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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.

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.