Sorafenib Functions to Potently Suppress RET Tyrosine Kinase Activity by Direct Enzymatic Inhibition and Promoting RET Lysosomal Degradation Independent of Proteasomal Targeting*

Received for publication, April 25, 2007, and in revised form, July 27, 2007 Published, JBC Papers in Press, July 30, 2007, DOI 10.1074/jbc.M703461200

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Germ line missense mutations in the RET (rearranged during transfection) oncogene are the cause of multiple endocrine neoplasia, type 2 (MEN2), but at present surgery is the only treatment available for MEN2 patients. In this study, the ability of Sorafenib (BAY 43-9006) to act as a RET inhibitor was investigated. Sorafenib inhibited the activity of purified recombinant kinase domain of wild type RET and RETV804M with IC50 values of 5.9 and 7.9 nM, respectively. Interestingly, these values were 6–7-fold lower than the IC50 for the inhibition of B-RAFV600E. In cell-based assays, Sorafenib inhibited the kinase activity and signaling of wild type and oncogenic RET in MEN2 tumor and established cell lines at a concentration between 15 and 150 nM. In contrast, inhibition of oncogenic B-RAF- or epidermal growth factor-induced ERK1/2 phosphorylation required micromolar concentrations of Sorafenib demonstrating the high specificity of this drug in targeting RET. Moreover, prolonged exposure to Sorafenib resulted in inhibition of cell proliferation and RET protein degradation. Using lysosomal and proteasomal inhibitors, we demonstrate that Sorafenib induces RET lysosomal degradation independent of proteasomal targeting. Furthermore, we provide a structural model of the Sorafenib-RET complex in which Sorafenib binds to and induces the DFGout conformation of the RET kinase domain. These results strengthen the argument that Sorafenib may be effective in the treatment of MEN2 patients. In addition, because inhibition of RET is not impaired by mutation of the Val804 gatekeeper residue, MEN2 tumors may be less susceptible to acquired Sorafenib resistance.

The receptor tyrosine kinase RET2 is expressed and required during early development for the formation of neural crest-derived lineages, kidney organogenesis, and spermatogenesis (1–3). To date a family of glial-derived neurotrophic factor (GDNF) ligands, which include GDNF, ARTN, NRTN, and PSPN and a family of GPI-linked co-receptors, GFRA1–4, have been identified (4). Ligand-co-receptor-RET complex formation results in transient RET dimerization and activation of the RET tyrosine kinase domain, followed by transphosphorylation of intracellular tyrosine residues. Phosphotyrosine residues 905, 981, 1015, 1062, and 1096 are the docking sites for Grb7/10, c-Src, phospholipase Cγ, and Grb2/Shc/IRS1–2/FRS2/DOK1–4, respectively. This docking of adaptor proteins triggers the activation of downstream pathways, which include activation of ERK1/2 mitogen-activated protein kinase, phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, p38, ERK5, and cAMP-responsive element-binding protein (5–7).

Germ line missense activating point mutations in the RET oncogene cause the cancer syndrome multiple endocrine neoplasia type 2 (MEN2) (1, 8). Three mutation-specific disease phenotypes can be recognized: MEN2A, MEN2B, and a familial form of medullary thyroid carcinoma (FMTC). Medullary thyroid carcinoma (MTC), the cancer arising from the calcitonin-secreting C cells of the thyroid, is the common clinical feature of the three clinical variants. MEN2A patients develop, in addition to MTC, pheochromocytoma (the tumor arising from the adrenal medulla cells) and hyperplasia of the parathyroid (HPT). MEN2B patients develop MTC and pheochromocytoma; however, instead of HPT, they develop a more complex and aggressive phenotype with neuromas in tongue, lips, and eyelids; intestinal ganglioneuromas; thickening of corneal nerves; and marfanoid habitus. In papillary thyroid carcinoma, the

* This work was supported by the Breakthrough Breast Cancer, Ministry of Education and Science of Spain, the Medical Research Council, the Italian Association for Research on Cancer, and European Union Prokinase Network Grant 503467. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: RET, rearranged during transfection; GDNF, glial cell line-derived neurotrophic factor; MEN2, multiple endocrine neoplasia, type 2; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MTC, medullary thyroid carcinoma; FMTC, familial form of MTC; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; ARTN, artemin; NRTN, neurturin; PSPN, persephin.
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tumor arises from the follicular cells of the thyroid and is caused by chromosomal inversions or translocations in which the 5' end of various genes are fused in frame with the tyrosine kinase domain of the RET gene (9). The resulting chimera oncoproteins called RET-PTCs, of which more than 10 variant forms have been identified, display constitutive tyrosine kinase activity caused by formation of homodimers between the N-terminal domains.

