Toll-like Receptor 9 Agonist IMO Cooperates with Cetuximab in K-Ras Mutant Colorectal and Pancreatic Cancers

Roberta Rosa1,3, Davide Melisi2,4, Vincenzo Damiano1, Roberto Bianco1, Sonia Garofalo1, Teresa Gelardi5, Sudhir Agrawal8, Federica Di Nicolantonio9,10, Aldo Scarpa6,7, Alberto Bardelli9,10, and Giampaolo Tortora5

Abstract

Purpose: K-Ras somatic mutations are a strong predictive biomarker for resistance to epidermal growth factor receptor (EGFR) inhibitors in patients with colorectal and pancreatic cancer. We previously showed that the novel Toll-like receptor 9 (TLR9) agonist immunomodulatory oligonucleotide (IMO) has a strong in vitro activity in colorectal cancer models by interfering with EGFR-related signaling and synergizing with the anti-EGFR monoclonal antibody cetuximab.

Experimental Design: In the present study, we investigated, both in vitro and in vivo, the antitumor effect of IMO alone or in combination with cetuximab in subcutaneous colon and orthotopic pancreatic cancer models harboring K-Ras mutations and resistance to EGFR inhibitors.

Results: We showed that IMO was able to significantly restore the sensitivity of K-Ras mutant cancer cells to cetuximab, producing a marked inhibition of cell survival and a complete suppression of mitogen-activated protein kinase phosphorylation, when used in combination with cetuximab. IMO interfered with EGFR-dependent signaling, modulating the functional interaction between TLR9 and EGFR. In vivo, IMO plus cetuximab combination caused a potent and long-lasting cooperative antitumor activity in LS174T colorectal cancer and in orthotopic AsPC1 pancreatic cancer. The capability of IMO to restore cetuximab sensitivity was further confirmed by using K-Ras mutant colorectal cancer cell models obtained through homologous recombination technology.

Conclusions: We showed that IMO markedly inhibits growth of K-Ras mutant colon and pancreatic cancers in vitro and in nude mice and cooperates with cetuximab via multiple mechanisms of action. Therefore, we propose IMO plus cetuximab as a therapeutic strategy for K-Ras wild-type as well as for K-Ras mutant, cetuximab-resistant colorectal and pancreatic cancers. Clin Cancer Res; 17(20); 6531–41. ©2011 AACR.

Introduction

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor (TKR) playing a key role in the development and progression of several human epithelial cancers. Therefore, EGFR targeting has become a successful approach for cancer treatment (1). Two anti-EGFR monoclonal antibodies (mAb), cetuximab and panitumumab, and 2 small-molecule selective tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, have been approved for the treatment of several tumors (2–5). However, an emerging issue in cancer patients is the occurrence of constitutive resistance or the development of acquired refractoriness to anti-EGFR drugs by several different mechanisms (6). These include specific mutations or loss of EGFR, alternative signaling by different TKRs, constitutive activation of downstream signal transducers, such as the serine/threonine kinase Akt or the small GTPase Ras, and induction of angiogenesis by tumor-derived factors such as the VEGF (1, 7). Particularly, mutations of the K-Ras gene, most frequently in codon 12 of exon 2, produce a single amino acid change resulting in mutant Ras proteins that are insensitive to the function of GTPase—activating protein and are constitutively active. These mutations are among the most common genetic alterations in human cancers, occurring in 30% of colorectal cancers, 75% to 90% of pancreatic cancers, 50% of cholangiocarcinomas, and 20% of lung carcinomas (1, 8, 9). K-Ras mutations leading to constitutive activation of the Ras/MAPK signaling
Translational Relevance

In this study, we show the antitumor effect of the combination of immunomodulatory oligonucleotide (IMO) and cetuximab in colon and pancreatic cancer models harboring K-Ras mutations and resistance to EGFR inhibitors, both in vitro and in vivo. We show that IMO significantly restores cetuximab sensitivity in both intrinsically mutated and knocked-in cells for mutant K-Ras gene, suppressing activated expression of mitogen—activated protein kinase when used in combination with cetuximab. A possible mechanism for this effect is the capability of IMO to modulate a structural/functional interaction between TLR9 and EGFR. The antitumor effect of the combination of IMO and cetuximab was confirmed also in vivo in clinically relevant tumor models harboring mutant K-Ras: a subcutaneous colorectal and an orthotopic pancreatic cancer model. Our data support IMO plus cetuximab as a novel therapeutic strategy to be tested in a clinical setting for K-Ras mutant, cetuximab-resistant colon and pancreatic cancer patients.

