Cellular effects of imatinib on medullary thyroid cancer cells harboring multiple endocrine neoplasia Type 2A and 2B associated RET mutations

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Background. Activating mutations in the RET gene, which encodes a tyrosine kinase receptor, often cause medullary thyroid carcinoma (MTC). Surgical resection is the only curative treatment; no effective systemic treatment is available. We evaluated imatinib, a tyrosine kinase inhibitor currently used to treat chronic myelogenous leukemia and gastrointestinal stromal tumors, as a potential drug for systemic treatment of MTC, in 2 MTC-derived cell lines expressing multiple endocrine neoplasia–associated mutant RET receptors.

Methods. We determined RET expression and Y1062 phosphorylation using Western blot analysis and quantitative polymerase chain reaction. We determined the effects on cell proliferation by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, and we used fluorescence-activated cell sorter analysis with annexin V/propidium iodide staining to study imatinib-induced cell-cycle arrest, apoptosis, and cell death.

Results. Imatinib inhibited RET Y1062 phosphorylation in a dose-dependent manner after 1.5 hours of exposure. After 16 hours both RET Y1062 phosphorylation and protein expression levels were affected. Dose-dependent decreases in cell proliferation of both cell lines after exposure to imatinib with inhibitory concentration of 50% levels of 23 μmol/L and 25 μmol/L were seen. These values are high, compared with those for chronic myelogenous leukemia and gastrointestinal stromal tumors. We further could show that imatinib induced cell-cycle arrest, and apoptotic and nonapoptotic cell death.

Conclusions. Imatinib inhibits RET-mediated MTC cell growth affecting RET protein levels in vitro in a dose-dependent manner. The concentration of imatinib necessary to inhibit RET in vitro, however, makes it impossible to conclude that imatinib monotherapy will be a good option for systemic therapy of MTC. (Surgery 2006;139:806-14.)
thyroid carcinomas) and by single-point mutations. Mutations in the extracellular cysteine–rich domain of the RET protein are encountered in patients having the cancer syndrome multiple endocrine neoplasia type 2A (MEN 2A) and familial MTC (FMTC), while mutations in the tyrosine kinase domain are found in patients with FMTC and MEN 2B.8–10 In MEN 2A, the most frequently found mutation is C634R, whereas in MEN 2B, the most frequently observed activating mutation in RET is M918T.10 This mutation also is found in approximately 40% of sporadic MTC cases.10,11 These mutations and translocations lead to ligand-independent RET autophosphorylation, resulting in a constitutively active RET receptor. In the hereditary form of MTC, RET mutations always are observed, and RET expression is characteristic for both the sporadic and hereditary type of MTC.

Small molecule drugs that can inhibit tyrosine kinases selectively have proven to be useful in the systemic treatment of a number of neoplastic diseases. Recently the selective tyrosine kinase inhibitor 571 (STI571, imatinib, Glivec, Gleevec, CGP57148B; Novartis Pharma, Basel, Switzerland) has been developed. It belongs to the 2-phenylaminopyrimidine class and is proven to target BCR-ABL, platelet derived growth factor receptor (PDGFR)12,13 and c-kit receptor tyrosine kinases.14,15 Currently, imatinib is used clinically to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors as well as dermatofibrosarcoma protuberans.16–21

Because both c-kit and RET belong to the same subfamily of tyrosine kinase receptors, we investigated whether RET tyrosine kinase (RET-TK) can be inhibited by imatinib as well. Dose-dependent growth inhibition induced by imatinib in the human MTC cell line TT has been reported.22 However, others could not reproduce these data.23 Furthermore, it remains unclear whether the antiproliferative effect of imatinib is caused by inhibition of RET autophosphorylation or by induction of apoptosis, or through other mechanisms; several questions have been raised concerning the relatively high concentrations of imatinib used.23,24 In addition, only 1 MTC-derived cell line has been used in previous studies.

