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Ponatinib is a potent inhibitor of wild-type and drug-resistant gatekeeper mutant RET kinase

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ABSTRACT

RET kinase is aberrantly activated in thyroid cancers and in rare cases of lung and colon cancer, and has been validated as a molecular target in these tumors. Vandetanib was recently approved for the treatment of medullary thyroid cancer. However, vandetanib is ineffective in vitro against RET mutants carrying bulky aminoacids at position 804, the gatekeeper residue, similarly to drug-resistant BCR–ABL mutants in chronic myeloid leukemia. Ponatinib is a multi-target kinase inhibitor that was recently approved for treatment-refractory Philadelphia-positive leukemia. We show here potent inhibition of oncogenic RET by ponatinib, including the drug-insensitive V804M/L mutants. Ponatinib inhibited the growth of RET+ and BCR–ABL+ cells with similar potency, while not affecting RET-negative cells. Both in biochemical and in cellular assays ponatinib compared favorably with known RET inhibitors, such as vandetanib, cabozantinib, sorafenib, sunitinib and motesanib, used as reference compounds. We suggest that ponatinib should be considered for the treatment of RET+ tumors, in particular those expressing vandetanib-resistant V804M/L mutations.

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1. Introduction

RET (REarranged during Transfection) proto-oncogene is a transmembrane tyrosine kinase receptor expressed in central and peripheral nervous system and neural crest-derived cells that transduces proliferative and survival signals in response to GDNF-family neurotrophic factors. Aberrant RET kinase activity is involved in the onset of hereditary and sporadic thyroid cancer (Mologni, 2011) and in rare cases of colon and lung cancers (Wood et al., 2007; Lipson et al., 2012). Germline activating mutations affecting the extracellular and the catalytic domains have been described in 100% of multiple endocrine neoplasia type 2A (MEN2A) and MEN2B patients, respectively, as well as in familial medullary thyroid carcinoma (FMTC) (Borrello et al., 2013). In addition, different mutations spanning the entire receptor are found in a variable proportion of sporadic MTC (30–50%). Moreover, several rearranged forms of RET have been identified in up to 80% of papillary thyroid cancer (PTC) patients, depending on age, exposure to radiation, and histological tumor variant. In these cases, the intracellular kinase domain is fused to the dimerization region of an activating gene. Whatever the mechanism, in all cases RET kinase activity is turned on independently of ligand binding and induces malignant transformation of cells. RET uncontrolled activity is both sufficient and necessary to cause neoplastic phenotype (Plaza-Menacho et al., 2006). Therefore, it represents an ideal target for molecular therapy.

Several small-molecule inhibitors are currently under clinical investigation for selective RET inhibition (Mologni, 2011). Among them, vandetanib (ZD6474, Zactima™) is a rather potent inhibitor of rearranged RET and of oncogenic RET mutants observed in thyroid cancer (Carlomagno et al., 2002; Vitagliano et al., 2011). After encouraging results of the ZETA trial (Wells et al., 2012), it was approved in 2011 for metastatic MTC. Unfortunately, vandetanib is inactive against the V804M gatekeeper mutant of RET (Carlomagno et al., 2004), which has variable frequency in MEN2 families from different countries and is typically associated with FMTC and atypical MEN2 (Pinna et al., 2007; Machens and Dralle, 2008; Shifrin et al., 2009). The gatekeeper residue is a key aminoacid within the active site of tyrosine kinases (Zuccotto et al., 2010). It controls access of small-molecules to a hydrophobic cavity also known as the selectivity pocket. Therefore, mutations at this position are

Abbreviations: CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; RET, REarranged during Transfection; GDNF, glial-derived neurotrophic factor; TKI, tyrosine kinase inhibitor; MTC, medullary thyroid carcinoma; FMTC, familial MTC; PTC, papillary thyroid carcinoma; MEN2, multiple endocrine neoplasia type 2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide.

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therapeutically intractable, as they significantly affect the binding of several type II inhibitors (Quintas-Cardama and Cortes, 2008; Oxnard et al., 2011). More recently another drug, cabozantinib, was approved for advanced MTC (Hart and De Boer, 2013). Ponatinib (AP24534) was developed as a potent inhibitor of wild-type and mutant ABL kinase and was recently approved for the treatment of patients with Philadelphia-positive leukemia who are resistant or intolerant to prior tyrosine kinase inhibitor (TKI) therapy (O'Hare et al., 2009; Cortes et al., 2012). Interestingly, ponatinib was specifically designed to target the T315I gatekeeper mutant of ABL. In particular, it has been shown to overcome the bulky isoleucine at position 315 via a straight ethynyl linker (Zhou et al., 2011). Therefore, it is a candidate inhibitor of mutant kinases harboring a large gatekeeper residue. In addition, ponatinib has shown activity against other clinically relevant oncogenic kinases (Gozgit et al., 2011; Gozgit et al., 2012; Smith et al., 2013).

In this study, we report that ponatinib causes potent and specific inhibition of both wild-type and mutant RET kinase, including the drug-resistant V804M/L mutants. Ponatinib inhibited RET-dependent cells growth at low nanomolar concentrations, similar to BCR–ABL-expressing CML cells. In parallel, ponatinib blocked RET autophosphorylation at corresponding doses. A comparison with anti-RET activity of vandetanib and other RET inhibitors is also presented.

2. Materials and methods

2.1. Cell lines and inhibitors

All cell lines were purchased from the American Type Culture Collection, where they are routinely verified using genotypic and phenotypic testing to confirm their identity. All cells were grown in RPMI supplemented with 10% FBS, unless otherwise specified. TPC-1 cells carry a complex t(1;10;21) translocation (Ishizaka et al., 1989) leading to high expression of the RET/PTC1 fusion gene. TT is a human MTC cell line harboring the MEN2A-associated C634W substitution in the RET extracellular domain (Carlomagno et al., 1995). MZ-CRC-1 cells derive from a MTC carrying the MEN2B-RET^{M918T} mutation (Cooley et al., 1995). TPC-1 and MZ-CRC-1 cells were maintained in DMEM plus 10% FBS. RET-negative cell lines used in this study include: RWPE-1 human immortalized prostate epithelial cells, which were cultured in keratinocyte-serum free medium supplemented with epidermal growth factor and bovine pituitary extract (Magistroni et al., 2011); the colon cancer cell lines Ls174T and HT-29; the BCR–ABL-expressing CML cell line K562. Human embryonic kidney HEK293 cells were grown in DMEM supplemented with 10% FBS and were stably transfected with pCMV vector alone or pCMV vector containing the MEN2A mutant RET^{C634R}, the MEN2B mutant RET^{M918T} and the gatekeeper mutants RET^{V804M} and RET^{V804L}. Transfectants were selected with 1 mg/ml G418 (Plaza-Menacho et al., 2007, 2011). Ponatinib (AP-24534) was kindly provided by Ariad, Inc. Vandetanib (ZD6474), cabozantinib (XL-184), motesanib (AMG-706), sorafenib (BAY-43-9006) and sunitinib (SU-11248) were all purchased from Selleck Chemicals. The compounds were dissolved in dimethylsulfoxide (DMSO) and aliquoted. Small aliquots were stored at −20 °C until use to avoid repeated freezing/thawing.

2.2. Proliferation assay

Semi-logarithmic dilutions of inhibitors were prepared in DMSO and added to 96-well plates containing 10,000 cells/well in complete culture medium (DMSO final concentration = 0.5%). Cell proliferation was measured at 72 h using the tritiated-thymidine incorporation assay as described previously (Gambacorti-Passerini et al., 1997). Each data point was done in triplicate.

2.3. Western blotting

The cells were seeded at 40% confluence and treated with inhibitors. After 4 h, the cells were harvested and lysed as described (Mologni et al., 2006). Total cell extracts were loaded on SDS–PAGE, transferred to a nitrocellulose membrane and probed with the indicated primary antibodies overnight at 4 °C. Proteins were revealed by chemiluminescence after incubation with HRP-conjugated secondary antibodies (GE Healthcare, diluted 1:2500). Primary antibodies recognizing total RET (clone C31B4) and ERK1/2 proteins, or the phosphorylated forms of RET (pY905) and ERK1/2 (pT202/pY204), were from Cell Signaling Technology and used 1:1000 as recommended. Anti-lamin B antibody was purchased from AbCam (1:1000). The anti-actin antibody was from Sigma–Aldrich and diluted 1:2000.

2.4. Production of recombinant proteins and in vitro kinase assay

Recombinant wild-type and V804M mutant RET kinase domains were produced in Baculovirus and purified as described previously (Sala et al., 2006). Kinase activity was measured in the absence or presence of inhibitors using an ELISA-based kinase assay, as described (Mologni et al., 2005).

2.5. Cell cycle analysis

The cells were treated with vehicle or ponatinib 1 μM for 72 h, or with staurosporine for 24 h, and then fixed with 75% ethanol overnight. Then, the cells were washed with PBS and resuspended in PBS containing RNase 100 μg/ml and propidium iodide 50 μg/ml, incubated for 1 h at 37 °C and analyzed by FACScan flow cytometer (Beckton Dickinson).

2.6. Statistical analyses

All graphs were built by GraphPad Prism4 software, using the sigmoidal dose–response equation. Data were normalized over vehicle-treated controls. IC50 values indicate the concentration of inhibitor that gives half-maximal inhibition. Significance was calculated by 1-way ANOVA test, with Dunnett's post-test comparison.

3. Results

Ponatinib was tested in an ELISA-based in vitro kinase assay using a purified recombinant RET kinase domain, as described (Mologni et al., 2005). Dose-dependent inhibition of RET was observed, with an IC50 value in the low nanomolar range (Fig. 1A). The anti-proliferative effects of ponatinib were analyzed by thymidine incorporation assay in three RET-dependent tumor cell lines representing three common RET-driven diseases: TT for MEN2A, MZ-CRC-1 (MEN2B) and TPC-1 (PTC). Since ponatinib is approved as a clinical ABL inhibitor, a BCR/ABL-driven CML cell line was used as a reference (K562). As shown in Fig. 1B, the drug inhibited RET+ cells growth in a dose-dependent manner with low nanomolar IC50 values, comparable to those obtained in K562 cells. This result suggests similar sensitivity of RET-driven cells compared to BCR/ABL+ cells. In contrast, the sensitivity of three ABL- and RET-negative cell lines (two colorectal cancer cell lines, HT-29 and Ls174T, and one prostate epithelium cell line, RWPE-1) to ponatinib was 10–1000-fold lower, indicating high specificity toward RET-dependent proliferation.

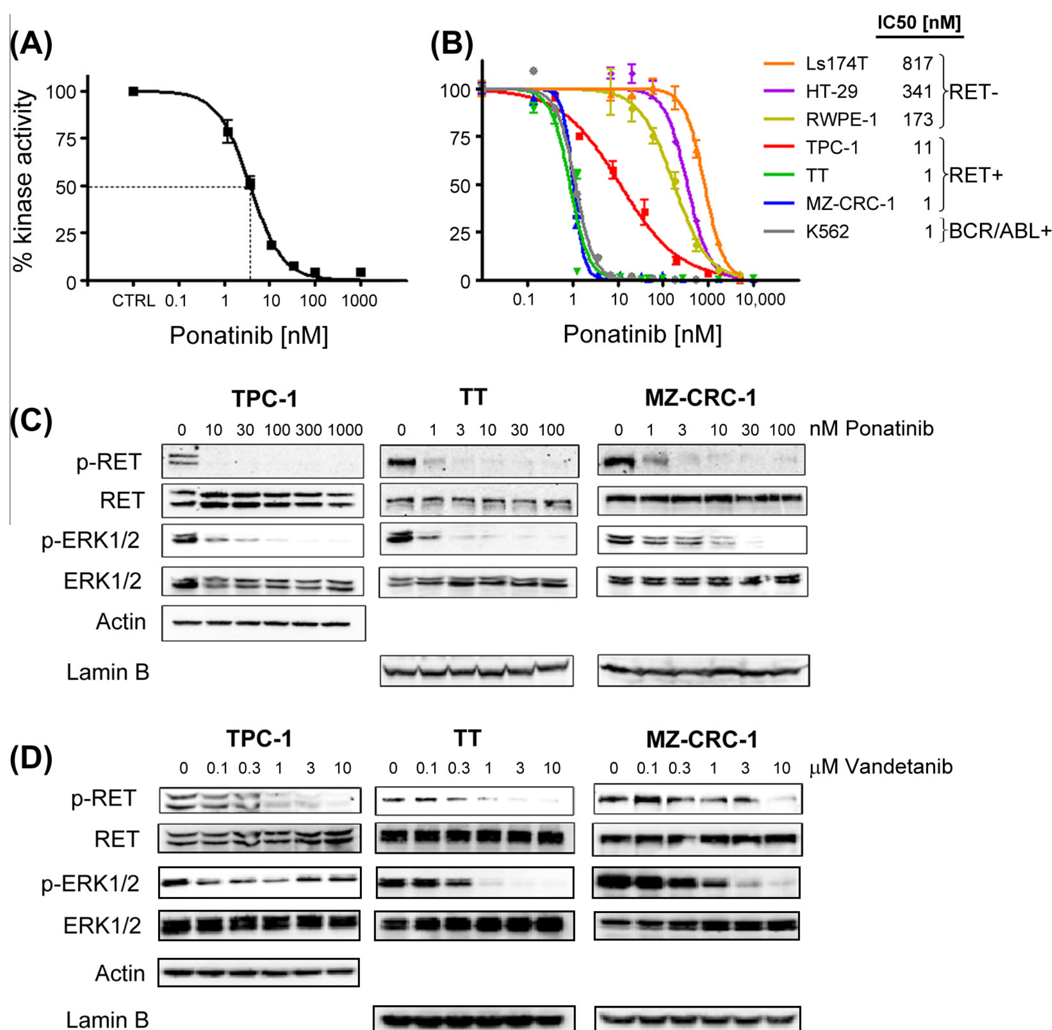

Fig. 1. Characterization of RET inhibition by ponatinib. (A) Dose–response curve of RET inhibition by ponatinib in kinase assay. Residual kinase activity (control = 100%) was plotted as a function of ponatinib concentration. The dashed line identifies the IC50. (B) Three RET+ and three RET- cell lines were treated for 72 h with increasing ponatinib concentrations. A BCR-ABL+ cell line (K562) was included as a control. Proliferation was measured by tritiated-thymidine incorporation assay. Sigmoidal dose–response curves were generated by GraphPad software using non-linear fitting of experimental data points. IC50 values [nM units] are reported in the legend. (C and D) The indicated cell lines were treated for 4 h with ponatinib (C) or vandetanib (D) and lysed. Total lysates were run in SDS–PAGE and probed with the indicated antibodies. Actin or Lamin B were used as loading controls on stripped membranes. All data are representative of two or three experiments.

To correlate growth inhibition data with molecular effects, the phosphorylation status of RET was evaluated in the same cell lines (Fig. 1C). Ponatinib caused potent suppression of oncogenic RET phosphorylation in all three thyroid cancer cell lines at nanomolar concentrations. A concomitant inactivation of downstream signal was noted, as indicated by loss of ERK1/2 phosphorylation. Using lower (down to picomolar) concentrations of ponatinib revealed that in TPC-1 cells RET phosphorylation dropped dramatically between 1 and 3 nM, while inTT and MZ-CRC-1 cells the signal was lost between 0.1 and 1 nM (Supplementary Fig. S1), in agreement with cell proliferation data. As a comparison, vandetanib (Wells et al., 2012), the first approved drug for MTC, caused substantial inhibition of RET and ERK phosphorylation only at micromolar concentrations (Fig. 1D). In order to evaluate whether the observed proliferative block was accompanied by cell death, the proportion of live and dead cells after 3 days treatment with ponatinib was assessed. Staurosporine was used as a positive control (Supplementary Fig. S2). Ponatinib caused a rather modest induction of cell death at low concentrations (10–100 nM), while the effect was more pronounced at 1 μM, especially in TT and MZ-CRC-1 cells (approximately 25% dead cells). Induction of apoptosis at high dose was confirmed by cell cycle analysis: the sub-G1 population increased significantly at 1 μM ponatinib compared to vehicle control, and was comparable to that caused by staurosporine in TT and MZ-CRC-1 cells (Supplementary Fig. S2, panels B, E, H). Thus, ponatinib exerts mainly a cytostatic effect at nanomolar concentrations that are sufficient to arrest proliferation, while it causes cell death at micromolar doses. This result is in line with previous reports on RET inhibition (Carlomagno et al., 2006; Mologni et al., 2006). The phosphorylation status of AKT was also determined, as the PI3K/AKT/mTOR axis lies downstream of RET and may contribute to cell survival (Segouffin-Cariou and Billaud, 2000). AKT was efficiently dephosphorylated in all cells, at all tested concentrations (Supplementary Fig. S2C, 2F, 2I). Therefore, inactivation of AKT does not seem to correlate with induction of apoptosis in these cells.