pathway represent a strong predictive biomarker for resistance to EGFR inhibitors. Several studies reported that mutations in the K-Ras gene are associated with lack of response to anti-EGFR mAbs in advanced colorectal cancer and to EGFR TKIs in patients with non-small-cell lung cancer (10–12). It has been reported that K-Ras mutations could be a predictive marker for erlotinib resistance also in pancreatic cancer (13, 14).

Toll-like receptor 9 (TLR9) participates in vertebrate immune system recognizing unmethylated CpG dinucleotides, common in bacterial and viral DNA but not in vertebrate DNA, and initiating a cascade of potent Th1-type innate and adaptive immune responses (15, 16). TLR9 agonists are synthetic oligodeoxynucleotides containing CpG motifs, developed as immunoprotective or antiallergic agents, vaccine adjuvants, or antitumor agents (16). These agents are able to potentiate the antitumor immune responses through the activation of natural killer, dendritic, and cytotoxic T cells, increased production of antitumor cytokines, and enhanced antibody-dependent cell-mediated cytoxicity (ADCC). Second-generation synthetic TLR9 agonists, referred to as immunomodulatory oligonucleotides (IMO), contain a novel structure with 2 accessible 5’-ends critical for TLR9 recognition and exhibit higher metabolic stability than conventional TLR9 agonists (17). TLR9 agonists, such as IMOAs, are showing promising results as anticancer agents in clinical studies; however, the molecular mechanisms by which they affect tumor growth and angiogenesis have not been fully elucidated.

We have previously contributed to clarify some of these mechanisms showing that a TLR9 agonist, IMO, potentiates the ADCC activity of anti-EGFR mAb cetuximab (18) and anti-HER2 mAb trastuzumab (19) in in vivo models of colorectal and breast cancers, respectively. In addition to this immunomodulating function, we showed that IMO acts also by impairing EGFR signaling and potently synergizes in vivo both with cetuximab or EGFR TKI gefitinib in EGFR-addicted colorectal cancer models (20). Finally, we showed that IMO cooperates in vivo with anti-VEGF mAb bevacizumab in EGFR-independent colorectal cancer models by affecting function of endothelial cells (18). These findings opened the path to the ongoing clinical studies combining TLR9 agonists with EGFR inhibitors in cancer patients (http://clinicaltrials.gov/ct2/show/NCT00719199).

Because K-Ras mutations are a major limitation for using EGFR inhibitors in patients with colorectal and pancreatic cancer, we investigated the effects of IMO alone and in combination with cetuximab in colon and pancreatic cancer models harboring a mutant K-Ras.

Methods

Compounds

The anti-EGFR mAb cetuximab was kindly provided by ImClone Systems. IMO, 5’-CTGACRTCTC-X-TCITRCAGTCT-5’ (X and R are glycerol linker and 2’-deoxy-7-deazaguanosine, respectively), was synthesized with phosphorothioate backbone, purified and analyzed as described previously (21).

Cell cultures

Human GEO, SW48, and LS174T colon carcinoma cell lines were obtained from the American Type Culture Collection. Human AsPC1/GLT pancreatic cancer cells were provided by Prof. A. Scarpa (Departments of Pathology, University of Verona). GEO-CR (cetuximab resistant) cells were generated through the homologous recombination technology as previously described (23). Human T3M4 and Mia PaCa-2 pancreatic carcinoma cell lines were kindly provided by Prof. A. Scarpa (Departments of Pathology, University of Verona). GEO-CR (cetuximab resistant) cells were established as previously described (24). Cells were maintained in McCoy’s media or Dulbecco’s Modified Eagle’s Media supplemented with 10% heat-inactivated FBS, 20 mmol/L HEPES (pH = 7.4), penicillin (100 U/I/ ml), streptomycin (100 mg/ml), and 4 mmol/L glutamine (ICN Biomedicals Ltd.) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Soft agar growth assay

Cells (104 cells per well) were suspended in 0.3% Difco Noble agar (Difco) supplemented with complete medium, layered over 0.8% agar-medium base layer, and treated with different concentrations of cetuximab or IMO. After 10 to 14 days, cells were stained with nitroblue tetrazolium (Sigma Chemical Co.) and colonies greater than 0.05 mm were counted using the Quantity One Software Package (Bio-Rad).