Aims of this study were (1) to investigate whether imatinib can inhibit the activity of oncogenic RET mutants in vitro at clinical achievable serum levels; (2) to determine whether imatinib induces growth arrest and/or cell death in 2 MTC cell lines: TT, a cell line reported to be derived from a sporadic MTC carrying a C634W RET mutation,25,26 and MZ-CRC-1, a cell line derived from a malignant pleural effusion from a patient with metastatic sporadic MTC carrying a M918T mutation26,27; and (3) to determine whether there is specificity of imatinib for particular RET mutations associated with MEN 2A and MEN 2B.

**MATERIAL AND METHODS**

The human MTC cell line MZ-CRC-1 was a gift of A. Knuth; the human MTC cell line TT and the human hepatocellular carcinoma cell line HepG2 were obtained from the American Type Culture Collection (Manassas, Va). RPMI 1640 medium, Dulbecco modified Eagle medium (DMEM), trypsin-EDTA, L-glutamine, and penicillin-streptomycin were purchased from Gibco (Invitrogen Corp, Breda, The Netherlands) and fetal calf serum (FCS) from Integro BV (Zaandam, The Netherlands). Antibodies against RET (H300), PY-RET (Y1062) and actin (C4) were used in dilutions of 1:1000, 1:250, and 1:10000, respectively. Antirabbit IgG-HRPO peroxidase-conjugated antibodies were used in dilutions of 1:2000. All antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, Calif), except antiactin, which was obtained from ICN Biomedicals (Aurora, Ohio). Imatinib kindly was provided by Novartis Pharma AG (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO). MG132 was purchased from Calbiochem (Merck Biosciences, Nottingham, UK) and dissolved in DMSO.

**Cell culture.** MZ-CRC-1 cells were cultured in DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. TT cells were cultured in RPMI 1640 supplemented with 15% (v/v) FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. HepG2 cells were maintained in DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained under a fully humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Western blot analysis.** MZ-CRC-1 cells and TT cells were grown at a density of 5 × 10⁵ cells/well for 48 hours and treated with different concentrations of imatinib for 90 minutes. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 100 µL of lysis buffer (20 mmol/L HEPES at pH 7.4, 2 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na₂VO₃ (ortho), 1% Triton X-100, 10% glycerol, 10 µg/mL leupeptin, 30 µg/mL aprotinin, and 0.4 mmol/L PMSF). Protein concentration was determined according to the Bradford method before SDS-polyacrylamide gel electrophoresis.28 Forty micrograms of total cell lysates were analyzed by Western blotting. Cell ly-
sates were boiled in Laemmli buffer (4% SDS, 250 mmol/L TRIS-Cl at pH 6.8, 20% glycerol, 0.01% bromphenol blue, and 10% 2-mercaptoethanol) before loading. Then they were separated on 12% SDS-polyacrylamide gels and electroblotted in TRIS-glycine buffer (39 mmol/L glycine, 48 mmol/L TRIS-Cl, 0.037% SDS, 20% methanol) onto 0.20 μmol/L nitrocellulose membranes (Bio-Rad Labs, Hercules, Calif).

Membranes were incubated in blocking solution (5% nonfat milk in TBS-T [25 mmol/L TRIS-Cl at pH 8.0, 137 mmol/L NaCl, 5 mmol/L KCl, 0.05% Tween]) for 1 hour at 22°C, followed by incubation with anti-RET (H300), anti-PY-RET (Y1062), or antiactin (C4) in fresh blocking solution at 4°C overnight. The membranes were then washed in TBS-T and incubated with a 1:2000 dilution of the indicated HRPO-conjugated secondary antibody for 2 hours at 22°C. Membranes were then washed with TBS-T. Antibody detection was performed with an enhanced chemiluminescence reaction (Pierce Chemical Co, Rockford, Ill) according to the manufacturer’s recommendations.

