Translational Oncology
www.transonc.com

Volume 4 Number 1 February 2011 pp. 38–46 38

Isolation of Fully Human Antagonistic RON Antibodies Showing Efficient Block of Downstream Signaling and Cell Migration[1]

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Abstract

RON belongs to the c-MET family of receptor tyrosine kinases. As its well-known family member MET, RON and its ligand macrophage-stimulating protein have been implicated in the progression and metastasis of tumors and have been shown to be overexpressed in cancer. We generated and tested a large number of human monoclonal antibodies (mAbs) against human RON. Our screening yielded three high-affinity antibodies that efficiently block ligand-dependent intracellular AKT and MAPK signaling. This effect correlates with the strong reduction of ligand-activated migration of T47D breast cancer cell line. By cross-competition experiments, we showed that the antagonistic antibodies fall into three distinct epitope regions of the RON extracellular Sema domain. Notably, no inhibition of tumor growth was observed in different epithelial tumor xenografts in nude mice with any of the antibodies. These results suggest that distinct properties beside ligand antagonism are required for anti-RON mAbs to exert antitumor effects *in vivo*.

Translational Oncology (2011) 4, 38–46

Introduction

Monoclonal antibodies (mAbs) are attractive therapeutic drug candidates in cancer therapy thanks to their potential for disease-specific targeting and low-toxicity profiles. Significant progress has been made in recent years in this field. However, there is substantial difficulty in predicting the efficacy of mAbs because the molecular pathways leading to their therapeutic effect are not clearly understood. Although many mechanisms have been proposed to account for the antitumor activities of therapeutic antibodies as a consequence of the interference with signaling pathways, antibody-dependent cell cytotoxicity stands also as a crucial component of the *in vivo* antitumor effect exerted by antibodies targeting tumor antigens [1].

Receptor tyrosine kinases (RTKs) are preferred target candidates for new mAbs because they fine-tune cellular pathways that control events involved in cellular growth and migration that are important in cancer development and progression.

The recepteur d'origine nantais (RON), also known as macrophage stimulating 1 receptor, is a member of the MET proto-oncogene family of RTKs and has been shown to be important in cancer [2–6]. It is a 180-kDa disulfide-linked heterodimeric protein composed of a 35-kDa extracellular α-chain and a 150-kDa transmembrane β-chain with intrinsic kinase activity and regulatory elements [2,7]. The extracellular segment contains a Sema domain that constitutes the distinctive structural and functional elements of semaphorins, their plexin receptors, and the RTK MET [8–10]. This domain seems to provide a common structural scaffold that can be adopted to mediate a diverse range of protein-protein interactions such as dimerization,

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[1]This article refers to supplementary materials, which is designated by Figures W1 to W6 and are available online at www.transonc.com.

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Received 5 August 2010; Revised 22 October 2010; Accepted 1 November 2010

1944-7124/11
DOI 10.1593/tlo.10211

semaphorin-plexin binding, and receptor-ligand binding. RON's Sema domain is considered to be the ligand-binding region [2,9,10].

RON is mainly expressed in epithelial cells and is highly expressed and activated in most primary breast, colon, and pancreatic samples and tumor cell lines [2,4,11–15]. Ligand of RON is the macrophage-stimulating protein (MSP), which is a 78-kDa growth and motility factor [12,16,17]. RON and its ligand MSP have been implicated in the progression and metastasis of tumors. On binding to its ligand MSP, RON becomes phosphorylated at key intracellular tyrosine residues that provide docking sites for downstream signaling adapter molecules and triggers activation of several signaling pathways that include the Ras/MAPK, PI3K/Akt, and FAK pathways [18,19]. On stimulation with MSP, RON enhances invasion and cell motility in various epithelial cell lines [13,20]. A recent study demonstrated RON as a novel molecular target of hypoxia-inducible factor 1 and suggested a potential therapeutic role for RON TK inhibitors in the blockade of RON tyrosine kinase–mediated invasion of carcinoma cells [21].

RON is in cross talk with other RTKs, cell surface proteins and growth factors such as epidermal growth factor receptor (EGFR), MET, integrins, and transforming growth factor β [22–26]. RON activation can enhance signaling through c-MET and EGFR, both associated with tumorigenesis [25,27]. The changes associated with the epithelial-to-mesenchymal transition due to RON activation together with the ability of MSP-activated RON to increase cellular migration further support a role for RON in metastasis [23]. Tissue-specific overexpression of RON resulted in aggressive tumor formation in transgenic mouse models of lung and breast cancer [28,29].

There is a body of evidence that suggest that RON should be considered as target for cancer therapy [30]. Efforts to develop a cancer therapeutic specific for this RTK have generated a variety of agents that block RON signaling and alter oncogenic phenotypes [3,6,9,31]. Recently, a mAb, IMC-41A10, was reported to bind human RON with high affinity and to inhibit MSP binding as well as MSP-mediated RON, MAPK, and AKT phosphorylation and MSP-dependent *in vitro* cell migration. IMC-41A10 inhibited growth of tumor xenografts in nude mice, and its antitumor efficacy was enhanced when administered in combination with Erbitux [12].

In this study, we have extensively characterized three mAbs against RON, which were identified through the screening of a human scFv phage displayed library. These novel mAbs bind with high affinity to human RON, recognize distinct sites on the Sema domain, block downstream signaling mediated by MSP binding, and inhibit MSP-driven migration of T47D cells. Despite these characteristics being similar to those of IMC-41A10, no *in vivo* efficacy was detected with any of our antagonistic RON mAbs. Our results indicate that different mechanism(s) other than ligand antagonism and inhibition of downstream signaling are needed for a RON mAb to have a therapeutic potential in cancer.

Materials and Methods

Cell Lines

T47D, BxPC3, ZR-75, HT29, MDA-MB-231, DU145, SW480, HPAC, HEK293, and 293-EBNA cells were purchased from ATCC (Milan, Italy) and grown as recommended by the manufacturer except T47D cells, which were grown in Dulbecco modified Eagle medium F-12 Glutamax medium (GIBCO-Invitrogen, Carlsbad, CA), enriched with 10% FBS at 37°C and 5% CO_2.

To generate cell lines that overexpress Ron receptor, complementary DNA (cDNA) encoding the full-length RON protein was cloned into the Gateway-pcDNA-Dest40 expression vector (Invitrogen, Carlsbad, CA) and into the doxycycline-inducible expression vector pCEP/TetO-MCS. CHO cells were transfected with Gateway-pcDNA-Dest40-RON construct, and HEK 293 cells were transfected with pCEP/TetO-MCS-RON construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells expressing high RON levels were FACS-sorted and grown under appropriate antibiotic selection to obtain stable clones (Geneticin for pDest 40 -RON, Hygromycin for pCEP/TetO-MCS-RON).