MTT survival assay

Cells (104 cells per well) were grown in 24-well plates and exposed to increasing doses of cetuximab or IMO, alone or in combination. The percentage of cell survival was
determined using the MTT assay according to manufacturer’s instructions.

**Immunoprecipitation and Western blot analysis**

Total cell lysates were obtained from cell cultures. The protein extracts were resolved by 4% to 15% SDS-PAGE and probed with anti-human, polyclonal pEGFR, polyclonal EGFR, monoclonal pMAPK, monoclonal MAPK (Santa Cruz Biotechnology), monoclonal pAkt, polyclonal Akt (Cell Signaling Technologies), and monoclonal actin (Sigma-Aldrich). Immunoprecipitations were conducted using monoclonal anti-TLR9 antibody (Calbiochem/EMD Biosciences) and blotting with the same antibody or polyclonal anti-EGFR antibody, following the procedures described above. As negative control, lysis buffer was mixed with anti-TLR9 antibody.

**Ras activation assay**

The analysis of Ras activation was conducted by an immunoprecipitation assay with GST-Raf1-RBD (Ras-binding domain), as previously described (23). The K-Ras protein was detected with Anti-K-Ras (F234) mAb (Santa Cruz Biotechnology).

**Nude mice cancer xenograft models**

Five-week-old Balb/cAntNChBR athymic (nu+/nu−) mice (Charles River Laboratories) maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee and in accordance with the Declaration of Helsinki were injected subcutaneously with LS174T human colon cancer cells (10⁷ cells per mice) resuspended in 200 μL of Matrigel (Collaborative Biomedical Products). After 7 days, tumors were detected and groups of 10 mice were randomized to receive the following treatments: IMO 1 mg/kg intraperitoneally 3 times a week for 4 weeks; cetuximab 10 mg/kg intraperitoneal twice a week for 3 weeks; 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, or vehicles only as control. Disease was considered bulky when the tumor burden was prominent in the mouse abdomen (tumor volume ≥ 2.0 cm³). When at least 3 of 5 mice per treatment group presented a bulky disease, the median survival duration was considered achieved for that group. At the median survival duration of the control group, the tumor growth in mice of all groups was evaluated using the bioluminescence emitted by the tumor cells. Bioluminescence imaging was conducted using a cryogenically cooled IVIS 100 imaging system coupled to a data acquisition computer running Living Image software (Xenogen). The mice were euthanized by CO₂ inhalation when evidence of advanced bulky disease was present, which was considered the day of death for survival evaluation.

**Statistical analysis**

The results of *in vitro* experiments were analyzed for statistical significance of differences by the Student *t* test or nonlinear regression analysis and were expressed as means, SDs, and 95% CIs for at least 3 independent experiments conducted in triplicates. The statistical significance of differences in tumor growth was determined by 1-way ANOVA and the Dunnett multiple comparison posttest, whereas the significance of the differences in survival was evaluated by a log-rank test. All reported *P* values were 2 sided. All analyses were conducted with the BMDP New System Statistical Package version 1.0 for Microsoft Windows (BMDP Statistical Software).

**Results**

**Characterization of a panel of colorectal and pancreatic cancer cell lines**

For this study, we selected a panel of colorectal and pancreatic human cancer cell lines, whose K-Ras status is reported in the catalogue of somatic mutations in cancer provided by Sanger Institute (COSMIC database,
Catalogue of Somatic Mutations In Cancer, http://www.sanger.ac.uk/) and in previous studies (26–28). Particularly, SW48 and T3M4 cells have a wild-type K-Ras gene. Both LS174T and MIA PaCa-2 exhibit K-Ras mutations (Gly12Asp for LS174T cells; Gly12Cys for MIA PaCa-2 cells). GEO cells were previously reported as positive for a K-Ras mutation (Gly12Ala; ref. 27), as we verified (data not shown).

