The Effects of Four Different Tyrosine Kinase Inhibitors on Medullary and Papillary Thyroid Cancer Cells

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Context: Medullary and papillary thyroid carcinoma (MTC and PTC) are two types of thyroid cancer that can originate from activating mutations or rearrangements in the RET gene. Therapeutic options are limited in recurrent disease, but because RET is a tyrosine kinase (TK) receptor involved in cellular growth and proliferation, treatment with a TK inhibitor might be promising. Several TK inhibitors have been tested in clinical trials, but it is unknown which inhibitor is most effective and whether there is any specificity for particular RET mutations.

Objective: We aimed to compare the effect of four TK inhibitors (axitinib, sunitinib, vandetanib, and XL184) on cell proliferation, RET and ERK expression and autophosphorylation, and ERK activation in cell lines expressing a MEN2A (MTC-TT), a MEN2B (MZ-CRC-1) mutation, and a RET/PTC (TPC-1) rearrangement.

Design: The three cell lines were cultured and treated with the four TK inhibitors. Effects on cell proliferation and RET and ERK expression and activation were determined.

Results: XL184 and vandetanib most effectively inhibited cell proliferation, RET autophosphorylation in combination with a reduction of RET expression, and ERK phosphorylation in MTC-TT and MZ-CRC-1, respectively. TPC-1 cells showed a decrease in RET autophosphorylation after treatment with XL184, but no effect was observed on ERK activation.

Conclusion: There is indeed specificity for different RET mutations, with XL184 being the most potent inhibitor in MEN2A and PTC and vandetanib the most effective in MEN2B in vitro. No TK inhibitor was superior for all the cell lines tested, indicating that mutation-specific therapies could be beneficial in treating MTC and PTC. (J Clin Endocrinol Metab 96: E991–E995, 2011)

Medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma (PTC) can be caused by activating mutations or rearrangements of the rearranged during transfection (RET) gene. MTC originates from the calcitonin-producing C cells of the thyroid and can occur sporadically (75%) or as part of a familial cancer syndrome (25%). The latter occurs as multiple endocrine neoplasia (MEN) type 2 syndrome (MEN2A and MEN2B) or as familial MTC (1–3). PTC is the most common thyroid cancer (80% of all thyroid cancers) and originates from the follicular epithelial cells of the thyroid. In 2.5–40% of PTC, a RET rearrangement is found, although this percentage is higher in patients exposed to radiation (1).

Total thyroidectomy and extensive lymph node dissection is the curative treatment for MTC and PTC, followed by radioiodine ablation in PTC. However, recurrent disease is often seen in sporadic MTC, and until recently, reoperation was the only therapeutic option. In iodine-refractory PTC, no effective adjuvant therapy is available...
as well (4, 5). New systemic therapies are therefore needed for both recurrent MTC and PTC.

With RET being the gene involved in a subset of MTC and PTC, it is logical to consider the encoded receptor as an important target for systemic therapy. RET is expressed in all tumor cells and continuous autophosphorylation on its tyrosine kinase (TK) residues caused by mutations (MEN2) or rearrangements (PTC) on RET results in a constant activation of downstream signaling pathways that ultimately lead to tumor growth (1). Therefore, inhibition of RET phosphorylation and its downstream pathways could be of great value.

Small-molecule inhibitors that selectively inhibit TK have been proven to be effective in the treatment of several neoplastic diseases (6–8). A number of these clinically useful inhibitors target TK receptors that belong to the same family group of proteins as RET (9). Several TK inhibitors have already been tested in vitro and evaluated in phase II clinical trials (Table 1). In a large number of patients (25–81%), a stable disease state can be established, and some patients even show a partial response (2–33%) (10–19).

Because most studies have focused on one particular TK inhibitor and have not looked for mutation specificity, it is hard to compare these compounds for the different patient groups. We therefore set out to compare the efficiency of four recently developed TK inhibitors, XL184, vandetanib, sunitinib, and axitinib, using three cell lines: MTC-TT reported to be derived from a sporadic MTC expressing a C634W RET mutation, and TPC-1 derived from a patient with PTC expressing a RET/PTC-1 rearrangement.

Materials and Methods

Cell culture
MZ-CRC-1, MTC-TT, TPC-1, and HEK293 (human embryonic kidney cells) cell lines were cultured as described in Supplemental Materials and Methods. All experiments were performed in triplicate.

Extraction and RT-PCR procedures are described in the Supplemental Materials and Methods. All experiments were performed according to the manufacturer’s instructions. The concentration that led to 50% growth inhibition (IC50) was determined using linear interpolation at r = 0.5 (Supplemental Table 1). If IC50 concentrations were between 0.5 and 5 μM, additional cell proliferation assays were performed (Supplemental Fig. 1, A–D). All experiments were performed in duplicate.