Identification of Human Anti-RON ScFv Antibodies from Phage Display Libraries

Human ScFv phage-display libraries with high complexity (>10^{11} different clones) were obtained from Cambridge Antibody Technology Group plc (CaT). Each CAT library has undergone two rounds of selection. The first round was carried out by panning the phage library on recombinant RON SEMA-PSI domain (R&D Systems, Minneapolis, MN) followed by a second-round selection with SW480 cells naturally expressing RON. Phage rescue and amplification were carried out between subsequent selections as described [32]. After two rounds of selection, individual phage clones were examined by phage ELISA for their binding capability to immobilized RON-Fc (Sigma-Aldrich, St Louis, MO) through the use of an anti-Fc Ab (immunoPure Goat anti hIgG [Fc]; Pierce, Milan, Italy). Bound phage was revealed with HRP-conjugated anti M1 monoclonal antibody (Amersham Biosciences, Milan, Italy) using tetramethylbenzidine (Sigma-Aldrich) as substrate. Positive clones were converted from an ScFv to an IgG1 format as previously described [33]. Positive clones were also tested by phage ELISA for their binding to human recombinant MET-Fc (R&D Systems) and to the recombinant mouse RON (R&D). The scFv VH and VL sequences were introduced into mammalian expression plasmids to produce IgG1 heavy or light chain using the Gateway technology (Invitrogen, Carlsbad, CA). The two plasmids were cotransfected into 293-EBNA cells using Lipofectamine (Invitrogen). Secreted IgG was purified from the medium by affinity chromatography on a Protein A Sepharose High Performance prepacked column (1 ml of HiTrap Protein A HP; GE Healthcare, Milan, Italy).

Identification of mAbs That Bind Selectively to RON Using FACS Analysis

Each purified RON mAb was tested for its binding to RON-positive cells (CHO/RON, HEK293/RON + doxycycline, SW480, T47D, HCT116) or RON-negative cells (CHO wt, HEK293/RON-doxycycline) using FACS analysis. MAbs were labeled by incubation with Zenon Human IgG Alexa Fluor 488 Labeling Kit (Molecular Probes/Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Cells were then incubated with 50 μg/ml prelabeled mAb solution for 30 minutes at room temperature (RT; 2 × 10^5 cells in 100 ml of phosphate-buffered saline (PBS), 2% BSA for 30 minutes), washed, and fixed with 1% paraformaldehyde in PBS. Analysis was done using BD FACS Canto II cytometer and BD Diva analysis software (BD Biosciences, Milan, Italy).

Immunoprecipitation/Mass Spectrometry Experiment—Identification of Target Receptors

Experiments was carried out as before [34]. Shortly, immunoprecipitation (IP) with RON IgGs 4, 7, and 10 was carried out on fluorescently

labeled (Cy5) membrane proteins. One-dimensional PAGE was followed by in-gel digestion of immunoprecipitated protein bands and identification of the protein bands using matrix-assisted laser desorption ionization–time of flight–mass spectrometer (MALDI-TOF-MS). Data were elaborated using the TurboSequest software (Thermo Electron Rodano, Milan, Italy). Candidates with Xcorr values greater than 2.5 were considered identified with high confidence.

K_d Determination Through FACS Analysis

A total of 4×10^5 T47D cells were incubated with related RON IgGs with increasing concentrations from 3.4×10^{-4} to 60 nM in FACS buffer (0.2% BSA, 10 mM HEPES) for 1 hour at RT followed by 40 minutes of incubation with APC/F(ab′)$_2$ fragment goat anti-human IgG Fcγ fragment specific (Jackson ImmunoResearch, Milan, Italy) at RT. Analysis was carried out using BD FACS Canto II cytometer and BD Diva analysis software. K_d values were determined using Sigma Plot 10 software (Systat Software, San Jose, CA).

Cross-competition Assay

A total of 5×10^5 T47D cells per sample were washed once with PBS 1% BSA and incubated with 100 μg/ml of competitor IgG or unrelated human IgG (IgG1, Lambda, from human myeloma plasma; Sigma) for 40 minutes at room temperature on an orbital shaker. The IgG to be tested was labeled with AlexaFluor 647 using Zenon AlexaFluor 647 for human IgG (cat. no. Z25408; Molecular Probes). After a wash with PBS 1% BSA, cells saturated for the first IgG were incubated with different concentrations of the labeled antibody (330, 66, 13, 2.6, 0.5, and 0 nM) for 40 minutes at RT on an orbital shaker. After a wash with PBS 1% BSA, cells were fixed in 1% formaldehyde in PBS. Analysis was done using BD FACS Canto II cytometer and BD Diva analysis software. Binding curves were prepared using Sigma Plot 10 software.

Western Blot Experiments

Induction with MSP. Subconfluent RON-expressing cell lines (HT29, MDA-MB-231, Bx-Pc3, ZR-75, T47D, DU145, SW480, and HPAC) were serum-starved for 18 hours. Wild-type MSP (10 nM; R&D) was used to induce cells for 30 minutes at 37°C.

Testing RON IgGs' ability to block MSP-driven induction. After 18 hours of serum starvation, 100 μg/ml of RON IgGs was incubated with subconfluent BxPC3, T47D, and ZR-75 cell lines for 1 hour at 37°C before 30 minutes of induction with MSP. ID1 commercial blocking antibody CD136 (RON; Beckman Coulter, Milan, Italy) against RON was used as a positive control, and an unrelated antibody (IgG1, Lambda, from human myeloma plasma; Sigma) was used as a negative control.

RON IgGs dose-response experiment. Subconfluent T47D cells that were serum-starved for 18 hours were incubated with increasing concentrations of RON mAbs (2.4, 7.3, 22, and 66 nM) for 1 hour. MSP, 10 nM, was used to induce T47D cells for 30 minutes at 37°C.

Time course experiment with RON mAbs. A total of 4.5×10^5 T47D cells per well in a six-well plate for each point were used. The day after plating, cells were treated with 20 μg/ml of RON mAbs 4, 7, and 10 for 30 minutes, 1 hour, 4 hours, or 24 hours.