RNA extraction, preparation of complementary DNA and quantitative polymerase chain reaction. TT cells were cultured for 24 hours in RPMI 1640 supplemented with 10% FCS, and subsequently treated with or without 15 μmol/L imatinib for 16 hours. Total RNA was isolated with the use of the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. The RNA concentration was determined by spectrophotometry (Nanadrop). Thereafter, 1 μg of total RNA was transcribed in first-strand complementary DNA (cDNA) with the use of the RTG First Strand cDNA kit (Amersham Biosciences, ‘s-Hertogenbosch, The Netherlands) according to the manufacturer’s protocol. The cDNA synthesis was primed by the pd(N)6 Random Hexamer (Amersham). Relative changes in transcript level were determined on the Icycler (Bio-Rad) with the use of the Quantitect SYBR Green PCR Kit (Qiagen) according to the manufacturer’s instructions.

Primer sequences used in this study were as follows: hypoxanthine phosphoribosyltransferase (HPRT)-forward: cggccaaagggaaagtgtg; HPRT-reverse: agttctggccttgcgg; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-forward: caccactctcacttttg; GAPDH-reverse: ccacacctcctggctgtg; RET-forward: tgggaatcctcggagga; RET-reverse: tactccagtgggagagc. The polymerase chain reaction (PCR) efficiencies for all primers used were between 96% and 99%. Data are expressed as fold-induction corrected for GAPDH and HPRT. All experiments were performed in duplicate.

Proliferation assay. To determine cell proliferation, we used a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics, Almere, The Netherlands). Briefly, cells were seeded at a density of 1 × 10^5 cells/well in 96-well microtiter plates, grown overnight, and exposed to different concentrations of imatinib or 0.1% DMSO as a control. After 1, 2, 3, 4, and 5 days of incubation, respectively, 10 μL of MTT reagent was added to each well, and the plate was incubated at 37°C for 4 hours. Formazan products were solubilized with 100 μL/well of the supplied detergent buffer, and the plate was incubated overnight at 37°C. The optical density was determined at 595 nm wavelength. To determine the inhibitory concentration of 50% (IC_{50}) of imatinib (the concentration that causes 50% growth inhibition), a concentration range of imatinib was added to the wells (quadruplicates), and IC_{50} was determined by using linear interpolation at r = 0.5. All experiments were performed in triplicate.

Cell cycle and apoptosis analysis. For fluorescence-activated cell sorting (FACS) analysis, cells were cultured subconfluent and treated with 15 μmol/L imatinib for 24, 48, 72, and 96 hours. The percentage of apoptotic cells was assessed with the use of an annexin V staining kit (IQ Products, Groningen, The Netherlands) according to the manufacturer’s recommendations. Briefly, cells were harvested, resuspended in 100 μL calcium buffer (2.6 mg/mL HEPES, 0.28 mg/mL CaCl₂, 8.18 mg/mL NaCl) containing 5 μL annexin V, and incubated for 20 minutes at 4°C in the dark. Cells were washed with 5 mL calcium buffer and subsequently incubated in 300 μL calcium buffer containing 2.5 μL propidium iodide (PI) for 10 minutes in the dark. Finally, binding of fluorescein-conjugated annexin V (apoptotic death) and PI (necrotic death) was measured by FACS (FACSCalibur; Becton Dickinson, Sunnyvale, Calif). For assessing cell-cycle distribution, cells were harvested, washed with PBS, and incubated in PI solution (0.1 μg/μL Rhoe A, 0.96 mg/mL sodium citrate-dihydrate, 0.02 μg/μL PI, 0.1 % Triton X-100) for 20 minutes at room temperature. Binding of PI was measured by FACS, and cell-cycle analysis was performed with the use of ModFit LT.

Statistical analysis. Values shown are depicted as mean ± SD. The relationship between continuous variables was analyzed with the use of the Student t test. In all tests, P < .05 was considered significant.
RESULTS

Imatinib inhibits RET autophosphorylation in MZ-CRC-1 and TT cells in a dose-dependent manner. Lysates prepared from MZ-CRC-1 and TT cells treated with imatinib for 90 minutes were analyzed by Western blotting with an anti-PY-RET (Y1062) antibody. Inhibition of RET Y1062 autophosphorylation was observed in a dose-dependent manner, with complete inhibition observed at concentrations higher than 15 μmol/L in both cell lines (Fig 1).

