

Identification of a Novel Recepteur d'Origine Nantais/c-Met Small-Molecule Kinase Inhibitor with Antitumor Activity *In vivo*

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Abstract

Recepteur d'origine nantais (RON) is a receptor tyrosine kinase closely related to c-Met. Both receptors are involved in cell proliferation, migration, and invasion, and there is evidence that both are deregulated in cancer. Receptor over-expression has been most frequently described, but other mechanisms can lead to the oncogenic activation of RON and c-Met. They include activating mutations or gene amplification for c-Met and constitutively active splicing variants for RON. We identified a novel inhibitor of RON and c-Met, compound I, and characterized its *in vitro* and *in vivo* activities. Compound I selectively and potently inhibited the kinase activity of RON and c-Met with IC₅₀s of 9 and 4 nmol/L, respectively. Compound I inhibited hepatocyte growth factor-mediated and macrophage-stimulating protein-mediated signaling and cell migration in a dose-dependent manner. Compound I was tested *in vivo* in xenograft models that either were dependent on c-Met or expressed a constitutively active form of RON (RONΔ160 in HT-29). Compound I caused complete tumor growth inhibition in NIH3T3 TPR-Met and U-87 MG xenografts but showed only partial inhibition in HT-29 xenografts. The effect of compound I in HT-29 xenografts is consistent with the expression of the activating b-Raf V600E mutation, which activates the mitogen-activated protein kinase pathway downstream of RON. Importantly, tumor growth inhibition correlated with the inhibition of c-Met-dependent and RON-dependent signaling in tumors. Taken together, our results suggest that a small-molecule dual inhibitor of RON/c-Met has the potential to inhibit tumor growth and could therefore be useful for the treatment of patients with cancers where RON and/or c-Met are activated. [Cancer Res 2008;68(16):6680–7]

Introduction

Recepteur d'origine nantais (RON) is a receptor tyrosine kinase (RTK) normally expressed at low levels mostly on epithelial cells (1, 2). It is closely related to c-Met in terms of homology and function (3). Both RON and c-Met are activated in response to their

respective ligand: macrophage-stimulating protein (MSP) for RON (4, 5) and hepatocyte growth factor (HGF) for c-Met (6). The two RTKs induce an invasive program (7) consisting of cell proliferation, migration, and invasion, all of which are important at multiple points during tumorigenesis. RON and c-Met elicit these functions through a unique docking site located at their COOH terminus (8). On receptor activation, two tyrosine residues at the COOH terminus become phosphorylated and form a multifunctional docking site for SH2 domain-containing adaptor proteins. This event subsequently triggers a complex signaling cascade that includes activation of the phosphatidylinositol 3-kinase and the mitogen-activated protein kinase (MAPK) pathways and results in the characteristic functional responses associated with RON and c-Met (9, 10). The oncogenic potential of RON and c-Met has been shown *in vitro* as well as in transgenic animals (11–19).

Oncogene addiction (20) refers to the dependence of tumor cell survival on an activated oncogene, such as BCR-ABL, in chronic myelogenous leukemia. This concept suggests that inhibition of the activated oncogene is sufficient to obtain a clinical response. This has now been proven in the clinic with several kinase inhibitors, such as imatinib (21) and gefitinib (22). In the case of kinases, oncogene addiction may stem from constitutive activity that can arise as a result of gene translocation, mutation, or amplification (23–26).

c-Met and RON can be constitutively activated through ligand-independent mechanisms in tumor cells. Constitutively activated c-Met can result from gene amplification or activating mutations. The “oncogene addiction” to c-Met is exemplified by the recent identification of a subset of gastric cancer cell lines harboring *MET* gene amplification (26). These cells are dependent on c-Met for growth and survival and are sensitive to a c-Met inhibitor. Unlike c-Met, RON-activating mutations or gene amplification has not been described. Instead, constitutively active RON variants generated by alternative splicing (RONΔ165, RONΔ160, and RONΔ155) or by methylation-dependent promoter usage [short form RON (sfRON)] have been identified (14, 27, 28). Cells expressing these RON proteins show greater scatter activity, focus formation, anchorage-independent growth, and tumor formation in nude mice compared with cells expressing wild-type RON (14, 27, 28). Among these RON variants, RONΔ160 is located at the plasma membrane, whereas RONΔ165 and RONΔ155 are retained in the cytoplasm. sfRON lacks almost all of the extracellular domain and is incapable of ligand binding. These variants have been detected in primary colon cancers and established colon cancer cell lines as well as in gastric and lung cancer cell lines. Whether cancer cells are addicted to the activated RON kinases, including RONΔ160, is currently unknown.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Recently, a monoclonal antibody against RON was described and shown to partially inhibit tumor growth in several tumor xenograft models (29, 30). The exact mechanism by which this antibody inhibits tumor growth has not been shown, but it may act by blocking MSP-mediated RON activation. In the case of tumors where RON is activated in a ligand-independent manner, an antibody against RON would presumably not be effective. However, a small-molecule kinase inhibitor should have broader application in RON-driven cancers.

In this article, we characterize a RON/c-Met dual kinase inhibitor and show that this molecule inhibits both RON-dependent and c-Met-dependent signaling and cell migration *in vitro* and tumor growth *in vivo*. Together, our data show for the first time that constitutively activated RON can be targeted by a small-molecule kinase inhibitor and that targeting both c-Met and RON could represent a useful strategy to treat patients whose cancers are dependent on RON and/or c-Met.

Materials and Methods

Compounds. Compound I [IUPAC name: *N*-(3-fluoro-4-((7-methoxy-4-quinolinyl)oxy)phenyl)-1-(2-hydroxy-2-methylpropyl)-5-methyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazole-4-carboxamide] was synthesized at Amgen, Inc. The MAPK/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) inhibitor U0126 was obtained from Calbiochem.

Cells. KATOIII (gastric), PC3 (prostate), HT-29 (colorectal), Colo205 (colorectal), BxPC3 (pancreatic), and U-87 MG (glioblastoma) cancer cell lines were obtained from the American Type Culture Collection. NIH3T3 TPR-Met or NIH3T3 RON cells were generated by stable transfection of TPR-Met, a constitutively active, ligand-independent form of c-Met (31) or wild-type RON in NIH3T3 cells. Cells were grown as monolayers using standard cell culture conditions.

