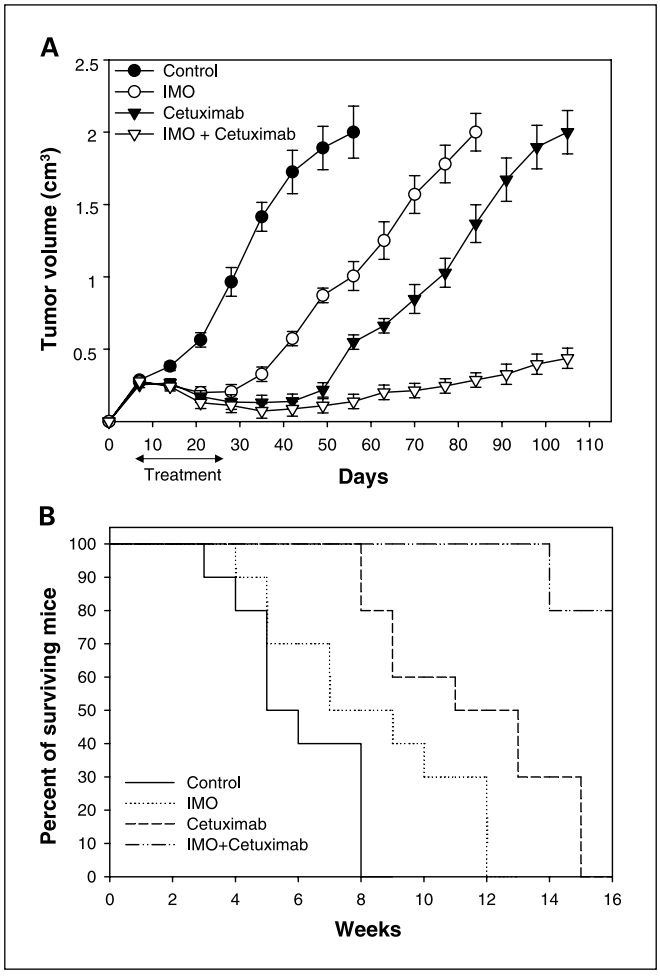

Fig. 1. Cooperative effect of IMO and cetuximab on tumor growth and survival of mice bearing human colon cancer xenografts. *A,* after 7 days following tumor injection, 10 mice were randomized to receive the following treatments (i.p. IMO 1 mg/kg thrice weekly for 4 weeks, i.p. cetuximab 10 mg/kg twice weekly for 3 weeks, or the combination of these agents) on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Two mice were sacrificed at day 25 to perform biochemical and histochemical analyses. The Student's *t* test was used to compare tumor sizes among different treatment groups at day 56 following GEO cell injection. Statistically significant difference for IMO + cetuximab versus control (two-sided $P < 0.0001$), versus IMO alone (two-sided $P < 0.0001$), and versus cetuximab alone (two-sided $P < 0.0001$). *B,* mice survival. Median survival was 6 weeks in untreated mice, 9 weeks in IMO-treated group (log-rank test versus controls; $P < 0.0001$), and 13 weeks in the cetuximab treated group (log-rank test versus controls; $P < 0.0001$; log-rank test versus IMO; $P < 0.0001$). No median survival could be calculated for IMO + cetuximab group, because at the end of the experiments 80% of mice were still alive. Bars, SD.