The expression of EGFR and TLR9 proteins in colorectal and pancreatic cell lines was evaluated through Western blot analysis (Supplementary Fig. S1A). GEO, SW48, and LS174T human colon cancer cells exhibit a differential expression of EGFR, higher in SW48 than in GEO and LS174T cells. Among the pancreatic cancer cells, T3M4 showed higher EGFR levels than MIA PaCa-2 cells. Colorectal and pancreatic cancer cells expressed different levels of TLR9, resulting higher in GEO than in SW48 and LS174T cells, and similar in T3M4 and MIA PaCa-2 cells.

Then, we characterized the sensitivity of these different cell lines to the anti-EGFR drugs cetuximab and erlotinib or the TLR9 agonist IMO (Supplementary Fig. S1B), as measured through soft agar assays. GEO and SW48 colon cancer cells were responsive to both cetuximab (IC_{50} = 7 and 35 nmol/L, respectively) and erlotinib (IC_{50} = 0.5 and 1 μmol/L, respectively). Conversely, K-Ras mutant LS174T colon cancer cells were resistant to these agents (IC_{50} > 140 nmol/L for cetuximab and >5 μmol/L for erlotinib). Whereas K-Ras wild-type T3M4 pancreatic cancer cells showed a marked sensitivity to both cetuximab (IC_{50} = 7 nmol/L) and erlotinib (IC_{50} = 1 μmol/L), K-Ras mutant MIA PaCa-2 cells were only modestly inhibited even when high doses were used (IC_{50} = 140 nmol/L for cetuximab and >5 μmol/L for erlotinib). IMO moderately inhibited the soft agar growth of all the cell lines, independently of TLR9 expression levels and K-Ras status (Supplementary Fig. S1B).

IMO restores sensitivity of K-Ras mutant colorectal and pancreatic cancer cell lines to cetuximab

Because we have previously shown that IMO is able to interfere with EGFR-related signaling and has a synergistic antitumor effect with EGFR inhibitors (20), in this study, we evaluated the ability of IMO to restore cetuximab sensitivity in K-Ras mutant, cetuximab-resistant cancer cells. We first tested the combination of IMO and cetuximab on soft agar growth of colorectal and pancreatic cancer cells. As shown in Fig. 1, IMO significantly potentiated the activity of cetuximab in all of the cell lines studied (cetuximab IC_{50} GEO plus IMO, 8.21E-10, 95% CI = 3.3038e-010 to 2.0416e-009 vs. GEO control, 8.93E-09, 95% CI = 6.1600e-009 to 1.2948e-008; SW48 plus IMO, 3.24E-09, 95% CI = 1.4267e-009 to 7.3485e-009 vs. SW48 control, 3.29E-08, 95% CI = 2.4814e-008 to 4.3536e-008; LS174T cells to cetuximab.
plus IMO, 4.08E-08, 95% CI = 2.6385e-008 to 6.2935e-008 vs. LS174T control, 6.77E-07, 95% CI = 2.0322e-007 to 2.2525e-006, T3M4 plus IMO, 9.91E-10, 95% CI = 5.3450e-010 to 1.8375e-009 vs. T3M4 control, 1.38E-08, 95% CI = 9.7619e-009 to 2.5507e-008, MIA PaCa-2 plus IMO, 3.07E-08, 95% CI = 1.7744e-008 to 5.2965e-008 vs. MIA PaCa-2 control, 2.45E-07, 95% CI = 1.1057e-007 to 5.4341e-007; all P < 0.001). In particular, at higher and more clinically relevant doses of cetuximab, this effect was more relevant in K-Ras mutant LS174T and MIA PaCa-2 cells, cetuximab reduced the EGFR-induced EGFR phosphorylation but was unable to interfere with phosphorylation of MAPK, the main Ras downstream signal transducer. A variable effect of cetuximab on Akt phosphorylation was detected on these cell lines. The combination IMO plus cetuximab inhibited EGFR-dependent signaling in the K-Ras mutant LS174T and MIA PaCa-2 cells, producing a total suppression of MAPK phosphorylation (Fig. 2).

These data show that IMO partly restores cetuximab sensitivity of K-Ras mutant colorectal and pancreatic cancer cell lines, producing a significant inhibition of cell growth and survival and a strong interference with EGFR-dependent signal transduction when used in combination with cetuximab.