Antibodies and reagents. Antibodies against c-Met (C-12), RON (C-20), and actin (1615-R) were acquired from Santa Cruz Biotechnology. Antibodies against phospho-c-Met (Y1234/1235), phospho-Gab1 (Y627), phospho-ERK1/2 (T202/Y204), and phospho-AKT (S473) were acquired from Cell Signaling Technology, Inc. The antibody against phosphotyrosine (clone 4G10) was purchased from Millipore. Recombinant human HGF was generated by expression in Chinese hamster ovary cells and purified by heparin sulfate affinity chromatography. MSP and MSP C672A were acquired from R&D Systems. MSP was used in Western blotting experiments as indicated, whereas MSP C672A was used in the *in vitro* wound-healing assay because MSP was no longer available commercially.

Kinase assays. IC₅₀ measurements of compound I for c-Met were measured by homogeneous time-resolved fluorescence as previously described with minor modification (32). A modified kinase reaction buffer was used that included 60 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 20 mmol/L MgCl₂, and 5 mmol/L MnCl₂. Compound I was tested in a 10-point serial dilution using an ATP concentration of two-third *K_m* value that was determined for c-Met and calculated using the Eadie-Hofstee and Lineweaver-Burke methods. The fluorescence ratios were read on a RubyStar instrument (BMG Labtech, Inc.). To measure the IC₅₀ of compound I for RON, the Invitrogen LanthaScreen procedure was followed (PV4314; Invitrogen) and the fluorescein-poly-GT substrate was used. The fluorescence ratios were read on a Tecan Safire II (Tecan).

Western blot analysis. Cells were grown to confluence, serum starved overnight, and then treated with various concentrations of compound I for 1 h. To investigate the ability of compound I to inhibit ligand-dependent receptor activation, serum-starved cells were treated with increasing concentration of compound I for 1 h and then stimulated with recombinant human HGF for 10 min (250 ng/mL) or MSP for 30 min (800 ng/mL) in the presence of compound I. These respective concentrations of growth factor were chosen based on previous dose-response studies indicating that these concentrations maximally activated their receptor.

Immunoprecipitation. Phosphorylated RON and RONΔ160 were immunoprecipitated from NIH3T3 RON cells and HT-29 cells, respectively,

from 1 mg of cellular protein per sample using anti-phosphotyrosine 4G10 antibody. RON/RONΔ160 was subsequently detected with the RON C-20 antibody by Western blotting.

Monolayer scratch assay. NIH3T3 RON and NIH3T3 TPR-Met cells were seeded in six-well plates and grown until confluent. Cells were serum starved overnight and then treated with various concentrations of compound I for 1 h. A gap was introduced with a P200 pipette tip. In the case of NIH3T3 RON cells, cells were stimulated to migrate across the gap with 160 ng/mL of MSP C672A alone or MSP C672A and compound I, whereas NIH3T3 TPR-Met cells were treated with compound I only. After overnight incubation, the cells were examined by light microscopy and photographed. Magnifications ($\times 40$) are shown.

Animals. Female CD1 *nu/nu* mice ages 6 to 8 wk were obtained from Charles River Laboratories. Animals were housed in sterilized caging and received Harlan Teklad sterilized rodent diet and water *ad libitum*. All of the procedures were conducted in accordance with the guidelines of the Amgen Animal Care and Use Committee. The laboratory housing the animals met all Association for Assessment and Accreditation of Laboratory Animal Care specifications.

Xenograft studies. NIH3T3 TPR-Met (1×10^6), U-87 MG (5×10^6), HT-29 (2×10^6 with Matrigel at a ratio of 2:1), or Colo205 (2×10^6 with Matrigel) cells were injected s.c. in the right flank of female CD1 *nu/nu* mice ($n = 10$ per group). Compound I treatment began either 1 d after tumor implantation or when tumors were established (~ 200 mm³). Compound I was formulated in 2% hydroxypropylmethylcellulose 1% Tween 80 in water (pH 2.2 adjusted with HCl). Mice were dosed by oral gavage either once or twice daily. Tumor volume was measured twice weekly with a Pro-Max Fowler Digital Ultra Caliper (Fred Fowler Co., Inc.) as length (mm) \times width (mm) \times height (mm) and expressed as cubic millimeters. Data are expressed as mean \pm SE for each group and plotted as a function of time. The statistical significance of observed differences between growth curves was evaluated by repeated measures ANOVA followed by Scheffe post hoc test. To assess the pharmacodynamic effect in tumors, mice bearing established (~ 300 – 400 mm³) NIH3T3 TPR-Met or HT-29 xenograft tumors were treated with a single dose of compound I at 100 mg/kg. Tumors were harvested ($n = 3$ per time point) at the indicated times after treatment and immediately frozen in liquid nitrogen. Western blot analysis was performed

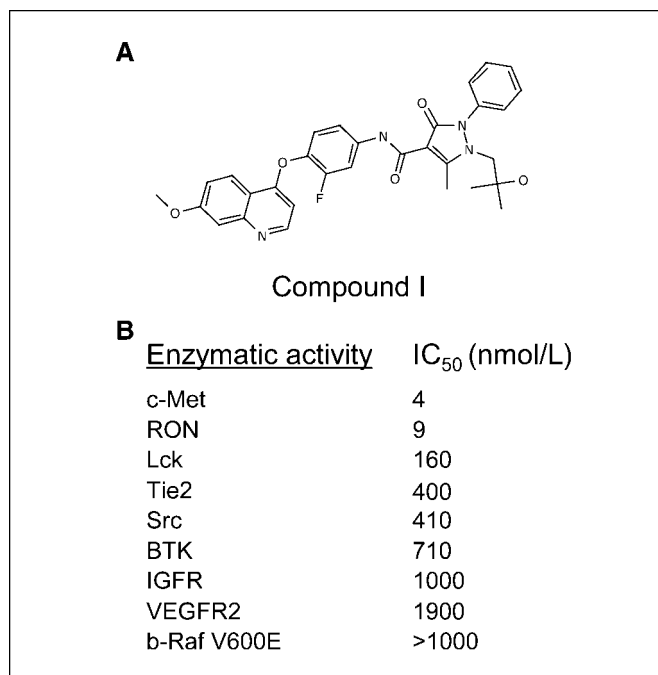


Figure 1. Compound I selectively inhibits the kinase activity of RON and c-Met. A, structure of compound I. B, effect of compound I on the enzymatic activity of several kinases. Data represent the mean of three or more experiments.