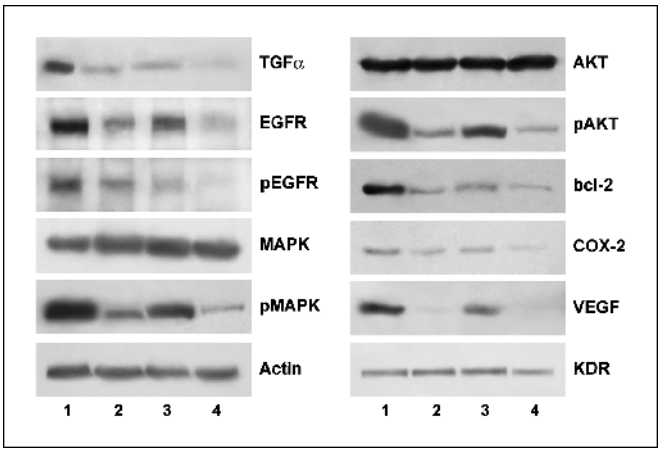

Fig. 2. Western blotting and immunohistochemical analysis of GEO tumors. Western blotting was done on total lysates from tumor specimens of two mice sacrificed on day 25 and treated as in Fig. 1. Lane 1, untreated control; lane 2, IMO; lane 3, cetuximab; lane 4, IMO + cetuximab. Doses and time of administration are indicated in Materials and Methods.

Combined treatment inhibits signaling protein expression and angiogenesis. We then analyzed the effect of treatment on the expression of a variety of proteins playing a critical role in cancer cell proliferation, apoptosis, and angiogenesis. Western blotting and/or immunoprecipitation analysis were done on cell lysates from tumors removed at the end of the third week of treatment on day 25. As shown in Fig. 2, IMO was able to inhibit the expression of both total and phosphorylated/activated EGFR and of its ligand transforming growth factor-α. IMO did not affect the total amount of MAPK and Akt but inhibited its activated forms, pMAPK and pAkt, respectively. Moreover, IMO inhibited the expression of cyclooxygenase-2 and, at higher degree, bcl-2. Interestingly, VEGF expression was completely suppressed, whereas VEGF receptor type 2, KDR, was unaffected. Treatment with cetuximab also showed an inhibitory activity on the same signaling proteins although to a lesser degree compared with IMO. When the two agents were used in combination, a more potent inhibition or complete suppression was observed on the expression of the proteins affected by each single agent, including ~30% inhibition of KDR expression (Fig. 2).

Table 1. Histochemical analysis from tumor specimen in Figure 2

	Tumor volume (cm^3)	Proliferative activity (Ki67)	Vessels (CD34)	Tumor infiltrating lymphocytes
Control	0.56	70%	30 ± 1	+
IMO	0.20	50%	20 ± 2	++
Cetuximab	0.17	10%	8 ± 2	++
IMO + cetuximab	0.13	3%	1	massive

NOTE: Antibodies are described in Materials and Methods. The massive tumor infiltration of lymphocytes observed in the combination treatment correspond to >20-fold increase compared with untreated control and 12-fold compared with each single agent. Bars, SD.

Immunohistochemical analysis of tumor specimens revealed an inhibition of proliferative activity, as measured by Ki-67, by IMO and, at a higher degree, by cetuximab alone. Combination caused >90% inhibition of Ki-67 staining. Analysis of microvessels showed ~30% inhibition of CD34-stained host vessels in the animals treated with IMO and ~70% inhibition following cetuximab treatment. In the specimen from mice treated with the two agents in combination, vessel formation was almost suppressed (Table 1). We also measured the tumor-infiltrating lymphocytes observing a discrete induction by either IMO or cetuximab alone and a striking enrichment with the two agents together, resulting >12-fold higher than achieved by each single agent (Table 1).

We then measured the effect of treatment on nuclear factor-κB activity. Band shift analysis of nuclear translocation of nuclear factor-κB did not show any specific effect of either IMO or cetuximab alone but ~30% reduction with the two agents in combination (data not shown).

***IMO effects are not present* in vitro.** To verify whether IMO may have a direct effect on cell signaling and interfere with cell growth, we have measured the dose- and time-dependent effect of IMO on the growth of GEO cells *in vitro* and evaluated the effect of treatment on protein expression by Western blot analysis. No antiproliferative effect was obtained at any dose tested (data not shown) and no inhibition of total EGFR or phosphorylated EGFR expression was observed (Fig. 3). Moreover, no expression of TLR9 was detected on GEO cells (data not shown).