**IMO modulates a functional interaction between TLR9 and EGFR in colorectal and pancreatic cancer cells**

We investigated whether the inhibition of EGFR signaling by IMO could be related to a modulation of the interaction between TLR9 and EGFR, similar to what we previously described in breast cancer cells (19). To this aim, we conducted a time-course experiment over a 25-hour window. Cells cultured in complete medium were treated with IMO (1 μmol/L) at 0 and 24 hours, and cell lysates were obtained at 1, 24, and 25 hours. We immunoprecipitated GEO, SW48, LS174T, and MIA PaCa-2 cell lysates with an anti-TLR9 antibody. In both cetuximab-sensitive GEO and SW48 and cetuximab-resistant LS174T and MIA PaCa-2 cells, TLR9 coimmunoprecipitated with EGFR, similar to what we previously described in breast cancer cells (19). To this aim, we conducted a time-course experiment over a 25-hour window. Cells cultured in complete medium were treated with IMO (1 μmol/L) at 0 and 24 hours, and cell lysates were obtained at 1, 24, and 25 hours. We immunoprecipitated GEO, SW48, LS174T, and MIA PaCa-2 cell lysates with an anti-TLR9 antibody and blotted with an anti-EGFR antibody. As negative control, lysis buffer was mixed with monoclonal anti-TLR9 antibody. In both cetuximab-sensitive GEO and SW48 and cetuximab-resistant LS174T and MIA PaCa-2 cells, TLR9 coimmunoprecipitated with EGFR. IMO modulated this interaction, which was reduced 1 hour after each IMO treatment, and completely restored within 24 hours from IMO treatment (Fig. 3). The kinetics of this modulation is consistent with the EGFR signaling inhibition induced by IMO, suggesting that the disruption of TLR9/EGFR interaction may play a critical role in the ability of IMO to interfere with EGFR-dependent signal transduction.
IMO restores sensitivity to cetuximab in colorectal cancer cells knocked-in for mutant K-Ras gene

To further confirm the activity of IMO on K-Ras mutant cancer cells, we used isogenic wild-type and Gly12Val K-Ras mutant SW48 colon cancer cells obtained through homologous recombination. This technology allows to introduce individual or multiple cancer mutations into the genome of cancer cells; as a result, the heterozygous-mutated genes are expressed under their endogenous promoters, thus closely recapitulating the lesions observed in human tumors (29). Accordingly to their K-Ras status, the mutant SW48 cells were resistant to cetuximab compared with their wild-type control cells (Fig. 5A). However, combining IMO with increasing doses of cetuximab significantly reversed this resistance, although not to the same level as that of the parental cell line, both in soft agar and MTT assays (Fig. 5B and C).

Then, we compared the effects of IMO and cetuximab on the activation of EGFR-dependent signal transduction in wild-type and K-Ras mutant SW48 cells. In the cetuximab-sensitive SW48 K-Ras wild-type parental cells, cetuximab efficiently inhibited EGFR signaling, suppressing phosphorylation/activation of EGFR, Akt, and MAPK. Conversely, in the cetuximab-resistant K-Ras mutant SW48 cells, cetuximab reduced the EGFR-induced EGFR phosphorylation but was unable to inhibit MAPK phosphorylation and slightly reduced Akt phosphorylation. Although IMO alone did not reduce pMAPK in both wild-type and K-Ras mutant SW48 cells, a complete suppression of MAPK phosphorylation was achieved by the combined treatment with IMO plus cetuximab in Gly12Val K-Ras mutant SW48 cells (Fig. 5D). We analyzed whether the combination of IMO and cetuximab could interfere with activation of K-Ras. The results (shown in Supplementary Fig. S3) seem to rule out this hypothesis. These data confirm that IMO is able to restore cetuximab sensitivity in Gly12Val K-Ras mutant colorectal cancer cells, but further studies are required to fully elucidate the mechanism by which this is achieved.
IMO is ineffective in a colorectal cancer model of acquired resistance to cetuximab due to Akt overactivity