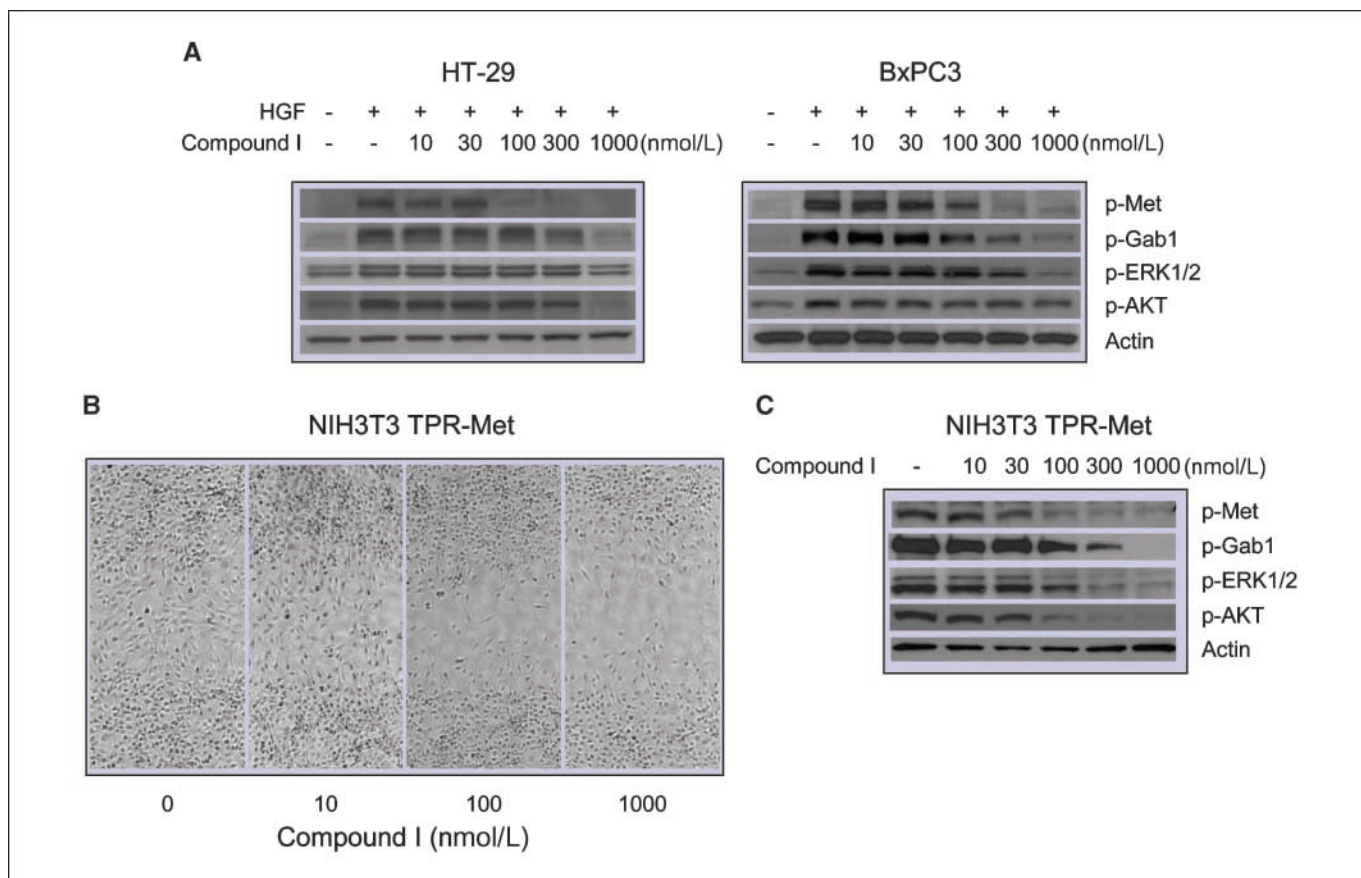


Figure 2. Compound I inhibits c-Met-mediated signaling and function. **A**, HT-29 and BxPC3 cells were treated with compound I for 1 h followed by stimulation with HGF. The effects of compound I on c-Met phosphorylation and on the phosphorylation of signaling molecules downstream of c-Met were analyzed by Western blotting. Each antibody detected a single band of the expected size [phospho-Met (*p-Met*), 145 kDa; phospho-Gab1 (*p-Gab1*), 110 kDa; phospho-AKT (*p-AKT*), 60 kDa; phospho-ERK1/2 (*p-ERK1/2*), 42 and 44 kDa; actin, 43 kDa]. **B**, *in vitro* wound-healing assay in NIH3T3 TPR-Met cells in the presence of compound I. Cells were photographed 20 h after wounding. Magnification, $\times 40$. Experiment was done twice with similar results. **C**, NIH3T3 TPR-Met cells were treated with increasing concentrations of compound I for 1 h. The effects of compound I were analyzed by Western blotting.

to determine the effect of treatment on the phosphorylation of c-Met and Gab1, an adaptor protein immediately downstream of c-Met, or ERK1/2, which is downstream of RON.

Pharmacokinetics. Mouse plasma samples (20 μ L) were extracted by using protein precipitation to isolate compound I and internal standard. Extracted samples were separated by reversed-phase liquid chromatography on a Varian Pursuit C18 analytic column (30 \times 2.0 mm, 5 μ m). The reagents were water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). Gradient at a flow rate of 0.6 mL/min was used, with a total run time of 2 min. Compound I concentrations were determined in mouse samples by liquid chromatography-tandem mass spectrometry using ion atmospheric pressure ionization with multiple reaction monitoring in the positive ion mode. Peak areas were integrated by the Sciex program Analyst, version 1.4.1. The data were then exported to the software package Small Molecules Discovery Assay Watson (version 6.4.0.02, Thermo Electron Corp.) where concentrations were determined by a weighted ($1/\times^2$) linear regression of peak area ratios (peak area of compound I/peak area of internal standard) versus the theoretical concentrations of the calibration standards. Overall precision and accuracy for the calibration standards and QC samples were determined by Small Molecules Discovery Assay Watson.

Results

Compound I inhibits the kinase activity of RON and c-Met. The structure of compound I is shown in Fig. 1A. *In vitro* kinase

assays showed that compound I is a potent inhibitor of human RON and c-Met with IC_{50} s of 9 and 4 nmol/L, respectively. To determine its selectivity profile, compound I was tested against a panel of tyrosine and serine/threonine kinases. Compound I had weak inhibitory activity on Lck, Tie2, Src, and BTK with IC_{50} s ranging from 160 to 710 nmol/L (Fig. 1B) and had IC_{50} s of >1 μ mol/L on all other kinases tested (>20 kinases; Fig. 1B; data not shown). These data suggest that compound I is a selective inhibitor of RON and c-Met.