IMO cooperates also with EGFR tyrosine kinase inhibitor gefitinib. To evaluate whether the inhibitory effect on EGFR signaling induced by IMO could favor a cooperative effect also with another anti-EGFR agent, devoid of immunologic activity, we used the small-molecule tyrosine kinase inhibitor gefitinib. BALB/c nude mice xenografted with GEO tumors were treated with IMO or gefitinib alone and in combination (Fig. 4A). Compared with all untreated mice that reached the maximum allowed tumor size of ~2 cm^3 after 8 weeks from tumor injection, mice treated with IMO alone or with gefitinib alone reached that size 12 and 14 weeks after tumor injection, respectively. When the IMO and gefitinib were used in combination, a cooperative antitumor activity was observed, because at the end of the experiment, 15 weeks after tumor injection and 11 weeks after treatment withdrawal, these tumors were still ~1.4 cm^3 (Fig. 4A). Tumor size was significantly different between the different groups as calculated by the Student's *t* test.

Western blotting analysis done on cell lysates from tumors removed at the end of the third week of treatment on day 25 showed that gefitinib is able to inhibit pMAPK, pAkt, and VEGF although at a lesser degree compared with IMO. The combination of IMO and gefitinib caused a much stronger inhibition of all the three signaling proteins examined (Fig. 4B).

IMO is ineffective in cetuximab-resistant GEO-CR xenografts but still cooperates with cetuximab. To evaluate the relevance of EGFR-dependent and EGFR-independent activity of IMO and cetuximab, we have used cetuximab-resistant GEO-CR xenografts (26). We have shown that IMO alone is ineffective, whereas cetuximab causes a modest inhibitory activity (Fig. 5A) likely due to the formerly described ADCC mechanism (24). When IMO was used in combination with cetuximab, a cooperative inhibitory effect was observed, because at the end of experiment, 6 weeks after treatment withdrawal, tumors were still ~1.0 cm^3 (Fig. 5A). Inhibition of growth induced by each treatment was statistically different as well as the comparison of tumor size among different treatment groups evaluated by the Student's *t* test. Western blotting analysis of protein extracts did not reveal any substantial change in the expression of EGFR and the other signaling proteins examined (Fig. 5B).

Tumor eradication by IMO in combination with cetuximab and irinotecan. We then evaluated whether IMO could increase also the activity of irinotecan, a drug commonly used in the treatment of colorectal cancer, alone and in combination with cetuximab. Treatment of tumor-bearing mice with cetuximab plus irinotecan, a regimen active in colon cancer patients, caused an inhibition less effective than IMO plus cetuximab or IMO plus irinotecan, showing the ability of IMO to markedly enhance also the activity of irinotecan. When IMO, cetuximab, and irinotecan were used in combination altogether, a potent cooperative antitumor effect was obtained, and at the end of the experiment, tumor growth was completely inhibited.

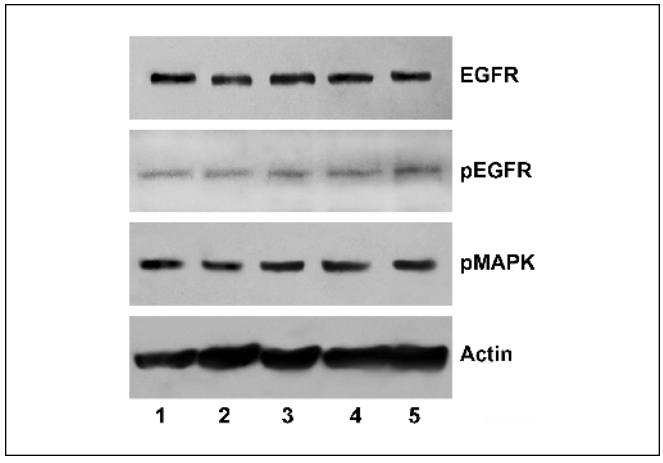

Fig. 3. Western blotting of GEO cells treated *in vitro*. Cell lysates of GEO cells treated *in vitro* on days 0 and 2 were collected on day 5. Lane 1, untreated control; lane 2, IMO (0.1 μmol/L); lane 3, IMO (1 μmol/L); lane 4, IMO (2.5 μmol/L); lane 5, IMO (5 μmol/L). Bars, SD.

