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“Antitumor activity of a novel TLR9 agonist and cooperativity with targeted agents”

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Antitumor activity

of a novel TLR9 agonist and

cooperativity with targeted agents

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. **TLR9 agonist acts by different mechanisms synergizing with bevacizumab in sensitive and cetuximab-resistant colon cancer xenografts.**
   Damiano V, Caputo R, Garofalo S, Bianco R, Rosa R, Merola G, Gelardi T, Racioppi L, Fontanini G, De Placido S, Kandimalla ER, Agrawal S, Ciardiello F, Tortora G.
   Proc Natl Acad Sci USA 2007;104:12468-73.

2. **Inhibition of mTOR pathway by everolimus cooperates with EGFR inhibitors in human tumours sensitive and resistant to anti-EGFR drugs.**
   Bianco R, Garofalo S, Rosa R, Damiano V, Gelardi T, Daniele G, Marciano R, Ciardiello F, Tortora G.
   Br J Cancer 2008;98:923-30.

3. **VEGF Receptor 1 contributes to resistance to anti-EGFR drugs in human cancer cells.**
   Bianco R, Rosa R, Damiano V, Daniele G, Gelardi T, Garofalo S, Tarallo V, De Falco S, Melisi D, Benelli R, Albini A, Ryan A, Ciardiello F, Tortora G.
   Clin Cancer Res 2008;14:5069-80.

4. **EGFR-targeting agents in oncology.**
   Garofalo S & Rosa R, Bianco R, Tortora G.
   Expert Opin Ther Pat 2008;18:1-13.

ABSTRACT

Novel modified Toll-like receptor 9 (TLR9) agonists, termed immune modulatory oligonucleotides (IMOs), exhibit antitumor activity and are currently investigated in cancer patients. Intriguingly, their mechanisms of action on tumor growth and angiogenesis are still incompletely understood. Using an IMO recognizing human and murine TLR9, we recently discovered that IMO acts by impairing Epidermal Growth Factor Receptor (EGFR) signalling, cooperates with anti-EGFR drugs and boosts the antibody-dependent cell-mediated cytotoxicity (ADCC) of anti-EGFR antibodies. IMO in fact cooperates with anti-EGFR antibody cetuximab in GEO human colon cancer xenografts, through the potentiation of cetuximab ADCC and the inhibition of EGFR signalling. IMO is instead ineffective in VEGF overexpressing cetuximab-resistant GEO-CR tumors.

Therefore, we used IMO and the anti-VEGF antibody bevacizumab as tools to study IMO's role on EGFR and angiogenesis and to explore its therapeutic potential in GEO, LS174T and GEO-CR cancer xenografts. We found that bevacizumab has no ADCC and IMO is unable to enhance it. Nevertheless, IMO plus bevacizumab combination cooperatively inhibits the growth of GEO and LS174T preceded by inhibition of signalling proteins expression, microvessels formation and human, but not murine, VEGF secretion. Interestingly also the growth of GEO-CR tumors is cooperatively inhibited by this drug combination. Moreover, IMO shows a direct antiangiogenic activity, inhibiting survival, adhesion, migration and capillary formation of VEGF-stimulated endothelial cells. The antitumor activity was irrespective of the TLR9 expression on tumor cells. Therefore, considering that IMO creates the opportunity to take advantage of multiple chance for cooperativity, involving EGFR- and ADCC-dependent and -independent mechanisms and neoangiogenesis, we have evaluated IMO ability to revert mechanisms of resistance in different tumor models from colon cancer. Since intrinsic and acquired resistance to anti-ErbB2 antibody trastuzumab is becoming an increasingly relevant issue in breast cancer, we investigated whether the antitumor, antiangiogenic and immunostimulatory activity of IMO could enhance trastuzumab effect in trastuzumab-resistant breast cancers. We found that IMO potentiates trastuzumab-mediated ADCC on KPL-4 and JIMT-1 cells, naturally resistant to trastuzumab *in vitro*. IMO inhibits the growth of KPL-4 and JIMT-1 xenografts, and potentiates the inhibitory effect of trastuzumab, with an almost complete suppression of tumor growth and expression of several ErbB-related signal transducers by the combined treatment. The combination produces also a cooperative direct antiangiogenic effect. Surprisingly, TLR9 is expressed in KPL-4 cells and seems to functionally interact with EGFR.

These studies demonstrate that TLR9 agonists interfere with tumor and its microenvironment by multiple mechanisms providing a strong rationale to combine IMO with several targeted agents in cancer patients.

1. BACKGROUND

1.1. Targeted antitumor therapy and mechanisms of resistance

The "targeted therapy" has been defined as a new strategy based on antitumor agents that are able to interfere with a specific molecular target, typically a protein, believed to have a critical role in tumor growth or progression. Most of these targets are represented by kinases controlling cell homeostasis. Tyrosine kinase receptors (TKRs) are able to mediate the effect of growth factors and sustain signal transduction pathways. One of the most investigated TKRs family is the erbB/HER-family, which includes four members defined as ErbB-1/EGFR/HER1, ErbB-2/HER2/neu, ErbB-3/HER3 and ErbB-4/HER4 (Citri and Yarden 2006). The EGFR gene encodes a 170 kDa trans-membrane glycoprotein containing 1186 amino acids, which consists of an extracellular domain that recognizes and binds to specific ligands, a hydrophobic trans-membrane domain, involved in interactions between receptors within the cell membrane, and an intracellular domain that serves as the site of protein kinase activity. Ligand-binding induces either homo- or heterodimerization between EGFR and other members of the HER-family and EGFR function and activity are strictly regulated by these interactions (Lemmon and Schlessinger 1994). While ErbB-2 exists in an activated and ligand-independent three-dimensional structure, ErbB3 seems to be a non-autonomous receptor, since its kinase activity is defective, but it is able to form heterodimeric complexes with other ErbB receptors generating potent cellular signals. The ligands of the ErbB receptors belong to the EGF-family of growth factors, usually divided into three groups: EGF, transforming growth factor alpha (TGF-α) and amphiregulin (AR), bind to the EGFR; betacellulin (BTC), heparin-binding growth factors (HB-EGF), epiregulin (EPR) and heregulins (HRG) have dual binding specificity for EGFR and ErbB4; the neuregulins (NRGs) are able to bind ErbB3 and/or ErbB4 (Yarden 2001). EGF-related growth factors have different affinity for the members of HER-family and are characterized by the presence of an EGF-like domain composed of three disulfide-bonded intramolecular groups conferring binding specificity, and additional structural motifs such as immunoglobulin-like domains, heparin-binding sites and glycosylation sites (Sebastian et al. 2006). Ligand-binding induces a conformational change of the receptor leading off the dimerization process (Ferguson 2004). The autophosphorylated receptor initiates the recruitment to the plasma-membrane and activates trough phosphorylation other cytoplasmic substrates, which, in turn, mediate the activation of different signal transduction pathways depending upon the type of ligand, levels of receptor expression and partners of dimerization. The most studied downstream pathways include the PI3K/Akt, Ras/ERK and PLCγ/PKC signalling cascades (Scaltriti and Baselga 2006).

Aberrant activity of ErbB signalling is associated with cancer development and growth and is initiated by several events, such as altered ligand production, receptor mutations, deletions, or persistent activation. High levels of EGFR or ErbB2 expression are a common feature of the malignant phenotype in many solid human tumors, and may result from a variety of mechanisms including increased gene transcription or gene amplification (Milanezi et al. 2008). Several studies have demonstrated that EGFR or ErbB2 expression correlates with reduced disease-free and overall survival, poor prognosis, increased risk of disease recurrence, advanced tumor stage, and increased risk of metastasis (Scaltriti and Baselga 2006). High expression of ErbB ligands in conjunction with increased expression of ErbB receptors may facilitate the development of an autocrine/paracrine growth pathway, contributing to carcinogenesis. Gene amplification leading to EGFR or ErbB2 overexpression is a frequent feature of human cancers (Milanezi et al. 2008), often accompanied by other structural rearrangements that cause in-frame deletions in the extra-cellular domain of the receptor. The most frequent deleted form of the human EGFR is the type-III variant (EGFRvIII), characterized by a deletion in the extra-cellular domain that leads to constitutive activation of its TK domain (Kuan et al. 2001). Besides perturbations in EGFR or ErbB2 expression, mutations and ligand production, downstream intracellular signalling pathways under receptors control are frequently altered in tumor cells, ensuring survival, metastatic spread and resistance to therapies: Ras, PTEN and STAT mutations are often present in human cancers (Bianco et al. 2003; Friday and Adjei 2005; Cochet et al. 2005).

The role of ErbB-related signal transduction pathways in cancer development induced pharmaceutical companies to devote efforts to the development of inhibitors, producing remarkable results in several human malignancies. Among the ErbB targeting agents, there are monoclonal antibodies (MAbs) that bind the extra-cellular domain of the receptor and compete with endogenous ligands; small-molecules tyrosine kinase inhibitors (TKIs) that bind the intracellular portion of the receptor, generally by competing with ATP and inhibiting receptor autophosphorylation; immunotoxin conjugates that deliver toxins; antisense oligonucleotides or siRNA that decrease the expression of the receptor; soluble ligand trap; receptor decoys or polypeptides; drugs targeting transduction molecules downstream to the ErbB signalling (Hynes and Lane 2005).

The most promising and well studied ErbB inhibitors are MAbs and TKIs. They share the same target but display different mechanisms of action and different specificity for the target; in fact, MAbs are exclusively specific, while TKIs are relatively specific. Moreover, MAbs are able to induce receptor internalization, down-regulation, degradation and activation of host immune response via antibody-dependent cell-mediated cytotoxicity (ADCC). These features may contribute to the observed differences in efficacy and toxicity profiles (Ciardiello and Tortora 2008). Among EGFR inhibitors, two anti-EGFR monoclonal antibodies (cetuximab and panitumumab) and two small-

molecules, reversible EGFR tyrosine kinase inhibitors (gefitinib and erlotinib) have been approved in several countries for the treatment of various human cancer types, but more than ten EGFR-targeting agents are actually in advanced clinical development (Garofalo et al. 2008). Approximately 20% to 25% of invasive breast cancers exhibit overexpression of ErbB2. As elevated ErbB2 levels are associated with reduced disease-free and overall survival in metastatic breast cancer (MBC), therapeutic strategies are being developed to target this oncoprotein (Nahta and Esteva 2006). Trastuzumab, a recombinant humanized monoclonal antibody directed against an extracellular region of ErbB2, was the first ErbB2-targeted therapy approved by the United States Food and Drug Administration (FDA) for the treatment of HER2-overexpressing MBC. Trastuzumab with adjuvant chemotherapy (in sequence or in combination) significantly improved disease-free and overall survival rates in patients with early stage ErbB2-overexpressing breast cancer (Romond et al. 2005).

A relevant issue in cancer patients is the development of primary and secondary resistance to anti-ErbB drugs. Primary or constitutive resistance refers to patients who either do not achieve stable disease or who progress within 6 months after an initial clinical response, whereas secondary or acquired resistance typically occurs after prolonged treatment. However, it is not possible to define the molecular basis of each type of resistance. The mechanisms of resistance to targeted agents are similar to cytotoxic agent resistance such as inactivating metabolism, poor absorption, reduced drug availability or defective immune system-mediated functions. However, most relevant causes of targeted drug resistance are specific mutations or loss of the target, activation of alternative TK receptors that bypass the pathway targeted by the specific agent, independent or constitutive activation of intracellular molecular effectors downstream to the target protein, and activation of tumor-induced angiogenesis (Tortora et al. 2007).

The rate of primary resistance to single-agent trastuzumab for ErbB2-overexpressing MBC is 66% to 88%, therefore, elucidating the molecular mechanisms underlying primary or acquired trastuzumab resistance is critical in order to improve the survival of MBC patients whose tumors overexpress ErbB2 and to identify novel therapeutic targets toward the goal of increasing the magnitude and duration of response to trastuzumab-based treatment. (Nahta and Esteva 2006; Friedländer et al. 2008). A potential mechanism by which resistance to targeted antibodies may develop is via disruption of the interaction between the therapeutic agent and the target protein. Resistance to trastuzumab was associated with increased expression of the membrane-associated glycoprotein MUC4 that bind and sterically hinder ErbB2 from binding to trastuzumab. MUC4 interacts directly with ErbB2 through its EGF-like domain and serves as a ligand for ErbB2, resulting in increased phosphorylation of the receptor on the residue Tyr1248 (Nagy et al. 2005). The JIMT-1 trastuzumab-resistant cell line was established from a breast cancer patient showing *her2* gene amplification and primary resistance to trastuzumab.

Using this model, the authors demonstrated that the level of MUC4 protein was inversely correlated with the trastuzumab binding capacity, and showed that knockdown of MUC4 increased the sensitivity of JIMT-1 cells to trastuzumab. Thus, the authors proposed that elevated MUC4 expression masks the trastuzumab binding epitopes of ErbB2 that is also unable to interact with other proteins, such as EGFR or ErbB3, because of epitope masking by MUC4 (Tanner et al. 2004). Trastuzumab resistance has been also associated with increased signalling from the insulin-like growth factor-I receptor (IGF-IR). Increased expression of IGF-IR was shown to reduce trastuzumab-mediated growth arrest of ErbB2-overexpressing breast cancer cells. Crosstalk occurs between IGF-IR and ErbB2, and showed that IGF-IR physically interacts with and phosphorylates ErbB2 in trastuzumab-resistant cells, but not in trastuzumab-sensitive parental cells (Lu et al. 2001). It has been demonstrated that p27kip1 is a critical mediator of trastuzumab response, and that its downregulation may occur subsequent to increased signalling from growth factor receptors such as IGF-IR, promoting resistance to trastuzumab (Nahta et al. 2004). Moreover, also PTEN and PI3K signalling are associated with trastuzumab resistance. Constitutive PI3K/Akt activity, resulting fom the loss of PTEN phosphatase, was previously shown to inhibit cell-cycle arrest and apoptosis mediated by trastuzumab. Patients with PTEN-deficient ErbB2-overexpressing breast tumors have a much poorer response to trastuzumab-based therapy (Nagata et al. 2004). Finally, another mechanism of trastuzumab resistance is related to serum ErbB2 extracellular domain. The full-length 185 kDa ErbB2 protein has been reported to be cleaved by matrix metalloproteases into a 110 kDa extracellular domain (ECD), which is released into cell culture media or circulating in serum *in vivo* (Colomer et al. 2000), and a 95 kDa amino-terminally truncated membrane-associated fragment with increased kinase activity, defined as p95HER2 (Christianson et al. 1998). Elevated serum levels of ErbB2 ECD correlate with poor prognosis in patients with advanced breast cancer. Of potential importance, trastuzumab blocked ErbB2 ECD proteolytic cleavage *in vitro* (Molina et al. 2001). ErbB2-targeted monoclonal antibodies bind to circulating ECD, competing away binding to membrane-bound ErbB2. Hence, signalling from the receptor form of ErbB2 continued in the presence of ErbB2 antibodies, indicating that ErbB2 ECD promoted resistance to ErbB2-targeted antibody therapy (Zabrecky et al. 1991). The p95HER2 protein may be generated by different, nonmutually exclusive, mechanisms, including not only the extracellular domain shedding, but also alternative RNA processing (Scott et al. 1993) or alternative initiation of translation from different methionines within the *her2* sequence (Anido et al. 2006). However, the presence of these truncated forms of ErbB2 may promote resistance to trastuzumab.