Compound I inhibits c-Met-mediated signaling and functional activity. To confirm its activity on c-Met, compound I was evaluated for its ability to inhibit HGF-mediated c-Met phosphorylation and downstream signaling. HT-29 and BxPC3 cells were used throughout these studies because they both express c-Met and RON (Fig. 4A). In both cell lines, exogenous HGF stimulated c-Met phosphorylation and downstream signaling as evidenced by the phosphorylation of Gab1, ERK1/2, and AKT (Fig. 2A). Compound I inhibited HGF-mediated c-Met phosphorylation and downstream signaling in a dose-dependent manner. Furthermore, compound I inhibited c-Met phosphorylation with similar potency in both cell lines (Fig. 2A).

c-Met is known to mediate cell migration (33). To confirm that compound I can inhibit c-Met function, we tested the ability of compound I to inhibit migration using an *in vitro* monolayer

scratch assay. We used NIH3T3 cells stably expressing the ligand-independent, constitutive form of c-Met, TPR-Met (NIH3T3 TPR-Met), as a model. As shown in Fig. 2B, compound I inhibited NIH3T3 TPR-Met cell migration in a dose-dependent manner. There was a close correlation between the concentrations of compound I required to inhibit cell migration and those required to block c-Met phosphorylation in HT-29, BxPC3 (Fig. 2A), and NIH3T3 TPR-Met cells (Fig. 2C), suggesting that the inhibitory effect was mediated by inhibition of the constitutively active TPR-Met. Compound I also inhibited the proliferation of NIH3T3 TPR-Met cells (Supplementary Fig. S1). Collectively, these data show that compound I inhibits c-Met-mediated signaling and function.

Compound I inhibits RON-mediated signaling and functional activity. Because compound I also inhibits RON kinase activity, we evaluated its ability to block MSP-mediated signaling. Because none of the commercially available antibodies to phospho-RON recognized the appropriate molecular weight band by Western blotting (data not shown), we performed immunoprecipitation to test the effect of compound I on MSP-induced RON phosphorylation in NIH3T3 RON or BxPC3 cells. Phospho-RON was immunoprecipitated from the lysates of cells treated with MSP and/or compound I using the anti-phosphotyrosine 4G10 antibody. RON was then detected by Western blotting using an antibody against the COOH terminus of the receptor. We found that compound I inhibited RON phosphorylation in a dose-dependent manner in both cell lines (Fig. 3A and B, top). We then tested the effect of MSP and/or compound I on downstream signaling, including the phosphorylation of Gab1, ERK1/2, and AKT, in NIH3T3 RON cells or in BxPC3.

Treatment with exogenous MSP resulted in robust phosphorylation of Gab1, AKT, and ERK1/2 in NIH3T3 RON cells (Fig. 3A), whereas MSP treatment resulted in weaker phosphorylation of Gab1 and had little effect on AKT and ERK1/2 phosphorylation in BxPC3 cells (Fig. 3B). Because the only known receptor for MSP is RON, it is reasonable to conclude that the activation of Gab1, AKT, and ERK1/2 is mediated by RON. Compound I inhibited MSP-induced phosphorylation of Gab1, ERK1/2, and AKT in a dose-dependent manner in NIH3T3 RON cells (Fig. 3A) and MSP-mediated phosphorylation of Gab1 in BxPC3 cells (Fig. 3B). Compared with HGF/c-Met signaling (Fig. 2A), MSP/RON signaling is weak in BxPC3 cells and has a minor effect on ERK1/2 and AKT phosphorylation (Fig. 3B).

Like c-Met, RON has been implicated in the migration and invasion of epithelial cells (7). To further validate that compound I specifically inhibits RON function, we used the scratch assay in NIH3T3 RON cells. We showed that MSP, but not HGF, significantly induced NIH3T3 RON cell migration (Fig. 3C), and compound I inhibited this MSP-induced migration in a dose-dependent manner (Fig. 3D). Moreover, the close correlation of a dose response for inhibiting MSP-induced RON signaling and the inhibitory effect of compound I on cell migration suggest that compound I inhibited NIH3T3 RON cell migration by blocking RON signaling.

HT-29 cells express a constitutive form of RON that signals via the MAPK pathway. Both c-Met and RON are implicated in tumorigenesis; however, the two receptors seem to use different mechanisms for constitutive signaling: amplification and activating mutations for c-Met and alternative splicing for RON (9, 34).