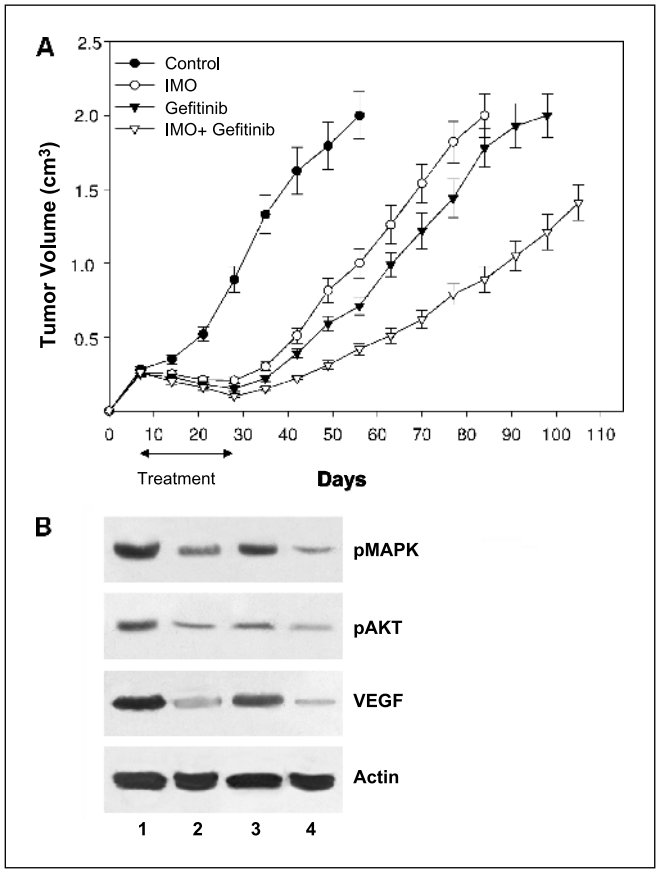

Fig. 4. Cooperative effect of IMO and gefitinib on tumor growth of mice bearing GEO xenografts and Western blotting analysis of tumors. *A,* after 7 days following tumor injection, 10 mice were randomized to receive i.p. IMO 1 mg/kg thrice weekly for 4 weeks, oral gefitinib 210 mg/kg 5 days weekly for 3 weeks, or the combination of these agents on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Tumor size was significantly different between the IMO + gefitinib – treated group versus the control group, the IMO alone group, and the gefitinib alone – treated group (two-sided $P < 0.0001$ for each comparison). The Student's t test was used to compare tumor sizes among different treatment groups at day 56, resulting statistically significant for IMO + gefitinib versus control (two-sided $P < 0.0001$), versus IMO alone (two-sided $P < 0.0001$), and versus gefitinib alone (two-sided $P < 0.0001$). Two mice were sacrificed at day 25 to perform biochemical analyses. *B,* Western blotting done on total lysates from tumor specimens of two mice sacrificed on day 25. Lane 1, untreated control; lane 2, IMO; lane 3, gefitinib; lane 4, IMO + gefitinib. Doses and time of administration are indicated in Materials and Methods. Bars, SD.

Comparison of tumor sizes in surviving mice at the end of the experiment, on day 105, by Student's t test showed a statistically significant difference among all treated groups. Remarkably, pathologic analysis of mice showed that seven of eight mice were tumor free in absence of evident organ toxicity (Fig. 6).

Discussion

CpG DNAs represent a novel and promising therapeutic strategy in different diseases and the recent demonstration of their antitumor activity due to activation of immune cells and induction of cytokines is promoting their evaluation in clinical trials in cancer patients in combination with conventional treatments. Potential enhancement of ADCC is also regarded with interest, and studies are ongoing in combination with the anti-CD20 antibody rituximab (12, 13) in lymphomas and have been proposed with trastuzumab in breast cancer patients.