Surgical resection, radioiodine treatment, and hormone replacement are the standard treatment for papillary thyroid tumors. In the case of MEN2 patients, early diagnosis is crucial because the disease does not respond to standard chemotherapy or conventional radiotherapy. Surgery is at present the only curative treatment for MTC, consisting of a total thyroidectomy. However, more than 50% of patients have persistent or recurrent disease after surgery and are prone to develop distal metastasis, for which there is no systemic treatment (1). More recently attention has focused on the use of small molecule inhibitors to target RET in MEN2-related tumors (6). Indeed, various compounds have been reported to inhibit oncogenic RET including PP1 (10) and PP2 (11), RPI-1 (12), CEP-701, CEP-751 (13), ZD-6474 (11, 14), Gleevec (15), SU5416 (16), and NVP-AST487 (17). Sorafenib (BAY 43-9006) was designed originally as a RAF inhibitor (18) but in addition to RAF-1 and B-RAF is also able to inhibit VEGFR-2, VEGFR-3, PDGFR, c-Kit, and Flt-3 (18, 19). A recent study (20) reported that Sorafenib could inhibit the kinase activity and signaling of wild type and oncogenic RET. Here we have extended these studies to further investigate: (i) the effect of Sorafenib against the enzymatic activity of purified recombinant kinase domain of RETWT and RETV804M, (ii) the specificity of RET inhibition by Sorafenib, (iii) the mechanism of oncogenic RET degradation induced by Sorafenib, and (iv) the structural insights of the mechanism by which Sorafenib inhibits RET kinase activity.

MATERIALS AND METHODS

Cell Lines and Reagents—HEK293 (human embryonic kidney) cells were transfected with pCMV vector alone or pCMV vector containing wild type RET (RETWT), the MEN2A mutant RETC634R, the MEN2B mutant RETM918T, or the FMTC mutant RETS891A. Transfected cells were selected in 500 μg/ml of G418 (Sigma-Aldrich), screened by Western blotting and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 500 μg/ml of G418. MTC-TT cells (human medullary thyroid carcinoma cell line harboring the MEN2A-RET C634W oncoprotein) were grown in RPMI medium (Invitrogen) containing 20% fetal bovine serum. MZ-CRC-1 (human medullary thyroid carcinoma cell line harboring the MEN2B-RET M918T oncoprotein) and TGW-1 (human neuroblastoma cell line expressing wild type RET and GFRα1 co-receptor) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. BAY 43-9006 was provided by Bayer HealthCare Pharmaceuticals (West Haven, CT) and made up as a 10 mM stock solution in Me2SO. STI571 (Novartis, Basel, Switzerland) and PP1 (Promega, Madison, WI) were made up as 10 and 3 mM stock solutions in Me2SO, respectively. Concanaamycin A, lactacystin, and MG132 were purchase from Calbiochem and dissolved in Me2SO to give stock solutions of 5 μM, 10 mM, and 50 mM, respectively.

The antibodies were used: RET (H-300), RET (C-19), and phospho-Tyr1062RET (Santa Cruz, Palo Alto, CA), ERK1/2 and phospho-ERK1/2 (Cell Signaling Technology, New England Biolabs, UK), anti-HisG (Invitrogen), LAMPI (clone H4A3, Abcam Laboratories), Alexa 488 anti-rabbit Ig, Alexa 555 anti-mouse, and Alexa 647-conjugated phalloidin (Molecular Probes).

Cell Proliferation Assays—For cell number assays, MTC-TT (2.5 × 104 cells), MZ-CRC-1 (2.5 × 104 cells), TPC-1 (1 × 104 cells), and established HEK293 cells (1 × 105 cells) were plated in triplicate in 30-mm dishes in the presence of Sorafenib or vehicle (Me2SO 1:1000) on day 0. Cell number was counted after trypsinization using a standard hemocytometer at days 1, 2, 4, and 8.