We then evaluated whether ponatinib is able to inhibit the vandetanib-resistant RET^{V804M} gatekeeper mutant in biochemical assays and compared ponatinib to five clinically relevant RET inhibitors (vandetanib, cabozantinib, sorafenib, sunitinib and motesanib). Interestingly, while RET^{V804M} was insensitive to vandetanib and motesanib up to 10 μM and greatly affected cabozanti-

Table 1
Inhibitory activity of ponatinib in comparison with other RET inhibitors.

	RET-WT	RET-V804M	TPC-1	TT	MZ-CRC-1
Ponatinib	7	12	11	0.7	0.7
Vandetanib	122	>10,000	82	221	50
Cabozantinib	164	4094	21	48	20
Motesanib	634	>10,000	189	1273	661
Sunitinib	30	55	40	164	47
Sorafenib	4	12	1938	134	61

Notes: IC50 values are reported in the table, in nanomolar units. RET-WT and RET-V804M refer to inhibition of wild-type and V804M mutant RET, respectively, in the in vitro kinase assay. TPC-1, TT and MZ-CRC-1 refer to inhibition of cell proliferation, as measured by [3H-methyl]-thymidine incorporation assay.

nib activity, it was efficiently inhibited by ponatinib, as well as by sorafenib and sunitinib at nanomolar concentrations, with IC50 values close to the wild-type enzyme (table 1). In cell growth assays, ponatinib proved to be the most potent inhibitor of RET-dependent proliferation, showing at least 1-log higher potency when compared to the other compounds in TT and MZ-CRC-1 cells.

In order to explain why ponatinib binding is maintained while vandetanib cannot bind to the mutant RET^{V804M} kinase, we modeled the gatekeeper V804 M mutation using the published crystal structure of RET bound to vandetanib (Knowles et al., 2006) and superimposed it to the available structure of ABL^{T315I} in complex with ponatinib (O'Hare et al., 2009). In both kinases, the gatekeeper residue is mutated into a bulkier one, suggesting a reduction of the available space within the active site. According to our analysis (Supplementary Fig. S3) ponatinib binds and induces the DFG-out conformer in ABL^{T315I} mutant kinase that precludes ATP binding. The compound is flexible enough through its ethynyl linker to accommodate itself in the active site without being sterically compromised by isoleucine substitution at the gatekeeper position. In the case of RET kinase, this hypothetical arrangement by ponatinib is not possible as it would sterically clash with the activation loop in the DFG-in conformation. However, from our biochemical and biological characterization of ponatinib on RET kinase activity, we assume it will also bind and induce a DFG-out conformation, allowing as in the case of ABL^{T315I} the accommodation of the compound deep in the active site, overcoming steric constrains from the gatekeeper mutation. In the case of RET-vandetanib structure, the inhibitor binds to an active conformer of the activation loop (DFG-in mode) but is most likely to be sterically impeded by the V804M substitution in the gatekeeper residue, hence explaining the differences in RET kinase activity against wild-type and the drug resistant mutant V804M.

Finally, in order to confirm in a different cell model the activity of ponatinib against oncogenic RET, we engineered HEK293 stable clones expressing four disease-associated oncogenic mutants of the RET receptor tyrosine kinase, namely RET^{C634R} (frequently observed in MEN2A patients), RET^{M918T} (typically associated with MEN2B), RET^{V804L} and RET^{V804M} (both found in FMTC patients). The four transgenes were expressed at similar levels (Fig. 2A) and were all significantly inhibited by ponatinib at nanomolar concentrations (Fig. 2C), although with varying efficiency. Among the different oncogenic RET mutants used, RET^{C634R} showed the strongest phosphorylation inhibition, already evident at 30 nM. Interestingly, ectopic expression of RET mutants sensitized HEK293 cells to the anti-proliferative effects of ponatinib: when challenged in a proliferation assay, all four transfected cell lines showed increased sensitivity to the compound compared to empty-vector control cells (Fig. 2B; $p = 0.0229$). Despite variable degree of phosphorylation inhibition, the cell growth IC50 was similar among the four transfectants ($p = 0.9904$), in the 125–150 nM range. Therefore, based on these results we suggest that ponatinib is a potent pan-RET inhibitor.

4. Discussion

RET is a well-defined clinical target in thyroid cancer, as recently demonstrated by vandetanib and cabozantinib, two multikinase inhibitors that were approved for the treatment of advanced MTC. Although it is possible that anti-VEGFR and anti-EGFR activities of vandetanib may contribute to its clinical efficacy, as described in a preclinical study with motesanib (Coxon et al., 2012), the results of a large phase III trial (Wells et al., 2012) showed that RET-positive patients had a significantly better hazard ratio for progression-free survival compared to RET-negative ones. Therefore, its anti-RET activity is directly linked to efficacy. Moreover, single EGFR inhibition with gefitinib did not yield clinical responses in MTC (Pennell et al., 2008).

Kinase inhibitors have emerged in the last decade as the most promising class of targeted drugs in oncology. However, despite enormous success of TKI therapy, resistance to a single agent almost invariably develops as a result of Darwinian selection. Therefore, it is necessary to have a wide arsenal of selective inhibitors targeting an oncogenic kinase. For example, it takes at least five second/third-generation ABL inhibitors to counteract all clinically relevant imatinib-resistant clones (Redaelli et al., 2012). In order to enlarge the family of anti-RET drugs, we tested ponatinib as a candidate second-generation RET inhibitor, in view of its rather broad specificity and its ability to avoid steric clash with bulky gatekeeper residues. We found that ponatinib inhibits RET kinase activity at low nanomolar concentrations in enzymatic assays and in cells. The high potency shown by this compound against RET-dependent cells was comparable to its activity on BCR/ABL+ cells, with 10 to 100-fold selectivity versus RET-negative cells. Interestingly, ponatinib compared favorably to vandetanib, cabozantinib, motesanib, sorafenib and sunitinib in both biochemical and cell growth assays. Although no in vivo or ex vivo data are reported here, this study suggests that ponatinib may be evaluated as a clinical RET inhibitor, after further verification of its activity on clinical samples. In this regard, we note that ponatinib has been extensively investigated in recent and ongoing trials and possesses a well-defined bioavailability and safety profile (Cortes et al., 2012).