Cell lysates and Western blot analysis
MTC-TT and MZ-CRC-1 cells were plated in 200 μl medium at concentrations of 4 × 104 cells per well. TPC-1 and HEK293 cells were plated at a density of 2 × 105 cells per well. After overnight incubation, increasing concentrations of TK inhibitor solutions (0, 0.005, 0.05, 0.1, 0.5, and 5 μM) were added. A concentration of 0.1% DMSO was used in all experiments. Control cells were grown without DMSO or TK inhibitor. Proliferation was measured at 1, 4, and 7 d using a cell proliferation kit (MTT assay; Roche, Almere, The Netherlands) according to the manufacturer’s instructions. The concentration that led to 50% growth inhibition (IC50) was determined using linear interpolation at r = 0.5 (Supplemental Table 1). If IC50 concentrations were between 0.5 and 5 μM, additional cell proliferation assays were performed (Supplemental Fig. 1, A–D). All experiments were performed in triplicate.

RNA extraction and RT-PCR
MTC-TT and MZ-CRC-1 cells, treated with 0, IC50, and maximal concentrations of the different TK inhibitors for 0, 2, and 5 d. Cell lysates were prepared as described in Supplemental Materials and Methods, and supernatants were stored at −80°C before they were further processed for SDS-PAGE followed by Western blot analysis. The antibodies used are described in Supplemental Materials and Methods. All experiments were performed in duplicate.

Table 1. Molecular targets and clinical trials performed with TK inhibitors in MTC patients

<table>
<thead>
<tr>
<th>Molecular targets</th>
<th>No. of patients</th>
<th>Tumor response [% (n)]</th>
<th>Stable disease [% (n)]</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>Bcr-Abl, PDGFR-α and -β, c-Fms, c-Kit, RET</td>
<td>24</td>
<td>0</td>
<td>38 (9)</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>RAF, VEGFR2 and -3, PDGFR, RET</td>
<td>21</td>
<td>10 (2)</td>
<td>86 (18)</td>
</tr>
<tr>
<td>Motesanib</td>
<td>VEGFR1, -2, and -3, PDGFR, c-Kit, RET</td>
<td>91</td>
<td>2 (2)</td>
<td>81 (74)</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>EGFR</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>VEGFR, EGFR, RET</td>
<td>30</td>
<td>20 (6)</td>
<td>53 (16)</td>
</tr>
<tr>
<td>Axitinib</td>
<td>VEGFR1, -2, and -3</td>
<td>19</td>
<td>16 (3)</td>
<td>53 (10)</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>RET, VEGFR, PDGFR</td>
<td>11</td>
<td>18 (2)</td>
<td>27 (3)</td>
</tr>
<tr>
<td>XL184</td>
<td>MET, VEGFR2, RET</td>
<td>37 (35 with measurable disease)</td>
<td>29 (10)</td>
<td>41 (15)</td>
</tr>
</tbody>
</table>

* Clinical trial performed in advanced thyroid cancer patients, not only MTC patients.

** Phase 1 trial.
Statistical analysis

The statistical analysis was performed using the software program SPSS version 16.0.

Results

Effect of different TK inhibitors on cell proliferation

A dose-dependent decrease in cell proliferations was seen for all four inhibitors (Fig. 1, A–C, and Supplemental Fig. 1, A–D). In contrast, HEK293 cells, who do not endogenously express RET, showed only minor effects on proliferation at concentrations lower than 0.5 µM (Supplemental Fig. 2A). Based on the IC₅₀ values (Supplemental Fig. 2B and Supplemental Table 1), we observed that XL184 was the most effective inhibitor of MTC-TT (IC₅₀ = 0.04 µM) and TPC-1 (IC₅₀ = 0.06 µM) proliferation, whereas for MZ-CRC-1, vandetanib inhibited cell proliferation at the lowest concentration (IC₅₀ = 0.26 µM). DMSO did not seem to have a significant negative effect on cell growth of any of the cell lines tested (Supplemental Table 2).

Effect of XL184 and vandetanib on RET autophosphorylation

Inhibition of RET autophosphorylation on tyrosine 1062 was observed for the three cell lines tested after 2 d of treatment with XL184 (MTC-TT and TPC-1) and vandetanib (MZ-CRC-1). However, only vandetanib was able to induce this effect with IC₅₀ levels (Fig. 1D). For MTC-TT and TPC-1, 5 d exposure to IC₅₀ concentrations of XL184 were necessary to reduce RET autophosphorylation levels (Fig. 1, E and F).

FIG. 1. Effect of XL184, vandetanib, sunitinib, and axitinib on cell proliferation. A–C, Dose-response curves of MZ-CRC-1 (A), MTC-TT (B), and TPC-1 (C) cell lines incubated with different concentrations of XL184, vandetanib, sunitinib, and axitinib; D–F, effect of XL184 and vandetanib on RET expression, RET autophosphorylation, ERK expression, and ERK phosphorylation: MZ-CRC-1 cells treated with IC₅₀ concentration and 5 M vandetanib for 2 and 5 d (D), MTC-TT (E), and TPC-1 (F) cells treated with IC₅₀ concentration and 5 M XL184 for 2 and 5 d. Error bars shown correspond to SD.
To investigate whether the decrease in RET autophosphorylation was due to reduced levels of RET expression, the membranes were stripped and probed for RET. MTC-TT and MZ-CRC-1 cells showed a dose-dependent response after 5 d incubation with XL184 and vandetanib, respectively. At IC_{50} values, a marked decreased in RET expression was observed, and at maximal concentrations, RET was no longer detectable (Fig. 1, D and F). To determine whether this effect was due to inhibition of RET transcription, RET expression levels were determined. For MTC-TT, no effect on RET transcription levels was observed. However, for MZ-CRC-1, 5 d exposure to maximal concentrations of vandetanib led to a decrease in RET expression (Supplemental Fig. 3, A–C). For TPC-1 cells, the total amount of RET remained relatively unchanged even after 5 d exposure to maximal concentrations of XL184 (Fig. 1F).

**Effect of XL184 and vandetanib on RET downstream signaling pathways**

RET is involved in the activation of several signaling pathways, including the MAPK pathway (20). Therefore, we evaluated the effect of XL184 and vandetanib in ERK activation. For MTC-TT and MZ-CRC-1, ERK phosphorylation was markedly reduced with IC_{50} levels of XL184 and vandetanib, respectively, and was totally inhibited when maximal concentrations were used (Fig. 1, D and E). However, XL184 was able to exert this effect after only 2 d exposure. Interestingly, this reduction of ERK activation was related to a decrease in ERK expression (Fig. 1, D and E). For TPC-1, no effect on ERK expression and activation was observed, even when maximal concentrations of XL184 were used (Fig. 1F).

**Discussion**

We compared the effects of four TK inhibitors, XL184, vandetanib, sunitinib, and axitinib, on cell proliferation and RET inhibition and looked for mutation specificity using cell lines harboring a MEN2A mutation (MTC-TT), a MEN2B mutation (MZ-CRC-1), and a RET/PTC rearrangement (TPC-1). Our results showed that all four TK inhibitors are capable of reducing cell proliferation to some extent. However, XL184 was found to be the most efficient inhibitor for MEN2A- and PTC-derived cell lines, whereas vandetanib proved to be the most potent inhibitor for MEN2B.

We also showed that XL184 and vandetanib were able to decrease RET autophosphorylation and expression levels in MTC-TT and MZ-CRC-1 cells, respectively. However, only vandetanib exerted this effect by inhibiting RET transcription. It is possible that for XL184, lysosomal or proteasomal degradation is involved, as was described for other inhibitors (20). For TPC-1 a marked decrease in RET phosphorylation levels was detected, but surprisingly, RET/PTC expression levels increased after exposure to XL184. This dual effect might be the result of a negative feedback mechanism to compensate a reduction in RET activation. Furthermore, it shows that XL184 exerts its effect in PTC by direct inhibition of RET autophosphorylation and that lysosomal or proteasomal degradation may not be effective due to the presence of the fusion protein.

Finally, we explored the effects of these drugs in a downstream signaling pathway directly activated by RET, the MAPK pathway. For MTC-TT and MZ-CRC-1, exposure to XL184 and vandetanib, respectively, induced a marked decrease in ERK phosphorylation. Interestingly, a reduction on ERK expression was also observed for these cell lines, suggesting a possible effect of XL184 and vandetanib on RET transcription. For the TPC-1 PTC model system, no change in ERK phosphorylation was detected after exposure to XL184. ERK phosphorylation through other TK receptors and TK effector molecules, e.g., BRAF, are likely to exert a stronger effect in ERK phosphorylation than RET. It is possible that for PTC, a combination of different inhibitors targeting RET and, e.g., BRAF (serine/threonine-protein kinase B-Raf), could circumvent this problem and thus result in an even more effective treatment for this type of cancer.

Because no TK inhibitor was superior for the cell lines tested, our in vitro results suggest that mutation-specific therapies could be beneficial for the treatment of MTC and PTC. However, because only three different mutations were analyzed, additional mutational studies are necessary to confirm this specificity.

To date, no distinction has been made between the different RET-related mutational subtypes (MEN2A/MEN2B/PTC) in clinical trials, and it is known that aspecific targeting of TK inhibitors can also contribute to antitumor effects. However, the risk of severe side effects for the patients in combination with the development of resistance to the TK inhibitors incorrectly used reinforces the need of mutation-specific therapies. Furthermore, the combined use of different TK inhibitors for multiple targeting will also benefit from this knowledge, because only then optimal combinations of inhibitors can be chosen.

In conclusion, our results are in general agreement with the outcome of the clinical trials, supporting the idea that XL184 and vandetanib are two potent inhibitors for tumor response in MTC. We also showed that there is specificity of these inhibitors for the treatment of different RET mutations, which suggest that mutation-specific therapies...
will likely improve the outcome of ongoing studies. Thus, reanalysis of already performed trials based on mutation status is more than worthwhile.

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