Western Blotting—The cells were lysed on ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 2 mM dithiothreitol, 1 mM sodium vanadate, 87% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride). Protein lysates (10 μg) were resolved on SDS-PAGE and analyzed using Western blotting and ECL (Roche Applied Science). Densitometric analysis was performed using ImageJ software.

In Vitro Kinase Assay—Baculovirus expressed recombinant RET, ALK, and Abl kinases were purified and employed in an enzyme-linked immunosorbent assay-based kinase assay, as described previously (16, 21). The data shown are from three independent experiments performed in triplicate.

Confocal Microscopy—The cells were plated on glass coverslips for 24 h, after which cells were fixed in 4% paraformaldehyde for 1 h at room temperature, followed by permeabilization in 0.5% Triton X-100 in phosphate-buffered saline for 10 min. Immunostaining with primary antibodies for 1 h was followed by incubation with Alexa 486 anti-rabbit Ig and Alexa 555 anti-mouse Ig for 45 min. Alexa 647-phalloidin conjugated was used to stain actin filaments, and 4′,6′-diamino-2-phenylindole was used to stain the nucleus.

RNA Extraction, cDNA Synthesis, and Quantitative Real Time PCR—RNA extraction was performed using the RNeasy kit (Qiagen) under the manufacturer’s instructions. For cDNA synthesis, 200 ng of mRNA were used with the Omniscript RT kit (Qiagen) following the manufacturer’s instructions using oligo(dT)20 primer (Qiagen). 1 μl of cDNA was used per qPCR. Each analysis reaction was performed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control throughout all experimental analysis. Gene expression analysis was performed using TaqMan® gene expression assays on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Analysis was performed using the ΔΔCt method, which determines fold changes in gene expression relative to a comparator sample (control). qPCR primers were purchased from Applied Biosystems. The Assays-on-Demand references for RET and glyceraldehyde-3-phosphate dehydrogenase are Hs00240887_m1 and 4310884E, respectively.

Structural Model Analysis—To generate a model of RET complex with Sorafenib, we assumed that Sorafenib would bind.
to the DFGout conformation of the kinase. A model of the RET-Sorafenib complex was therefore derived from the unphosphorylated RET kinase structure (Protein Data Bank code 2IVS) (22) with its activation segment substituted (DFG to APE motif, Asp892–Ala919) with that of the c-Kit-Gleevec (STI1571) complex (Protein Data Bank code 1T46) (23), with mutations to convert the c-Kit activation segment sequence to that of RET. Superimposing this model onto the B-RAF complex (Protein Data Bank code 1UWH) (24) allowed a fit of RET by Sorafenib to the RET protein model. Further changes to the model included a small shift of the C-helix and of residues of the Gly loop to conformations observed in the c-Kit-STI-571 crystal structure. The resultant model provides a stereochemically plausible structure of the RET-Sorafenib complex. All modeling work was performed using COOT (CCP4), and the figures were generated with PyMOL (DeLano Scientific; www.pymol.org).

Statistical Analysis—Statistical analysis was performed using Prism software. The cell number assays were compared using one-way analysis of variance for repeating measures and the Dunnet test. Differences were considered statistically significant when \( p < 0.05 \).