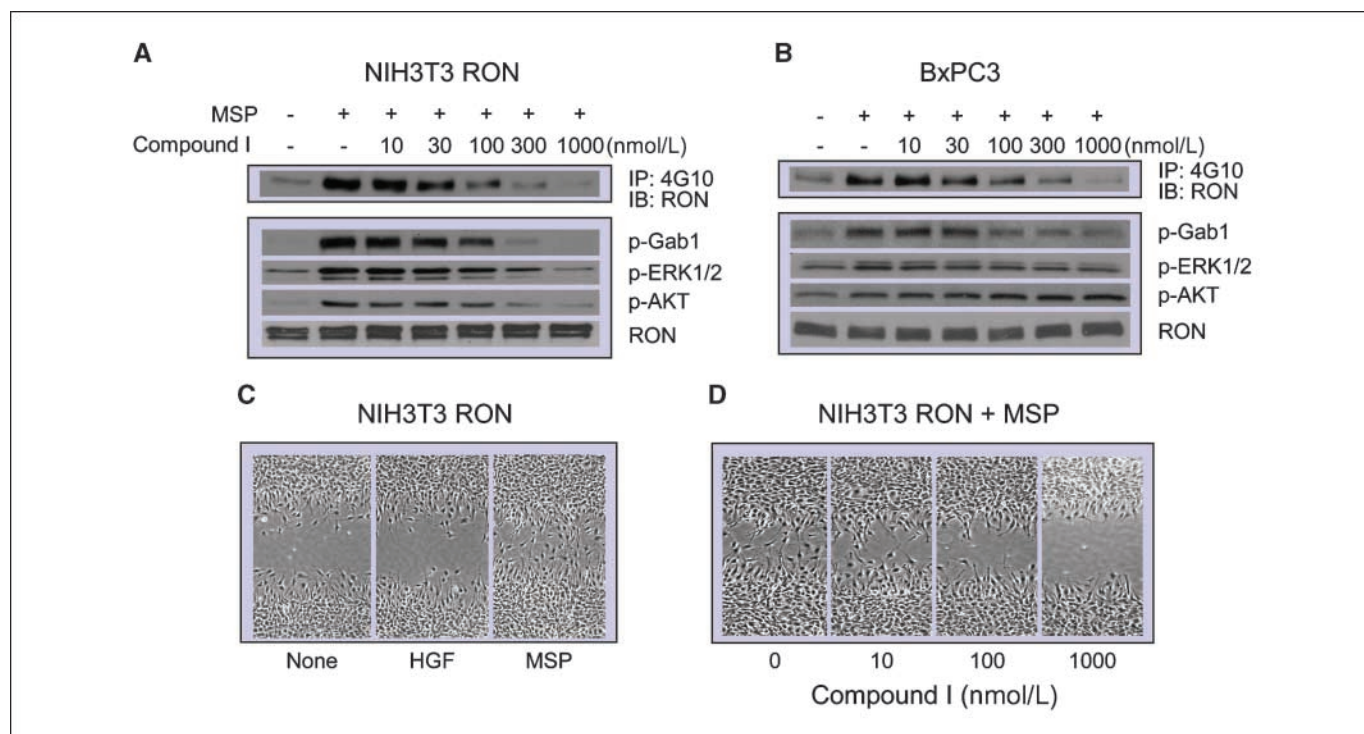


Figure 3. Compound I inhibits RON-mediated signaling and function. NIH3T3 RON (A) and BxPC3 (B) cells were treated with compound I followed by stimulation with MSP. The effects of compound I were analyzed by Western blotting. In A and B, phospho-RON was detected with the RON C-20 antibody as a single band of 150 kDa after immunoprecipitation with the 4G10 antibody. Total RON expression was used as a loading control [the RON C-20 antibody detects pro-RON (170 kDa) and the mature β -chain of RON (150 kDa)]. All other antibodies used in A and B detect a single band of the expected size. C, *in vitro* wound-healing assay in NIH3T3 RON cells in the presence of HGF or MSP C672A. D, *in vitro* wound-healing assay in NIH3T3 RON cells in the presence of compound I and MSP C672A. Data shown in C and D were generated in the same experiment; note that the MSP panel in C is the same as in D (left). Cells were photographed 30 h after wounding. Magnification, $\times 40$. Experiments were done twice with similar results. IP, immunoprecipitation; IB, immunoblotting.

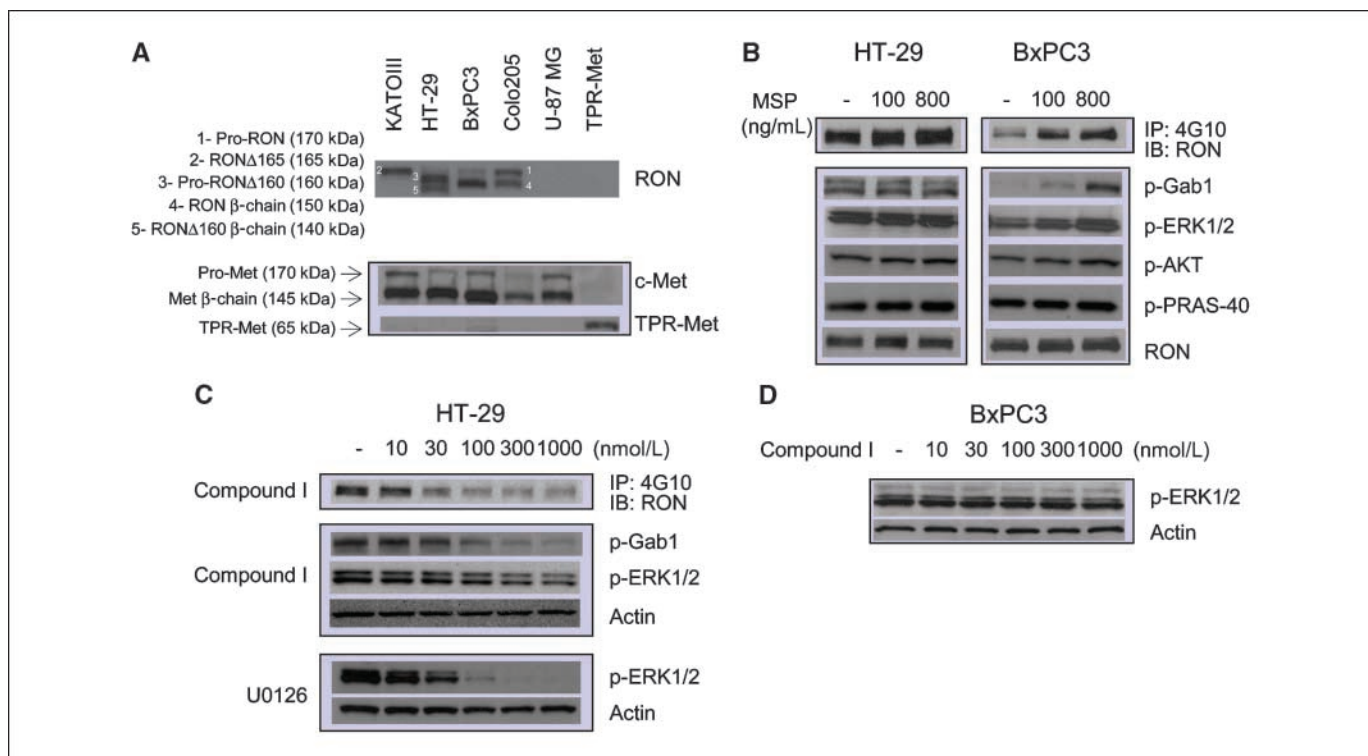


Figure 4. HT-29 cells express a constitutively active form of RON. *A*, RON and c-Met expression pattern in various cell lines. Wild-type pro-RON (170 kDa) and the mature RON β -chain (150 kDa) are expressed in BxPC3 and Colo205 cells, a truncated RON protein of 140 kDa (RON Δ 160 β -chain) and its precursor (Pro-RON Δ 160, 160 kDa) are expressed in HT-29 cells, and a single-chain RON protein of 165 kDa (RON Δ 165) is present in KATOIII cells. U-87 MG and NIH3T3 TPR-Met cells did not express detectable levels of RON protein. Wild-type c-Met protein (170 kDa precursor and 145 kDa mature β -chain) is expressed in KATOIII, HT-29, BxPC3, Colo205, and U-87 MG cells. TPR-Met is a 65-kDa protein. *B*, HT-29 and BxPC3 cells were treated with increasing concentrations of MSP to fully activate RON. Phospho-RON was detected as described in the legend of Fig. 3. *C*, HT-29 cells were treated with increasing concentrations of compound I (*top*) or the MEK1/2 inhibitor U0126 (*bottom*). The effects of treatment were determined by immunoprecipitation/Western blotting for phospho-RON and Western blotting for all other proteins. *D*, same as *C* (*top*), except in BxPC3 cells. In contrast with its effect in HT-29 cells, compound I had no effect on the levels of phospho-ERK1/2 in BxPC3 cells.