Cell lysate preparation and Western blot analysis. Cells were lysed using lysis buffer (50 mM Tris, 1% Triton, 150 mM NaCl, complete protease inhibitor cocktail (Roche, Milan, Italy), halt phosphatase inhibitor cocktail [Thermo Scientific, Milan, Italy]). Total protein concentration was determined by Bradford method, 20 μg of total protein per sample was loaded on 4% to 12% NuPage BisTris Gels, and SDS-PAGE was performed. Proteins were transferred on to a 22-μm nitrocellulose membrane that was then blocked with 5% nonfat dry milk in 0.05% Tween in TBS. For determination of extracellular signal–regulated kinase (ERK) (mitogen-activated protein kinase, MAPK) and AKT phosphorylation, p44/42 MAPK Thr202/Tyr204 rabbit mAb (1:1000; Cell Signaling, Milan, Italy) and phospho–Akt/Ser473 (1:500; Cell Signaling) were used. Protein-blotted membranes were incubated overnight at 4°C with primary antibodies in 5% BSA in 0.05% Tween in TBS. HRP-conjugated rabbit (Abcam, Cambridge, UK) and mouse (Promega, Madison, WI) secondary antibodies were used as 1:1500 and 1:3000, respectively, carrying out 1 hour of incubation at RT. To verify equivalent sample loading, blots were probed for β-actin (NeoMarkers, Fremont, CA). Blots were developed using ECL reagent (GE Healthcare, Milan, Italy). Images were acquired by luminescent image analyzer LAS3000 and then quantified by MultiGauge version 2.2 software (FUJIfilm, Düsseldorf, Germany). To determine expression levels of total ERK and total AKT, these membranes were stripped with Restore Western Blot Stripping Buffer for 15 minutes at room temperature (Pierce) and reprobed with p44/42 MAPK (1:1000; Cell Signaling) or anti-AKT antibody (1:2000; Cell Signaling). After washing and incubating with the secondary antibody, development and image analysis were carried out as before. For determination of RON protein levels, Ron (C-20) sc-322 rabbit polyclonal IgG and HRP-conjugated rabbit (Abcam) were used.

Cell Migration Assay

Twenty-four–well HCT FluoroBlok Insert Systems with fluorescence blocking PET membranes were used as migration chambers (BD Biosciences). T47D cells were serum starved for 18 hours and then plated as 5×10^4 cells per well in serum-free medium on previously collagen coated Fluoroblok chambers (rat tail collagen type I; BD Biosciences). Recombinant hMSP C672A (R&D Systems) was added at a final concentration of 2 nM to the bottom chambers. (Recombinant MSP C672A has increased bioactivity than recombinant MSP [35] because of the limited incorrect disulfide bond formation, so the concentration used in comparison to the previous experiments is lower than previously made signaling experiments as we were furnished with MSP C672A by R&D only during half of this study.) RON mAbs or an unrelated human IgG (IgG1, Lambda, from human myeloma plasma; Sigma) were added in both upper and lower chambers at a final concentration of 20 μg/ml before stimulation with MSP. After 2-nM MSP stimulation, cells are allowed to migrate for 24 hours at 37°C and 5% CO_2. Migrated cells were quantified after fluorescent labeling with 4 μg/ml Calcein-AM (Fluka, Milan, Italy) and by using fluorescence plate reader VICTOR^2 (Perkin Elmer, Waltham, MA).

Cell Cytotoxicity Assay

T47D cells were serum-starved for 18 hours and were plated in 96-well plates that had been previously coated with collagen (rat tail collagen type I; BD Biosciences) Ron mAbs or an unrelated human IgG in combination with MSP (2-nM recombinant hMSP C672A; R&D Systems) were added to the cells in serum-free medium. After 24 hours of incubation at 37°C and 5% CO_2, ATP levels of cells

were measured using ATPlite 1 step reagent (Perkin Elmer) using Perkin Elmer TopCount microplate luminescence counter.

In Vivo *Mouse Models*

In the T47D model, 17β-estradiol pellets (0.36 mg per pellet, 60-day release) were implanted in the dorsal midline of 5-week-old female NOD/SCID mice. After 1 week, 2×10^6 T47D cells in Matrigel were injected in the fourth mammary fat pad. Nineteen days after injection, mice with tumors greater than 30 mm^3 were allocated randomly in five groups. Mice were treated weekly with 25 mg/kg intraperitoneally with RON mAbs and isotypic control human IgG1. On day 29 after allocation, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

In the following two *in vivo* models, 5-week-old female athymic nude mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 5×10^6 BxPC3 cells in Matrigel or intraperitoneally with HT29 cells in Matrigel. After tumors reached an average size of 150 to 200 mm^3, 25-mg/kg intraperitoneal treatment with RON mAbs or histidine buffer was carried out biweekly. At day 26 after treatment, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

Results

Identification and Characterization of RON Specific mAbs

To identify RON-specific mAbs, we screened a human scFv phage-displayed library obtained from CaT with the following selection strategy: a first round of selection was carried out on the recombinant RON Sema-PSI domain, and the recovered phages were screened by a second round of selection on SW480 cells that express high levels of endogenous RON on the cell surface. Finally, individual scFv phages specific for RON were screened for binding to the closely related family member human recombinant MET-Fc and to the recombinant mouse RON. The restricted pool of RON-specific phages was found to bind selectively to human RON but not to mouse RON or human MET (data not shown). Individual phage clones were converted into a human type 1 IgGs and subjected to a further round of screening in a whole-cell binding FACS assay using RON-positive and -negative cell lines. IgG4, IgG7, and IgG10 were found to bind specifically to CHO/RON when compared with CHO wt (Figure W1*B*). We further confirmed the specificity of RON mAbs by IP of fluorescently labeled membrane proteins with RON mAbs followed by in-gel digestion of protein bands and peptide identification by MALDI-TOF-MS mass spectrometry (IP-MS). All RON mAbs specifically immunoprecipitated two major bands in polyacrylamide gel, which were identified as RON α and β chains (Figure W1*A*).

IgG4, IgG7, and IgG10 Bind RON with High Affinity

To determine the mAbs' affinity toward RON receptor, we performed whole-cell FACS binding assays on T47D cells endogenously expressing RON. IgG4, IgG7, and IgG10 all displayed high affinity toward RON (Figure 1) with subnanomolar K_d values of 0.17, 0.43, and 0.64 nM, respectively.