We previously showed that IMO is unable to inhibit in vivo growth in GEO-CR (cetuximab resistant) tumors, a colon cancer model of acquired resistance to cetuximab (20). In the present study, we found that combining IMO with increasing doses of cetuximab did not reverse cetuximab resistance in GEO-CR cells, both in soft agar and MTT assays (Fig. 6A and B). Moreover, whereas IMO inhibited EGFR-dependent signal transduction in GEO original cells, it was ineffective in reducing phosphorylation/activation of EGFR signal transducers, such as Akt and MAPK, in GEO-CR cells. As previously showed (20), treatment of GEO-CR cells with cetuximab reduced EGFR and MAPK phosphorylation but not Akt phosphorylation. The combination IMO plus...
cetuximab was unable to reduce Akt phosphorylation in GEO-CR cells (Fig. 6C). Importantly, we identified a TLR9/EGFR interaction in both GEO and GEO-CR cells, and IMO was able to interfere with such interaction in GEO but not in GEO-CR cells, as shown by immunoprecipitation analysis (Fig. 6D). Probably, IMO inability to modulate the TLR9/EGFR interaction in GEO-CR cells could explain the lack of IMO interference with EGFR-dependent signal transduction in this cell line. Because we previously showed that acquired resistance to cetuximab in GEO-CR cells is related to Akt overactivity resulting from overexpression of the VEGF receptor 1 (VEGFR1/Flt1; ref. 24), it is conceivable that IMO activity is affected by the different molecular mechanisms of resistance to cetuximab.

**Discussion**

The constitutive or acquired resistance to EGFR inhibitors in cancer patients is a relevant clinical issue. A number of molecular abnormalities in tumor cells partially contribute to resistance to anti-EGFR mAbs or small molecules TKIs, including overexpression of other TKRs, constitutive activation of downstream transducers, and increased VEGF-mediated tumor angiogenesis (1). A critical role in the resistance to EGFR inhibitors of patients with colon, lung, and pancreatic cancer is played by continuous activation of the Ras/MAPK pathway because of mutations in codon 12 of the K-Ras gene. In this regard, the use of panitumumab- and cetuximab-based therapies is currently restricted only to
patients with wild-type K-Ras metastatic colorectal cancer (7). Therefore, there is an unmet need to identify and clinically validate novel therapeutic strategies based on combination of different targeted agents able to hinder the occurrence of resistance to EGFR antagonists.

In this study, we investigated the antitumor effect of the TLR9 agonist IMO as single agent or in combination with cetuximab in colorectal and pancreatic cancer models harboring the oncogenic K-Ras mutations conferring resistance to EGFR inhibitors.

We found a correlation between K-Ras status and sensitivity to EGFR antagonists in all the cell lines tested, with the exception of the human GEO colorectal cancer cells. Even though these cells bear a low-frequency K-Ras mutation (Gly12Ala), they maintain a measurable sensitivity to both cetuximab and erlotinib. This is in agreement with the evidence that different K-Ras mutations have different relevance in mediating resistance to EGFR inhibitors. In this regard, a small number of patients carrying K-Ras mutant tumors have been reported to respond to either cetuximab or panitumumab (23, 30–32).

We showed that IMO is able to restore sensitivity to cetuximab in K-Ras mutant cell lines. The combination of IMO plus cetuximab produced a significant inhibition of cell growth and survival and a strong interference with EGFR-dependent signal transduction in K-Ras mutant colorectal and pancreatic cancer cells, totally suppressing phosphorylation/activation of MAPK, the main signal transducer downstream to Ras. We have reported that the ability of IMO to interfere with EGFR-dependent signaling is associated with the modulation of the structural/functional interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19). In this study, we document that the kinetics of this modulation is consistent with the ability of IMO to inhibit EGFR signaling and disrupt the interaction between TLR9 and EGFR (19).
The antitumor effect of IMO on K-Ras mutant tumors was confirmed also in vivo, in subcutaneous LS174T colorectal and orthotopic AsPC1 pancreatic cancer models. In both models, cetuximab still produced a significant tumor growth inhibition despite the intrinsic resistance to this agent in vitro. This effect is likely due to its ADCC activity, as we previously reported (19). The antitumor activity of IMO was particularly evident in AsPC1 pancreatic cancer orthotopic model, with a reduction of the tumor burden of about 85%. We hypothesized that this event may depend on the capability of IMO to interfere with different cell populations of tumor microenvironment, whose contribution to tumor growth could be higher in the orthotopic than in the subcutaneous xenograft (37). The combination of IMO plus cetuximab caused a potent and long-lasting cooperative antitumor activity, with a significant inhibition of tumor growth and prolongation of mice survival.