Furthermore, an important finding of this study relates to the observed potent activity of ponatinib against the $RET^{V804M/L}$ gatekeeper mutants, which are gain-of-function mutants found in FMTC patients, that are resistant to vandetanib as well as to other RET inhibitors (Carlomagno et al., 2004). In fact, in our assays, ponatinib did not show preference for any of four different forms of the RET kinase: a fusion protein with wild-type sequence (RET/PTC1), the full-length receptor carrying an activating extracellular domain mutation (C634R), full-length protein with kinase domain M918T mutation, and the V804M/L gatekeeper mutants, thus suggesting that its inhibitory activity is not affected by single sequence changes. In line with this interpretation, ponatinib did not allow emergence of resistant clones in BCR/ABL+ cells (O'Hare et al., 2009). The mutants tested in this work represent major oncogenic forms of RET. Indeed, the RET/PTC1 fusion (expressed by TPC-1 cells) is one of the two most frequent rearrangements found in PTC. Together, RET/PTC1 and RET/PTC3 account for 90% of rearranged cases, which in turn represent 20–50% of sporadic and up to 80% of radiation-associated PTCs (Ciampi and Nikiforov, 2007; Borrello et al., 2013). The M918T mutation carried by MZ-CRC-1 cells represents >95% of MEN2B cases, while C634 substitutions are found in 80–90% of MEN2A patients and are the most frequent RET mutation in all thyroid cancers (Putzer and Drosten, 2004; Mologni, 2011). The gatekeeper V804 gain-of-function mutations, alone or in combination with other mutations, are usually associated with both familial and sporadic MTC, but can also be found in rare MEN2A, MEN2B and MEN2-unclassified patients (Carlo-

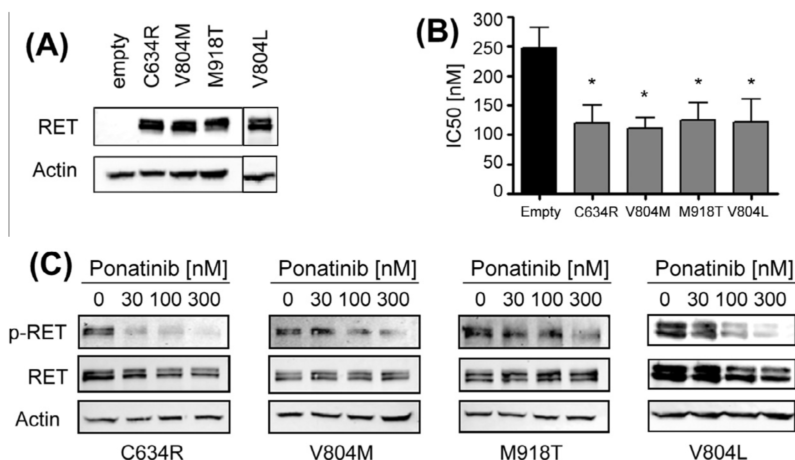

Fig. 2. Activity of ponatinib in HEK293-RET cells. (A) HEK293 cells stably transfected with empty vector or four disease-associated RET mutant constructs were established and confirmed to express the transgenes. (B and C) The transfectant cells were challenged with ponatinib: (B) dose–response proliferation assays after 72 h (as in Fig. 1A) yielded IC50 values reported in the bar graph (mean ± SEM). Data are the average of four independent experiments; $^{*}p < 0.05$ versus Empty. (C) Total lysates after 4 h treatment were tested for RET phosphorylation status.

magno et al., 2004; Cranston et al., 2006; Shifrin et al., 2009). In selected areas, V804M is the most common RET mutation (Pinna et al., 2007). Although it does not rank among the most frequent mutants overall (Machens et al., 2003), V804 position is clinically relevant because the observed mutants are predicted to be refractory to inhibition by vandetanib. We confirmed that V804M substitution confers resistance to vandetanib, motesanib and cabozantinib in vitro. It is important to note, however, that V804 mutations are not selected during treatment, but arise spontaneously and are likely to confer primary resistance to the drugs. Presently, it is not known whether drug-resistant mutations will be acquired during therapy.

In principle, although the compound was profiled here only on thyroid cancer cells, it may potentially be beneficial for other RET-mutated/translocated cancers as well, including the recently discovered cases of colon and lung cancer (Wood et al., 2007; Lipson et al., 2012). Although this needs to be proven, the current trend is to stratify patients based on driver mutations rather than by tumor histology. Finally, in support of our data, a similar work showing RET inhibition by ponatinib was published while our manuscript was under review (De Falco et al., 2013). The authors showed complete suppression of established TT xenografts in mice. Collectively, their and our data provide evidence that ponatinib, currently approved as a pan-ABL inhibitor, behaves as a potent and robust pan-RET inhibitor and may well be investigated in RET+ cancers, including V804 mutants.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.06.025.

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