Antagonistic RON mAbs Bind to Different Epitopes on Sema Domain

Because of the strategy used to select RON-specific antibodies, the novel RON mAbs IgG4, IgG7, and IgG10 bound to the Sema domain of RON. To ensure that these mAbs recognized different regions

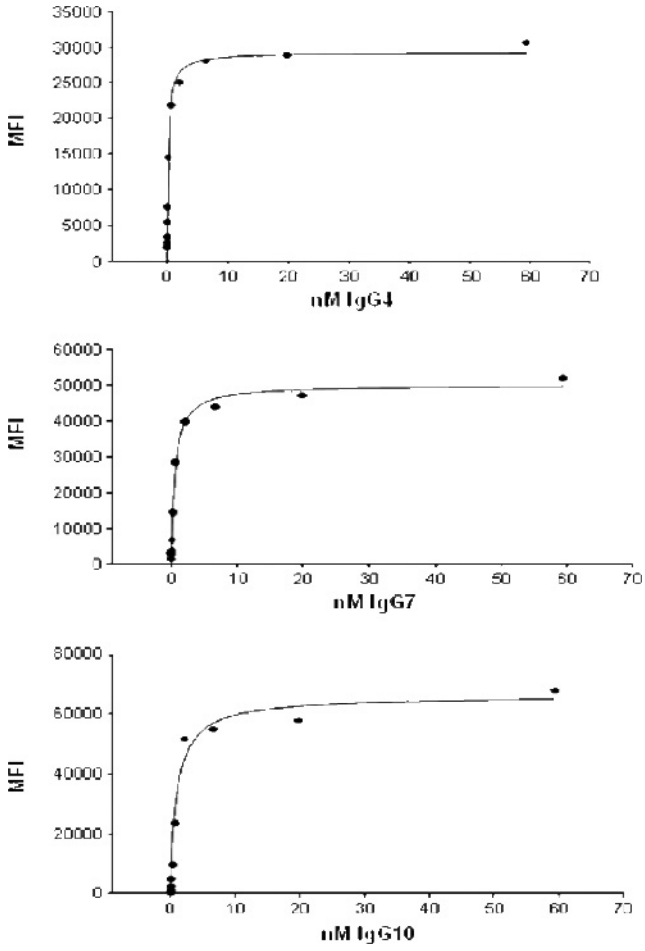

Figure 1. IgG4, IgG7, and IgG10 bind RON with high affinity. Binding saturation curves determined by whole-cell FACS binding assay are represented as mean fluorescence intensities (MFIs) over antibody concentration (nM). Calculated K_d values for IgG4, IgG7, and IgG10 were 0.17, 0.43, and 0.64 nM, respectively.

of the Sema domain, the epitope binding specificity was evaluated. A FACS-based cross-competition experiment was performed in which the binding of one tagged antibody to RON-displaying cells was measured in the presence of a potential competitor antibody or an unrelated IgG1 isotypic control at saturating concentration. As a reference, the commercially available blocking mAb ID1 was also included. This antibody is known to bind to the RON extracellular domain [36].

Notably, IgG4, IgG7, and IgG10 did not cross compete with each other, as the extent of binding was not altered by the presence of another mAb (Figure 2, *A* and *B*). Only mAb ID1 and IgG7 cross-competed with each other, as IgG7 binding to RON was strongly inhibited in the presence of ID1 (Figure 2*B*). As shown by the simplified cartoon in Figure 2*C*, these results indicate that IgG4, IgG7, and IgG10 bind to different regions of the RON Sema domain, whereas ID1 and 7 bind to the same or to closely positioned regions.

RON mAbs Block Downstream Signaling

Overexpression of the receptor as a consequence of transfection of the RON cDNA often results in autophosphorylation of this RTK regardless of MSP stimulation [37]. Therefore, to study the effect of RON mAbs on ligand-dependent receptor signaling, different cell

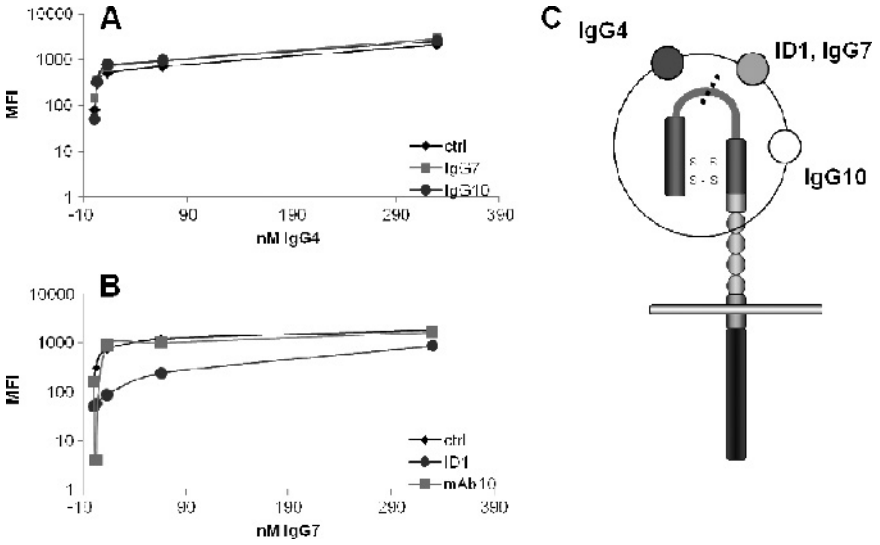

Figure 2. RON IgGs bind to different regions of the Sema domain. (A) T47D cells were incubated with increasing concentrations of labeled mAb4 in the presence of saturating amounts of mAb7, mAb10, or control isotypic IgG1 antibody (Ctrl IgG) as negative control. (B) T47D cells were incubated with increasing concentrations of labeled mAb7 in the presence of saturating amounts of mAb10, ID1, and control IgG. IgG4, IgG7, or IgG10 did not interfere with any other's binding, indicating that they recognize different regions, whereas ID1 blocks mAb7 binding, indicating that they recognize the same region. (C) Schematic cartoon summarizing the results of cross-competition experiments.

lines were used that endogenously express RON. In addition, to evaluate the ability of the RON mAbs to inhibit phosphorylation of ERK and AKT, an extensive screening of tumor cell lines was carried out to identify cells that expressed RON and at the same time showed ERK and AKT phosphorylation on MSP binding. As shown in Figure 3*A*, the pancreatic cancer cell line BxPC3 and the breast cancer cell lines ZR-75 and T47D exhibited a strong MSP-dependent phosphorylation of AKT and ERK. Kinetics of the treatment of RON-expressing cells with the ligand MSP showed that the maximum phosphorylation of ERK and AKT was reached after 30 minutes of incubation (Figure W2). To measure the inhibitory potency of the different antibodies, dose-response experiments were carried out in the T47D cells. Increasing concentrations of IgG4, IgG7, and IgG10 or unrelated IgG1 isotypic control were incubated with T47D cells before MSP stimulation. All mAbs inhibited both ERK (Figure 2*B*) and AKT (data not shown) phosphorylation in a dose-dependent manner. Because the intensity of the pERK signal was stronger than pAKT, the half-maximal inhibitory concentration (IC_{50}) for each IgG was calculated on the basis of the extent of pERK inhibition. The measured IC_{50} was 3 nM for IgG4 and IgG10 and 50 nM for IgG7. Thus, these data demonstrate that IgG4, IgG7, and IgG10 are high-affinity antibodies, specific for human RON, which have ligand antagonistic activity. Finally, we investigated the cytotoxic potential of the antagonist RON mAbs 4, 7, and 10 in *in vitro* proliferation assays on T47D cells. Treatment with the antibodies did not induce an antiproliferative effect, whereas a known cytotoxic drug, cisplatin, clearly reduces proliferation (Figure W6).