Finally, we confirmed the ability of IMO to overcome cetuximab resistance associated with K-Ras mutations by using targeted homologous recombination to introduce (knock-in) Gly12Val K-Ras-mutated allele in human colon cancer cells. This technology allows the construction of models able to accurately recapitulate the genetic alterations present in human cancers, useful to identify genotype and tumor-specific pharmacologic responses (29, 38). While IMO alone did not produce any effect on EGFR signaling in this model, this agent was able to restore sensitivity to cetuximab, producing a significant inhibition of cell growth and survival and a strong interference with EGFR-dependent signal transduction when used in combination with cetuximab. Taken together, our results show for the first time that IMO is effective in restoring cetuximab antitumor activity in K-Ras mutant colon and pancreatic cancers. This effect is likely due to multiple mechanisms of action, including interference with EGFR signaling by modulation of the TLR9/EGFR interaction, and enhancement of cetuximab ADCC.

The combination of IMO plus cetuximab is currently under clinical investigation, and the results of the present study suggest that the combination with IMO could allow the use of cetuximab also in a set of patients with K-Ras mutant tumors, where this agent would be otherwise ineffective.

Disclosure of Potential Conflicts of Interest

S. Agrawal has employment (other than primary affiliation, e.g., consulting) and G. Tortora has commercial research grant. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

Human T3M4 and MiaPaCa-2 pancreatic carcinoma cell lines were kindly provided by Prof. A. Scarpa (Department of Pathology and Diagnostics, University of Verona).

Grant Support

This study was supported in part by Associazione Italiana per la Ricerca sul Cancro (AIRC), Ministero della Salute, Ministero dell’Università e della Ricerca (PRIN), and Regione Campania grants to G. Tortora. This work was also supported in part by AIRC (Start-Up grant no. 10129), American-Italian Cancer Foundation, and The Sassi Foundation for Medical Research grants to D. Melisi. V. Damiano was supported by a fellowship from AIRC. This study was supported partly also by grants from Italian AIRC IG 2009-2011 (A. Bardelli), AIRC “2010 Special Program Molecular Clinical Oncology 5 × 1000” Project no 9970 (A. Bardelli), Regione Piemonte (F.D. Nicolantonio and A. Bardelli), Italian Ministry of University and Research (A. Bardelli), European Union Seventh Framework Programme grant agreement 239015 (A. Bardelli), FPRC, Fondazione Piemontese per la Ricerca sul Cancro Onlus (F.D. Nicolantonio and A. Bardelli).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 21, 2010; revised August 9, 2011; accepted August 11, 2011; published OnlineFirst September 2, 2011.

References

12. Van Cutsem E, Rougier P, Kohne C, Stroh C, Schluechting M, Bokemeyer C. A meta-analysis of the CRYSTAL and OPUS studies combining cetuximab with chemotherapy (CT) as 1st-line treatment for patients (pts) with metastatic colorectal cancer (mCRC). Results
according to KRAS and BRAF mutation status. Eur J Cancer 2009;7: S345, abst 6077.
Toll-like Receptor 9 Agonist IMO Cooperates with Cetuximab in K-Ras Mutant Colorectal and Pancreatic Cancers

Roberta Rosa, Davide Melisi, Vincenzo Damiano, et al.

*Clin Cancer Res* 2011;17:6531-6541. Published OnlineFirst September 2, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-3376</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://clincancerres.aacrjournals.org/content/suppl/2011/09/02/1078-0432.CCR-10-3376.DC1">http://clincancerres.aacrjournals.org/content/suppl/2011/09/02/1078-0432.CCR-10-3376.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 38 articles, 21 of which you can access for free at: <a href="http://clincancerres.aacrjournals.org/content/17/20/6531.full#ref-list-1">http://clincancerres.aacrjournals.org/content/17/20/6531.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 1 HighWire-hosted articles. Access the articles at: <a href="http://clincancerres.aacrjournals.org/content/17/20/6531.full#related-urls">http://clincancerres.aacrjournals.org/content/17/20/6531.full#related-urls</a></td>
</tr>
</tbody>
</table>

E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |

Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |

Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |