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

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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
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 isoform 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. CPC-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 C634R mutation in the RET extracellular domain (Carломagno et al., 1995). MZ-CRC-1 cells derive from a MTC carrying the MEN2B-RET(M918T) mutation (Cooley et al., 1995). CPC-1 and MZ-CRC-1 cells were maintained in DMEM plus 10% FBS. RET-negative cell lines used in this study included: K562, a human immortalized erythroleukemia cell line derived from a patient with chronic myeloid leukemia (Kiel et al., 1976) representing three common RET-driven diseases: TT for MEN2A, V804M and RET

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-thymi-
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 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 in TT 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<sub>V804M</sub> gatekeeper mutant in biochemical assays and compared ponatinib to five clinically relevant RET inhibitors (vandetanib, cabozantinib, sorafenib, sunitinib and motesanib). Interestingly, while RET<sub>V804M</sub> was insensitive to vandetanib and motesanib up to 10 µM and greatly affected cabozanti-
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 conformation, as measured by [3H-methyl]-thymidine incorporation assay.

As in the case of ABL

T315I mutant kinase that precludes ATP binding.

The compound is flexible enough through its ethylm 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

S634F (frequently observed in MEN2A patients), RET

M918T (typically associated with MEN2B), RET

V804M and RET

V804D (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

S634F showed the strongest phosphorilation inhibition, already evident at 30 nM. Interestingly, 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 multi-kinase 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 (Carломagno 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 Niki forov, 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-

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<thead>
<tr>
<th>RET-WT</th>
<th>RET-V804M</th>
<th>TPC-1</th>
<th>TT</th>
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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.
ently, it is not known whether drug-resistant mutations will be pre-
viously and are likely to confer primary resistance to the drugs. Pres-
relevance because the observed mutants are predicted to be refrac-
mutants overall (Machens et al., 2003), V804 position is clinically
selected areas, V804M is the most common RET mutation (Pinna
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 se-
In principle, although the compound was profiled here only on thy-
In the present study, we investigated RET inhibition by ponatinib: (A) 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.

References
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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.

Funding
This work was supported by the Italian Association for Cancer Research (AIRC) and by Lombardy Region (Grants ID-16871 and ID-14546A to CGP). The funding sources had no involvement in the study design, in the collection, analysis and interpretation of data, in the writing of the report.

Acknowledgment
The authors are grateful to Ariad, Inc. for providing ponatinib and for reviewing the manuscript.

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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