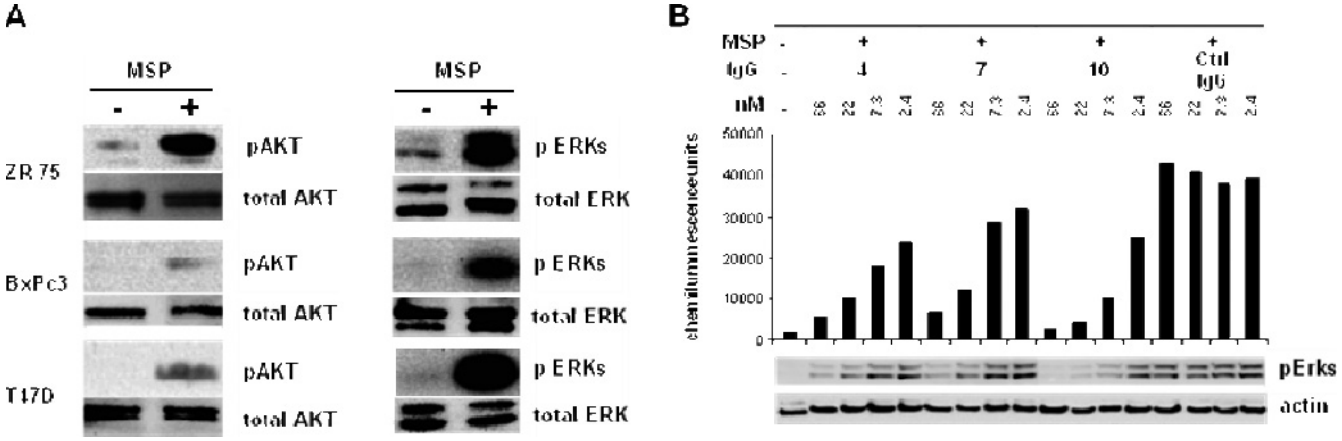

Figure 3. RON IgGs block MSP-activated signaling. (A) Cell lysates of ZR75, T47D, and BxPC3 were assayed by Western blot analysis with anti–phospho-Akt and anti–phospho-ERK antibodies. Antibodies directed to total Akt and total ERK were used as loading controls. (B) Western blot analysis of cell lysates of T47D cells treated with increasing concentration of IgG4, IgG7, and IgG10 before MSP stimulation. Signal intensities were quantified by densitometric scanning and expressed as chemiluminescence units. An antibody directed to actin was used as a loading control.