One of the most relevant causes of EGFR/ErbB-2 inhibitors resistance is the activation of tumor-induced angiogenesis. Angiogenesis is crucial for local tumor growth and in the development of distant metastasis (Folkman 1971; Kerbel and Folkman 2002). Different growth factors, such as basic

fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor-α (TGFα), have been identified as positive regulators of angiogenesis, and are secreted by cancer cells to stimulate normal endothelial cell growth through paracrine mechanisms (Ferrara et al. 2003). VEGF is a potent and specific mitogen for endothelial cells, activates the angiogenic switch *in vivo* and enhances vascular permeability (Ferrara et al. 2003). VEGF mRNA expression is rapidly and reversibly induced in cells exposed to hypoxia, occurring in poorly vascularized tumors, via the Hypoxia-inducible factors (HIF), a heterodimeric protein consisting of HIF-1α and HIF-1β, which, by binding to hypoxia-response elements, controls the transcription of genes involved in cell proliferation/survival, invasion/metastasis and angiogenesis, including VEGF (Melillo 2007). In mammals, the vascular endothelial growth factor (VEGF) family consists of five members: VEGF-A (thereafter called VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). In addition, alternative exon splicing results in generation of four main VEGF isoforms denoted VEGF121, VEGF165, VEGF189, and VEGF206. Moreover, VEGF165 can be cleaved by plasmin and various metalloproteinases at the COOH terminus, generating VEGF110 or VEGF113, two bioactive NH2-terminal fragments (Kowanetz and Ferrara 2006). Members of the VEGF family show different affinities for one of the three VEGF tyrosine kinase receptors: VEGFR-1/Flt-1, VEGFR-2/KDR, and VEGFR-3/Flt-4. These receptors are overexpressed on activated endothelial cells, where they regulate cell permeability, proliferation, and differentiation, as well as on hematopoietic stem cells, osteoblasts and monocytes (Ferrara et al. 2003). VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGFR-2 appears mostly restricted to vascular endothelial cells while VEGFR-1 is present in both vascular endothelial and macrophage-like cells and may promote inflammation, tumor growth and metastasis (Shibuya 2006). Interestingly, subsets of liquid and solid tumor cells were found to express VEGFR-1 and VEGFR-2 (Kowanetz and Ferrara 2006). In fact, enhanced VEGF expression is found in human cancer cell lines and in cancer patients with different malignancies, including colorectal (CRC), breast, non-small-cell lung (NSCLC) and ovarian cancers and is directly correlated with increased neovascularization as measured by microvessel count (MVC) within the tumor (Fontanini et al. 1997). In 1971, Folkman (Folkman 1971) proposed that antiangiogenesis may be a novel anticancer strategy. Several pharmacologic approaches to inhibit the VEGF axis have been described and are considered another good example of targeted therapy for tumors (Kowanetz and Ferrara 2006). The first antiangiogenic agent approved by the FDA is bevacizumab, a humanized version (Presta et al. 1997) of an anti-VEGF monoclonal antibody used in early proof-of-concept studies (Kim et al. 1993). In 2004, bevacizumab was approved for the treatment of previously untreated metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens (Ferrara et al. 2004). Bevacizumab is presently being tested in several phase III studies in combination with chemotherapy, in relapsed metastatic colorectal

cancer, non–small cell lung cancer, and previously untreated or relapsed metastatic breast cancer. Preliminary evidence of clinical benefit has been observed in all cases. Currently, other anti-VEGF agents are at various stages of clinical development. These include the following: VEGFTrap, a soluble receptor targeting VEGF, VEGF-B, and placental growth factor; an antisense oligonucleotide, VEGF-AS, targeting VEGF, VEGF-C, and VEGF-D; and several small-molecule tyrosine kinase inhibitors some of which have been recently approved by the FDA for clinical use (Kowanetz and Ferrara 2006). It has been previously shown that tumors acquiring resistance to anti-EGFR/ErbB2 drugs overexpress and secrete the main proangiogenic factor VEGF, that acts as an escape pathway, overcoming receptor blockade (Viloria-Petit et al. 2001). Moreover, it has been shown that VEGF overexpression markedly impairs the activity of dendritic cells and the antitumor immune response (Gabrilovich et al. 1998). These studies and the demonstration of a cooperative effect of cetuximab with a selective inhibitor of VEGF (Ciardiello et al. 2000) have provided the basis for the ongoing clinical studies combining inhibitors of EGFR and of VEGF/VEGFRs (Sandler and Herbst 2006). Interestingly, the VEGF blockade by bevacizumab recovers the activity of dendritic cells improving their antitumor function (Gabrilovich et al. 1999).

1.2. TLR9 agonists as new antitumor agents

Vertebrates are endowed with two complementary immune systems, the innate and the adaptive. The adaptive immune system is mediated by the highly sophisticated B and T cells, which specifically target the invader, and provide a memory response to prevent a repeat of the infection. The innate immune system, which is evolutionarily more ancient than adaptive immunity, must accomplish four fundamental tasks: it must rapidly detect any infectious agent, categorize the type of invading infectious agent, eradicate or at least temporarily contain the infection and finally induce the appropriate type of adaptive immune response to eliminate the infection and prevent its recurrence (Krieg 2008). The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of Pattern-Recognition Receptors (PRRs) that can recognize pathogen-expressed molecules, such as lipopolysaccharide, viral RNA or bacterial DNA, defined as Pathogen-Associated Molecular Patterns (PAMPs). The most well understood of these receptors are the Toll-like receptors (TLRs), of which a family of 10 related molecules has been identified in humans. TLRs are transmembrane receptors comprising an extracellular leucine-rich repeat and a cytoplasmic TIR [Toll/IL-1 (interleukin-1) receptor] domain, connected through a transmembrane domain. Of the ten TLRs identified in humans, TLR3, 7, 8 and 9, referred to as intracellular TLRs, are expressed on the membranes of the endosomes and recognize nucleosides, nucleotides and oligo- and poly-nucleotides derived from intracellular viral and bacterial pathogens, while the

other six TLRs are referred to as cell-surface TLRs and recognize cell-wall constituents of extracellular pathogens (Agrawal and Kandimalla 2007). The immune role of TLR9 has been studied most extensively in plasmacytoid dendritic cells (pDCs) and B cells, which may be the only human immune cells to constitutively express TLR9 (Krieg 2008). Cellular activation is reported to induce TLR9 expression in additional cell types, including human neutrophils (Hayashi et al. 2003), monocytes and monocyte-derived cells (Saikh et al. 2004; Siren et al. 2005) and CD4 T cells (Gelman et al. 2006), but the biologic role for this is less well understood. TLR9 expression has also been reported in some human non-immune cells, including pulmonary epithelial cells and lung cancers (Li et al. 2004; Platz et al. 2004; Droemann et al. 2005), keratinocytes (Lebre et al. 2007), intestinal epithelium (Pedersen et al. 2005; Lee et al. 2006), endometrial epithelium/stroma with a menstrual cycle-dependent regulation (Aflatoonian et al. 2007), and in gastric mucosa, where TLR9 polarization seems to be a process dynamically influenced by Helicobacter pylori infection (Schmausser et al. 2004). TLR9 recognizes and is activated by unmethylated cytosine-phosphate-guanine (CpG) dinucleotides, which are relatively common in bacterial and viral DNA but are suppressed and methylated in vertebrate DNA (Krieg 2006). Synthetic oligodeoxynucleotides (ODNs) that contain CpG motifs that are similar to those found in bacterial DNA, stimulate a similar TLR9 activation and are currently defined as TLR9 agonists. They are rapidly internalized by immune cells, perhaps involving class III phosphatidylinositol 3-kinase (PI3K), and they interact with TLR9 that is present in endocytic vesicles. The interaction between TLR9 agonists and TLR9 transduces an intracytoplasmic activation signal. The signal initiates with the recruitment of myeloid differentiation primary response gene 88 (MyD88) to the Toll–interleukin-1 receptor (TIR) domain of TLR9, followed by activation of the IRAK (IL-1 receptor-activated Kinase) – TRAF6 (tumor-necrosis factor receptor-associated factor 6) complex. This leads to the activation of both the mitogen-activated protein kinase (MAPK: JNK1/2 and p38) and inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) complexes, culminating in the upregulation of transcription factors, including NF-κB and activating protein 1 (AP1) (Klinman 2004). Recently, the concept that only unmethylated CpG motifs are thought to drive TLR9 activation and thus act as PAMPs has been challenged. In fact, it has been suggested that the sugar backbone determined DNA recognition by TLR9. Homopolymeric, base-free phospho-diester (PD) 2’ deoxyribose acted as a basal TLR9 agonist as it bound to and activated TLR9. This effect was enhanced by DNA bases, even short of CpG motifs. In contrast, phospho-thioate (PS)-modified 2’ deoxyribose homopolymers acted as TLR9 antagonists. Only CpG-motifs introduced to inhibitory PS 2' deoxyribose converted the antagonistic activity into powerful agonistic function. Therefore, these new data restricted the CpG-motif dependency of TLR9 activation to the promising group of TLR9 agonists that are based on PS modified synthetic DNA, while natural PD DNA drives TLR9 activation sequence-independently. Thus evolutionary pressure might have exiled nucleic

acid recognizing TLRs such as TLR9 to endosomes in order to avoid activation by host (self) derived nucleic acids (Haas et al. 2008). Moreover, it has been demonstrated that the ectodomain of TLR9 is cleaved in the endolysosome, such that no full-length protein is detectable in the compartment where ligand is recognized. Notably, although both the full-length and cleaved forms of TLR9 are capable of binding ligand, only the processed form recruits MyD88 on activation, indicating that this truncated receptor, rather than the full-length form, is functional. So this process prevents TLR9 from responding to self nucleic acids (Ewald et al. 2008).

At a cellular level, activation of TLR9 by TLR9 agonists initiates a cascade of potent Th1-type innate and adaptive immune responses. All of the cellular immune effects of TLR9 agonists in humans are thought to result directly and indirectly from activating TLR9-expressing pDC and B cells. TLR9 agonists activate pDCs to secrete type I interferon (IFN) and to express increased levels of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2). This is believed to initiate a range of secondary effects, including secretion of cytokines/chemokines, such as monocyte chemoattractant protein-1 (MCP-1) or IFN-γ-inducible 10kDa protein (IP-10), activation of natural killer (NK) cells, through both IFN-dependent and IFN-independent pathways, and expansion of T-cell populations, particularly type 1 helper T (Th1) cells and cytotoxic T lymphocytes (CTLs), that are capable of destroying tumor cells. Opposing these immune-boosting effects, pDCs activated through TLR9 also mediate immune-suppressive effects through counterregulatory factors such as the generation of Tregs. In B cells, TLR9 stimulation results in the secretion of proinflammatory cytokines, such as IL-6, and in the release of immune regulatory cytokines that might limit the intensity of the inflammatory response, such as IL-10. TLR9 activation of B cells confers a greatly increased sensitivity to antigen stimulation and enhances their differentiation into antibody-secreting plasma cells, potentially promoting ADCC. On balance, these immune effects of TLR9 agonists generally promote strong Th1 CD4+ and CD8+ T cell responses. However, the concurrent activation of counterregulatory pathways such as the induction of Tregs limit TLR9-induced immune activation, offering a potential for enhancing the therapeutic efficacy of TLR9 agonists by coadministration of antagonists of one or more of these inhibitory pathways (Krieg 2007; Krieg 2008).

An understanding of the immune cascade initiated by TLR9 activation has prompted the clinical development of several TLR9 agonists in the fields of infectious disease, vaccines, asthma/allergy and cancer. The innate immune response elicited by CpG motifs has developed to protect the host from infectious pathogens. Therefore, TLR9 agonists might be used as stand-alone agents to reduce susceptibility to infection. When combined with allergen, these agonists stimulate an antigen-specific T helper 1 (Th1)-cell response that inhibits the development of Th2-cell-mediated allergic asthma. TLR9 agonists also improve the function of professional antigen-presenting cells (APCs), and create a cytokine/chemokine milieu that is conducive to the development of an

adaptive immune response to co-administered vaccines (Klinman 2004). The rationale for investigating TLR9 agonists as anticancer agents is based on the hypothesis that the innate immune activation may have direct antitumor effects and that the enhanced tumor antigen presentation in a Th1-like cytokine and chemokine milieu will promote an antitumor immune response (Krieg 2008). In particular, *in vivo* dendritic cell activation through TLR9 has a key role in the switching on antitumor immunity. In fact, malignant tumors suppress immune function, and create an environment that favours the maintenance of T-cell tolerance, preventing the development of antitumor immunity. Dendritic cell activation creates a Th1-like cytokine and chemokine milieu and can up-regulate the expression of co-stimulatory molecules on the pDCs, shifting T cells from tolerance, to a strong cytotoxic T-lymphocyte response against the tumor antigens (Krieg 2006).

The initial impetus to develop TLR9 agonists as anticancer drugs came from several preclinical studies demonstrating antitumor activity in a wide variety of tumor models, both in monotherapy or in combination with traditional anticancer therapies, such as radiotherapy and chemotherapy, or other immunotherapies (Baines and Celis 2003; Weigel et al. 2003; Milas et al. 2004). Mice and humans have a different TLR9 expression pattern, and exposure to CpG motifs stimulates a more narrow profile of cytokines/chemokines in humans; thus, preclinical results cannot be considered predictive of clinical findings (Krieg 2007). However, based on the strength of the extensive preclinical data, several TLR9 agonists are now being developed as anticancer agents. Because the phosphodiester bond of native DNA is rapidly degraded by endonucleases, these investigational TLR9 agonists use a nuclease-resistant phosphorothioate backbone that improves the half-life in the body from just a few minutes (for unmodified native DNA) to about 48 h (Krieg 2008). Coley Pharmaceutical Group (Wellesley, MA, USA) has developed a TLR9 agonist known as CPG 7909 that is being investigated as a vaccine adjuvant in several tumor types. CPG 7909 was licensed by Pfizer Pharmaceuticals Inc. (New York, NY, USA) for clinical investigation as a single agent or in combination with other therapeutic approaches, and is now known as PF-3512676 when it is not being used as a vaccine adjuvant. Other TLR9 agonists in clinical development for cancer include ISS 1018 (Dynavax Technologies, Berkeley, CA, USA) and CpG-28 (University of Paris, France) (Krieg 2008).

A distinguishing characteristic of TLR9 agonists is their ability to induce strong CD4+ and CD8+ T-cell responses and rapid production of antigen-specific antibodies when used as a vaccine adjuvant with many types of antigen (Krieg 2007). These TLR9 agonists adjuvants have been heavily studied for the treatment of infectious diseases, and several clinical trials are also investigating their activity as tumor vaccines. Phase I and II trials have been investigating CPG 7909 as a vaccine adjuvant in patients with melanoma, breast cancer and renal cell carcinoma (RCC). The most advanced cancer vaccine program is a phase III, randomized, controlled, clinical trial of the

tumor antigen MAGE-A3 combined with CPG 7909 for the treatment of patients with early-stage (stage IB, II or IIIA), completely resected NSCLC whose tumors express the antigen (Krieg 2008).

Monotherapy with TLR9 agonists has been evaluated in patients with hematologic malignancies, skin cancers and other solid tumors, such as advanced renal cell carcinoma (RCC) or recurrent glioblastoma. TLR9 agonists have a variety of effects on B cells that may be relevant in the treatment of hematologic malignancies. Activation of TLR9 on primary malignant B cells upregulates expression of major histocompatibility complex molecules and other surface receptors, thereby increasing their capacity to stimulate T cells. This may result in an enhanced T-cell-mediated response to tumor antigens on the malignant B cells. Furthermore, in patients with advanced cutaneous T-cell lymphoma (CTCL), NK cells and CD8+ T cells were activated following culturing of peripheral blood mononuclear cells with TLR9 agonists, and there was a marked increase in IFN-α production. This could enhance the antitumor immune response by activating NK cells, or may induce a direct antiproliferative effect (Krieg 2008). Chronic lymphocytic leukemia (CLL) cells, which express TLR9, are induced by TLR9 agonists to undergo apoptosis, in contrast with normal primary B cells in which TLR9 activation protects against apoptosis (Jahrsdorfer et al. 2005). Several clinical studies of single-agent TLR9 agonists have been completed in patients with hematologic malignancies, indicating that TLR9 agonists may be useful in the treatment of these tumors (Link et al. 2006). Because melanoma is a highly immunogenic tumor that has been shown to respond to immunotherapy, it is a logical malignancy in which to explore the activity of TLR9 agonists. Two general approaches have been investigated using monotherapy with PF-3512676: local therapy with intra- or perilesional injection and systemic therapy with s.c. injection. These studies demonstrated that PF-3512676 is generally well tolerated as a single agent in patients with melanoma or basal cell carcinoma (BCC) and is associated with antitumor activity (Molenkamp et al. 2007; Hofmann et al. 2008). Monotherapy with TLR9 agonists has also been evaluated in phase I trials in patients with advanced RCC or recurrent glioblastoma. A minor response was observed in 2 of 24 patients with recurrent glioblastoma receiving CpG-28 in a phase I trial (Carpentier et al. 2006). In a phase I trial in 31 patients with advanced RCC receiving PF-3512676, one patient (3%) had a PR and nine patients (29%) had SD (Krieg 2008).

Combination therapy with TLR9 agonists has been evaluated above all in patients with Non-Hodgkin's lymphoma and NSCLC. Preclinical studies have indicated that the combination of a TLR9 agonist and rituximab, a MAb against CD20, may be more effective than rituximab alone. Two phase I clinical trials have investigated the combination of a TLR9 agonist with rituximab in patients with relapsed/refractory NHL, obtaining an overall response of 24% and 32% respectively. Combination therapy with TLR9 agonists is being further evaluated for antitumor activity in a phase I/II trial in combination with radiation therapy and in a phase II trial in combination with

rituximab (Krieg 2008). Several preclinical models have also suggested that a TLR9 agonist can synergize with cytotoxic chemotherapy in NSCLC (Krieg 2007). Based on both preclinical and clinical proof-of-concept studies, a randomized controlled phase II trial was conducted in chemotherapy-naïve patients with stage IIIb/IV NSCLC, suggesting that the addition of PF-3512676 to taxane plus platinum chemotherapy for first-line treatment of NSCLC improves objective response and may improve survival (Manegold et al. 2008). Following completion of the phase II trials, two phase III studies were conducted exploring the combination of PF-3512676 with paclitaxel/carboplatin or gemcitabine/cisplatin versus chemotherapy alone as first-line treatment of patients with advanced NSCLC. No improvement in overall survival or progression-free survival was observed when PF-3512676 was added to standard platinum-based doublet chemotherapy in either trial. However, there was a higher frequency of grade 3/4 neutropenia and thrombocytopenia and a higher frequency of 'sepsis-like events' and 'septic deaths' reported as serious adverse effects in the PF-3512676 plus chemotherapy arms. Based on the recommendation of an independent data monitoring safety committee, these phase III studies were terminated. The reason for the failure of these trials is unclear, but possible factors include the advanced stage of the disease, the use of concomitant steroid therapy as premedication for the chemotherapy, and possible immune-suppressive effects of the repeated chemotherapy cycles (Krieg 2008).

Overall, TLR9 agonists are generally well tolerated in patients with cancer. The most common adverse effects observed with administration of TLR9 agonists are local injection-site reactions (erythema, edema, inflammation and pain) or systemic flu-like symptoms (headache, rigors, pyrexia, nausea and vomiting). These symptoms typically develop with 24 h of dosing and are transient, generally lasting for less than 2 days (Krieg, 2006). Clinical trials are ongoing in other settings and in combination with other therapeutic approaches, and the true therapeutic potential of TLR9 agonists for the treatment of cancer remains to be further determined (Krieg 2008).

Based on extensive structure activity relationship studies, second-generation synthetic agonists of TLR9 containing 3'-3'-attached novel structures (immunomers) and immunomodulatory CpR (R=2'-deoxy-7-dezaguanosine) dinucleotides, referred to as immune modulatory oligonucleotides (IMOs), have been synthesized. 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. IMOs contain two accessible 5'-ends and have demonstrated distinct cytokine profiles *in vitro* and *in vivo*, compared with conventional TLR9 agonists and higher metabolic stability due to the novel structure present in them (Kandimalla et al. 2003; Yu et al. 2002). Previous studies have demonstrated potent antitumor activity of IMO-2048, containing a mouse-specific immunostimulatory sequence cross-reacting with human TLR9, as monotherapies and in combination with chemotherapeutic agents and MAbs (Kandimalla et al. 2003; Damiano et al.

2006). Currently, a human analogue of this IMO, IMO-2055 (Idera Pharmaceuticals Inc., Cambridge, MA, USA), is under clinical evaluation in combination with chemotherapy and other agents in cancer patients. IMO-2055 has been evaluated for its safety and immunological activity in Phase I studies involving healthy subjects and refractory cancer patients, and is being evaluated in phase I/II trial in combination with gemcitabine and carboplatin in patients with refractory solid tumors. Moreover, IMO-2055 is being evaluated at two dose levels, with subcutaneous administration, in a Phase II trial for metastatic or recurrent renal cell carcinoma (RCC). The first part of the study (Stage A) including treatment naïve patients and patients who have received one prior therapy, has been completed. The primary objective, tumor response based on RECIST (Response Evaluation Criteria In Solid Tumors), was not achieved in the study. Median progression-free survival among the arms ranged from 2 to 4 months. IMO-2055 treatment was generally well-tolerated with good dose intensity in all arms of the study.

Although the TLR9 immunologic mechanisms are fairly well understood and the clinical development of TLR9 agonists is very encouraging, the mechanisms by which they affect signalling proteins involved in tumor growth and angiogenesis, thus leading to tumor growth inhibition, are yet to be elucidated. We have hypothesized that the novel TLR9 agonists IMO could impair EGFR signalling. If this is the case, IMO could greatly enhance the activity of EGFR antagonists, including cetuximab, with an EGFR-dependent mechanism, additional to the possible boost of ADCC, which has been shown previously to be an important component of the antitumor activity of cetuximab (Clynes et al. 2000). Using IMO-2048 recognizing human and murine TLR9, we recently discovered that IMO acts by impairing EGFR signalling, cooperates with anti-EGFR drugs and boosts the ADCC of anti-EGFR antibodies (Damiano et al. 2006). 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 TGF-α, total and activated EGFR, activated pMAPK and pAkt, cyclooxygenase-2, and bcl-2, almost suppressing VEGF, without affecting its receptor VEGFR-2/KDR. Moreover, IMO causes about 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 signalling proteins although at a degree surprisingly lower than that obtained with IMO, whereas inhibition of microvessel formation was about 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 VEGFR-2/KDR, and an almost completely suppressed vessels formation. It should be noticed that the inhibition of these signalling proteins and of angiogenesis by IMO may be a valuable tool to prevent the occurrence of cetuximab resistance. We have also analyzed the

presence of tumor-infiltrating lymphocytes, which are considered a favorable prognostic factor in colorectal cancer (Compton et al. 2000). 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. The impairment of EGFR signalling 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 immunologic activity, showed again a cooperative antitumor effect and inhibition of critical signalling proteins although at a lesser degree compared to the combination 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 signalling becomes EGFR independent (Bianco et al. 2008). 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, IMO plus cetuximab had a cooperative inhibitory effect, suggesting that IMO is able to boost the previously reported immunologic activity of cetuximab (Fan et al. 1993). 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 signalling. Finally, we have evaluated the antitumor activity of IMO in combination with cetuximab and the topoisomerase I–selective drug irinotecan, demonstrating that this drug combination is able to eradicate the tumors in 90% of animals without evidence of major side effects or organ toxicity in the pathologic examination. In conclusion, our study has shown 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 signalling and involved in tumor cell proliferation, apoptosis, and angiogenesis; (b) this effect contributes to greatly enhance the antitumor activity of 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 (Damiano et al. 2006). These new findings have opened the path to clinical studies combining TLR9 agonists with EGFR inhibitors. An ongoing clinical trial in NSCLC is addressing the safety and potential clinical benefit of PF-3512676 combined with erlotinib (Krieg 2008). In December 2007, a Phase Ib trial of IMO-2055 in combination with erlotinib and bevacizumab has been initiated in patients with NSCLC who have progressed on first line chemotherapy. A second Phase Ib clinical study of IMO-2055 in combination with cetuximab and irinotecan in patients with advanced colorectal cancer is planned.

2. AIM OF THE STUDY

The recent findings on the major role played on tumor progression by host immune system and tumor microenvironment suggest that they may be critical factors also in the failure of targeted therapies. The yet lacking integration of these findings with the information guiding the clinical use of targeted drugs would fill a major gap, greatly improving the efficacy of targeted agents, reducing the chance for resistance and favouring the development of other effective drugs. It is now necessary to investigate the mechanisms of resistance to targeted agents and to design new combined targeted treatments in a wider framework, taking into account also angiogenesis, tumor/host immune balance and microenvironment.

The aim of this study is to provide new insights into the mechanism of action of the novel antitumor TLR9 agonist IMO on signalling and angiogenesis, in order to design optimal treatment combination and schedule strategies to be easily transferred into clinical practice.

In the former study we observed a potent inhibitory effect of IMO and cetuximab on VEGF and angiogenesis in wild type but not in GEO-CR colon tumors, suggesting that the anti-VEGF effect may be only EGFR-dependent. Therefore, in this study we have studied whether: a) the potent inhibitory effect on VEGF and angiogenesis observed with IMO and cetuximab is mainly due to interference on EGFR pathway or it is independent; b) the dependance of IMO on an integral EGFR pathway may affect its combination with an anti-VEGF agent, such as bevacizumab; c) the ADCC mechanism is necessary to obtain a cooperative effect with MAbs. Therefore, we used IMO and the anti-VEGF antibody bevacizumab as tools to study IMO's role on EGFR and angiogenesis and to explore its therapeutic potential in colon cancer xenografts both sensitive and resistant to anti-EGFR drugs.

Moreover, considering that IMO creates the opportunity to take advantage of multiple chance for cooperativity, involving EGFR- and ADCC-dependent and -independent mechanisms and neoangiogenesis, we have evaluated IMO ability to revert mechanisms of resistance to different targeted agents from EGFR inhibitors in different tumor models from colon cancer. Since intrinsic and acquired resistance to anti-ErbB2 antibody trastuzumab is becoming an increasingly relevant issue in breast cancer, we investigated whether the antitumor, antiangiogenic and immunostimulatory activity of IMO could enhance trastuzumab effect in trastuzumab-resistant breast cancers.

3. MATERIALS AND METHODS

Compounds. IMO, 5'-TCTGACRTTCT-X-TCTTRCAGTCT-5' (X and R are glycerol linker and 2'-deoxy-7-deazaguanosine, respectively), was synthesised with phosphorothioate backbone, purified and analyzed as described previously (Kandimalla et al. 2003). The anti-VEGF MAb bevacizumab was kindly provided by Genentech (South San Francisco, CA). The anti-ErbB2 MAb trastuzumab was kindly provided by Roche (Basel, Switzerland).

Cell cultures. GEO, LS174T, GEO cetuximab-resistant (GEO-CR) (Ciardiello et al. 2004) colon cancer cells, BT474, KPL-4, JIMT-1 breast cancer cells and HUVEC (human umbilical vein endothelial cells) were maintained respectively in McCoy's or RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

ADCC assay. Non-adherent fraction of human peripheral blood mononuclear cells (PBMCs) were used as effector cells. Briefly, human PBMCs were isolated by density gradient centrifugation and re-suspended in RPMI medium. Prior to the assay, the PBMCs were cultured 1 hour on plastic dishes to remove adherent cells (monocytes) and then the peripheral blood lymphocytes (PBLs) were incubated for 24 hours in presence or absence of IMO (5µM). The target cells (chronic erythroid leukemia K562 and breast cancer MDA468, JIMT-1 or KPL-4) were loaded with the fluorescence enhancing ligand (DELFIA BATDA reagent, PerkinElmer, Wellesley, MA) and, after washing, were incubated in presence or absence of antibodies bevacizumab, cetuximab or trastuzumab 10 µg/ml. Target cells were mixed with effector cells at varying cell concentration for 2 hours at 37°C and centrifuged. Supernatants were added to Europium solution and the signal was measured, as previously described (Blomberg et al. 1996).

Xenografts in nude mice. Five weeks old Balb/cAnNCrlBR 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. GEO, LS174T, GEO-CR, KPL-4 or JIMT-1 human cancer cells (10^7 cells/mice) were resuspended in 200 µL of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected subcutaneously (sc) in mice. After 7 days, tumors were detected and groups of 10 mice were randomized to receive the following treatments: intraperitoneal (ip) IMO, 1 mg/kg three times a week for 4 weeks; ip bevacizumab 5 mg/kg, twice a week for 3 weeks; ip trastuzumab 3,75 mg/kg, twice a week for 3 weeks; or the combination of these agents, on days 7-11, 14-18 and 21-25, continuing only IMO on days 28-32. Tumor volume

was measured using the formula $\pi/6$ x larger diameter x (smaller diameter)^2 as previously reported (Ciardiello et al. 1996). Two mice were sacrificed on day 25 to perform biochemical analysis.

Immunoprecipitation and Western blot analysis. Total cell lysates were obtained from cellular lysates or homogenized tumor specimens removed on day 25. The protein extracts were resolved by 4-15% SDS-PAGE and probed with anti-human, polyclonal Akt, monoclonal pAkt, (Cell Signaling Technologies, Beverly, MA), monoclonal actin (Sigma-Aldrich, Milan), monoclonal pEGFR, polyclonal EGFR, polyclonal pErbB2, monoclonal ErbB2, monoclonal VEGF, monoclonal pMAPK and monoclonal MAPK (Santa Cruz, Santa Cruz, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL), as described previously (Ciardiello et al. 2004). Co-immunoprecipitation analysis were performed by immunoprecipitation using a monoclonal anti-TLR9 antibody (Calbiochem/EMD Biosciences, La Jolla, CA) and blotting with polyclonal anti-EGFR antibody or with monoclonal anti-ErbB2 antibody, following procedures described above.

Elisa assay. Anti-hVEGF or anti-mVEGF polyclonal antibody (R&D Systems, Minneapolis, MN), diluted at 1 μg/ml in PBS, pH 7.5, was used to coat a 96-well plate, 100 μl/well, overnight at 4 °C. Washings, dilutions of standards (recombinant hVEGF or mVEGF) and samples (serum of sacrificed mice or conditioned media from cultured cells), biotinylation and mix with preformed avidin and biotinylated HRP macromolecular complex (Vectastain kit) were previously described (Errico et al. 2004). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Hercules, CA). VEGF concentrations were determined by interpolation of the standard curve using linear regression analysis.

Cell Survival Assay. Cells were grown in 24-well plates and exposed to drugs. The percentage of cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's instructions.

Growth in soft agar Assay. Cells (10^4 cells/well) were suspended in 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete medium, layered over 0.8% agar-medium base layer and treated with different concentrations of trastuzumab. After 10–14 days, cells were stained with nitro blue tetrazolium (Sigma Chemical Co., Milan, Italy) and colonies >0.05 mm were counted (Ciardiello et al. 2001).

Adhesion assay. 96-microwell bacterial culture plates were precoated with 50 μl/well of serum-free medium containing 0.1% BSA (SFM, negative control) or Matrigel (1 mg/ml in water). After 1 hour, all coating solutions

were removed and HUVECs (20,000 cells/well) were plated in SFM, in presence or absence of IMO 1 μM After incubation cells were analyzed as previously described (Benelli et al. 2003).

Wound healing assay. Monolayers of HUVEC cells were grown on gridded plastic dishes and scratched as previously reported (Bennett et al. 2007) with or without doxorubicin 10 ng/ml or IMO 1 μM (0 h), which cause the same antiproliferative effect. Since doxorubicin did not interfere with cell migration, it was used as a negative control. The migration distances between the edges of the cells in the wound were photographed (10X magnification) at 0 and 24 hours, quantified and compared using Adobe Photoshop ver 8.0.1 (Bennett et al. 2007).

Vascular endothelial cell capillary tube and network formation. Five hundred μl of diluted Matrigel was added into a 30-mm culture dish and incubated at 37°C for 30 min. After the Matrigel was solidified, HUVECs (4×10^5) in 1 ml of RPMI medium were added in each dish, in presence or absence of IMO and/or trastuzumab, incubated at 37°C and photographed (10X) at 0 and 24 hours, to monitor the process of vascular endothelial cell tube and network formation. As positive control, Matrigel was mixed with VEGF 100 ng/ml (R&D Systems, Minneapolis, MN, USA).

Immunofluorescence and confocal microscopy analysis. 5×10^4 cells/ml were plated in complete medium on 12 mm diameter glass coverslips. 48 hours later, cells were fixed and permeabilized, as previously described (Ulianich et al. 2008). Cells were then incubated for 1 hour with the primary antibodies diluted in 0.5% BSA (Sigma) in PBS: polyclonal rabbit anti-human EGFR (Santa Cruz, Santa Cruz, CA) and monoclonal mouse anti-human TLR9 (Calbiochem/EMD Biosciences, La Jolla, CA). After three washes with 0.2% gelatin in PBS-CM, cells were incubated for 20 minutes with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:50 in 0.5% BSA in PBS. After final washes with PBS, the coverslips were mounted on a microscope slide and examined with a Zeiss 510 confocal laser scanning microscope. Samples were observed by three investigators, without knowledge of the experimental conditions.

Statistical analysis. The Student's *t* test was used to evaluate the statistical significance of the results. All reported P values were two-sided. All analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

4. RESULTS AND DISCUSSION

Bevacizumab has no ADCC activity and IMO is unable to affect it. Since bevacizumab binds the ligand and not the membrane receptor, we have verified whether it has any ADCC activity and whether IMO affects it. To investigate the capability of bevacizumab to activate an ADCC reaction in cancer cells and the influence of IMO in a combination treatment, we performed an *in vitro* cytotoxic assay using the conventional target MDA-468, a human breast cancer line which expresses both EGFR and VEGFRs. As expected, in the absence of antibodies, freshly isolated non-adherent human peripheral blood lymphocytes (PBLs) were able to kill the standard NK-target K562 cells, but did not induce any detectable lysis of MDA468 cells (Fig. 1). The same result was obtained when PBLs were incubated with IMO. Conversely, the anti-EGFR MAb cetuximab, that possesses a formerly described ADCC mechanism (Fan et al. 1993), caused a 40% induction of MDA-468 lysis. Pre-incubation of PBLs with IMO potentiated up to 60% of the MDA-468 killing induced by cetuximab (Fig. 1). On the contrary, pre-incubation of PBLs with IMO did not affect the bevacizumab inability to produce the lysis of MDA-468 cells. In fact, bevacizumab as well as the combination of IMO and bevacizumab have no ADCC. Therefore, we have shown that while the basal ADCC activity of cetuximab is enhanced by IMO, thus contributing to cetuximab activity with an EGFR-independent mechanism, bevacizumab has no ADCC activity and IMO is unable to affect it.

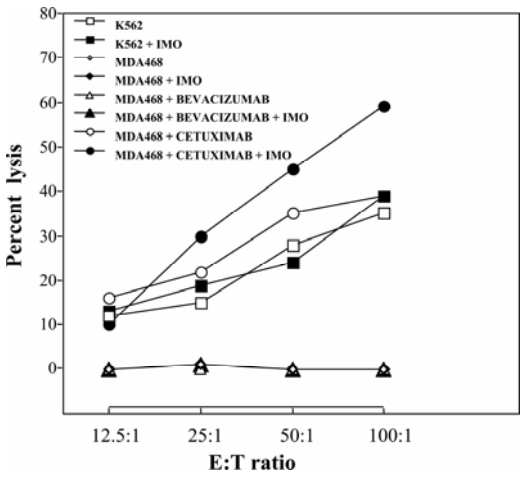

Fig. 1. ADCC Assay. The effector cells (human PBLs) were incubated in presence or absence of IMO and then mixed with the target cells (K562 and MDA468), which were incubated in presence or absence of the antibodies cetuximab and bevacizumab, at different effector/target ratio (E:T ratio). Doses and time of incubation are indicated in Materials and Methods Section.

Combination of bevacizumab with IMO synergistically inhibits GEO and LS174T colon cancer xenografts. BalbC nude mice xenografted with GEO and LS174T tumors were treated with IMO or bevacizumab, alone and in combination (Fig. 2A and 2B). On day 56, 8 weeks after tumor injection, all untreated mice xenografted with GEO cells reached the maximum allowed tumor size of about 2 cm^3, while mice treated with IMO alone exhibited a 50% growth inhibition, having a size of about 1 cm^3. Treatment with bevacizumab produced a 35% inibition, since tumors measured 1.3 cm^3 at the same time point. The combination of IMO plus bevacizumab caused a potent cooperative antitumor activity, with over 95% growth inhibition (tumor size of only 0.12 cm^3) (Fig. 2A). Similar effects were observed in LS174T xenografts. The maximum allowed size of about 2 cm^3 was reached on day 35 in the untreated mice. On day 56, at the end of the experiment, mice treated with IMO or bevacizumab alone measured about 1.6 and 1 cm^3, respectively, while in those treated with the two agents in combination showed a potent cooperative tumor growth inhibition of about 95% compared to untreated animals, resulting in a tumor size of 0.16 cm^3 (Fig. 2B). No treatment-related side effects were observed in either tumor model. Comparison of tumor sizes among different treatment groups, evaluated by the Student's *t* test, was statistically significant both in GEO and LS174T tumors (Fig. 2).

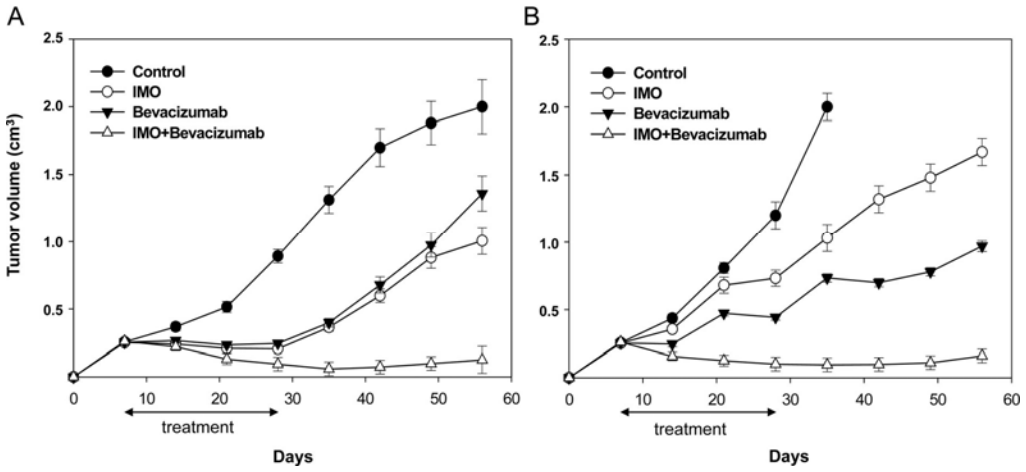

Fig. 2. Cooperative effect of IMO and bevacizumab on tumor growth of mice bearing human colon cancer xenografts. After 7 days following tumor cell injection, mice were randomized (10/group) to receive IMO, bevacizumab or the combination, as indicated in Materials and Methods Section. The Student's t test was used to compare tumor sizes among different treatment groups at day 56 following GEO (A) and LS174T (B) cell injection. They resulted statistically significant for IMO plus bevacizumab versus control (two sided $P < 0.0001$), versus IMO alone (two sided $P < 0.0001$), and versus bevacizumab alone (two sided $P < 0.0001$) in both experiments. Bars, SD.

Combination of bevacizumab with IMO inhibits the expression of signalling proteins and angiogenesis in GEO and LS174T xenografts and reduces the levels of hVEGF, but not of mVEGF, in mice serum. We analyzed the effect of treatment on the expression of a variety of proteins playing a critical role in cancer cell proliferation and angiogenesis. Western blotting analysis was performed on cell lysates from tumors removed at the end of the third week of treatment, on day 25. As shown in Fig. 3A and 3B, IMO did not affect the total amount of MAPK and Akt, but inhibited their activated forms pMAPK and pAkt and the VEGF expression. Bevacizumab inhibited the same signalling proteins, although to a lesser degree compared to IMO. When the two agents were used in combination, a more potent inhibition was observed on protein expression. To confirm the effect of combination of bevacizumab with IMO on human VEGF levels, we performed ELISA on the serum obtained from LS174T bearing mice (Fig. 3C and D). Since bevacizumab recognizes only human VEGF, as expected it caused a reduction in the levels of circulating human VEGF (hVEGF) in the serum. The treatment with IMO alone reduced the secreted hVEGF levels and the combination with bevacizumab caused a more potent inhibition of circulating hVEGF levels compared to treatment with single agents (Fig. 3C). On the contrary, neither single agent nor the combination affected murine VEGF (mVEGF) compared to untreated mice (Fig. 3D). Therefore, analysis of the secreted VEGF in the serum of sacrificed mice confirmed that bevacizumab, as expected, reduces the hVEGF levels and that also the combination of IMO and bevacizumab cooperates in reducing the levels of hVEGF, but not of mVEGF. These results suggest that the murine-dependent immune mediated effects of IMO enhance the activity of bevacizumab only on the human tumor cells.

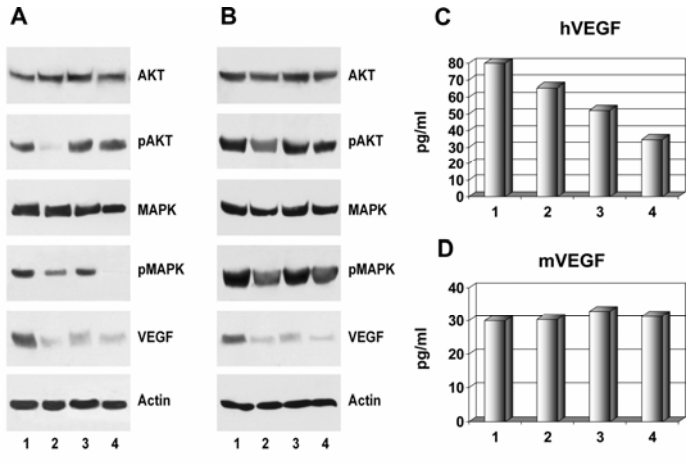

Fig. 3. Western blot analysis of GEO (A) and LS174T (B) tumors and ELISA on mice serum (C-D). Western blotting (A and B) was performed on total lysates from tumor specimens of two mice sacrificed on day 25. ELISA for hVEGF and mVEGF (C and D) were performed on serum of two mice sacrificed on day 25. Lane 1, Untreated control; lane 2, IMO; lane 3, bevacizumab; lane 4, IMO plus bevacizumab.

Combination of bevacizumab with IMO causes a potent antitumor activity in cetuximab resistant GEO-CR xenografts. We evaluated whether a cooperative effect could be obtained in cetuximab-resistant GEO-CR tumors, in the absence of an ADCC effect. We have shown that IMO alone is ineffective, while bevacizumab markedly inhibited GEO-CR growth (Fig. 4A). When IMO was used in combination with bevacizumab a cooperative inhibitory effect was observed, since at the end of the experiment, tumors were still about 0.15 cm^3 (Fig 4A). With the exception of the mice treated with IMO alone, the Student's *t* test demonstrated that the growth inhibition caused by each treatment in comparison to untreated mice as well as the tumor size among different treatment groups were statistically different (Fig. 4). Western blotting analysis of protein extracts from GEO-CR tumors did not reveal any substantial changes in the expression of pAkt, pMAPK and VEGF in tumor specimens treated with IMO alone, while a marked inhibition was seen in those treated with bevacizumab or with the combination IMO plus bevacizumab (Fig. 4B).

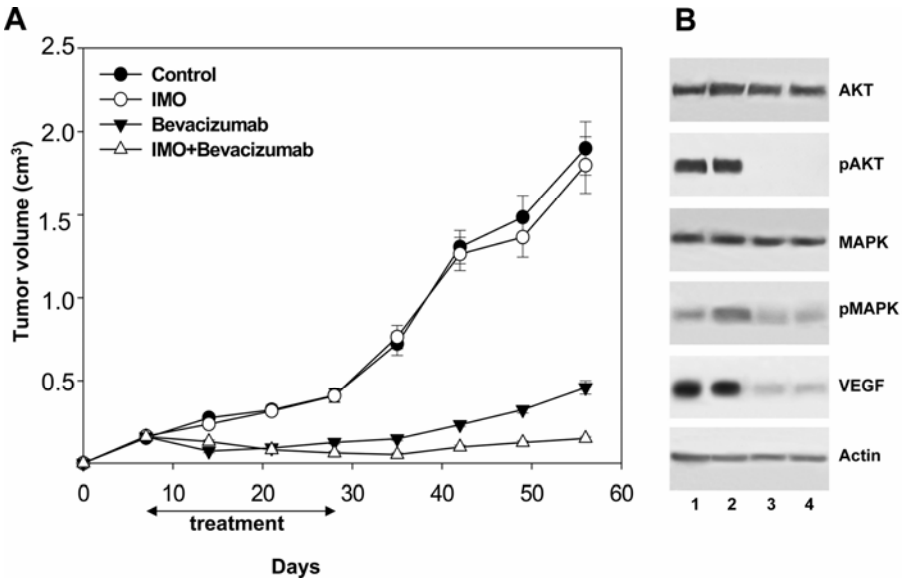

Fig. 4. Effect of the combination of IMO with bevacizumab in mice bearing cetuximab-resistant GEO-CR tumor xenografts. (A) After 7 days following GEO-CR tumor injection mice were randomized (10/group) to receive IMO, bevacizumab or the combination, as indicated in Materials and Methods Section. Inhibition of growth was significantly different in the IMO plus bevacizumab-treated group versus the control group, the IMO alone group and the bevacizumab alone group (two sided $P < 0.0001$ for each comparison). Bars, SD. (B) Western blotting was performed on total lysates from tumor specimens of two mice sacrificed on day 25. Lane 1, Untreated control; lane 2, IMO; lane 3, bevacizumab; lane 4, IMO plus bevacizumab.

Therefore, we have demonstrated that combination of bevacizumab with IMO is active also in anti-EGFR resistant tumors in an ADCC-independent fashion, suggesting that other mechanisms, not strictly EGFR- and ADCC-dependent, take place. In support of this notion, the two agents in combination cooperatively inhibit the expression of proteins used by tumors as escape pathways to acquire resistance to targeted therapies, such as pMAPK, pAkt and VEGF (Bianco et al. 2005) and inhibit neoangiogenesis in all three tumor types.

IMO inhibits survival, adhesion to matrix, migration and capillary formation capability of human endothelial cells. An important mechanism of antiangiogenic therapy is the blockade of the VEGF-dependent proliferation of endothelial cells in the tumor. In an attempt to provide a clue to explain the non EGFR-dependent cooperative antiangiogenic effects obtained with IMO and bevacizumab, we measured IMO activity on several functions of endothelial cells. We performed a cell survival assay on HUVEC cells. At doses of 1 and 5 μM, IMO caused a moderated inhibition of HUVEC cell survival, which was increased by the addition of lipofectamine, an agent that facilitates cellular uptake of IMO (Fig. 5A). We then analyzed the effects of IMO on HUVEC cell adhesion and migration using a cell adhesion assay to matrigel and a wound healing assay. At 1 μM concentration, IMO showed a potent inhibitory activity on adhesion and migration of endothelial cells (Fig. 5B and C). Finally, we examined the effects of IMO on VEGF-stimulated capillary tube and network formation and observed that this process is strongly inhibited by IMO (Fig. 5 D). Therefore, we demonstrated for the first time that IMO inhibits proliferation, adhesion and migration of HUVEC endothelial cells and, importantly, the VEGF-stimulated capillary tube and network formation. It is likely that the well documented inhibitory effect of bevacizumab on vessels formation, due to VEGF inhibition, combined with the interference of IMO on critical functions of tumor endothelial cells, may finally be responsible for the cooperative effect observed. In addition, since it has been reported that colon cancer cell lines, including GEO, express VEGFRs (Fan et al. 2005), the combined effect of IMO and bevacizumab may have a direct impact also on tumor cells. Altogether these data may also help to explain, at least in part, the hemorragic necrosis observed in the LS174T tumors. Since IMO is able to directly inhibit *in vitro* endothelial cells functions, we have suggested that, beside the immune activation, TLR9 agonists have a much broader range of mechanisms, involving not only the EGFR-dependent pathway but also the neoangiogenesis. It has been demonstrated that tumor microenvironment is a complex system made of many cell types participating in tumor progression and including endothelial cells and their precursors, pericytes, fibroblasts, granulocytes, mast cells, T, B and natural killer (NK) lymphocytes, and antigen-presenting cells such as macrophages and dendritic cells (DCs). Moreover, recent evidence shows that complex endothelial-immune cell cross-

talk regulate vessel function and that multiple immune cells polarizations are involved in a dichotomous activity towards angiogenesis induction or inhibition. The factors imposing immune cell polarization appear to be a function of the microenvironment (Noonan et al. 2008). In this respect, it could be interesting to study IMO anti-angiogenic effect also on other cells of tumoral/microvascular microenvironment and to evaluate IMO potential activity on these pro-or anti-angiogenic polarizations.

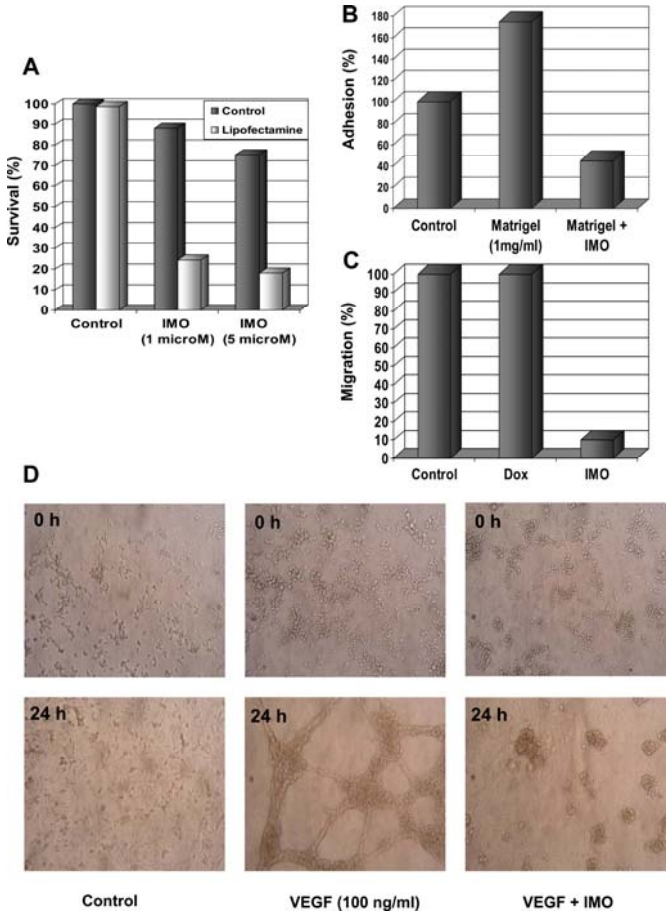

Fig. 5. IMO effects on HUVEC cell survival (A), adhesion to matrix (B), migration (C), and tube formation (D). (A) HUVEC cells were treated with IMO in presence or absence of Lipofectamine (2 µg/ml). The results are statistically significant for each dose of IMO versus control (two sided P<0.0001). (B) HUVEC cells plated in presence or absence of IMO 1 µM in serum-free medium (negative control) or Matrigel. The results are statistically significant for Matrigel versus negative control and for IMO versus Matrigel (two sided P<0.0001). (C) HUVEC cells monolayers were wounded in absence or presence of doxorubicin 10 ng/ml or IMO 1 µM (0h). The results are statistically significant for IMO versus control and versus doxorubicin (two sided P<0.0001). (D) HUVEC cells were incubated on diluted Matrigel in presence or absence of IMO 1 µM. Matrigel mixed with VEGF 100 ng/ml was used as positive control. Photographs were taken at 0 and 24 hours.

Trastuzumab is unable to inhibit growth and ErbB-related signalling pathway in KPL-4 and JIMT-1 human breast cancer cell lines. Considering that IMO creates the opportunity to take advantage of multiple chance for cooperativity, involving EGFR- and ADCC-dependent and -independent mechanisms and neoangiogenesis, we have evaluated IMO ability to revert mechanisms of resistance in different tumor models from colon cancer. Since intrinsic and acquired resistance to anti-ErbB2 antibody trastuzumab is becoming an increasingly relevant issue in breast cancer, we investigated whether the antitumor, antiangiogenic and immunostimulatory activity of IMO could enhance trastuzumab effect in trastuzumab-resistant breast cancers. To this aim, we have identified/selected human breast cancer cell lines with different sensitivity to trastuzumab (Kurebayashi et al. 1999; Tanner et al. 2004). As shown in Figure 6A, we have verified in a soft agar cell growth assay that BT474 cells are sensitive to trastuzumab (IC_{50} <0.1 μg/ml), while KPL-4 and JIMT-1 are insensitive to trastuzumab at doses up to 50 μg/ml. In fact, KPL-4 and JIMT-1 are described as two human estrogen receptor-negative and ErbB2-overexpressing breast carcinoma cell lines, recently established as experimental models for the study of new trastuzumab-resistance mechanisms. KPL-4 cell line was isolated from the malignant pleural effusion of a breast cancer patient with an inflammatory skin metastasis (Kurebayashi et al. 1999). JIMT-1 cell line was established from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab (Tanner et al. 2004). On BT474, KPL-4 and JIMT-1 cells, we have evaluated the expression levels of EGFR and ErbB2. All cells show similar levels of EGFR expression while ErbB2 levels are higher in KPL-4 and JIMT-1 than in BT474 cells. Moreover, we have demonstrated that KPL-4 cells overexpress the NH2 terminally truncated ErbB2 receptor, p95ErbB2 (Fig. 6B), that has been correlated to trastuzumab.resistance in breast tumors (Scaltriti et al. 2007). We have compared for the first time the effect of trastuzumab on the activation of ErbB signal transduction, in cancer cells sensitive and resistant to trastuzumab. We have demonstrated that trastuzumab is able to inhibit the activation of signalling proteins critical for cancer cell proliferation, survival and angiogenesis in sensitive but not in resistant cell lines. In fact, as shown in fig. 6C, trastuzumab reduces the phosphorylation/activation of MAPK and Akt in BT474 cell line but not in KPL-4 and JIMT-1 cell lines. Similarly, trastuzumab inhibits the secretion of the main pro-angiogenic factor VEGF in BT474 cells but is totally ineffective in KPL-4 and JIMT-1 cells (Fig. 6D).

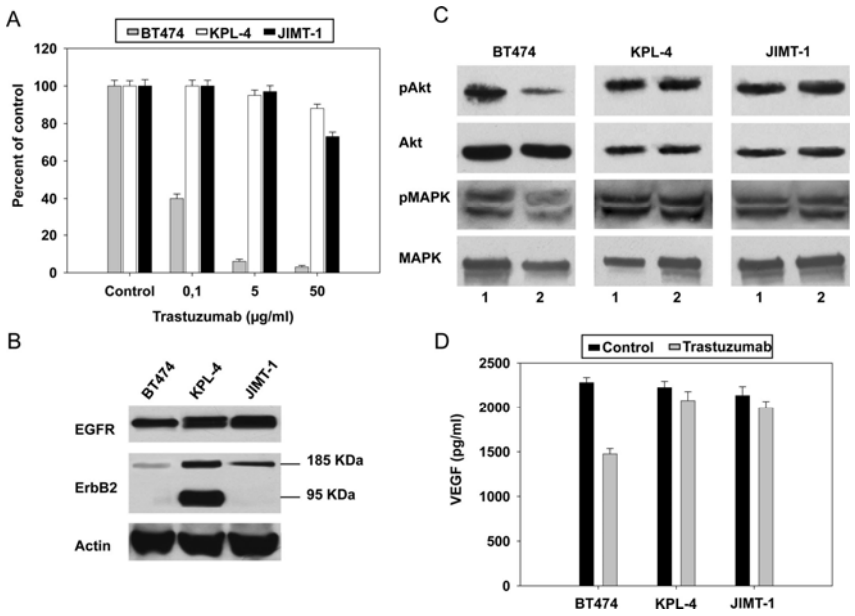

Fig. 6. Characterization of human breast cancer cell lines sensitive or resistant to trastuzumab. (A) Effects of trastuzumab on the soft agar growth of BT474, KPL-4 and JIMT-1 cells. Cells were treated with the indicated concentrations of trastuzumab each day for 3 consecutive days. Colonies were counted after 10–14 days. Data represent the mean (±SD) of three independent experiments, each performed in triplicate, and are presented relative to untreated control cells. The effects of trastuzumab were statistically significant versus control for BT474 cell line (2-sided $P<0.0001$), but not for KPL-4 and JIMT-1 cell lines. (B) Western blot analysis of EGFR and ErbB2 protein expression on total cell lysates from BT474, KPL-4 and JIMT-1 cell lines. (C) Western blotting on total cell lysates from cells treated with 5µg/ml trastuzumab. Lane 1: untreated control; lane 2: trastuzumab. (D) ELISA assays for hVEGF on conditioned media from cancer cells treated with trastuzumab (5µg/ml) each day for 3 consecutive days. Data represent the mean (±SD) of three independent experiments, each performed in triplicate. The effects of trastuzumab were statistically significant versus control for BT474 cell line (2-sided $P<0.0001$), but not for KPL-4 and JIMT-1 cell lines.

Combination of trastuzumab with IMO synergistically inhibits KPL-4 and JIMT-1 breast cancer xenografts. We have evaluated IMO capabilities to inhibit the growth and to enhance the activity of trastuzumab on KPL-4 and JIMT-1 xenografts. BalbC nude mice xenografted with KPL-4 or JIMT-1 tumors were treated with IMO or trastuzumab, alone and in combination (Fig.7). On day 56, 8 weeks after tumor injection, all untreated mice xenografted with KPL-4 or JIMT-1 cells reached the maximum allowed tumor size of about 2 cm^3. Treatment with trastuzumab produced a similar effect in both tumor models, causing on day 49, 3 weeks after treatment withdrawal, about 75% and 65% inhibition in KPL-4 and JIMT-1 tumor growth, respectively. However, starting from this time point, tumors treated

with trastuzumab alone resumed an exponential growth, reaching 2 cm^3 tumor size on day 98. Our data agree with a recent report demonstrating that trastuzumab is able to inhibit the outgrowth of macroscopically detectable JIMT-1 xenografted tumors despite the intrinsic resistance to trastuzumab *in vitro* and that this effect is likely to be mediated via ADCC (Barok et al. 2007); however, about three weeks after treatment withdrawal, tumors treated with trastuzumab resum an exponential growth, therefore at this time point trastuzumab is able only to reduce the number of circulating and disseminated tumor cells (CTCs and DTCs) but no more to inhibit the growth of primary tumor (Barok et al. 2008). IMO treatment inhibited tumor growth both in KPL-4 and in JIMT-1 xenografts, that reached the 2 cm^3 tumor size on day 77 and day 84, respectively. The combination of IMO plus trastuzumab caused a potent and long-lasting cooperative antitumor activity, with 75% growth inhibition (tumor size of 0.5 cm^3) until the end of the experiment, on day 105, 11 weeks after treatment withdrawal. No treatment-related side effects were observed in either tumor model studied. Comparison of tumor sizes among different treatment groups, evaluated by the Student's *t* test, was statistically significant both in KPL-4 and JIMT-1 tumors (Fig. 7A and 7B). The median survival of mice treated with IMO or trastuzumab was 8 and 9,5 weeks, respectively, compared with 5,5 weeks in control mice. Difference among the groups were calculated by log-rank test. IMO plus trastuzumab group did not reach a median survival, since 80% of the mice were still alive at the end of the esperiment (Fig. 7C and 7D). Interestingly, the mechanisms involved in trastuzumab resistance of KPL-4 and JIMT-1 human breast cancer cell models are different. KPL-4 overexpress p95ErbB2, the amino terminally truncated carboxyl terminal fragment of ErbB2, known also as C-terminal fragment, frequently found in ErbB2-expressing breast cancer cell lines and tumors and related to poor clinical outcome as well as reduced effectiveness of some therapeutic treatments. In particular, the presence of p95ErbB2 has been associated with trastuzumab-resistance. In fact, it has been shown that cells stably expressing p95ErbB2 are resistant to trastuzumab but remain sensitive to the antiproliferative effects of the TKI lapatinib, both *in vitro* and *in vivo*. Furthermore, in a series of patients with ErbB2-positive advanced breast cancer who were treated with trastuzumab, the presence of p95ErbB2 is associated with clinical resistance to trastuzumab, whereas tumors expressing only the full-length receptor exhibit a high response rate to trastuzumab (Scaltriti et al. 2007). Moreover, it has been suggested that trastuzumab resistance may be mediated in part by the selection of p95ErbB2-expressing breast cancer cells capable of exerting potent growth and prosurvival signals through p95ErbB2-ErbB3 heterodimers (Xia et al. 2004). Conversely to KPL-4, JIMT-1 cell line shows a decreased accessibility of ErbB2 and a diminished trastuzumab binding due to the expression of MUC4, a membrane-associated mucin that contributes to the partial masking of ErbB2. In fact, if the expression profile of ErbB proteins and trastuzumab-induced ErbB2 internalization/down-regulation in JIMT-1 are similar to those in trastuzumab-sensitive lines, the mean number

of trastuzumab binding sites in JIMT-1 is 1/5 that of the expressed ErbB2 molecules, although 5% to 10% of the cells showed a approximately 10-fold higher trastuzumab binding than the main population. The expression of MUC4 is higher in JIMT-1 than in trastuzumab-sensitive lines and knockdown of MUC4 expression by RNA interference increased the binding of trastuzumab (Nagy et al. 2005). Our data have demonstrated that the combination of IMO plus trastuzumab caused a potent and long-lasting cooperative antitumor activity, until the end of the experiment, 11 weeks after treatment withdrawal, when 80% of the mice were still alive. This potent effect is similar both in KPL-4 and in JIMT-1 xenograft models demonstrating the strong therapeutic impact of the IMO-trastuzumab combination regardless to the mechanism of trastuzumab resistance.

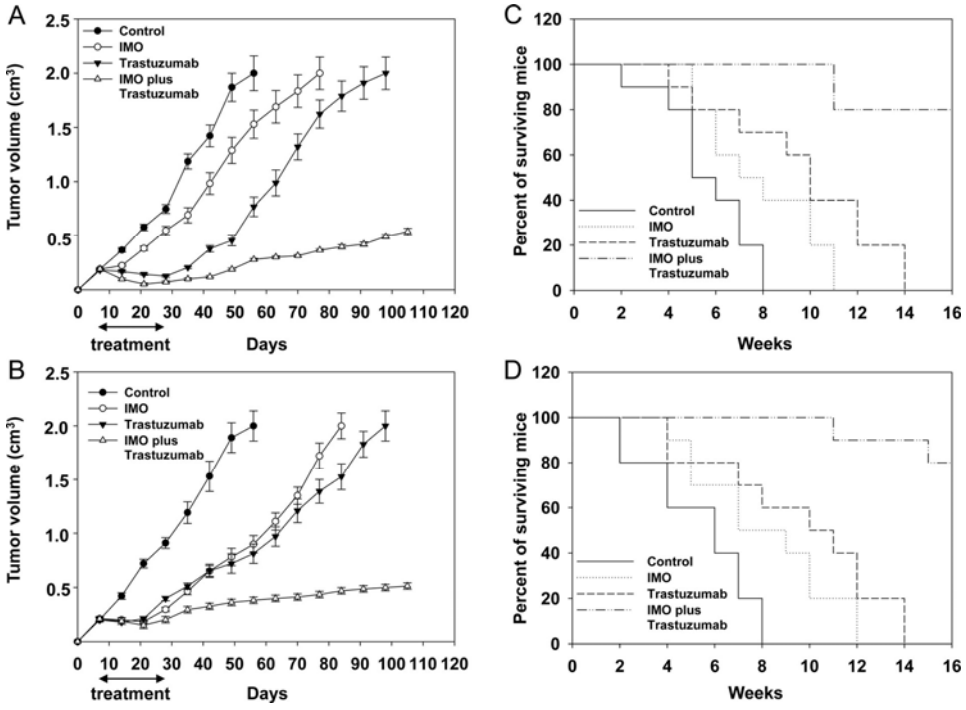

Fig. 7. Cooperative effect of IMO and trastuzumab on tumor growth (A and B) and survival (C and D) of mice bearing KPL-4 or JIMT-1 human breast cancer xenografts. After 7 days following tumor cell injection, mice were randomized (10/group) to receive IMO, trastuzumab or the combination, as indicated in Materials and Methods Section. The Student's t test was used to compare tumor sizes among different treatment groups at day 56 following KPL-4 (A) and JIMT-1 (B) cell injection. They resulted statistically significant for IMO plus trastuzumab versus control (two sided $P < 0.0001$), versus IMO alone (two sided $P < 0.0001$), and versus trastuzumab alone (two sided $P < 0.0001$) in both experiments. Bars, SD. Both in KPL-4 (C) and in JIMT-1 (D) xenografted mice, median survival was 5,5 weeks in untreated mice, 8 weeks in IMO-treated group (log-rank test versus controls; $P < 0.0001$), and 9,5 weeks in trastuzumab-treated group (log-rank test versus controls; $P < 0.0001$). No median survival could be calculated for IMO plus trastuzumab group. Bars, SD.

IMO, alone or in combination with trastuzumab, inhibits the expression of signalling proteins in KPL-4 and JIMT-1 xenografts. We analyzed the effect of treatment on the expression of a variety of proteins playing a critical role in cancer cell proliferation and angiogenesis. Western blotting analysis was performed on cell lysates from tumors removed at the end of the third week of treatment, on day 25. In KPL-4 tumors, IMO induced a moderate reduction of phospho-EGFR and a more pronounced reduction of phospho-ErbB2. Moreover, IMO strongly inhibited the activated forms of MAPK and Akt as well as the VEGF expression. This signalling inhibition was not observed with trastuzumab treatment, indeed trastuzumab seemed to induce a slight activation of EGFR- and ErbB2-dependent signal transduction. IMO in combination with trastuzumab suppressed trastuzumab-induced signalling activation, producing a moderate inhibition compared to control. No treatment affected the total amount of EGFR, ErbB2, MAPK and Akt (Fig. 8A). In JIMT-1 tumors IMO did not affect the phosphorylation/activation of EGFR, ErbB2 and their downstream effectors, while trastuzumab weakly inhibited the same signalling proteins. When the two agents were used in combination, a potent inhibition was observed on protein expression, with an almost total suppression of phosphorylation/activation of EGFR, MAPK, Akt and VEGF. The combination of IMO with trastuzumab also reduced the total amount of ErbB2 rather than the phospho-ErbB2 expression levels (Fig. 8B).

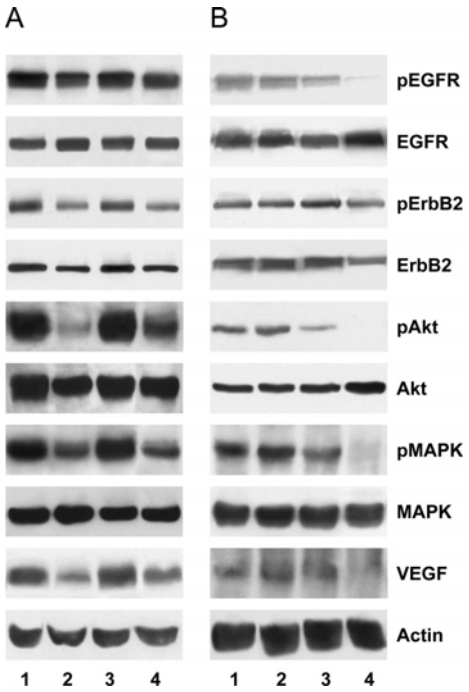

Fig. 8. Western blot analysis of KPL-4 (A) and JIMT-1 (B) tumors. Western blotting was performed on total lysates from tumor specimens of two mice sacrificed on day 25. Lane 1, Untreated control; lane 2, IMO; lane 3, trastuzumab; lane 4, IMO plus trastuzumab.

IMO enhances ADCC activity of trastuzumab on KPL-4 and JIMT-1 cells. To investigate the capability of trastuzumab to activate an ADCC reaction in KPL-4 and JIMT-1 cells cancer cells and the influence of IMO in a combination treatment, we performed a cytotoxic assay using as targets both cell lines. As expected, in the absence of antibodies, freshly isolated non-adherent human PBLs were able to kill the standard NK-target K562 cells, but did not induce any detectable lysis of KPL-4 or JIMT-1 cells (Fig. 9A and 9B). Similar results were obtained when PBLs were incubated with IMO. Conversely, trastuzumab, that possesses a formerly described ADCC mechanism (Mimura et al. 2005), caused a 85% induction of KPL-4 lysis (Fig. 9A), and a 35% induction of JIMT-1 lysis (Fig. 9B). This difference could be explained on the basis of the lower ErbB2 expression levels and the masking of the receptor by the membrane-associated mucin MUC4 in JIMT-1 (Nagy et al. 2005). Pre-incubation of PBLs with IMO potentiated KPL-4 and JIMT-1 cells killing induced by trastuzumab (Fig. 9A and 9B). Therefore, IMO potentiates trastuzumab-mediated ADCC on KPL-4 and JIMT-1 cells *in vitro*. Moreover, since it has been demonstrated that VEGF partially inhibits the ADCC of human monocytes mediated by trastuzumab, and this inhibition is mainly mediated by VEGFR1/Flt-1 (Mimura et al. 2007), the reduction of human VEGF levels induced by IMO could overcome such interference and further explain IMO-mediated potentiation of trastuzumab ADCC *in vivo*.

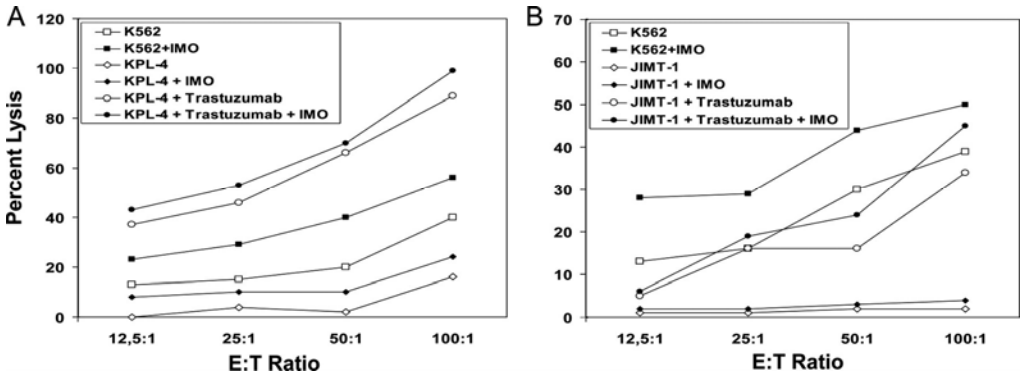

Fig. 9. ADCC assay on KPL-4 (A) or JIMT-1 (B) cells. The effector cells (human PBLs) were incubated in the presence or absence of IMO and then mixed with the target cells (K562 and KPL-4 or JIMT-1), which were incubated in the presence or absence of trastuzumab, at different effector/target ratio (E:T ratio). Doses and time of administration are indicated in Materials and Methods Section.