Targeted therapies against EGFR are among the most used strategies in clinical development, and different agents, such as the antibody cetuximab, have provided a valuable contribution in the treatment of several types of cancer. Cetuximab has shown important results in patients affected by colorectal cancer, both as single agent and in combination with chemotherapeutics like irinotecan or oxaliplatin (22, 23). A relevant issue may be to establish the specific contribution given to the antitumor activity of cetuximab as well as of other antibodies, by the immunologic structure, as inducer of ADCC, and by the EGFR-specific inhibition (24, 25).

In this study, we addressed the issue of the possible interference of second-generation IMO with EGFR signaling and with other critical proteins for growth and angiogenesis as basis to explain IMO antitumor activity. In addition, we have used IMO as a tool to evaluate the mode of action of cetuximab with the final aim to design a novel therapeutic strategy.

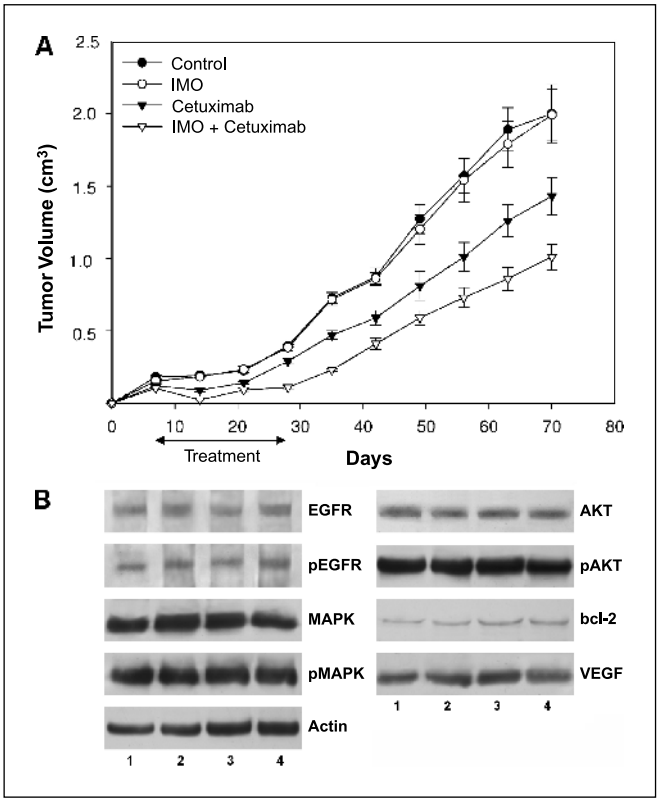

Fig. 5. Effect of the combination of IMO with cetuximab in mice bearing cetuximab-resistant GEO-CR tumor xenografts. *A,* after 7 days following GEO-CR tumor injection, 10 mice were randomized to receive i.p. IMO 1 mg/kg thrice weekly for 4 weeks, i.p. cetuximab 10 mg/kg twice weekly for 3 weeks, or the combination of these agents on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Inhibition of growth was significantly different in the IMO + cetuximab – treated group versus the control group, the IMO alone group, and the cetuximab alone group (two-sided $P < 0.0001$ for each comparison). Student's t test used to compare tumor sizes among different treatment groups at day 56 following GEO cell injection resulted statistically significant for IMO + cetuximab versus control (two-sided $P < 0.0001$), versus IMO alone (two-sided $P < 0.0001$), and versus cetuximab alone (two-sided $P < 0.0001$). *B,* Western blotting was done on total lysates from tumor specimens of two mice sacrificed on day 25 and treated as in Fig. 1. Lane 1, untreated control; lane 2, IMO; lane 3, cetuximab; lane 4, IMO + cetuximab. Doses and time of administration are indicated in Materials and Methods. Bars, SD.

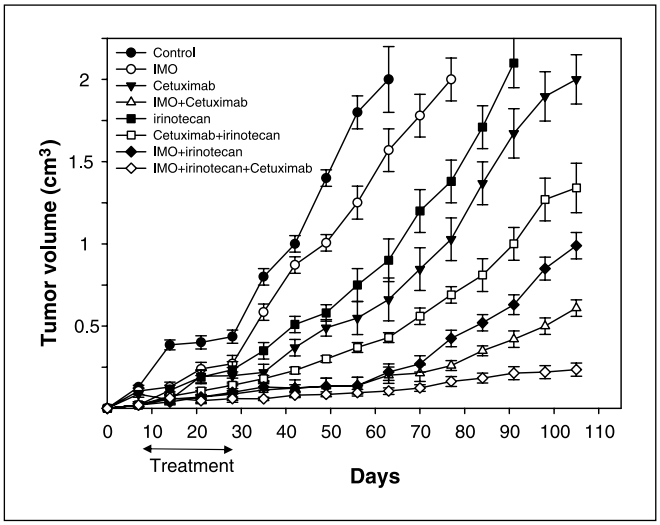

Fig. 6. Cooperative effect of IMO with cetuximab and irinotecan on human colon cancer xenografts. Treatments were i.p. IMO 1 mg/kg thrice weekly for 4 weeks, i.p. cetuximab 10 mg/kg twice weekly for 3 weeks, i.p. irinotecan 50 mg/kg once weekly for 3 weeks, or the combination of these agents on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Student's *t* test used to compare tumor sizes in surviving mice at the end of the experiment, on day 105, showed a statistically significant difference in mice treated with IMO + cetuximab + irinotecan compared with mice treated with IMO + cetuximab (two-sided $P < 0.0001$), IMO + irinotecan (two-sided $P < 0.0001$), and cetuximab + irinotecan (two-sided $P < 0.0001$). Bars, SD.

We and others have shown that cetuximab can cooperate with several chemotherapeutics, radiotherapy, and targeted novel agents (19–21). However, cancer cells may acquire resistance to cetuximab as well as to other EGFR inhibitors by increasing the expression of several downstream signaling proteins, including pMAPK, pAkt, and cyclooxygenase-2 that favor the overexpression and secretion of VEGF, representing a major escape pathway to EGFR inhibition (26, 29). On these bases, several therapeutic strategies can be devised to block also VEGF, or its receptors, in addition to EGFR blockade (26, 30, 31).

We have shown that IMO alone has an antitumor effect on GEO human colon cancer xenografts and that markedly inhibits the expression of EGFR ligand transforming growth factor-α, total and activated EGFR, activated pMAPK and pAkt, cyclooxygenase-2, and bcl-2, almost suppressing VEGF, without affecting its receptor KDR. Moreover, IMO causes ~30% inhibition of microvessel formation. Treatment with cetuximab caused an antitumor effect moderately better than IMO and, as expected from previous studies, accompanied by an inhibition of expression of the same signaling proteins although at a degree surprisingly lower than that obtained with IMO, whereas inhibition of microvessel formation was ~70%. When the two agents were used in combination, a potent cooperative antitumor effect was obtained, because a marked inhibition was still present 11 weeks after treatment withdrawal and tumors just recovered their growth rate. Moreover, the antitumor effect was accompanied by a marked inhibition or suppression of all the above proteins, including KDR, and an almost completely suppressed vessels formation as documented histochemically. It should be noticed that the inhibition of these signaling proteins and of angiogenesis by IMO may be a valuable tool to prevent the occurrence of cetuximab resistance.

These data may help to clarify an interesting issue raised by a former study reporting an induction of bcl-2 and VEGF in a macrophage cell line by a CpG DNA (32). Our results show that the expression of bcl-2 and VEGF in the tumor is inhibited, which is in agreement with the inhibition of vessels formation and the potentiation of cetuximab-dependent KDR inhibition. Taken together, these results may suggest that immunostimulatory oligonucleotides can induce activation of bcl-2 and VEGF in certain immune cells and, at the same time, also cause the release by other immune cells of cytokines that, in turn, efficiently interfere with expression and function of proangiogenic and antiapoptotic proteins in the tumor.