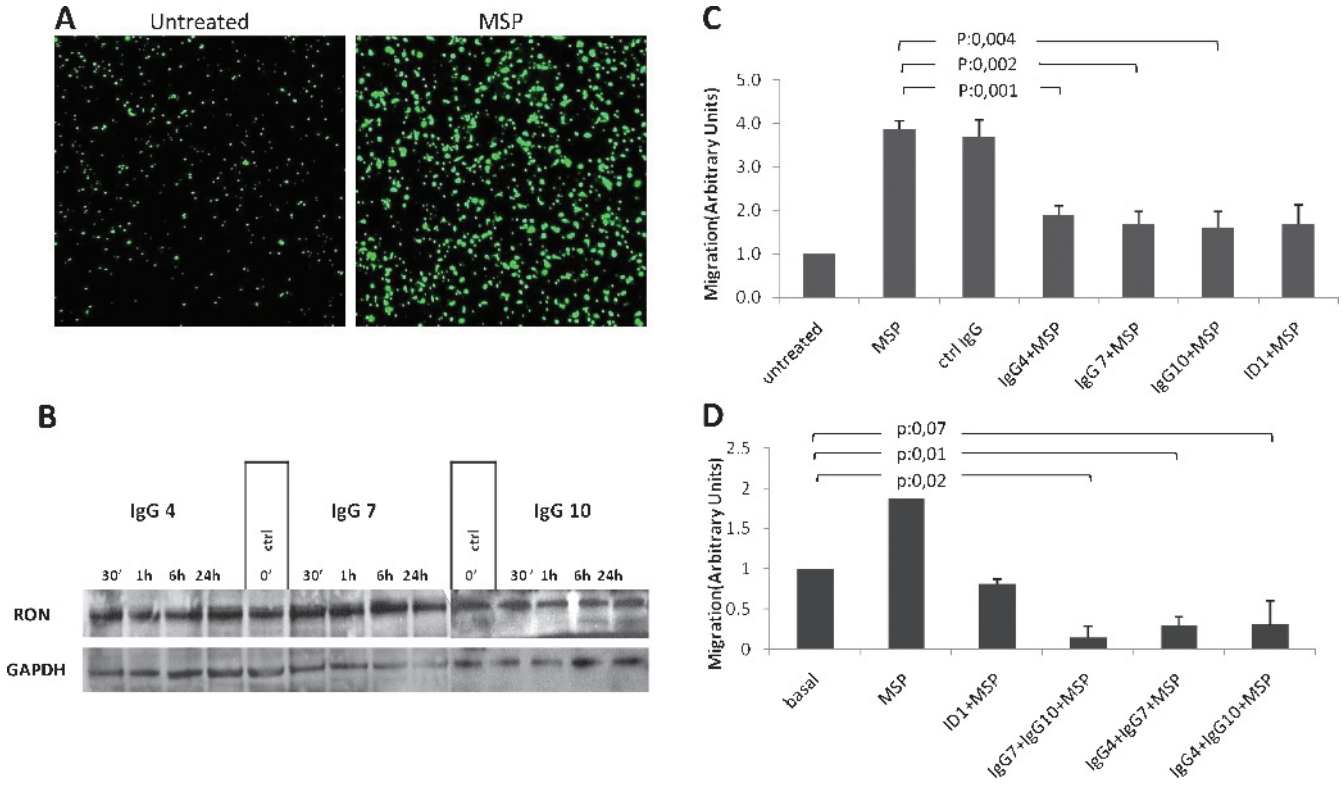

Figure 4. RON IgGs significantly block MSP-driven migration. (A) Representative images of fluorescently labeled T47D migrated to the lower side of the PET membrane in a Boyden chamber after 24 hours with or without MSP stimulation. (B) RON IgG4, IgG7, and IgG10 do not downregulate the RON receptor. T47D cells were treated with RON IgGs for 30 minutes, 1 hour, 4 hours, and 24 hours. (C) Migration activity of T47D cells was measured by a 24-well HCT FluoroBlok assay and expressed as arbitrary units. MSP stimulation (2 nM) caused up to a four-fold increase in migration with respect to untreated control. IgG4, IgG7, and IgG10 significantly blocked MSP-driven migration ($P = .001$, $P = .002$, and $P = .004$, respectively) comparable to the commercially available mAb ID1. (D) Combination of RON IgGs leads to an impairment of T47D spontaneous motility. Combination of IgG7-IgG10 and IgG4-IgG7 MSP inhibited spontaneous motility of T47D cells by 75% to 80% ($P = .02$ and $P = .01$, respectively). Combination of IgG4-IgG10 also reduces the motility of T47D although not significantly ($P = .07$). The experiments shown in the figures are representative of at least three different experiments.

RON IgG4, IgG7, and IgG10 Inhibit MSP-Driven Migration

It has previously been shown that on stimulation with MSP, RON enhances invasion and cell motility in various epithelial cell lines [13,20,27]. To assess the effect of the selected mAbs on cell motility, a fluorimetric transwell migration assay was set up with T47D cells where cells were allowed to migrate to the bottom chamber for 24 hours through collagen-coated membranes in the presence or absence of MSP. When the ligand was absent, a limited number of cells spontaneously migrated to the bottom chamber after 24 hours because of intrinsic cell motility. MSP stimulation caused up to a four-fold increase in the extent of migration with respect to the unstimulated condition. Migration of the cells to the bottom chamber was equally inhibited by IgG4, IgG7, and IgG10 when the mAbs were incubated with cells before MSP stimulation (Figure 4, *A* and *C*).

Because the three mAbs bind to the Sema domain, which is involved in RON, cross talk with other membrane proteins and because they do not interfere with each other's binding, we tested the effect of antibodies when used in combination. Notably, when IgG4, IgG7, and IgG10 were used in combination, they showed a stronger inhibiting effect on T47D migration, which not only was limited to inhibition of MSP-driven migration but also blocked the spontaneous motility of T47D cells. A significant effect was observed with IgG7-IgG10 or IgG4-IgG7 combinations, whereas IgG4-IgG10 exerted a lesser inhibitory effect. Nonetheless, all three combinations were more efficient than ID1 alone (Figure 4*C*). These results suggest that RON has a role in migratory machinery of T47D cells also independently from MSP stimulation. To exclude the possibility that the RON mAbs inhibitory effect on migration was due to receptor internalization, we carried out a time course experiment. T47D cells were treated for 30 minutes, 1 hour, 4 hours, or 24 hours with RON mAbs, and whole-cell lysates were evaluated for RON protein amount with Western Blot. As clearly shown in Figure 4*B*, there was no reduction in RON protein amounts during treatment with any of the RON mAbs. To control that the strong inhibitory effect was not due to mAb's induced cytotoxicity, cell viability in the migration assay conditions was measured in the presence of the mAbs either as single agents or in combination. No effect on cell viability due to the presence of the antibodies was observed (Figure W5). Thus, we concluded that the combinatorial effect of the RON mAbs was due to an enhanced block in receptor signaling.

IgG4, IgG7, and IgG10 Do Not Display Antitumor Activity In Vivo

We have generated an orthotopic mammary tumor xenograft model with implanted T47D cells to study the efficacy of IgG4, IgG7, and IgG10 *in vivo*. Groups of seven mice were randomly allocated at

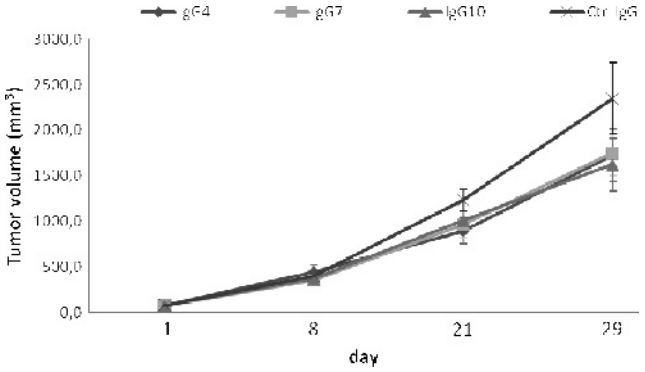

Figure 5. IgG4, IgG7, and IgG10 do not display antitumor efficacy in orthotopic T47D xenograft. Groups of 10 mice were treated weekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm^3. The minor reduction observed was not statistically significant with *P* values of .17 for IgG4, .23 for IgG7, and .23 for IgG10 with respect to control IgG on day 29.

day 19 after inoculation based on mean tumor sizes of 150 to 200 mm^3. Mice were treated weekly with 25 mg/kg of RON mAbs or an isotypic control IgG. On day 29 after treatment, the experiment was terminated. The minor reduction observed was not statistically significant with *P* values of .17 for IgG4, .23 for IgG7, and .23 for IgG10, with respect to control IgG on day 29 (Figure 5).

To verify whether the lack of antitumor effect was not limited to a single xenograft model, the selected IgGs were tested in mice injected with BxPC3 or HT29 cell lines. Recent studies have shown that mAb IMC-41A10 that blocks RON-mediated signaling also inhibits growth of these tumor cell lines *in vivo* [12]. Groups of 10 mice were treated with 25 mg/kg of the antibody. A control group was treated with histidine buffer. Antibody treatment was initiated when the tumors were 85 to 130 mm^3, and intraperitoneal injections were repeated twice weekly during the study. Dosing schedule was established based on preliminary mAb pharmacokinetic analyses in mice where the trough level of the antibodies remained above the concentration needed for receptor saturation in serum withdrawn 72 hours after mAb administration (data not shown). In the BxPC3 xenograft, we could use an antihuman IGF1R antibody as an internal positive control, which has antitumor activity in this xenograft model. Unlike the reported antitumor effects observed with mAb IMC-41A10, none of the antagonistic RON mAbs displayed an antitumor effect in either HT29 or BxPC3 xenograft, whereas the positive control antihuman IGF1R successfully inhibited tumor growth (Figures W3 and W4). The histopathology and the weight of the tumors from treated and untreated mice were also not different.

Discussion

Although less extensively studied than the family member MET, accumulating evidence suggest that RON RTK is important for cancer progression [2,3,12,14,38]. The pathways by which RON conveys signals to the intracellular environment have been studied; however, the relationships of these different pathways to the specific biologic responses that are relevant to tumor formation are still poorly defined [11].

RON and MET tyrosine kinase domains exhibit 63% sequence identity [39]. Because of the high similarity with MET tyrosine kinase domain, the development of potent and selective inhibitor drugs targeting RON has so far been very challenging [3,40,41]. Biologic drugs such as monoclonal antibodies or small interfering RNA (siRNA) are therefore highly desirable for the high specificity for the target. Biodrugs that target the RON receptor are in the early stage of development. The extracellular region of the RON receptor containing the Sema domain was able to block the binding of MSP to RON and to inhibit the growth of HCT116 colon cancer cells [9]. Silencing using siRNA against RON reduced cancer cell proliferation and motility, increased apoptotic susceptibility of the cells *in vitro*, and significantly reduced lung metastasis *in vivo* [31]. Neutralizing monoclonal antibodies that block the interaction of RON with MSP and diminish RON phosphorylation and downstream signaling have been developed [12,36,40]. Nevertheless, so far, only one humanized antibody against RON (IMC-41A10) has been found to significantly decrease tumor growth of murine xenografts from subcutaneously injected lung, colon, and pancreatic cancer cell lines in nude mice [12,42].

In this study, we describe the selection and characterization of three fully human mAbs that are potent antagonists of the human RON RTK. All three mAbs are highly specific for the human RON receptor as shown by IP followed by mass spectrometry (IP-MS) and highly selective; in fact, they do not cross react with mouse RON nor recognize human MET. Moreover, they all show high affinity binding to RON with measured K_d in the low-nanomolar range (mAb4 = 0.17 nM, mAb7 = 0.43 nM, and mAb10 = 0.64 nM; Figure 1) and do not compete with each other for receptor binding, indicating that they recognize different regions of the RON extracellular domain (Figure 3).

AKT and ERK are key downstream effector molecules very important in cancer progression and are also known to be modulated through RON receptor signaling [3,12]. For this reason, we were keen to evaluate whether the novel RON mAbs could inhibit AKT and ERK phosphorylation and subsequent activation. Consistent with their high affinity to RON, we found that IgG4, IgG7, and IgG10 potently inhibited MSP-induced ERK phosphorylation in the human mammary tumor cell line T47D in a dose-dependent manner (Figure 2).

The effect of the selected IgGs on RON-mediated signaling was assessed in transwell migration assays. All the three RON mAbs strongly inhibited MSP-dependent migration of T47D cells, giving a functional confirmation of their antagonistic activity (Figure 4). Blocking different epitopes in a recognition site can modulate the inhibitory effect on receptor-mediated signaling. Because our mAbs bind to different epitopes, we hypothesized that, by occupying different sites present on the receptor, we might interfere with the interaction of RON with other cell membrane elements and obtain a greater antimigratory effect. Indeed, when cells were treated with a combination of two mAbs, a significant reduction of T47D spontaneous motility was observed (Figure 5). Importantly, no effect on cell viability was observed on treatment of the cells with the antibodies alone or in combination. A recent work supporting the role of RON in spontaneous cell motility showed that siRNA-mediated knockdown of RON resulted in inhibition of ligand-independent motility of human colon cancer cells [31]. Inhibition of the spontaneous motility by combinatorial treatment with Sema-binding mAbs might be due to a more efficient blocking of the Sema domain leading to an impairment of RON RTK interaction with different surface proteins or receptors leading to reduction of spontaneous migration as well as MSP-driven migration. This conclusion is supported by the observation that, in addition to its ligand, RON may be activated through

cross talk with other surface proteins such as MET, integrins, type B plexins, and EGFR [22,25,26,43].

Because RON IgG4, IgG7, and IgG10 shared similar characteristics but bound to distinct regions of the receptor, they were tested *in vivo* to assess their antitumor potency. To this purpose, we established different xenograft models used based on cell lines derived from breast (T47D), colon (HT29), or pancreas (BxPC3) tumors. Despite the potent inhibitory effect on RON signaling, none of the mAbs had therapeutic efficacy in any of the three xenograft models. This observation leads us to conclude that blocking MSP-dependent signal transduction is not sufficient to inhibit tumor growth. There are several possible explanations for this finding. In the first instance, it is entirely possible that RON signaling *in vivo* is activated by the interaction with other membrane receptors. Previous studies had shown that the physical interaction between RON and EGFR is ligand independent and that transphosphorylation of both receptors can be induced on stimulation with either EGF or MSP [25,26]. Thus, for an antitumor activity, not only antagonistic ability but also interfering RON's interaction with other surface proteins such as EGFR might be needed. A second possibility is that the primary role of the RON receptor in tumors might not be the activation of antiapoptotic pathways but rather the control of motility and migration. This hypothesis cannot be easily ruled out in the xenograft models used in this study where tumor growth at the primary site of inoculation is the main parameter followed, but it could be better studied in mouse models of metastasis formation. Indeed, we plan to test the efficacy of the combinatorial administration of RON mAbs in metastatic models. Finally, it should be kept in mind that, for *in vivo* activity of therapeutic mAbs, Fc receptor–dependent mechanisms such as antibody-dependent cell-mediated cytotoxicity are important contributors [1]. We cannot exclude that RON IgG4, IgG7, and IgG10 are not efficient in activating these mechanisms, which might render them less effective *in vivo*.

In conclusion, RON IgG4, IgG7, and IgG10, although being potent antagonists of RON, do not seem to inhibit receptor-specific signaling that may be relevant for tumor progression. These results have important implications for future selection strategies of therapeutic mAbs for RON and, more in general, for RTK involved in tumor development and progression, in that properties other than blocking receptor signaling and inhibiting *in vitro* functions of RON are needed to generate IgGs with *in vivo* antitumor activity.