We hypothesized that cancer cells with constitutive c-Met or RON activity may be addicted to these pathways. To test this, we used HT-29 cells that have been shown to express a constitutive form of RON (RON Δ 160; ref. 35). We first profiled several cancer cell lines to confirm their c-Met and RON expression. We detected a wild-type pro-RON (170 kDa) and a mature RON β -chain (150 kDa) in BxPC3 and Colo205 cells, whereas we detected a truncated RON protein at 140 kDa (RON Δ 160 β -chain) and its precursor (Pro-RON Δ 160, 160 kDa) in HT-29 cells as previously documented (Fig. 4A). We observed a single-chain RON protein of 165 kDa (RON Δ 165) in the gastric cancer cell line KATOIII (28). U-87 MG and NIH3T3 TPR-Met cells did not express detectable levels of RON protein. Wild-type c-Met protein (170 kDa precursor and 145 kDa mature β -chain) was expressed in KATOIII, HT-29, BxPC3, Colo205, and U-87 MG cells (Fig. 4A). In NIH3T3 TPR-Met cells, the antibody to c-Met detected a band of 65 kDa as expected.

We decided to test our hypothesis that cancer cells might be addicted to the activated RON proteins in HT-29 cells because down-regulation of RON Δ 160 by small interfering RNA in these cells has previously been shown to have an inhibitory effect on cell growth, migration, and colony formation (36), suggesting that RON Δ 160 possesses oncogenic properties. We first tested whether exogenous MSP could induce RON activity in HT-29 cells. Exogenous MSP only slightly increased the phosphorylation levels of RON and had little effect on Gab1, ERK1/2, or AKT phosphorylation levels in HT-29 cells. In contrast, MSP induced RON, Gab1, AKT,

and ERK1/2 phosphorylation in a dose-dependent manner in BxPC3 cells (Fig. 4B). The effect of MSP in BxPC3 cells is consistent with previous reports (30, 37). Moreover, these data suggest that RON is strongly activated in HT-29 cells in the absence of ligand. HT-29 cells were treated with increasing concentration of compound I. Compound I inhibited RON and Gab1 phosphorylation in a dose-dependent manner and partially inhibited the phospho-ERK1/2 signal in a dose-dependent manner (Fig. 4C, *top*), suggesting that RON Δ 160 is constitutively active and contributes, at least partially, to signaling via the MAPK pathway in these cells. In contrast, basal levels of ERK1/2 phosphorylation in BxPC3 cells were unaffected even at high concentrations of compound I (Fig. 4D).

The fact that compound I only partially inhibited ERK1/2 phosphorylation in HT-29 cells might be explained by the expression of the activated b-Raf mutation V600E in these cells because this mutation is a strong inducer of ERK1/2 phosphorylation (38). The MEK1/2 inhibitor U0126, which acts downstream of b-Raf, completely inhibited ERK1/2 phosphorylation in HT-29 cells in a dose-dependent manner (Fig. 4C, *bottom*). Together, these results confirm that HT-29 cells express a constitutive form of RON that contributes to signaling via the MAPK pathway. HT-29 cells are therefore probably not entirely dependent on RON, but it is possible that RON Δ 160 contributes to the oncogenic properties of these cells.

Compound I inhibits the growth of c-Met-dependent and constitutively active RON-expressing tumors. Although cell line

models that depend on c-Met activity have been identified, few, if any, equivalent models are known for RON. Expression of c-Met, RON, or even their coexpression does not necessarily confer dependence on these pathways for tumor growth. Our goal was to identify models that were dependent on c-Met or expressed a constitutively active form of RON to investigate the *in vivo* antitumor activity of compound I.

Compound I was assessed in two c-Met-dependent xenograft models: U-87 MG and NIH3T3 TPR-Met. Both models are well documented for their dependence on c-Met activity (39, 40). Moreover, they both lack RON expression (Fig. 4A), eliminating the possibility that tumor growth inhibition might be mediated by blocking RON activity.

Treatment of NIH3T3 TPR-Met tumor-bearing mice once daily with compound I resulted in a dose-dependent inhibition of tumor growth compared with vehicle-treated control animals (Fig. 5A). Compound I significantly inhibited tumor growth at doses of 30 or 100 mg/kg once daily or at 30 mg/kg twice daily ($P < 0.02$). Furthermore, complete tumor growth inhibition was observed at a dose of 100 mg/kg once daily. Treatment with compound I did not adversely affect body weight (data not shown). To confirm that tumor growth inhibition occurred as a result of inhibition of c-Met activity, mice bearing established NIH3T3 TPR-Met tumors were treated with a single dose of compound I at 100 mg/kg. Tumors were harvested from the mice at different times after dose and analyzed for TPR-Met and Gab1 phosphorylation. Phosphorylation levels of TPR-Met and Gab1 were profoundly reduced through 12 h and returned to near basal levels by 24 h when compared with tumors from untreated mice (Fig. 5B). Plasma levels of compound I in mice from experiments shown in Fig. 5A and B were evaluated. In both studies, mice treated with 100 mg/kg had high concentrations of compound I through 12 h but significantly lower concentrations at 24 h (Fig. 5C; data not shown). These data show that phospho-Gab1 and phospho-Met inhibition correlates with plasma levels of compound I, and in turn, these variables also correlate with NIH3T3 TPR-Met tumor growth inhibition. Compound I had no direct effect on Src phosphorylation *in vivo* despite the high concentration achieved in mice (Supplementary Fig. S2B).

To extend the finding that compound I inhibits the growth of c-Met-dependent tumor models, compound I was tested against established U-87 MG tumor xenografts. As shown in Fig. 5D, compound I inhibited the growth of these tumors in a dose-dependent manner. Similar to the data in the NIH3T3 TPR-Met model, compound I significantly inhibited tumor growth at doses of 100 mg/kg once daily or 30 mg/kg twice daily ($P < 0.0001$).