RESULTS

Sorafenib Induces RET Lysosomal Degradation—In order to determine the inhibition of RET by Sorafenib, the His-tagged tyrosine kinase domain (amino acids 700–1020) from wild type RET was expressed in Sf9 cells and purified using a two-step chromatographic protocol (Fig. 1A, inset). In an in vitro kinase assay, Sorafenib blocked enzymatic activity with an IC\(_{50}\) = 5.9 ± 1.2 \( \mu \)M (Fig. 1A). To compare this potent inhibitory capacity of Sorafenib with other known RET tyrosine kinase inhibitors, the in vitro kinase assay was repeated in the presence of Gleevec (15). Gleevec blocked the kinase activity of wild type RET with an IC\(_{50}\) = 12 ± 2 \( \mu \)M compared with an IC\(_{50}\) = 0.3 ± 0.06 \( \mu \)M for the well-characterized Gleevec target, Abl (Fig. 1B). Next, the specificity of Sorafenib was investigated by in vitro kinase assay with recombinant RET, Abl, and ALK kinase domains (Fig. 1C). Sorafenib blocked the enzymatic activity of RET, Abl, or ALK with IC\(_{50}\) = 0.006, 25, and >500 \( \mu \)M, respectively. Finally, to assess the activity of the Sorafenib used in these experiments, an in vitro kinase assay with recombinant B-RAF\(^{V600E}\) was performed (Fig. 1D). Sorafenib inhibited B-RAF\(^{V600E}\) with an IC\(_{50}\) = 45 \( \mu \)M, which is essentially identical to that previously reported (25). The \( K_i \) values for Sorafenib inhibition of wild type RET and B-RAF\(^{V600E}\) were 0.45 and 5.6 \( \mu \)M, respectively. The lower \( K_i \) value for RET indicates a high binding affinity of the drug to the protein. Together with the lower IC\(_{50}\) values, this indicates that in vitro Sorafenib is a more potent inhibitor of RET tyrosine kinase rather than oncogenic B-RAF.

In Vivo Inhibition of RET Phosphorylation, Downstream Signaling, and Cell Proliferation by Sorafenib—It has recently been demonstrated that Sorafenib can inhibit RET activation and cell proliferation in tumor cells harboring a MEN2A-RETC634W mutation (MTC-TT cell line) or a RET/PTC1 oncogene (TPC1 cell line) (20). Here we have extended these studies to also examine the effects of Sorafenib on tumor cells expressing a MEN2B-RET\(^{M918T}\) mutation (MZ-CRC-1 cell line) or wild type RET together with the GFRα1 co-receptor (TGW-1 cell line). The cells were treated with Sorafenib for 90 min, and the levels of RET phosphorylation were assessed by Western blotting using an antibody that recognizes RET only when phosphorylated at Tyr\(^{1062}\). For the TGW-1 cells expressing wild type RET, GDNF (10 ng/ml) was added 30 min after drug treatment. In all of the tumor cell lines examined, a reduction in RET Tyr\(^{1062}\) phosphorylation was observed at a concentration of Sorafenib between 15 and 150 \( \mu \)M (Fig. 2A). Moreover, this decrease in RET phosphorylation was accompanied by a decrease in ERK1/2 phosphorylation. In cell growth assays it was demonstrated that Sorafenib treatment at concentrations as low as 0.1 \( \mu \)M resulted in a significant reduction in cell number (Fig. 2B). The in vitro kinase assays demonstrated that RET is...
more sensitive than oncogenic B-RAF\textsuperscript{V600E} to Sorafenib inhibition (Fig. 1).

The tumor cell lines expressing the different oncogenic forms of RET were derived from different patients and will therefore have very different genetic backgrounds. Consequently, to assess whether the observed inhibitor effects of Sorafenib were specific to RET, the assays were repeated using established HEK293 cells expressing wild type or different oncogenic RET variants (RET\textsuperscript{C634R}, RET\textsuperscript{M918T}, and RET\textsuperscript{S891A}). Again an inhibition of RET Tyr 1062 phosphorylation was detected at concentrations of Sorafenib between 15 and 150 nM (Fig. 3, A and B). As expected HEK293 cells transfected with vector alone showed no expression of RET and a very low level of ERK1/2 phosphorylation (Fig. 3B). Similarly, treatment of cells expressing oncogenic RET variants with 0.1 or 1 \textmu M Sorafenib over an 8-day period resulted in a significant decrease in cell number, whereas no effect of Sorafenib was observed on HEK293 cells transfected with vector alone (Fig. 3C). Sorafenib Induces Oncogenic RET Lysosomal Degradation—To further investigate the mechanisms by which Sorafenib impairs proliferation in RET-expressing cells, HEK293 cells expressing RET\textsuperscript{C634R} or RET\textsuperscript{M918T} were treated with Sorafenib for 48 h, and the level of RET protein was monitored by Western blotting. In both cell lines, treatment with Sorafenib resulted in a significant decrease in RET protein levels, and it was noted that this effect was stronger for RET\textsuperscript{M918T} compared with RET\textsuperscript{C634R} (Fig. 5A). Loss of the RET-PTC1 fusion protein following Sorafenib treatment was also observed in the TPC1 cell line (data not shown). Real time quantitative PCR analysis revealed no change in RET\textsuperscript{C634R} or RET\textsuperscript{M918T} mRNA levels following Sorafenib treatment (Fig. 5B), suggesting that the decrease in RET protein levels does not result from transcriptional regulation. To uncover the mechanism of oncogenic RET degradation, the cells were treated with Sorafenib for 48 h, and then lysosome or proteasome inhibitors were added for 8 h
**FIGURE 3.** *In vivo* inhibition of RET phosphorylation, downstream signaling and cell proliferation by Sorafenib in established human embryonic kidney cells expressing oncogenic and wild type RET. 

**A**. Protein lysates from serum-starved (overnight) established HEK293 cells treated with increasing amounts of Sorafenib for 90 min or Me2SO (control) were analyzed by Western blotting using the indicated antibodies; HEK293-RET<sup>C634R</sup> cells were transiently transfected with GFRα1 and stimulated for 30 min with GDNF (10 ng/ml) after drug treatment. 

**B**. Protein lysates from serum-starved (overnight) established HEK293 cells expressing empty vector (pCMV), RET<sup>C634R</sup> or RET<sup>S891A</sup> treated with increasing amounts of Sorafenib for 90 min or Me2SO (control), were analyzed by Western blotting using the indicated antibodies.

**C**. HEK293-RET<sup>C634R</sup>, HEK293-RET<sup>M918T</sup>, and HEK293-pCMV (Vector) cells were incubated with vehicle (dimethyl sulfoxide, DMSO) or 0.1 μM or 1 μM of Sorafenib, and the number of cells was counted at days 1, 2, 4, and 8. Each point represents the mean value of counted cells ± S.E. of three independent experiments performed in triplicate. ***, p < 0.01.***
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Sorafenib induces RET<sup>V804M</sup> kinase activity with similar efficacy as wild type RET—Mutations affecting the active domain of tyrosine kinase receptors can confer resistance to small molecule inhibitors (26). In the case of RET, mutations at the gatekeeper residue, Val<sup>804</sup>, have been reported to confer resistance to PP1, PP2, and ZD6474 (11). Similarly a novel MEN2B tandem mutation involving Val<sup>804</sup> (V804M/E805K) also confers resistance to the PP1 inhibitor (27). However, in <em>in vitro</em> kinase assays using immunoprecipitated RET, the IC<sub>50</sub> for Sorafenib inhibition of RET<sup>C634R</sup>, which contains a wild type kinase domain, was only two-3-fold lower then for RET<sup>V804M</sup> or RET<sup>V804I</sup> (20). Here we have performed <em>in vitro</em> kinase assays using recombinant wild type RET and RET<sup>V804I</sup> kinase domains. Both kinase domains showed a similar IC<sub>50</sub> of Sorafenib inhibition (Fig. 7A). As a control, kinase activity was assessed in the presence of PP1 inhibitor. Consistent with previous reports (11), wild type RET kinase activity, but not the activity of the RET<sup>V804I</sup> mutant, was inhibited by PP1 (Fig. 7B).

Structural Model of RET in Complex with Sorafenib—A model for RET in complex with Sorafenib was generated guided by x-ray crystal structures of the B-RAF-Sorafenib complex (24), the unphosphorylated state of RET (22), and the structure of c-KIT in complex with Gleevec (STI-571) (23). In the B-RAF-Sorafenib crystal structure, Sorafenib spans the interface between the N- and C-lobes of the kinase domain. The proximal trifluoromethyl phenyl ring (ring A) of the inhibitor engages a pocket between the C and E α-helices, whereas its urea moiety forms two hydrogen bonds with the protein: one via its amide nitrogen atom to the carbonylate side chain of the catalytic Glu<sup>501</sup> residue and the second via its carbonyl moiety to the main chain nitrogen of Asp<sup>598</sup> of the DFG motif (24). Together, these interactions promote the inactive conformation of the kinase with the DFG motif adopting the DFG<sub>out</sub> conformation, and the N-terminal region of the activation segment swung out toward the Gly loop of the kinase. This structure of the B-RAF-Sorafenib complex is reminiscent of the inactive conformation of the c-AbL tyrosine kinase domain in complex with Gleevec (28, 29) and with the mitogen-activated protein kinase-BIRB 796 complex (30). Specifically, Gleevec and Sorafenib induce the DFG<sub>out</sub> conformation of their cognate kinases via similar mechanisms: displacement of the phenyl ring of the DFG motif Phe residue via an aromatic moiety augmented by hydrogen bonds between the urea group of the inhibitor and the DFG motif.