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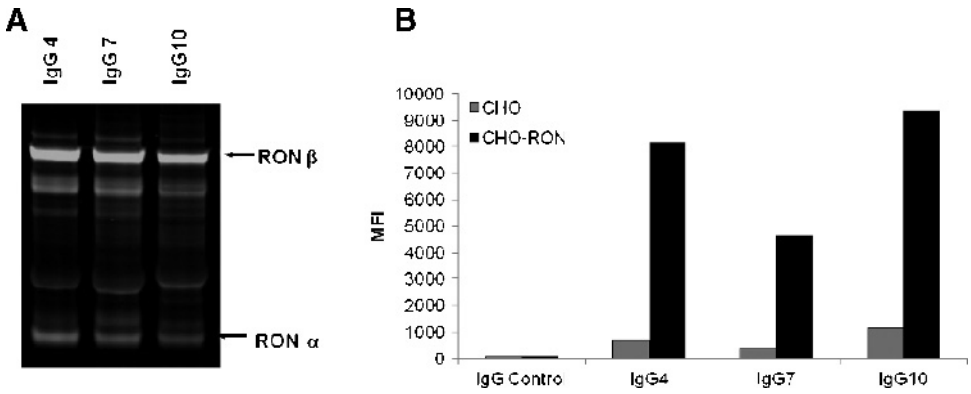

Figure W1. (A) RON IgGs are able to immunoprecipitate RON. Immunoprecipitation with RON IgG4, IgG7, and IgG10 was carried out on fluorescently labeled membrane proteins. One-dimensional PAGE was followed by in-gel digestion of immunoprecipitated protein bands and identification of the protein bands using MALDI-TOF-MS. Data were elaborated using the TurboSequest software. Candidates with Xcorr values greater than 2.5 were considered identified with high confidence. (B) Whole-cell binding FACS analysis was carried out for CHO wt or CHO-RON cells that are stably expressing RON full-length construct. IgG4, IgG7, and IgG10 specifically binds to CHO-RON cells, whereas unrelated IgG used as control does not bind any of the cells as expected.

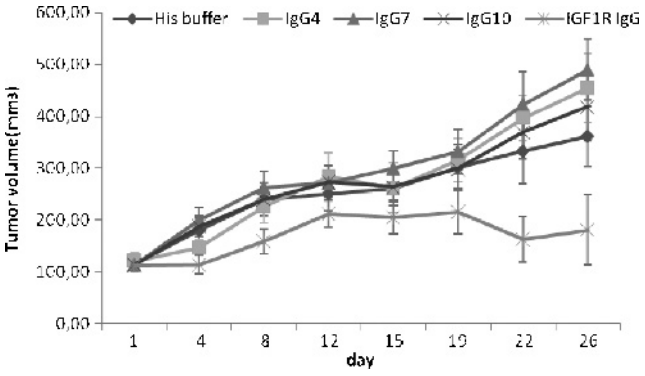

Figure W2. ZR-75 cells time course experiment for ERK and AKT phosphorylation in response to MSP stimulation. The highest phosphorylation levels were obtained at 30 minutes.

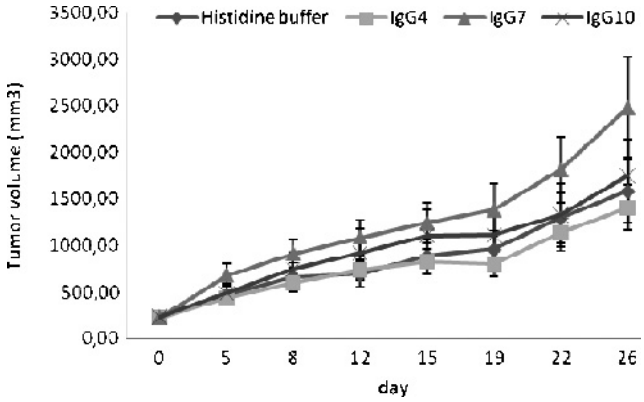

Figure W3. RON IgGs do not have antitumor activity in BxPC3 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm^3. An internal positive control MK1285, anti–IGF-1R antibody, has antitumor activity in this model.

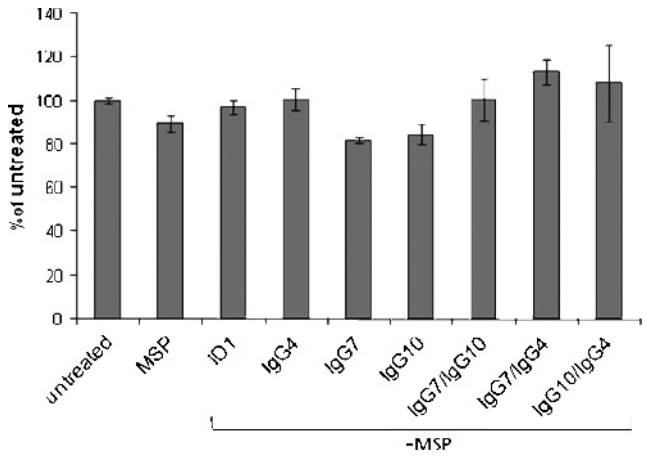

Figure W4. RON IgGs do not have antitumor activity in HT29 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm^3.

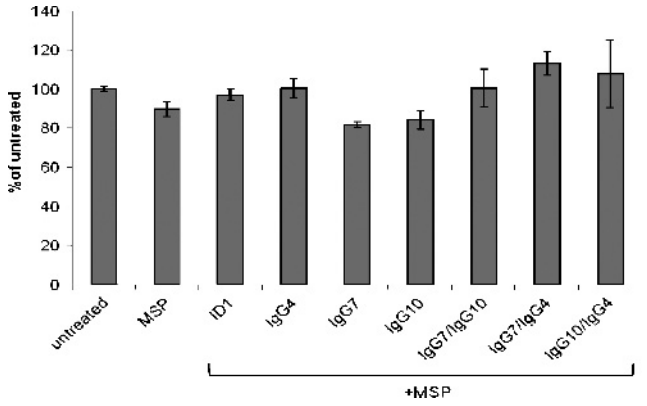

Figure W5. RON IgGs do not interfere with cell viability when used alone or in combination. T47D cell viability was determined using ViaLight Assay (Lonza, Milan, Italy), and values were expressed as percentages relative to untreated control.

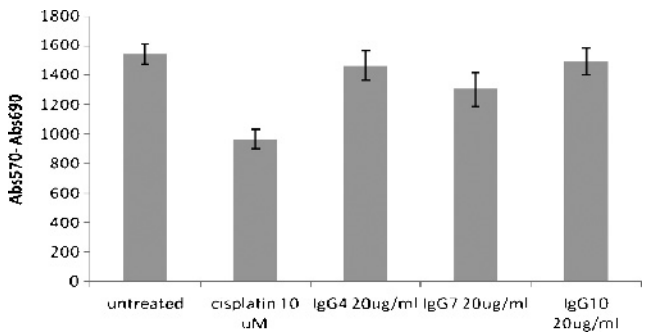

Figure W6. RON IgGs do not block T47D cell proliferation *in vitro*.