We have also analyzed the presence of tumor-infiltrating lymphocytes, which are considered a favorable prognostic factor in colorectal cancer (33). Both IMO and cetuximab induced an infiltration of the tumor by lymphocytes, but the combination of the two agents resulted in an impressive massive lymphocyte infiltration. Analysis of nuclear factor-κB, which is induced by TLR9 agonists in immune cells and may be modulated during tumor growth and angiogenesis (34) and EGFR activation (35), did not reveal relevant changes by IMO or cetuximab alone at the doses used in this GEO model, whereas the two agents in combination caused ~30% inhibition of nuclear factor-κB nuclear translocation.

We have ruled out the possibility that IMO effect on tumor proliferation and EGFR signaling may be due to a direct inhibitory effect of IMO on the EGFR expression on tumor cells. In fact, IMO is unable to affect GEO cell growth as well as the expression of EGFR *in vitro*. In addition, GEO cells, as the great majority of tumors, do not express TLR9. Because it has been shown previously that IMO activity is strictly TLR9 dependent (18), it is likely that the potential effector of the EGFR-related signaling inhibition is interleukin-12. In fact, interleukin-12, together with IFN-γ, is potently induced by IMO in naive BALB/c (14) and in colon cancer–bearing mice (15), whereas interleukin-12 knockout mice lack the IMO-induced antitumor effects (15).

Regardless of the identity of the effector, the impairment of EGFR signaling and the consequences on growth and angiogenesis seem to represent a critical function of IMO. In fact, IMO in combination with the small-molecule EGFR tyrosine kinase inhibitor gefitinib, which is devoid of potential immunologic activity, showed again a cooperative antitumor effect and inhibition of critical proteins pAkt, pMAPK, and VEGF although at a lesser degree compared with the combinations with cetuximab. Remarkably, IMO alone was completely inactive against GEO-CR xenografts, an established cetuximab-resistant tumor xenograft, in which the ability to activate pAkt and downstream signaling becomes EGFR independent (26).

On the other hand, the EGFR-independent boosting of immunologic activity by IMO was helpful to unravel the non-EGFR-dependent activity of cetuximab. In fact, whereas cetuximab exhibited a modest activity on cetuximab-resistant GEO-CR xenografts likely due to ADCC (24), IMO plus cetuximab had a cooperative inhibitory effect, suggesting that IMO is able to boost the previously reported immunologic activity of cetuximab (24).

These data further suggest that the antitumor effects observed with the combination are due part to the boost of immunologic properties of the mAb and part to the EGFR signaling.

Finally, we have conjugated the recent evidence of cooperation between a non-modified CpG and topotecan (10) with the proven activity shown by cetuximab in combination with the topoisomerase I drug irinotecan in the treatment of colon cancer patients (22, 23) by combining IMO with irinotecan and/or cetuximab. IMO alone enhanced the activity of irinotecan, prolonging mice survival. The combination of IMO, cetuximab, and irinotecan, although unintentionally the size of tumors was slightly smaller at the time of randomization, produced a dramatic antitumor effect, resulting in the absence of tumor recovery at 11 weeks after treatment withdrawal and in tumor eradication in >90% of mice without evidence of major side effects or organ toxicity in the pathologic examination.

In conclusion, the results of our study show for the first time that (*a*) IMO is able to interfere with the expression and function of a set of critical proteins related to EGFR signaling and involved in tumor cell proliferation, apoptosis, and angiogenesis; (*b*) this effect contributes to greatly enhance the antitumor activity of the anti-EGFR mAb cetuximab, helping to dissect its mechanism of action; and (*c*) the combination of IMO with cetuximab and irinotecan has a potent antitumor activity producing a high rate of cure in mice bearing colon cancer xenografts. These results open the path to the investigation of new mechanisms of action for these novel immunostimulatory agents and, above all, represent a strong rationale to translate this therapeutic strategy in cancer patients.

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