Recently, a structure of Gleevec in complex with c-KIT revealed a mode of inhibition similar to that observed for c-AbL. The kinase domains of RET and c-KIT are structurally very similar, sharing 41% sequence identity overall, increasing to 57% for residues of the activation segment. Such high sequence similarity would suggest that the activation segments of both kinases would adopt similar DFG<sub>out</sub> conformations in their inactive states. We based our model of a RET-Sorafenib complex on the assumption that the inhibitor would promote the DFG<sub>out</sub> conformation of the activation segment, similar to that of B-RAF in complex with Sorafenib, and c-AbL and c-KIT in complex with Gleevec. In all three
kinases, B-RAF, c-Kit, and RET, the relative interdomain arrangements are very similar. A model of the RET/Sorafenib complex was therefore derived using the RET kinase structure with its activation segment substituted with that of the c-Kit/Gleevec complex. RET residues were then substituted for c-Kit residues within the activation segment. Superimposing this model onto the B-RAF/Sorafenib complex allowed a fit of Sorafenib to the RET protein model (Fig. 8A). This provides a stereochemically plausible model of the RET/Sorafenib complex. Overall, nine residues that contact Sorafenib (defined as a contact distances less than 4 Å) differ between the two kinases (Fig. 8D). Residues of RET that interact with ring A and the urea group of the inhibitor are highly conserved with those in the B-RAF/Sorafenib complex, with only Val787 and Leu854 for Ile substitutions in RET. There are a total of six differences in the residues between B-RAF and RET that contact the central (B) and pyridyl (C) rings between the two kinases. These mainly comprise changes in van der Waals' contacts but also include a difference in the gatekeeper residue (Thr529 and Val804 in B-RAF and RET, respectively). In the B-RAF/Sorafenib complex, the hydroxyl group of the Thr529 side chain is buried by the central ring of the inhibitor. Substitution of Val804 for Thr in RET would potentially remove an unfavorable unsatisfied hydrogen bond present in the B-RAF/Sorafenib complex.

**DISCUSSION**

Tyrosine kinase receptors play a major role in cancer development. They control many biological processes in the cell, but when mutated, they become constitutively active or display altered signaling properties; hence they become oncoproteins. Understanding the molecular bases of cancer is necessary to gain a better insight into the genotype-phenotype correlations but also is crucial for the discovery of new therapeutic approaches. Indeed, tyrosine kinase receptor-based therapies have reached widespread clinical use in, for example, breast cancer (inhibition of HER2 by Herceptin), gastrointestinal stromal tumors (inhibi-
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domain in in vitro kinase assays, we have confirmed that Sorafenib is a potent RET kinase inhibitor. However, the IC₅₀ of inhibition (5.9 nM) was ~10-fold lower than previously reported. The reasons for this difference may reflect differences in the assays employed; however, it is also possible that the kinase activity of RET following immunoprecipitation may be altered by co-immunoprecipitation of RET associated kinases. A novel finding in our studies was that the IC₅₀ values of Sorafenib inhibition obtained with wild type RET were 6~7-fold lower than those obtained with B-RAFV600E. These findings were also supported by comparing their Kᵅ values: 0.45 and 5.6 nM for wild type RET and B-RAFV600E, respectively, indicating a higher affinity and efficacy of Sorafenib inhibition of wild type RET than for B-RAFV600E. Moreover, in cell-based assays, Sorafenib inhibited oncogenic B-RAF- or EGF-induced ERK1/2 activation at micromolar concentrations, giving evidence of the high specificity of RET inhibition by Sorafenib.