To exclude that the observed reduction in RET Y1062-phosphorylation was caused by decreased RET-protein levels, we stripped and reprobed the membrane with an anti-RET primary antibody. The total amount of expressed RET did not change after exposure to imatinib for 90 minutes indicating that imatinib inhibits RET autophosphorylation but does not affect the expression level of the RET protein (Fig 1).

Long-term exposure to imatinib induces RET oncoprotein levels. To investigate the effects of extended exposures to imatinib on RET expression and phosphorylation levels in MZ-CRC-1 and TT cells, we prepared cell lysates after 16 hours of exposure to imatinib and analyzed them by Western blotting. Complete inhibition of RET Y1062 autophosphorylation was observed in a dose-dependent manner, with complete inhibition observed at concentrations higher than 15 μmol/L in both cell lines (Fig 2). Reprobing the membrane with an anti-RET antibody showed that RET expression levels also were reduced by long-term imatinib treatment, indicating that reduced RET Y1062 phosphorylation levels are caused by reduced RET protein levels. In MZ-CRC-1 cells, expression levels of RET protein decreased dramatically, and, in TT cells, RET could not be detected at concentrations of 15 and 20 μmol/L (Fig 2). To determine whether equal amounts of protein were loaded, the blots were stripped and reprobed with an anti-actin antibody, and the total amount of actin did not change after exposure to imatinib.

We used the proteasome inhibitor MG132 to determine if RET was degraded by the proteasome-ubiquitin pathway under the influence of imatinib by adding 10 μmol/L MG132 to MZ-CRC-1 and TT cells in combination with increasing concentrations of imatinib. Cells were treated for 16 hours and whole-cell lysates were Western blotted and developed with an anti-RET antibody. In both cell lines, RET protein levels were still reduced by imatinib (Fig 3). MG132 did not have any influence on RET protein levels, and transcription was not altered by imatinib.

Therefore, we studied the effects of imatinib on RET gene expression by means of quantitative PCR (qPCR; Fig 4). Treatment of TT cells with 15 μmol/L imatinib for 16 hours did not result in a decreased synthesis at the transcription level. Therefore, we conclude that imatinib does not decrease RET protein levels by enhancing protein degradation through the proteasome-ubiquitin pathway or inhibition of RET gene transcription.

Imatinib inhibits proliferation of MZ-CRC-1 and TT cells. The effect of imatinib on cell growth of MTC-derived cell lines was measured by means of an MTT assay. MZ-CRC-1 and TT cells were incubated with increasing concentrations of imatinib for up to 5 days (Fig 5). To determine the IC50 of imatinib in MTC cell lines, MZ-CRC-1 and TT cells were grown in normal FCS-containing medium and exposed to increasing concentrations of imatinib (Fig 6). The concentrations of imatinib required inhibiting cell growth by 50% were 23 ± 2 μmol/L.
for MZ-CRC-1 cells and 25 ± 4 µmol/L for TT cells.

**Imatinib promotes cell cycle arrest and apoptosis.** We evaluated the contribution of cytotoxic and cytostatic components to imatinib-induced growth inhibition. First, simultaneous annexin V/PI staining was employed to determine whether cell death occurred and to clarify the nature of cell death in the treated cultures. FACS cell-cycle analysis showed that treatment with imatinib increased apoptotic cell death after 3 days of treatment (Fig 7, A). The fraction of apoptotic cells, however, is relatively small. Second, we determined the distribution over the different cell-cycle phases of MZ-CRC-1 cells treated with imatinib. For these experiments, only MZ-CRC-1 cells were used, as they proved to be diploid (data not shown) in contrast to TT cells, which were described as aneuploid (http://www.biotech.ist.unige.it/cldh/totcl4901.html). As shown in Figure 7, B, imatinib treatment increased the percentage of MZ-CRC-1 cells in the G0/G1-phase of the cell cycle. This increase of cells in the G0/G1-phase is accompanied by a reduced number of cells in the S-phase, supporting the observations obtained with the MTT assay. The analysis did reveal considerable significant (P < .001 for increase in S-phase; P = .003 for changes in G0/G1-phase) changes in the cell-cycle distribution pattern after 24 hours of incubation, compared with untreated cells. In conclusion, these data show that the main mechanism of imatinib-induced growth inhibition in vitro is through cell-cycle arrest in the G0/G1-phase, although induction of apoptosis plays a (small) role as well.