We next tested the contribution of RON Δ 160 in HT-29 tumor growth. When mice bearing established HT-29 tumors were dosed daily with compound I, a statistically significant, dose-dependent reduction in tumor growth was observed ($P < 0.02$; Fig. 6A). Mice bearing established HT-29 xenografts were also treated with a single dose of compound I at 100 mg/kg to assess the effect on

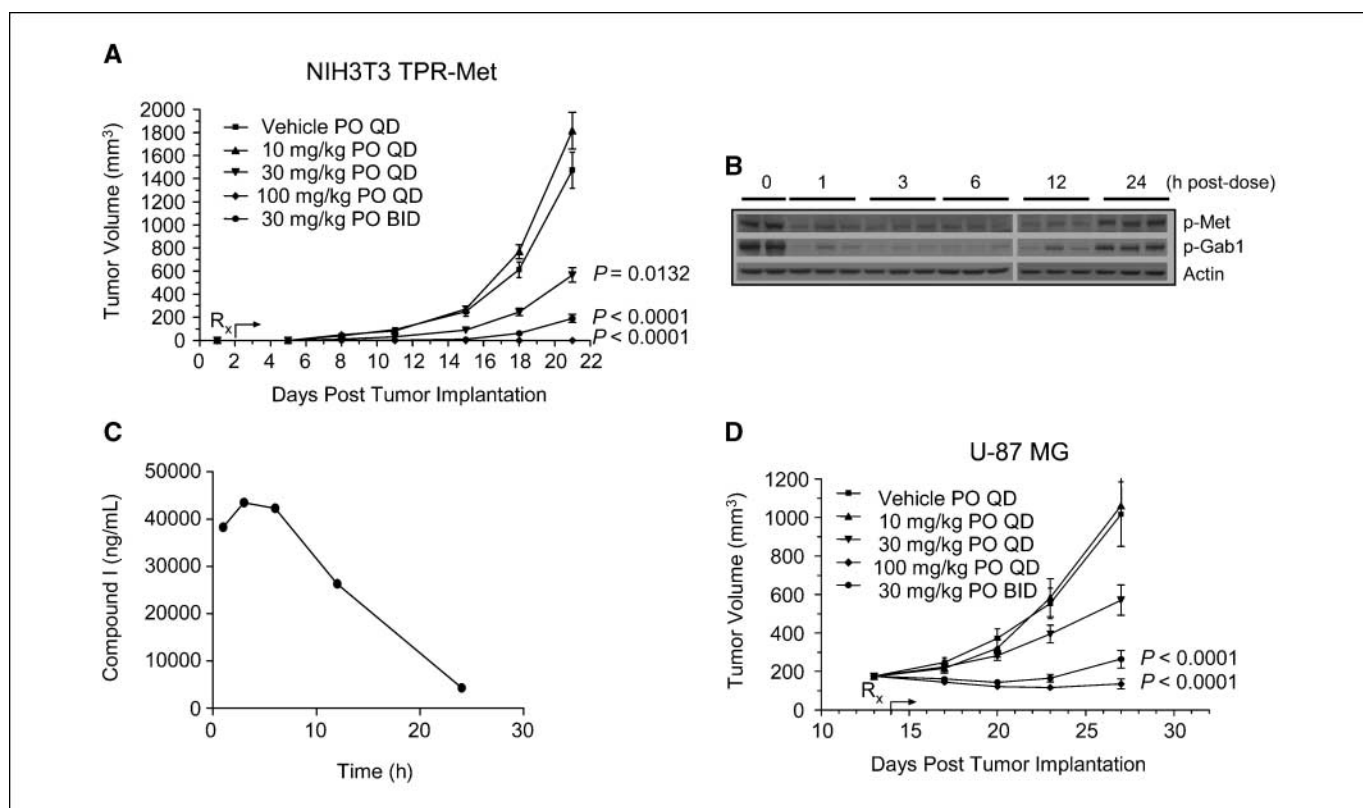


Figure 5. Compound I inhibits c-Met-dependent tumor growth *in vivo*. **A**, CD1 *nu/nu* mice bearing NIH3T3 TPR-Met s.c. tumors were treated with compound I beginning 1 d after tumor implantation. Mice were dosed orally as indicated. **B**, mice bearing established (~ 300 – 400 mm³) NIH3T3 TPR-Met tumors were treated with a single dose of compound I at 100 mg/kg. Tumors were harvested at the indicated times after treatment and immediately frozen in liquid nitrogen. c-Met and Gab1 phosphorylation was determined by Western blot analysis. **C**, plasma samples from **A** and **B** were analyzed to determine compound I concentrations at different time points. In both experiments, mice treated with 100 mg/kg showed similar plasma concentrations at each time point. **D**, mice bearing established (~ 200 mm³) U-87 MG tumors received compound I as indicated. P values denote statistical significance of observed differences between growth curves compared with the vehicle-treated group. Arrow and Rx, first day of dosing.