TLR9 is expressed in KPL-4 cells and seems to functionally interact with EGFR. Since in KPL-4 tumors IMO strongly inhibited ErbB-dependent signal transduction *in vivo*, we investigated whether this effect could be related to a direct modulation of ErbB-dependent signalling. Therefore, we examined the effect of IMO on KPL-4 cells grown *in vitro*. To investigate the potential functional/structural relationship between TLR9 and ErbB family receptors, and the role played by IMO, we performed a co-immunoprecipitation experiment. We immunoprecipitated KPL-4 cell lysates with the anti-TLR9 antibody and blotted with the anti-ErbB2 antibody or the anti-EGFR antibody. As the positive control, total proteins from KPL-4 cells were used; as the negative control, lysis buffer was mixed with monoclonal anti-TLR9 antibody. Therefore, we have demonstrated that ErbB2 and TLR9 co-immunoprecipitated but this interaction was only slightly affected by IMO treatment. Instead, EGFR and TLR9 co-immunoprecipitated and the observed interaction was strongly reduced after 1 hour of IMO treatment (Fig. 10A). Since we have shown an IMO-modulated interaction between TLR9 and EGFR, we performed an immunofluorescence and confocal microscopy analysis for EGFR and TLR9 on KPL-4 cells in order to further analyze the intracellular relationship between the two receptors. As expected, EGFR was prevalently localized on cell membranes, while TLR9 seemed widely distributed both in intracellular compartments and in juxtramembrane regions. Therefore, we observed a partial co-localization of EGFR and TLR9 in the merge staining (Fig. 10B). TLR9 distribution observed in KPL-4 cells is different from the distribution described in immune cells. In fact, it has been shown that human TLR9, both endogenously expressed in B cells or transfected in immortalized cell lines, are retained in the ER before CpG exposure and that, following treatment with TLR9 agonists, a special mechanism must exist for translocating TLR9 to the signalling compartments that contain the agonists. However, the same authors did not rule out that TLR9 may reside in a different cellular compartment depending upon the cell type examined, as previously described also for TLR4 (Leifer et al. 2004). Moreover, it has been shown that human intestinal epithelial cells respond to pathogenic bacterial DNA by increasing expression and surface localization of TLR9 (Ewaschuk et al. 2007). Finally we have verified whether IMO ability to modulate EGFR-TLR9 interaction could eventually produce an inhibition of EGFR signalling. As shown in Fig. 10C, IMO reduced the EGF-induced phosphorylation/activation of MAPK on KPL-4 *in vitro*, suggesting that IMO could directly affect EGFR signalling.

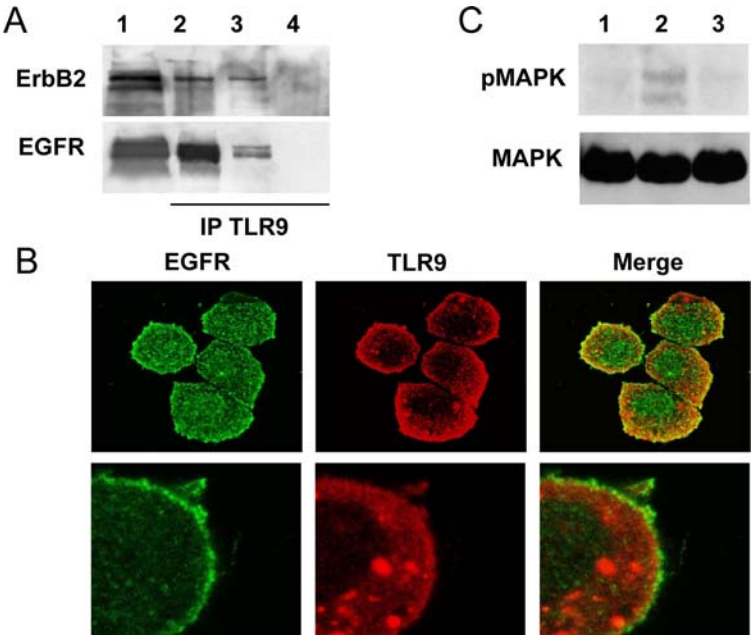

Fig. 10. Potential interaction between TLR9 and EGFR *in vitro*. (A) Immunoprecipitation using an anti-TLR9 antibody and blotting with an anti-ErbB2 or an anti-EGFR antibody on KPL-4 cells cultured in complete medium and treated for 1 hour with 1 μM IMO. Lane 1, positive control; lane 2, untreated control; lane 3, treatment with IMO; lane 4, negative control. (B) KPL-4 cells were grown on glass coverslips for 48 hours, then were double stained with anti-TLR9 and anti-EGFR primary antibodies and incubated with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit antibody. (C) Western blot analysis of protein expression in KPL-4 cells cultured in serum free medium, treated for 1 hour with 1 μM IMO, and stimulated for 15 minutes with EGF 50 ng/ml before protein extraction. Lane 1, unstimulated and untreated control; lane 2, stimulation with EGF; lane 3, treatment with IMO and stimulation with EGF.

Many interactions between EGFR and TLRs have been recently described; however, EGFR potential role in regulating human TLRs is not yet completely clarified. It has been demonstrated that EGFR acts as a negative regulator for TLR2 induction by the bacterium nontypeable Haemophilus influenzae (NTHi) via an Src-MKK3/6-p38 alpha/beta MAP kinase-dependent mechanism, attenuating host immune response. A NTHi-derived EGF-like factor appears to be responsible for the Direct activation of EGFR (Mikami et al. 2005). EGFR has been involved also in the induction of TLRs expression. It has been shown that lipid–pDNA complexes signal via EGFR-Erk to induce expression of TLR4 and TLR2, leading to an amplified inflammatory response after receptor engagement by PAMPs. EGFR inhibitor AG1478 completely inhibits pDNA-mediated upregulation of TLR4/2 levels and associated IL-8 release in response to TLR4/2 agonists (Liu et al. 2008). Moreover, multiple TLR ligands have shown to induce airway epithelial cell production of IL-8 and VEGF via a

signalling cascade involving EGFR. In fact, ligands for TLR 1, 2, 3, 5, and 6, bind to their receptors, activating Dual oxidase 1 (Duox1) with the consequent generation of reactive oxygen species (ROS). TNF-alpha-converting enzyme (TACE), activated by ROS, cleaves a proligand, releasing soluble TGF-α which binds to and activates EGFR, initiating signalling for IL-8 and VEGF production (Koff et al. 2008). Interestingly, we have demonstrated for the first time that TLR9 seems to functionally interact with EGFR and that this interaction is modulated by the TLR9 agonist IMO, eventually producing an inhibition of EGFR signalling in KPL-4 cells. The existence of a potential TLR9 coreceptor has been suggested previously (Sanjuan et al. 2006). The authors proposed that the recognition of A class CpG-DNA is a two-step process that can begin at the plasma membrane by a TLR9-independent mechanism. CpG-DNA stimulation of a CpG-sensing membrane receptor would activate two Src family Kinases (SFKs), Hck and Lyn, that would initiate a tyrosine phosphorylation cascade. One downstream effector of the SFK pathway, Syk, interacts with TLR9. The second CpG-induced activation event occurs at the endosome when internalized CpG-DNA binds TLR9 and initiates the MyD88-dependent cascade. Chloroquine, an inhibitor of CpG-triggered cytokine secretion, blocked TLR9/MyD88-dependent cytokine secretion as expected but failed to inhibit CpG-induced SFK activation and its dependent cellular responses. According to this view and to our data, it could be suggested a role for EGFR as the potential TLR9 coreceptor in cancer cells but further investigations are needed in order to dissect all the relationships between EGFR and TLR9.

Combination of IMO plus trastuzumab causes a direct antiangiogenic effect. To evaluate whether the strong cooperative effect of IMO plus trastuzumab combination on the growth of tumor xenografts could involve a direct antiangiogenic activity, we performed a cell survival assay and a capillary tubes and network formation assay on HUVEC cells. We confirmed our previous observation of IMO effects on HUVEC functions (Damiano et al. 2007), and demonstrated for the first time that also trastuzumab exhibits a direct antiangiogenic effect inhibiting both HUVEC survival (20% inhibition) and capillary formation capability. As shown in figure 11A, the combination of IMO and trastuzumab was more efficient in inhibiting HUVEC survival than the single agents. The VEGF-stimulated capillary tubes and network formation was almost totally suppressed by the combined treatment (Fig. 11B). Several reports have described the expression of both EGFR and ErbB2 receptors on HUVEC (Bueter et al. 2006; Russell et al. 1999) and a recent study has also suggested that the expression of TGF-alpha by tumor cells leading to the activation of EGFR in tumor-associated endothelial cells is a major determinant for the susceptibility of neoplasms to therapy by specific EGFR-TKIs (Kuwai et al. 2008). Therefore, we have verified whether the effect of IMO and trastuzumab on HUVEC functions could be related to a

direct inhibition of EGFR-ErbB2 signalling. We have demonstrated that IMO inhibits the EGF-induced phosphorylation/activation of EGFR, ErbB2, Akt and MAPK. Interestingly, also trastuzumab is able to inhibit to a similar extent the same signalling proteins and, when the two agents were used in combination, a more potent inhibition or complete suppression is observed on the expression of the proteins affected by each single agent (Fig. 11C). Therefore, our data demonstrate for the first time that trastuzumab exhibits a direct antiangiogenic effect inhibiting both endothelial cell survival and capillary formation capability. Therefore, the combination with IMO and trastuzumab produces a cooperative direct anti-angiogenic effect, also related to a direct inhibition of EGFR-ErbB2 signalling in endothelial cells.

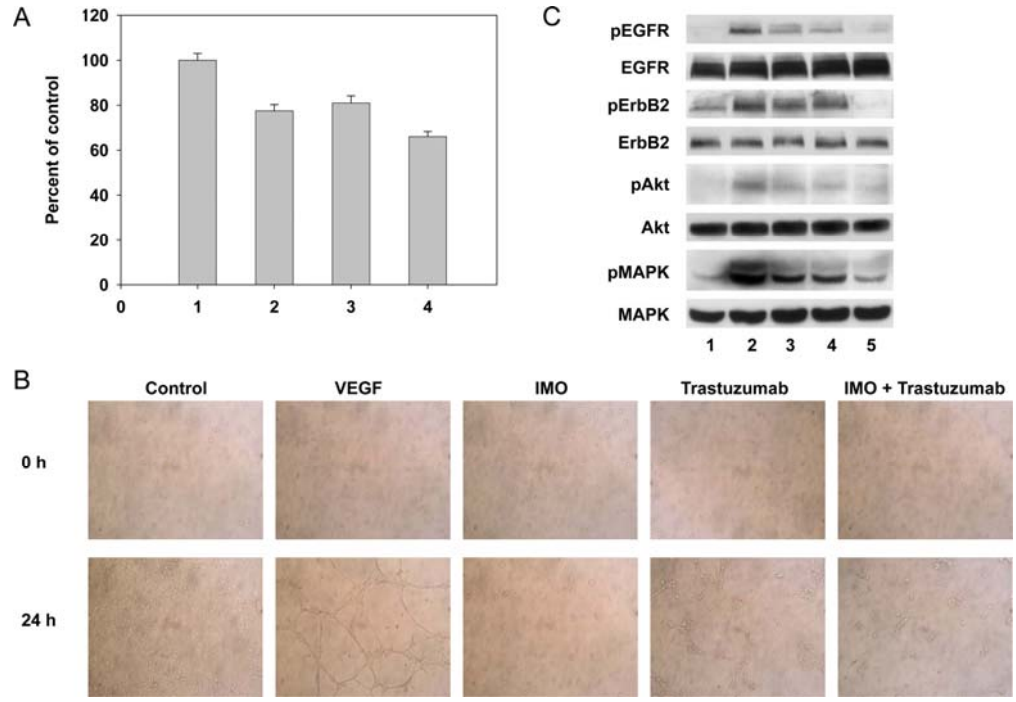

Fig. 11. Effects of IMO and trastuzumab on HUVEC cell survival (A), tube formation (B) and signal transduction (C). (A) HUVEC cells were treated with IMO 1 μM, trastuzumab 5 μg/ml, or the combination of these agents. The results are statistically significant for IMO, trastuzumab, and the combination versus control (two sided $P < 0.0001$). Lane 1, Untreated control; lane 2, IMO; lane 3, trastuzumab; lane 4, IMO plus trastuzumab. (B) HUVEC cells were added on matrigel in presence or absence of 1 μM IMO, 5 μg/ml trastuzumab, or the combination of these agents. As the positive control, the Matrigel was mixed with 100 ng/ml of VEGF. Photographs were taken at 0 and 24 hours. (C) Western blot analysis of protein expression in HUVEC cells cultured in serum free medium, treated for 1 hour with 1 μM IMO and/or for 24 hours with trastuzumab 5 μg/ml and stimulated for 15 minutes with EGF 50 ng/ml before protein extraction. Lane 1, unstimulated and untreated control; lane 2, stimulation with EGF; lane 3, treatment with IMO; lane 4, treatment with trastuzumab; lane 5, combined treatment with IMO and trastuzumab.

5. CONCLUSIONS

TLR9 agonists are a novel class of compounds with potent antitumor activity that have entered the clinical evaluation in cancer patients. However, the mechanisms by which they affect growth signalling and angiogenesis are yet poorly understood. We have recently demonstrated that a novel antitumor TLR9 agonist (IMO) acts via EGFR and cooperates with anti-EGFR MAb cetuximab or TKI gefitinib, synergistically inhibiting colon tumor xenografts growth and angiogenesis. Conversely, IMO is inactive against cetuximab-resistant tumors, further suggesting its dependance on EGFR signalling.

We have aimed to provide new insights into the mechanism of action of IMO on signalling and angiogenesis, in order to design optimal treatment combinations to be easily transferred into clinical practice. In order to analyze whether the potent inhibitory effect obtained with IMO and EGFR inhibitors on angiogenesis was dependent or independent on the interference in the EGFR pathway, we have studied the combination of IMO and anti-VEGF MAb bevacizumab. We have demonstrated that IMO synergizes potently with bevacizumab in cancer xenografts sensitive and resistant to anti-EGFR drugs, in spite of the fact that IMO is unable to influence the lack of ADCC activity of bevacizumab. Moreover, IMO inhibits survival, adhesion, migration, and capillary tubes formation capability of human endothelial cells and it is likely that this property may finally be responsible for the cooperative effect observed also in EGFR-resistant models. To evaluate IMO ability to revert mechanisms of resistance in different tumor models, we have analyzed the combination of IMO with the anti-ErbB2 MAb trastuzumab, demonstrating that IMO strongly synergizes with trastuzumab producing a potent and long-lasting growth inhibition of trastuzumab-resistant breast cancer xenografts. The synergism is related not only to the potentiation of trastuzumab-induced ADCC, but also to the direct antitumor activity of IMO, explained also through a functional modulation of ErbB-mediated signalling, and to the direct cooperative antiangiogenic activity of the drug combination. Therefore, we demonstrate that the antitumor, antiangiogenic and immunostimulatory activity of IMO enhances trastuzumab effect in trastuzumab-resistant breast cancers, suggesting that IMO plus trastuzumab treatment could be an effective therapeutic strategy in breast cancers.

This study provides new insights into the mechanisms of action of TLR9 agonists. In fact, it demonstrates that, although dependent on the immune activation, IMO has a much broader range of mechanisms, involving not only the EGFR-dependent pathway but also the neo-angiogenesis. These results provide a strong rationale to translate the combination with bevacizumab or trastuzumab into the clinical practice, as a potentially powerful and rationally-based therapeutic strategy. The possibility to combine IMO with inhibitors of EGFR, ErbB-2 and VEGF, creates the opportunity to take advantage of multiple chance for cooperativity, involving EGFR- and ADCC-dependent and -independent mechanisms and neoangiogenesis.

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TLR9 agonist acts by different mechanisms synergizing with bevacizumab in sensitive and cetuximab-resistant colon cancer xenografts

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Synthetic agonists of Toll-like receptor 9 (TLR9), a class of agents that induce specific immune response, exhibit antitumor activity and are currently being investigated in cancer patients. Intriguingly, their mechanisms of action on tumor growth and angiogenesis are still incompletely understood. We recently discovered that a synthetic agonist of TLR9, immune modulatory oligonucleotide (IMO), acts by impairing epidermal growth factor receptor (EGFR) signaling and potently synergizes with anti-EGFR antibody cetuximab in GEO human colon cancer xenografts, whereas it is ineffective in VEGF-overexpressing cetuximab-resistant GEO cetuximab-resistant (GEO-CR) tumors. VEGF is activated by EGFR, and its overexpression causes resistance to EGFR inhibitors. Therefore, we used IMO and the anti-VEGF antibody bevacizumab as tools to study IMO's role on EGFR and angiogenesis and to explore its therapeutic potential in GEO, LS174T, and GEO-CR cancer xenografts. We found that IMO enhances the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of cetuximab, that bevacizumab has no ADCC, and IMO is unable to enhance it. Nevertheless, the IMO-plus-bevacizumab combination synergistically inhibits the growth of GEO and LS174T as well as of GEO-CR tumors, preceded by inhibition of signaling protein expression, microvessel formation, and human, but not murine, VEGF secretion. Moreover, IMO inhibited the growth, adhesion, migration, and capillary formation of VEGF-stimulated endothelial cells. The antitumor activity was irrespective of the TLR9 expression on tumor cells. These studies demonstrate that synthetic agonists of TLR9 interfere with growth and angiogenesis also by EGFR- and ADCC-independent mechanisms affecting endothelial cell functions and provide a strong rationale to combine IMO with bevacizumab and EGFR inhibitory drugs in colon cancer patients.

angiogenesis | cancer therapy | growth factor receptors

Activation of Toll-like receptor 9 (TLR9) by DNA containing unmethylated CpG motifs, its natural ligand, produces potent Th1-type innate and adaptive immune responses (1). TLR9-stimulated B cells and plasmacytoid dendritic cells secrete a number of Th-1-promoting cytokines and chemokines, including IL-12, IL-6, IFN-γ, Type 1 IFNs, MIP-1, and IP-10 (2–4). Agonists of TLR9 have shown antitumor activity, alone and in combination with chemotherapy and radiotherapy, and ability to enhance the antibody-dependent cell-mediated cytotoxicity (ADCC) of mAbs in a number of preclinical and early clinical trials (3, 5).