**DISCUSSION**

The increasing knowledge of the critical signaling pathways responsible for the growth of malignancies has lead to the development of specific inhibitors of these pathways as new tools in cancer therapy. Treatment of patients with CML, gastrointestinal stromal tumor, and dermatofibrosarcoma protuberaus with imatinib is an example of the positive clinical effects of the increasing knowledge of cancer-specific signal transduction inhibition and the use of specific signal transduction inhibitors.16–21

A tumor for which no systemic treatment is yet available is MTC. MTC is a malignancy caused by mutations in the RET gene, which result in a constitutive active receptor tyrosine kinase (RET-TK). Recent reports have demonstrated that inhibition of constitutive active RET-TK has cytostatic or cytotoxic effects on MTC cells.22,29–32 As the tyrosine kinase domain of RET shows high homology with that of the tyrosine kinases, which can be inhibited by imatinib, we tested the effects of imatinib on RET.

Our analyses demonstrate that imatinib indeed has a dose-dependent inhibitory effect on cell proliferation of both MZ-CRC-1 and TT cell lines, the only 2 available MTC cell lines. In addition, imatinib does inhibit RET specifically, since RET Y1062 autophosphorylation of both cell lines is inhibited selectively after 90 minutes of exposure to imatinib, whereas RET protein levels are not affected. As reduction of phosphorylation of RET was seen in both cell lines, imatinib seems not to be selective for the particular RET mutations present in these cell lines (G634W and M918T).

Skinner et al23 recently reported no significant inhibition of TT cell proliferation at 10 µmol/L of
imatinib. In the present study, proliferation of both MZ-CRC-1 and TT cells is inhibited by 10 μmol/L of imatinib; similar results were obtained by Cohen et al.22 These discrepancies possibly are caused by differences in experimental procedures employed by the different research groups. Another possibility could be that imatinib is not as effective after more than 5 days and that the effect is reversible. We observed that, in concentrations higher than 20 μmol/L, MZ-CRC-1 and TT cells stop proliferating. This phenomenon also was observed in cell lines that are not dependent on constitutive tyrosine kinase activity, such as HepG2. Probably imatinib’s effects on cell growth are due to general cytotoxicity.

Although we proved that RET is a direct target of imatinib, we also have reason to believe that this inhibitor has other targets in TT and MZ-CRC-1. This conclusion is based on observed clear differences in the concentration necessary to inhibit RET phosphorylation, compared with the concentration needed to inhibit cell proliferation. The concentration of imatinib that decreased autophosphorylation and RET content was more than 10 and 15 μmol/L, respectively. However, cell proliferation was already inhibited with 1 and 5 μmol/L imatinib. This difference suggests that cell proliferation in MTC probably is not entirely dependent on RET-TK activity, implying that imatinib acts on other cellular pathways as well.