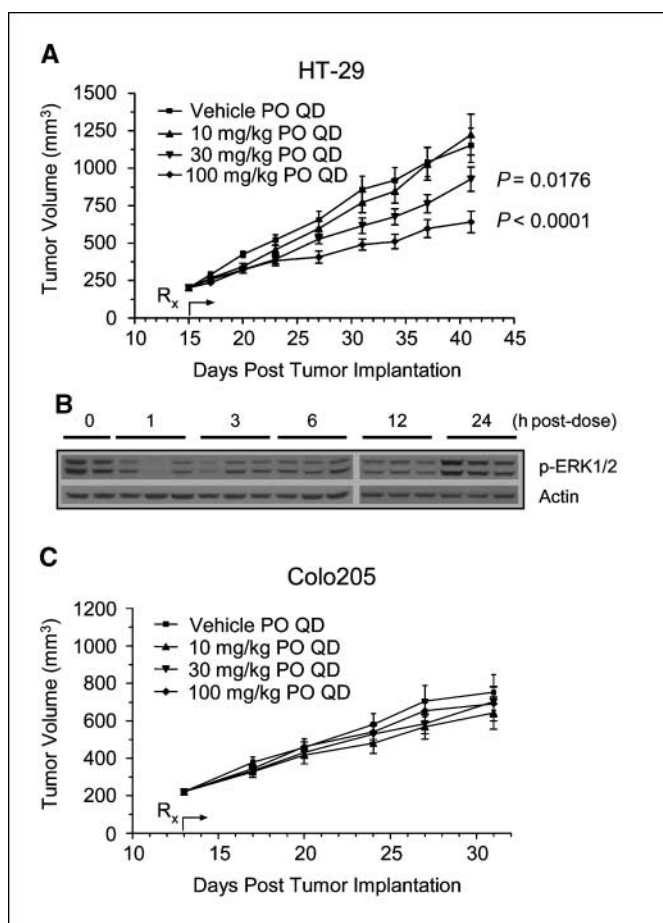


Figure 6. Compound I inhibits the growth of HT-29 tumors that express the constitutively active RON Δ 160. **A**, mice bearing established (~ 200 mm³) HT-29 tumors were treated as indicated. *P* values denote statistical significance of observed differences between growth curves compared with the vehicle-treated group. **B**, mice bearing established (~ 400 mm³) HT-29 tumors were treated with a single dose of compound I. Tumors were harvested at the indicated times after treatment and immediately frozen in liquid nitrogen. ERK1/2 phosphorylation was determined by Western blot analysis. **C**, mice bearing established (~ 200 mm³) Colo205 tumors were treated as indicated. No statistically significant differences between growth curves were observed. Arrow and *R_x*, first day of dosing.

ERK1/2 phosphorylation *in vivo*. ERK1/2 phosphorylation in the HT-29 xenografts was partially inhibited between 1 and 12 h following a single dose of compound I (Fig. 6B). The partial inhibition of ERK1/2 phosphorylation *in vivo* correlated with the partial inhibition of tumor growth (Fig. 6A) and with the partial inhibition of ERK1/2 phosphorylation observed *in vitro* (Fig. 4C). Interestingly, O'Toole and colleagues (30) had similar findings in HT-29 xenografts using a monoclonal antibody to human RON. Together, these data suggest that RON Δ 160 contributes to the oncogenic properties of HT-29 cells.

To confirm that compound I does not have a nonspecific anti-tumor effect, we tested it in Colo205 xenografts that express both wild-type c-Met and RON (Fig. 4A) but show no signs of constitutive activity from either receptor (data not shown). Moreover, these cells, like HT-29 cells, express the activated b-Raf mutant V600E (38). Figure 6C shows that the growth of Colo205 tumors was unaffected by compound I treatment at doses that significantly inhibited tumor growth in c-Met-dependent models and in a model expressing RON Δ 160.

Discussion

This report describes compound I, a novel dual inhibitor of RON and c-Met. We showed that compound I exhibits inhibitory effects on c-Met and RON kinase activity as well as on c-Met-mediated and RON-mediated signaling and function in cells. Furthermore, compound I inhibits c-Met-driven and RON-driven tumor growth *in vivo* by blocking their respective signaling. To our knowledge, this is the first time that a small-molecule inhibitor of RON is described. Our results show that inhibition of RON kinase activity constitutes a viable approach for an anticancer drug. Furthermore, targeting both RON and c-Met with a single molecule could benefit a larger patient population than an inhibitor directed only at RON or c-Met.

Targeted tumor therapy relies on the hypothesis that some cancers are addicted, or at least partially dependent, on specific genetic or molecular lesions. The discovery that constitutively active forms of RON and c-Met are present in human cancers provides a strong rationale for the development of targeted therapies against these RTKs. Several agents directed at c-Met are currently in early-phase clinical trials. c-Met has been recognized for some time now as a potentially important target for an anticancer drug. Indeed, many tumor types overexpress c-Met and some harbor activating mutations or amplification of the receptor. Recently, MET amplification emerged as an important mechanism of gefitinib resistance in non-small cell lung cancer cell lines and in tumors from patients initially responsive to the drug (41). Under these circumstances, inhibition of c-Met kinase activity restored sensitivity to gefitinib *in vitro*. These data provide a clear example where a c-Met kinase inhibitor would be clinically relevant. It will be interesting to see if MET amplification will become a common theme in the resistance to tyrosine kinase inhibitors in solid tumors.

The role of RON in human cancer is less well defined. Activating mutations or amplification has not been observed thus far. On the other hand, splicing variants of RON with constitutive activity have been described both in cell lines and in cancer specimens, including colorectal and gastric cancers (14, 27, 28), suggesting that RON may play a key role in these cancers and may constitute an important target for intervention. It is presently unclear whether an antibody against RON would inhibit the constitutive activity of RON Δ 160. O'Toole and colleagues have observed a partial inhibition of tumor growth in the HT-29 xenograft model with a RON neutralizing antibody, which is consistent with our findings. However, other active variants of RON are located in the cytoplasm (14) and would be inaccessible to an antibody therapy. For these reasons, a small-molecule approach to RON seems promising. Thus far, we have not been able to identify cancer cell line models where an active cytoplasmic RON contributes to tumor growth. KATOIII cells express RON Δ 165, but this form of RON does not have cell-transforming activities (28). A small-molecule inhibitor of RON will be an important tool to investigate the significance of the different RON variants in tumorigenesis *in vitro* and, more importantly, their potential role in human cancers.

In this study, we used HT-29 as a model where RON might contribute to the oncogenic properties of the cells due to the presence of the constitutive form of the receptor. We avoided using models where wild-type RON is expressed because (a) they would rely on mouse MSP to activate RON *in vivo* and (b) as seen with the Colo205 xenografts (Fig. 6C), expression of wild-type RON does not necessarily confer dependence on the pathway. In contrast, we confirmed that the constitutively activated RON Δ 160 contributes to the activation of the MAPK cascade in HT-29 cells and that compound I

showed a dose-dependent inhibition of ERK1/2 phosphorylation *in vitro* and *in vivo* and caused tumor growth inhibition. Whether human tumors expressing constitutive forms of RON will be addicted to this pathway will need to be determined in the clinic. It is likely that some of these tumors will be similar to the HT-29 model where multiple genetic defects are present. In these cases, a drug combination approach including a RON kinase inhibitor may be warranted.

In conclusion, we described the first RON/c-Met dual kinase inhibitor and showed that a single molecule might constitute an interesting strategy to interdict two different oncogenes that use different mechanisms of activation in human cancers.

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Disclosure of Potential Conflicts of Interest

All of the authors have an ownership interest in Amgen, Inc.

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Identification of a Novel Recepteur d'Origine Nantais/c-Met Small-Molecule Kinase Inhibitor with Antitumor Activity *In vivo*

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