Based on extensive structure–activity relationship studies, synthetic agonists of TLR9 containing novel DNA structures and synthetic dinucleotide motifs, referred to as immune modulatory oligonucleotides (IMOs), have been synthesized, demonstrating distinct cytokine profiles *in vitro* and *in vivo*, compared with conventional TLR9 agonists (4, 6, 7) and higher metabolic stability due to the novel DNA structure present in them (8–10). Previous studies have demonstrated potent antitumor activity of IMOs as monotherapies and in combination with chemotherapeutic agents and mAbs (11, 12). Currently, a synthetic agonist of TLR9, IMO-2055, is under clinical evaluation, in combination with chemotherapy and other agents in cancer patients.

Intriguingly, although the TLR9 immunologic mechanisms are fairly well understood, and the clinical development of TLR9 agonists is very encouraging, the mechanisms by which they affect signaling proteins involved in tumor growth and angiogenesis, thus leading to tumor growth inhibition, have yet to be elucidated.

The epidermal growth factor receptor (EGFR) plays a pivotal role in the control of cell growth, apoptosis, and angiogenesis (13), and EGFR blockade by mAbs or small-molecule tyrosine kinase inhibitors has now entered clinical practice in patients affected by different types of cancer, including colorectal (14, 15).

We have recently demonstrated that an IMO inhibits the expression and function of activated EGFR and of other critical downstream proteins (12). IMO exhibited a synergistic antitumor effect with the anti-EGFR mAb cetuximab or the tyrosine kinase inhibitor gefitinib in GEO human colon cancer xenografts, whereas it was ineffective against the cetuximab-resistant tumors GEO cetuximab-resistant (GEO-CR) (12). These findings have opened the path to clinical studies combining IMO with EGFR inhibitors in cancer patients.

We and others have previously shown that colon tumors, including GEO-CR, that acquire resistance to the anti-EGFR drugs cetuximab or gefitinib overexpress and secrete VEGF, which acts as an escape pathway, overcoming the EGFR blockade (16, 17). Moreover, it has been shown that VEGF overexpression markedly impairs the activity of dendritic cells and the antitumor immune response (18, 19). These studies and our demonstration of a cooperative effect of cetuximab with a selective inhibitor of VEGF (20) have provided the basis for the ongoing clinical studies combining inhibitors of EGFR and of VEGF/VEGF receptors (21, 22). Currently, the anti-VEGF mAb bevacizumab is successfully used in combination with

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Abbreviations: TLR9, Toll-like receptor 9; ADCC, antibody-dependent cell-mediated cytotoxicity; IMO, immune modulatory oligonucleotide; EGFR, epidermal growth factor receptor; PBL, peripheral blood lymphocyte; hVEGF, human VEGF; mVEGF, murine VEGF; HUVEC, human umbilical vein endothelial cells; GEO-CR, GEO cetuximab-resistant; PBMC, peripheral blood mononuclear cells.

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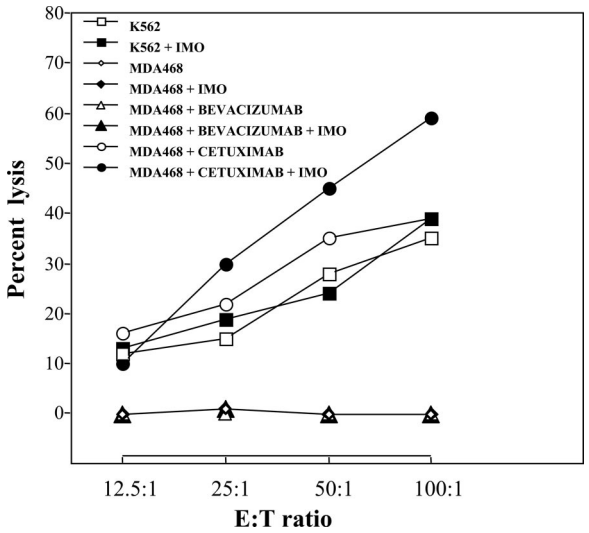

Fig. 1. ADCC assay. The effector cells (human PBLs) were incubated in the presence or absence of IMO and then mixed with the target cells (K562 and MDA468), which were incubated in the presence or absence of the antibodies cetuximab and bevacizumab. Doses and time of administration are indicated in *Materials and Methods*.

chemotherapy for the treatment of colorectal cancer (23) and has shown efficacy also in nonsmall-cell lung and breast cancer (24). Interestingly, the VEGF blockade by bevacizumab recovers the activity of dendritic cells, improving their antitumor function (18, 19). In the former study, we observed a potent inhibitory effect of IMO and cetuximab on VEGF and angiogenesis in wild-type but not in GEO-CR colon tumors, suggesting that the anti-VEGF effect is only EGFR-dependent. To address this issue, provide insight into the mechanism of action of IMO on signaling and angiogenesis, and explore the therapeutic potential of the association of IMO with a specific VEGF inhibitor, we have evaluated the effects of IMO in combination with bevacizumab on the growth, signaling, and angiogenesis of different wild-type and cetuximab-resistant colon cancer models.

Results

Bevacizumab Has No ADCC Activity, and IMO Is Unable to Affect It. To investigate the capability of bevacizumab to activate an ADCC reaction in cancer cells and the influence of IMO in a combination treatment, we performed a cytotoxic assay using the conventional target MDA-468, a human breast cancer line that expresses both EGFR and VEGF receptors. As expected, in the absence of antibodies, freshly isolated nonadherent human peripheral blood lymphocytes (PBLs) were able to kill the standard NK-target K562 cells but did not induce any detectable lysis of MDA468 cells (Fig. 1). The same result was obtained when PBLs were incubated with IMO. Conversely, the anti-EGFR mAb cetuximab, which possesses a formerly described ADCC mechanism (25), caused a 40% induction of MDA-468 lysis. Preincubation of PBLs with IMO potentiated up to 60% of the MDA-468 killing induced by cetuximab (Fig. 1). On the contrary, preincubation of PBLs with IMO did not affect the inability of bevacizumab to produce the lysis of MDA-468 cells. Therefore, bevacizumab, as well as the combination of IMO and bevacizumab, has no ADCC.

Combination of Bevacizumab with IMO Synergistically Inhibits GEO and LS174T Colon Cancer Xenografts. BALB/c nude mice xenografted with GEO and LS174T tumors were treated with IMO or bevacizumab, alone and in combination (Fig. 2). On day 56, 8 weeks after tumor injection, all untreated mice xenografted

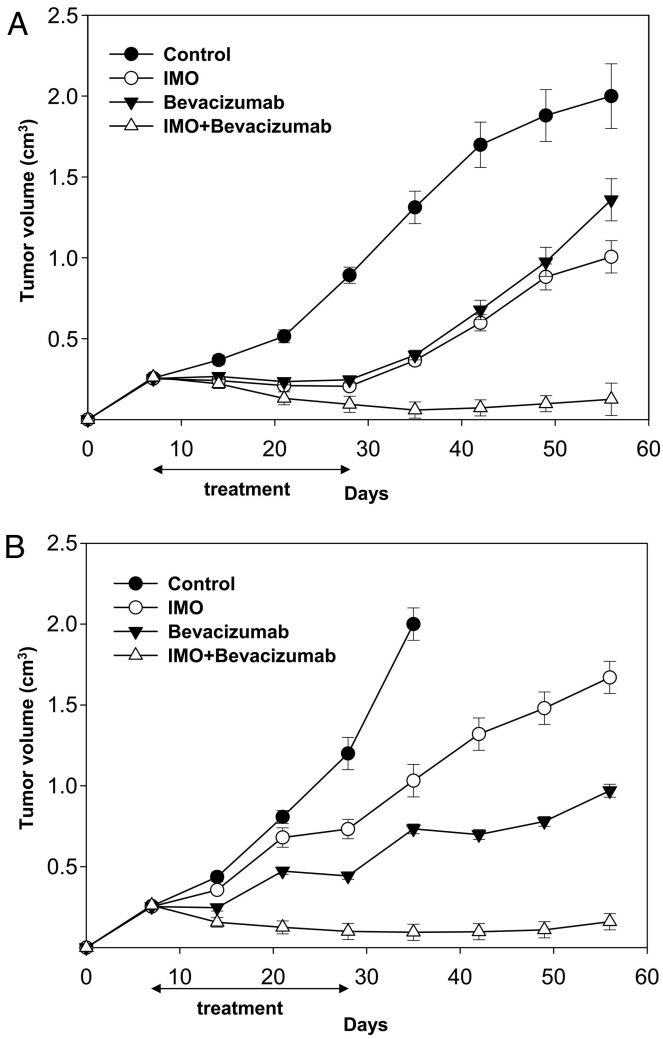

Fig. 2. Cooperative effect of IMO and bevacizumab on tumor growth of mice bearing human colon cancer xenografts. Seven days after tumor cell injection, mice were randomized (10 per group) to receive the following treatments: i.p. IMO, 1 mg/kg three times per week for 4 weeks; i.p. bevacizumab, 5 mg/kg, twice per week for 3 weeks, or the combination of these agents on days 7–11, 14–18, and 21–25, continuing only IMO on days 28–32. Two mice were killed at day 25 for biochemical and histochemical analyses. Student's *t* test was used to compare tumor sizes among different treatment groups at day 56 after GEO (*A*) and LS174T (*B*) cell injection. The results were statistically significant for IMO plus bevacizumab vs. control (two-sided $P < 0.0001$), vs. IMO alone (two-sided $P < 0.0001$), and vs. bevacizumab alone (two-sided $P < 0.0001$) in both experiments. Error bars indicate SD.

with GEO cells reached the maximum allowed tumor size of ≈2 cm^3, whereas mice treated with IMO alone exhibited 50% growth inhibition, having a size of ≈1 cm^3. Treatment with bevacizumab produced a 35% inhibition, because tumors measured 1.3 cm^3 at the same time point. The combination of IMO plus bevacizumab caused a potent cooperative antitumor activity, with >95% growth inhibition (tumor size of only 0.12 cm^3) (Fig. 2*A*).

Similar effects were observed in LS174T xenografts. The maximum allowed size of ≈2 cm^3 was reached on day 35 in the untreated mice. On day 56, at the end of the experiment, mice treated with IMO or bevacizumab alone measured ≈1.6 and 1 cm^3, respectively, whereas those treated with the two agents in combination showed a potent cooperative tumor growth inhibition of ≈95% compared with untreated animals, resulting in a

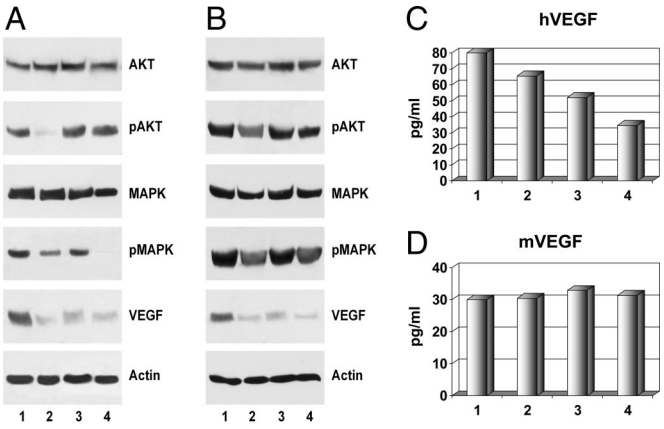

Fig. 3. Western blot analysis of GEO (*A*) and LS174T (*B*) tumors and ELISA on mice serum (*C* and *D*). (*A* and *B*) Western blot analysis was performed on total lysates from tumor specimens of two mice killed on day 25 and treated as in Fig. 1. (*C* and *D*) ELISA for hVEGF and mVEGF were performed on the serum of two mice killed on day 25 and treated as in Fig. 2. Doses and time of administration are indicated in *Materials and Methods*. Lane 1, untreated control; lane 2, IMO; lane 3, bevacizumab; and lane 4, IMO plus bevacizumab.

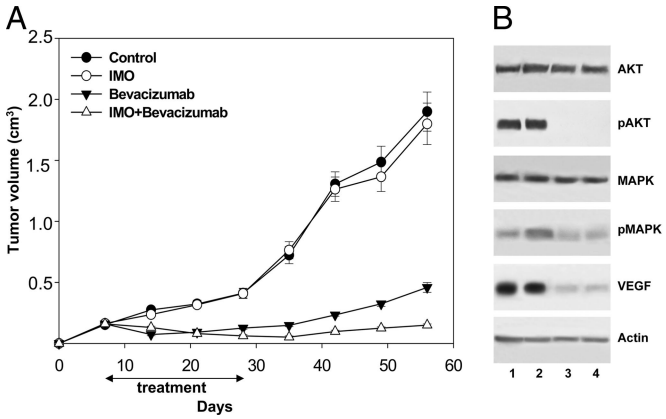

Fig. 4. Effect of the combination of IMO with bevacizumab in mice bearing cetuximab-resistant GEO-CR tumor xenografts. (*A*) Seven days after GEO-CR tumor injection, mice were randomized (10 per group) to receive i.p. IMO, 1 mg/kg three times per week for 4 weeks; i.p. bevacizumab, 5 mg/kg, twice per week for 3 weeks, or the combination of these agents, on days 7–11, 14–18, and 21–25, continuing only IMO on days 28–32. Inhibition of growth was significantly different in the IMO plus bevacizumab-treated group vs. the control group, the IMO alone group, and the bevacizumab alone group (two-sided $P < 0.0001$ for each comparison). (*B*) Western blot analysis was performed on total lysates from tumor specimens of two mice killed on day 25 and treated as in Fig. 2. Lane 1, untreated control; lane 2, IMO; lane 3, bevacizumab; and lane 4, IMO plus bevacizumab. Doses and time of administration are indicated in *Materials and Methods*. Error bars indicate SD.

tumor size of 0.16 cm^3 (Fig. 2*B*). No treatment-related side effects were observed in either tumor model studied. Comparison of tumor sizes among different treatment groups, evaluated by Student's *t* test, was statistically significant both in GEO and LS174T tumors (Fig. 2).

Combination of Bevacizumab with IMO Inhibits the Expression of Signaling Proteins and Angiogenesis in GEO and LS174T Xenografts and Reduces the Levels of Human VEGF (hVEGF), but Not of Murine VEGF (mVEGF), in Mice Serum. We analyzed the effect of treatment on the expression of a variety of proteins playing a critical role in cancer cell proliferation and angiogenesis. Western blot analysis was performed on cell lysates from tumors removed at the end of the third week of treatment, on day 25. As shown in Fig. 3 *A* and *B*, IMO did not affect the total amount of MAPK and Akt but inhibited their activated forms pMAPK and pAkt and VEGF expression. Bevacizumab inhibited the same signaling proteins, although to a lesser degree compared with IMO. When the two agents were used in combination, a more potent inhibition was observed on protein expression. To confirm the effect of a combination of bevacizumab with IMO on hVEGF levels, we performed ELISA on the serum obtained from LS174T-bearing mice (Fig. 3 *C* and *D*). Because bevacizumab recognizes only hVEGF, as expected, it caused a reduction in the levels of circulating hVEGF in the serum. The treatment with IMO alone reduced the secreted hVEGF levels, and the combination with bevacizumab caused a more potent inhibition of circulating hVEGF levels compared with treatment with single agents (Fig. 3*C*). On the contrary, neither single agent nor the combination affected mVEGF compared with untreated mice (Fig. 3*D*).

Immunohistochemical analysis of LS174T tumor specimens performed during the third week of treatment revealed a moderate induction of necrosis caused by IMO treatment and, at a higher degree, by bevacizumab. The combination of the two agents caused a massive hemorrhagic necrosis (in >90% of the tumor). Furthermore, analysis of microvessels demonstrated ≈25% inhibition of CD34-stained host vessels in the animals treated with IMO and ≈40% inhibition after bevacizumab treatment compared with untreated mice. The specimen from mice treated with the two agents in combination showed an inhibition of vessels formation up to 75%.

Combination of Bevacizumab with IMO Causes Potent Antitumor Activity in Cetuximab-Resistant GEO-CR Xenografts. We evaluated whether a cooperative effect could be obtained in cetuximab-resistant GEO-CR tumors, in the absence of an ADCC effect. We have shown that IMO alone is ineffective, whereas bevacizumab markedly inhibited GEO-CR growth (Fig. 4*A*). When IMO was used in combination with bevacizumab, a cooperative inhibitory effect was observed, because at the end of the experiment, tumors were still ≈0.15 cm^3 (Fig. 4*A*). With the exception of the mice treated with IMO alone, Student's *t* test demonstrated that the growth inhibition caused by each treatment, in comparison with untreated mice as well as the tumor size among different treatment groups, was statistically different (Fig. 4).

Western Blot Analysis of GEO-CR Tumors. Western blot analysis of protein extracts from GEO-CR tumors did not reveal any substantial changes in the expression of pAkt, pMAPK, and VEGF in tumor specimens treated with IMO alone, whereas a marked inhibition was seen in those treated with bevacizumab or with the combination IMO plus bevacizumab (Fig. 4*B*).

IMO Inhibits Survival, Adhesion to Matrix, Migration, and Capillary Formation Capability in Human Endothelial Cells. To evaluate whether antiangiogenic activity of IMO could be linked also to a direct effect on human endothelial cells, we performed a cell survival assay on human umbilical vein endothelial cells (HUVEC). At doses of 1 and 5 μM, IMO caused a moderated inhibition of HUVEC survival, which was increased by the addition of lipofectamine, an agent that facilitates cellular uptake of IMO (Fig. 5*A*). We then analyzed the effects of IMO on HUVEC adhesion and migration by using a cell adhesion assay to matrigel and a wound-healing assay. At 1 μM concentration, IMO showed a potent inhibitory activity on adhesion and migration of endothelial cells (Fig. 5 *B* and *C*). Finally, we examined the effects of IMO on VEGF-stimulated capillary tube and network formation and observed that this process is strongly inhibited by IMO (Fig. 5*D*).