Our structural model of a RET-Sorafenib complex predicts that the inhibitor will promote the DFG<sup>out</sup> conformation of the activation segment in the kinase domain. Consequently, the overall affinity of the inhibitor for RET will be determined by the overall energy difference between the DFG<sup>out</sup> and DFG<sup>in</sup> conformations and by the specific interactions between the protein and inhibitor. The inability of the Src kinase to adopt the DFG<sup>out</sup> conformation explains its insensitivity to Gleevec, despite sharing with c-Abl all the residues that contact the inhibitor (32). Our modeling indicates that the increased affinity of Sorafenib for RET could be due to a combination of more favorable interactions between the kinase and inhibitor and an equilibrium that favors the transition to the DFG<sup>out</sup> conformation. One of the nine Sorafenib contact residues that differ between RET and B-RAF is Ser<sup>891</sup>. Interestingly, a mutation at this residue to an alanine (RET<sup>S891A</sup>) is found in FMTC patients and, as illustrated in Fig. 3C, displays constitutive kinase activity as monitored by RET phosphorylation and downstream phosphorylation of ERK1/2. In cell-based assays, substitution of alanine for serine at residue 891 did not reduce sensitivity to Sorafenib inhibition (Fig. 3B). Similarly, substitution of Val<sup>804</sup> for Met in RET (RET<sup>V804M</sup>) did not alter the kinetics of Sorafenib inhibition in vitro (Fig. 7A). Together, these data indicate that the difference in sensitivity to Sorafenib observed between RET and B-RAF does not reside in a single amino acid interaction between the kinase domain and the drug rather than a combination of different amino acids along the interface between kinase and inhibitor.

The potent inhibition of RET by Sorafenib was confirmed in tumor cell lines and established HEK293 cells expressing wild type and oncogenic RET. In these cell lines, Sorafenib was found to inhibit RET tyrosine kinase activity and downstream signaling and to specifically inhibit RET-dependent cell proliferation. This confirms and extends the work of Carlomagno et al. (20). To further investigate the mecha-
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Sorafenib modeled into the RET kinase catalytic site. The chemical structure of Sorafenib is depicted on the right hand side. B, details of Sorafenib-RET kinase interactions. C, view similar to B showing equivalent B-RAF residues (blue). RET kinase residues, secondary structures, and functional motif are labeled. The figure was generated using the PyMOL Molecular Graphics System. D, table of residue differences at equivalent positions that contact Sorafenib.

nism of RET inhibition in these cells, the effects of Sorafenib on RET degradation were investigated. Exposure to the drug for 48 h had no effect on RET mRNA levels but resulted in a significant decrease in RET protein, indicating that Sorafenib has a 2-fold mechanism of receptor inhibition by both directly inhibiting RET kinase activity and promoting its degradation. The mechanism of Sorafenib-induced RET degradation was addressed by using proteasomally and lysosomally specific inhibitors in Western blotting experiments. Treatment with the lysosomal inhibitor concanamycin A reversed the Sorafenib-induced RETC634R degradation and provided a lesser, but still significant, rescue of RETM918T degradation. In contrast, no reversal was observed in the presence of two proteasome inhibitors, lactacystin and MG132, demonstrating that Sorafenib induces the lysosomal degradation of oncogenic RET independent of proteasome targeting. Similar data were obtained with tumor cell lines with endogenous expression of these oncogenic RET receptors. In these degradation experiments it should be noted that concanamycin A treatment does not rescue the level of RET protein in Sorafenib-treated cells to that found after treatment with concanamycin A treatment alone. The reason for this is most likely that cells were treated with Sorafenib for 48 h and that lysosomal or proteosomal inhibitors were only added for the final 8 h of the experiment because of their cytotoxic effects in longer term culture.