When MTC cells are exposed to imatinib for a longer period, not only reduced Y1062 phosphorylation but also reduced levels of RET protein were observed. This might indicate that the observed reduction in Y1062-phosphorylation after 16 hours of treatment is a consequence of reduced RET expression levels. The studies we performed with the proteasome inhibitor MG132 show that RET protein is not subjected to proteasomal degradation. Carniti et al.32 demonstrated that PP1 inhibitors induce degradation of RET oncoproteins through proteasomal targeting. Imatinib, however, induces RET degradation through other means. Another possible mechanism for this loss of RET could be a diminished RET expression. We, however, could show by qPCR that the expression was not altered after 16 hours of treatment with imatinib. The mechanism by which the inhibition of RET phosphorylation could give rise to such a drastic effect on RET expression levels is, therefore, yet unknown. Possible explanations for the reduced
RET expression are interference at translation level or increased proteolysis of the RET protein.

The main mechanism of imatinib-induced growth inhibition is that of cell-cycle arrest. In cell-cycle analysis, we observed that imatinib induced a change in the distribution of cells over different cell-cycle phases, primarily a G0/G1 arrest (Fig 7, B). Induction of apoptosis plays a (small) role as well. It should be noted that the population of MZ-CRC-1 and TT cells decreases after 5 days of exposure to imatinib (data not shown). It could be that nonapoptotic pathways play a role in cell death as well, since only about 9% of cells were apoptotic after 4 days of treatment with imatinib. It should be noted that, in these experiments, cells have been used that grow in culture for many years. It is possible that primary MTCs respond differently to imatinib.

The proliferation data show dose-dependent inhibition of cell growth by imatinib with IC50 of 23 ± 2 and 25 ± 4 µmol/L in MZ-CRC-1 and TT cells, respectively. These concentrations are relatively high, compared with the concentrations sufficient for the inhibition of BCR-ABL and c-kit in other cancers (Fig 8). Plasma concentrations of imatinib inducing hematologic and cytogenetic responses in patients with CML were in the range of 0.17 to 5.68 µmol/L. These patients were treated with 25 to 600 mg imatinib per day. The maximal achievable plasma levels of imatinib in patients are no higher than 6.78 µmol/L at maximal administered doses of 600 mg daily. Higher doses of imatinib in clinical practice are difficult to achieve, because of adverse effects that are difficult for patients to tolerate. Moreover, the lethal effects of imatinib on BCR-ABL expressing K562 cells are
recently the kinase inhibitors ZD6474, CEP-701 and CEP-751, inhibitors of the Trk receptor tyrosine kinases, also have been shown to directly inhibit RET at nanomolar levels. PP1 shows similar activity. The IC_{50} of imatinib for RET is around 4 times higher than the clinically relevant concentrations that can be achieved are not likely to inhibit RET activity enough to be beneficial in patients with MTC, although MTC can behave differently in an in vivo model, and plasma levels of imatinib do vary to a great extent among patients. Specific inhibition of the RET-TK is a very attractive treatment option for patients with MTC. Recently the kinase inhibitors ZD6474, CEP-701 and CEP-751, and PPI have been shown to inhibit RET-TK at clinically achievable levels. ZD6474, an inhibitor of the vascular endothelial growth factor receptor-2, has a reported IC_{50} of 100 nmol/L for RET. The indolocarbazole compounds CEP-701 and CEP-751, inhibitors of the Trk receptor tyrosine kinases, also have been shown to directly inhibit RET at nanomolar levels. PPI shows similar IC_{50} (80 nmol/L) for RET, but inhibits other kinases at similar or lower concentrations than RET as well. None of these agents, however, has been used in large-scale placebo-controlled trials in humans, and side effects are not yet known. Furthermore, in MTC, prolonged administration of these inhibitors probably is necessary. It would be preferable to use a tyrosine kinase inhibitor specific for RET to minimize the potential side effects.

CONCLUSION

We have shown that imatinib inhibits MTC cell proliferation and RET Y1062 phosphorylation, leading to decreased RET expression in cell lines harboring RET mutations associated with MEN 2A and MEN 2B. The concentration of imatinib necessary to inhibit RET in vitro, however, makes it impossible to conclude at this time that imatinib monotherapy will be a good option for systemic therapy of MTC. In vivo studies will be necessary to make firm conclusions concerning the clinical application of this tyrosine kinase inhibitor as an effective treatment for MTC.

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