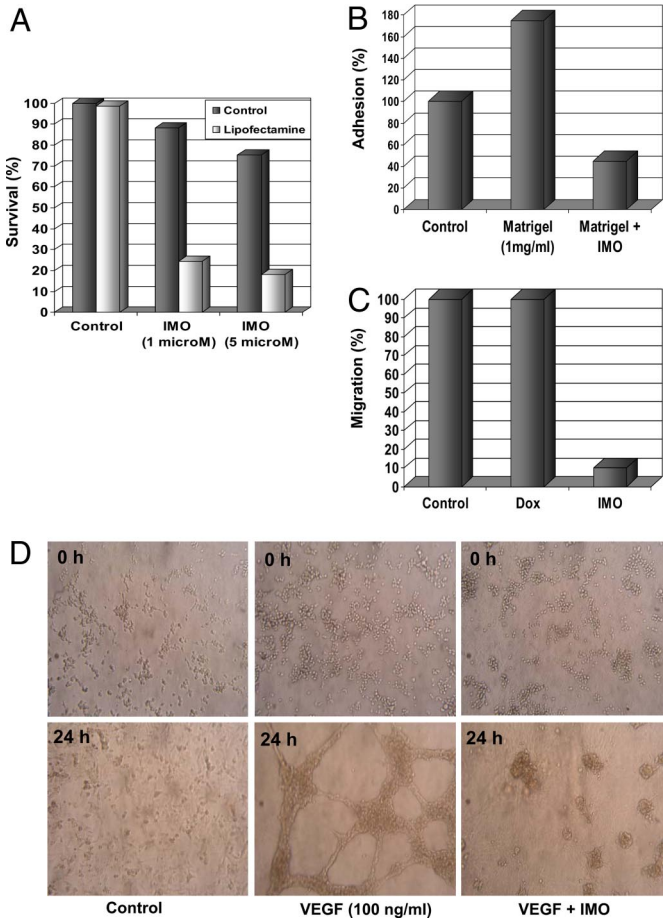

Fig. 5. IMO effects on HUVEC survival (*A*), adhesion to matrix (*B*), migration (*C*), and tube formation (*D*). (*A*) HUVEC were treated with IMO, 1 and 5 μM, in the presence or absence of Lipofectamine (2 μg/ml). The results are statistically significant for each dose of IMO vs. control (two-sided $P < 0.0001$). (*B*) HUVEC plated in the presence or absence of 1 μM IMO in serum-free medium (negative control) or Matrigel. The results are statistically significant for Matrigel vs. the negative control and for IMO vs. Matrigel (two-sided $P < 0.0001$). (*C*) HUVEC monolayers were wounded in the absence or presence of 10 ng/ml doxorubicin or 1 μM IMO (0 h). The results are statistically significant for IMO vs. control and vs. doxorubicin (two-sided $P < 0.0001$). (*D*) HUVEC were incubated with diluted Matrigel in the presence or absence of 1 μM IMO. Matrigel mixed with 100 ng/ml VEGF was used as positive control. Photographs were taken at 0 and 24 h.

TLR9 Is Expressed in LS174T, GEO, and GEO-CR Cells but Does Not Affect Their Growth *in Vitro*. We analyzed TLR9 expression in LS174T, GEO, and GEO-CR cell lines. In an earlier study, we were unable to detect TLR9 on GEO cells by using a direct Western blot assay (12). We have now used a more recent antibody (see *Materials and Methods*) and assayed each sample by immunoprecipitation followed by Western blot analysis, revealing a different degree of TLR9 protein expression in all cell lines. Further, the RT-PCR assay confirmed TLR9 mRNA expression in all of the cell lines examined (Fig. 6). We then investigated whether IMO could directly affect cell survival. Treatment of these cells with IMO at doses ranging from 0.1 to 1 μM, with or without lipofectamine, did not produce any effect on cell proliferation (data not shown).

Discussion

Synthetic agonists of TLR9 are a class of compounds with antitumor activity that have entered the clinical evaluation in cancer patients in combination with chemotherapy (3). Although

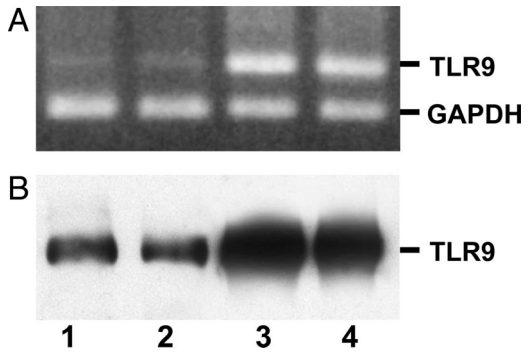

Fig. 6. Evaluation of TLR9 expression in human colon cancer cell lines. (*A*) TLR9 mRNA expression was evaluated in cultured LS174T, GEO, and GEO-CR colon cancer cell lines. (*B*) TLR9 expression was confirmed at the protein level by using immunoprecipitation and blotting with the same monoclonal anti-TLR9 antibody. PBMCs were included as positive control as well as RT-PCR targeting human GAPDH. Lane 1, PBMCs; lane 2, LS174T; lane 3, GEO; and lane 4, GEO-CR.

it is well documented that TLR9 agonists induce a potent innate and adaptive immune response and enhance the ADCC of mAbs, the mechanisms by which they affect growth signaling and angiogenesis are yet poorly understood.

We have recently hypothesized and demonstrated that a synthetic agonist of TLR9 (IMO) impairs EGFR and its downstream signaling proteins, including VEGF (12). The combination of IMO with anti-EGFR mAb cetuximab or tyrosine kinase inhibitor gefitinib, synergistically inhibits GEO tumor growth, the expression of several critical proteins and angiogenesis. Conversely, IMO is inactive against cetuximab-resistant GEO-CR tumors, further suggesting its dependence on EGFR signaling (12).

The EGFR pathway has a strong correlation with VEGF. In fact, EGFR can transactivate VEGF production (26, 27) and VEGF overexpression is a major escape pathway used by colon cancer, including GEO-CR tumors, to acquire resistance to EGFR antagonists (16, 21, 28, 29). This notion was recently confirmed in colon cancer patients failing treatment with cetuximab (30). On these bases, recently bevacizumab has been successfully used also in combination with EGFR inhibitors (22). Interestingly, VEGF impairs dendritic cell function, a major target of TLR9 agonists, and bevacizumab can overcome such interference (18, 19). We have previously shown that IMO fails to inhibit the growth as well as VEGF expression in GEO-CR tumors, suggesting that the interference of IMO with VEGF observed in wild-type tumors is actually an EGFR-dependent activity (12).

Based on the above studies, in the present work, we have analyzed the role played by IMO on these signaling pathways and its therapeutic implications. In particular, we have studied whether: (*i*) the potent inhibitory effect obtained with IMO and cetuximab on VEGF and angiogenesis depends on or is independent of interference in the EGFR pathway; (*ii*) the dependence of IMO on an integral EGFR pathway may affect its combination with an anti-VEGF agent, such as bevacizumab; and (*iii*) the ADCC mechanism and the expression of TLR9 are necessary to obtain a cooperative effect with mAbs.

To this aim, we combined IMO with bevacizumab, taking advantage of the fact that bevacizumab targets only the tumor-produced hVEGF and does not interfere with the mVEGF. Because bevacizumab binds the ligand and not the membrane receptor, we verified whether it has any ADCC activity, and whether IMO affects it. We have shown that the basal ADCC activity of cetuximab is enhanced by IMO in an *in vitro* assay, thus contributing to cetuximab activity with an EGFR-independent mechanism.

Conversely, bevacizumab has no ADCC activity, and IMO is unable to affect it. We have then demonstrated that the combination of bevacizumab with IMO causes a synergistic inhibition of tumor growth in human colon cancer xenografts GEO and LS174T and in the cetuximab-resistant GEO-CR, resulting in 90% of mice being tumor-free at pathologic analysis at the end of the experiment, 4 weeks after treatment withdrawal. Therefore, this combination treatment is also very effective in anti-EGFR-resistant tumors in an ADCC-independent fashion, suggesting that other mechanisms, not strictly EGFR- and ADCC-dependent, take place. In support of this notion, the two agents in combination cooperatively inhibit the expression of proteins used by tumors as escape pathways to acquire resistance to targeted therapies, such as pMAPK, pAkt, and VEGF (29) and inhibit neoangiogenesis in all three tumor types.

Analysis of the secreted VEGF in the serum of killed mice confirmed that bevacizumab, as expected, reduces the hVEGF levels, and also that the combination of IMO and bevacizumab cooperates in reducing the levels of hVEGF but not mVEGF. These results suggest that the murine-dependent immune-mediated effects of IMO enhance the activity of bevacizumab only on the human tumor cells. Interestingly, IMO and bevacizumab in combination caused a massive hemorrhagic necrosis, evaluated by pathological and immunohistochemical analysis, as early as the third week of treatment. An important mechanism of antiangiogenic therapy is the blockade of the VEGF-dependent proliferation of endothelial cells in the tumor. In an attempt to provide a clue to explain the non-EGFR-dependent cooperative antiangiogenic effects obtained with IMO and bevacizumab, we measured their activity on several functions of endothelial cells. We demonstrated that IMO inhibits proliferation, adhesion, and migration of HUVEC endothelial cells and, importantly, the VEGF-stimulated capillary tube and network formation. Therefore, it is likely that the well documented inhibitory effect of bevacizumab on vessel formation, due to VEGF inhibition, combined with the interference of IMO on critical functions of tumor endothelial cells, may finally be responsible for the cooperative effect observed. In addition, because it has been reported that colon cancer cell lines, including GEO, express VEGF receptors (31), the combined effect of IMO and bevacizumab may have a direct impact also on tumor cells. Together, these data may also help explain, at least in part, the hemorrhagic necrosis observed in the LS174T tumors.

Finally, we attempted to understand the relevance of TLR9 expression for the antitumor effects observed. Measurement of TLR9 by different techniques demonstrated the presence of mRNA and protein expression, to a different degree, in GEO, LS174T, and GEO-CR cells. Nevertheless, treatment of these cells *in vitro* with IMO, at different doses, with or without lipofectamine to improve cellular penetration, did not produce any effect on tumor growth or on EGFR expression. In addition, the *in vivo* effect of IMO with or without bevacizumab was not proportional to the degree of TLR9 expression. Therefore, the expression of TLR9 on these colon tumor cells is not directly responsible for the antitumor effects observed but is more likely related to the systemic immune responses produced by IMO.

Taken together, these studies provide insights into the mechanisms of action of synthetic agonists of TLR9. In fact, they demonstrate that, although dependent on the immune activation, IMO has a much broader range of mechanisms, involving not only the EGFR-dependent pathway but also the neoangiogenesis. These results provide a strong rationale to translate the combination with bevacizumab in the clinical practice, as a potentially powerful and rationally based therapeutic strategy. The possibility of combining IMO with inhibitors of EGFR and of VEGF creates the opportunity to take advantage of multiple chance for cooperativity, involving EGFR- and ADCC-dependent and -independent mechanisms and neoangiogenesis.

Materials and Methods

Compounds. IMO, 5′-TCTGACRTTCT-X-TCTTRCAGTCT-5′ (X and R are the glycerol linker and 2′-deoxy-7-deazaguanosine, respectively) was synthesized with phosphorothioate backbone, purified, and analyzed as described (6–8, 32). The mAb anti-VEGF bevacizumab was kindly provided by Genentech (South San Francisco, CA).

Cell Cultures. GEO, LS174T, GEO-CR (16) colon cancer cells, and HUVEC were maintained, respectively, in McCoy's or RPMI medium 1640 supplemented with 10% heat-inactivated FBS/20 mM Hepes, pH 7.4/penicillin (100 units/ml)/streptomycin (100 μg/ml)/4 mM glutamine (ICN, Irvine, CA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

ADCC Assay. A nonadherent fraction of human peripheral blood mononuclear cells (PBMCs) was used as effector cells. Briefly, human PBMCs were isolated by density gradient centrifugation and resuspended in RPMI medium 1640. Before the assay, the PBMCs were cultured for 1 h on plastic dishes to remove adherent cells (monocytes), and then the PBLs were incubated for 24 h in the presence or absence of IMO (5 μM). The target cells (chronic erythroid leukemia K562 and breast cancer MDA468) were loaded with the fluorescence-enhancing ligand (DELFIA BATDA reagent; PerkinElmer, Wellesley, MA) and, after washing, were incubated in the presence or absence of the antibodies bevacizumab or cetuximab (10 μg/ml). Target cells were mixed with effector cells at varying cell concentrations for 2 h at 37°C and centrifuged. Supernatants were added to Europium solution and the signal was measured, as described (33).

Xenografts in Nude Mice. Five-week-old BALB/cAnNCrlBR athymic (nu+/nu+) mice (Charles River Laboratories, Milan, Italy) were maintained in accordance with the institutional guidelines of the University of Naples Animal Care Committee in accordance with the Declaration of Helsinki. Wild-type GEO, LS174T, or GEO-CR human colon cancer cells (10^7 cells per mouse) were resuspended in 200 μl of 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, three times per week for 4 weeks; i.p. bevacizumab, 5 mg/kg, twice per week for 3 weeks; or the combination of these agents, on days 7–11, 14–18, and 21–25, continuing only IMO on days 28–32. Tumor volume was measured by using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported (34). Two mice were killed on day 25 for biochemical analysis.

Immunoprecipitation and Western Blot Analysis. Total cell lysates were obtained from homogenized tumor specimens removed on day 25. The protein extracts were resolved by 4–15% SDS/PAGE and probed with anti-human polyclonal Akt, monoclonal pAkt (Cell Signaling Technologies, Beverly, MA), monoclonal actin (Sigma-Aldrich, Milan, Italy), monoclonal VEGF, monoclonal pMAPK, and monoclonal MAPK (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL), as described (16). TLR9 protein levels were evaluated on total cell lysates by immunoprecipitation by using a monoclonal anti-TLR9 antibody (Calbiochem/EMD Biosciences, La Jolla, CA) and blotting with the same antibody, following the procedures described above.

Immunohistochemical Analysis. Immunocytochemistry was performed on formalin-fixed paraffin-embedded tissue sections (5 μm) of LS174T xenografts. Sections were processed, reacted with avidin-conjugated horseradish peroxidase H complex, and

stained as described (34). New blood vessels were detected by using a mAb against the CD34 antigen (Dako, Milan, Italy) at a dilution of 1:50 and stained with a standard immunoperoxidase method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Each slide was scanned at low power (10–100× magnification), and the area with the higher number of new vessels was identified (hot spot). This region was then scanned at 250× 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.

ELISA. Anti-hVEGF or anti-mVEGF polyclonal antibody (R&D Systems, Minneapolis, MN), diluted at 1 μg/ml in PBS, pH 7.5, was used to coat a 96-well plate, 100 μl/well, overnight at 4°C. Washings, dilutions of standards (recombinant hVEGF or mVEGF), and samples (serum of killed mice), biotinylation, and mix with preformed avidin and biotinylated HRP macromolecular complex (Vectastain kit) were described (35). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Hercules, CA). VEGF concentrations were determined by interpolation of the standard curve by using linear regression analysis.

RT-PCR. Total RNA was extracted by using the TRIzol reagent from Invitrogen Life Technologies (Grand Island, NY) and was quantified and used to create cDNA. Amplification of TLR9 was accomplished by using primers as published (36), and a portion of the PCR product was visualized by using ethidium bromide on an agarose gel. Human GAPDH was coamplified with TLR9 to verify the quality and expression level of the mRNA.

Cell Survival Assay. Cells were grown in 24-well plates and exposed to IMO with or without Lipofectamine 2000 (2 μg/ml) from Invitrogen Life Technologies. The percentage of cell survival was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions.

Adhesion Assay. Ninety-six-microwell bacterial culture plates were precoated with 50 μl/well of serum-free medium (SFM) containing 0.1% BSA (negative control) or Matrigel (1 mg/ml in water). After 1 h, all coating solutions were removed, and HUVEC (20,000 cells per well) were plated in SFM, in the presence or absence of 1 μM IMO. After incubation, cells were analyzed as described (37).

Wound-Healing Assay. Monolayers of HUVEC were grown on gridded plastic dishes and scratched as reported (38) with or without 10 ng/ml doxorubicin or 1 μM IMO (0 h), which have the same antiproliferative effect. Because doxorubicin did not interfere with cell migration, it was used as a negative control. The migration distances between the edges of the cells in the wound were photographed (10× magnification) at 0 and 24 h, quantified, and compared by using Adobe Photoshop, Ver. 8.0.1 (38).

Vascular Endothelial Cell Capillary Tube and Network Formation. Five hundred microliters of diluted Matrigel was added into a 30-mm culture dish and incubated at 37°C for 30 min. After the Matrigel was solidified, HUVEC (4×10^5) in 1 ml of RPMI medium 1640 were added in each dish, in the presence or absence of 1 μM IMO, incubated at 37°C, and photographed (10×) at 0 and 24 h. As a positive control, Matrigel was mixed with 100 ng/ml VEGF (R&D Systems).

Statistical Analysis. Student's *t* test was used to evaluate the statistical significance of the results. All reported *P* values were two-sided. All analyses were performed with the BMDP New System statistical package for Microsoft Windows (Version 1.0; BMDP Statistical Software, Los Angeles, CA).

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