Further, confocal microscopy analysis revealed that in untreated cells, a significant proportion of RETC634R co-localizes with LAMP1, suggesting that this active RET oncoprotein is constitutively internalized from the plasma membrane and targeted for lysosomal degradation. In support of this, treatment of cells with concanamycin A in the absence of Sorafenib resulted in a significant increase in level of RETC634R protein. It was notable in these experiments that concanamycin A was less effective at blocking Sorafenib-induced RETM918T degradation. In this respect, it is of interest that in untreated cells a lower proportion of RETM918T was found co-localized with LAMP1, indicating that the rate of constitutive RETM918T lysosomal degradation is lower than that for RETC634R. Again, this is supported by data showing that concanamycin A treatment in the absence of Sorafenib resulted in a statistically significant increase in RETM918T levels but that this increase was lower than that observed with RETC634R. The inhibitor studies and confocal analysis presented here support a model in which oncogenic RET, and in particular the MEN2A-RET(C634R) mutant, is constitutively internalized from the plasma membrane and that a proportion of this internalized receptor is targeted for degradation in the lysosomes. Treatment with concanamycin A prevents this lysosomal degradation and results in an accumulation of intracellular RET. Because treatment with Sorafenib accelerates the rate of degradation, it is likely that inhibition of the kinase activity shifts the equilibrium toward lysosomal degradation. Interestingly, in these experiments, concanamycin A treatment alone also increased the levels of wild type RET, indicating that in the absence of ligand, this receptor can be internalized and targeted to the lysosomes. However, it was notable that the effects of Sorafenib on receptor degradation were less striking that for oncogenic RET, suggesting that the majority of wild type RET in the absence of GDNF stimulation is kinase inactive and therefore shows little response to Sorafenib. This demonstration of a lysosomal degradation mechanism for RET is in keeping with data from other receptor tyrosine kinases that have been shown to be internalized via clathrin-coated pits and then trafficked through early and late endosome for either recycling back to the plasma membrane or targeting to the lysosome (33). However, to date there is conflicting data on the mechanism of wild type RET degradation following binding to its GDNF ligand. In cell lines it has been demonstrated that GDNF treatment results in clathrin-mediated internalization of RET into endosomes, suggesting that subsequent degradation will occur in the lysosome (34). In primary sympathetic neuron and transfected COS cells, activation with GDNF is rapidly followed by wild type RET ubiquitination and degradation, which can be blocked by proteasome inhibitors (35, 36). In contrast to the data reported here, Carniti et al. (10) have reported that treatment of cells expressing either RETC634R or RETM918T with the kinase inhibitor PP1 results in relatively rapid degradation of
the oncopgenic RET through proteasomal targeting. The reasons for the differences obtained with these two kinase inhibitors is not known, but it has been suggested that PP1 may induce structural perturbations in RET that result in the recruitment of the stress-inducible machinery and subsequent proteasomal degradation (10). If binding of Sorafenib does not induce such perturbations, then this would account for the observed differences in mechanism and time course of receptor degradation.

The effect of Sorafenib on the kinase activity of mutant RET<sup>Val804Met</sup> was also analyzed. This mutant is associated with the FMTC phenotype and affects the gatekeeper amino acid of the kinase domain. Importantly, mutations at this site have been shown to confer resistance to previously tested drugs (11, 20). Here we demonstrate that mutation of the gatekeeper residue (Val<sup>804</sup>) to Met desensitizes the kinase to inhibition by PP1 but has essentially no effect on the inhibition by Sorafenib (Fig. 5). Analysis of the RET-PP1 structure (Protein Data Bank code 2IVV) (22) indicates that the aliphatic side chain of Val<sup>804</sup> packs against the benzyl moiety of PP1; a bulky Met residue could only be accommodated by a shift of position of the PP1 inhibitor, and presumably this weakens dramatically its affinity for the kinase. Intriguingly, the model of the RET-Sorafenib complex indicates a similar close contact between the Val<sup>804</sup> side chain and the central ring of the inhibitor (Fig. 6). To accommodate a Met residue at position 804, the Sorafenib molecule would also have to be required to shift its position. Because inhibitory potency is maintained, we assume that compensatory new interactions are formed with the protein, possibly facilitated by conformational changes in RET. The different mode of binding of Sorafenib and PP1 to the DFG<sup>out</sup> and DFG<sup>in</sup> conformations of RET, respectively, and the more extensive contacts formed between Sorafenib and RET compared with PP1 and RET could account for these differences. In this respect, it is interesting that mutation of the gatekeeper residue Thr<sup>674</sup> in the FIP11-PDGF<sub>R</sub> oncogene conferred resistance to Gleevec but not to Sorafenib (26).

Taken together, the data presented here indicate that Sorafenib is indeed a potent RET kinase inhibitor and points toward its promising therapeutic potential for patients affected with MEN2-related tumors. In particular, the demonstration that Sorafenib is an effective inhibitor of oncogenic RET containing mutations at Val<sup>804</sup> indicates that tumors may not be able to acquire resistance to this drug by subsequent somatic mutation in this gatekeeper residue.

Acknowledgments—We thank Steven Whittaker (Institute of Cancer Research) for helping discussions.

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