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The *BRAF*^{T1799A} mutation confers sensitivity of thyroid cancer cells to the *BRAF*^{V600E} inhibitor PLX4032 (RG7204)

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Abstract

Aberrant signaling of the Ras-Raf-MEK-ERK (MAP kinase) pathway driven by the mutant kinase *BRAF*^{V600E}, as a result of the *BRAF*^{T1799A} mutation, plays a fundamental role in thyroid tumorigenesis. This study investigated the therapeutic potential of a *BRAF*^{V600E}-selective inhibitor, PLX4032 (RG7204), for thyroid cancer by examining its effects on the MAP kinase signaling and proliferation of 10 thyroid cancer cell lines with wild-type *BRAF* or *BRAF*^{T1799A} mutation. We found that PLX4032 could effectively inhibit the MAP kinase signaling, as reflected by the suppression of ERK phosphorylation, in cells harboring the *BRAF*^{T1799A} mutation. PLX4032 also showed a potent and *BRAF* mutation-selective inhibition of cell proliferation in a concentration-dependent manner. PLX4032 displayed low IC₅₀ values (0.115–1.156 μM) in *BRAF*^{V600E} mutant cells, in contrast with wild-type *BRAF* cells that showed resistance to the inhibitor with high IC₅₀ values (56.674–1349.788 μM). Interestingly, cells with *Ras* mutations were also sensitive to PLX4032, albeit moderately. Thus, this study has confirmed that the *BRAF*^{T1799A} mutation confers cancer cells sensitivity to PLX4032 and demonstrated its specific potential as an effective and *BRAF*^{T1799A} mutation-selective therapeutic agent for thyroid cancer.

Keywords

Thyroid cancer; PLX4032; RG7204; *BRAF* mutation; *BRAF* inhibitor; Genetic-dependent therapy

1. Introduction

Thyroid cancer is a common endocrine malignancy, which has seen a rapid rise in incidence in recent years, with 44,670 new cases and 1690 deaths estimated for the year 2010 in the United States [1]. Follicular epithelial cell-derived thyroid cancer histologically consists mainly of papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC) [2]. PTC is the most common type of thyroid cancer, accounting for 80% of all thyroid cancers. ATC is relatively uncommon but is a rapidly aggressive and

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lethal cancer. Although PTC, as a differentiated cancer, is usually curable in most patients with current surgical and radioiodine treatments, many cases of patients with this cancer fail these standard treatments. Therefore, new treatments are needed for patients currently with incurable thyroid cancer.

Among different thyroid cancers, the *BRAF*^{T1799A} mutation is exclusively found in PTC and ATC, occurring in 45% of cases of the former and 25% of cases of the latter [3]. This mutation leads to an amino acid change from valine to glutamic acid in position 600 of the *BRAF* protein kinase, resulting in the constitutively activated *BRAF*^{V600E} mutant. *BRAF*^{V600E} causes constant activation of the Ras-Raf-MEK-ERK pathway (MAP kinase pathway). MAP kinase pathway normally regulates a variety of molecular and cellular functions, including cell proliferation and growth. By over-activating this pathway, *BRAF*^{V600E} plays an important role in the pathogenesis and aggressiveness of thyroid cancer [4,5]. Thus, *BRAF* has become a popular target in the pursuit of new treatments for thyroid cancers [6].

BRAF inhibitors that were developed to selectively target *BRAF*^{V600E} for treatment of human cancers have recently received particular attention [7]. These *BRAF*^{V600E}-selective inhibitors are small-molecule ATP-competitive kinase inhibitors. They were shown to potently inhibit melanoma cells harboring the *BRAF*^{T1799A} mutation, much less so in melanoma cells harboring the wild-type *BRAF* gene [8,9]. Recent clinical studies reported exciting results on the therapeutic effects of *BRAF*^{V600E}-selective inhibitors in melanoma, as exemplified by PLX4032 [10,11]. Given the common occurrence of *BRAF*^{T1799A} mutation in thyroid cancer, it is expected that these inhibitors may also be effective drugs to treat patients with thyroid cancer harboring this mutation. In this study, we investigated the therapeutic potential of PLX4032 for thyroid cancer by examining its *BRAF*^{T1799A} mutation-dependent inhibition of the MAP kinase pathway and the proliferation of thyroid cancer cells.

2. Materials and methods

2.1. Cell cultures

Ten human thyroid cancer cell lines were used in this study, including OCUT1 originally from Dr. Naoyoshi Onoda (Osaka City University Graduate School of Medicine, Osaka, Japan); C643, Hth7, Hth74, and SW1736 from Dr. N.E. Heldin (University of Uppsala, Uppsala, Sweden); FTC133 from Dr. Georg Brabant (University of Manchester, Manchester, United Kingdom); K1 from Dr. David Wynford-Thomas (University of Wales College of Medicine, Cardiff, United Kingdom); KAT18 from Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY); WRO-82-1 from Dr. G.J.F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA); and BCPAP from Dr. Massimo Santoro (University of Federico II, Naples, Italy). These cells could be divided into three groups: Group I – OCUT1, SW1736, K1, and BCPAP harboring the *BRAF*^{T1799A} mutation; Group II – FTC133, KAT18, Hth74, and WRO harboring neither *BRAF* mutation nor *Ras* mutations; and Group III – C643 and Hth7 harboring *Ras* mutations. Except for FTC133, these cells were normally cultured and maintained in RPMI 1640 medium at 37 °C and 5% CO₂, supplemented with 10% fetal bovine serum (FBS), 5%

sodium pyruvate, 5% MEM nonessential amino acids, and 5% penicillin streptomycin. FTC133 cells were cultured in DMEM/HAM'S F-12 medium instead. When cells were treated with PLX4032 (Plexxikon Inc., Berkeley, CA 94710), 5% FBS was used in the medium instead of 10%.

2.2. Western blotting

Cells were lysed in RIPA buffer containing 1% phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). Cell lysates were kept on ice during the procedure and proteins were quantified using a spectrophotometer. Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% acrylamide, followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The PVDF membranes were then blotted with primary antibodies anti-phospho-ERK (sc-7383), anti-ERK1 (sc-94), or anti-Actin (sc-1616-R) (all from Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were subsequently washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit (sc-2204) or anti-mouse (sc-2005) secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were visualized using a chemiluminescent enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.3. Cell proliferation assay

Thyroid cancer cells grown in attachment to flask wall were freed by trypsin-EDTA treatment and resuspended in culture medium containing 10% FBS. An aliquot of 800 cells was transferred and seeded onto wells in triplicates on 96-well plates. During a 5-day treatment, cells were treated with 7 different concentrations of the BRAF^{V600E}-selective inhibitor PLX4032, including 0, 0.01, 0.03, 0.10, 0.30, 1.00, and 3.00 μ M under standard culture conditions. The culture medium containing the inhibitor at the required concentrations was replenished daily. During the treatment with PLX4032, medium was changed to contain 5% FBS, instead of 10%.

Cell proliferation was measured using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after 5 days of treatment with PLX4032, 10 μ L of 5 mg/mL MTT agent (Invitrogen Carlsbad, CA) was added to cells with a 4-h incubation. This was followed by an overnight incubation with 100 μ L of 10% sodium dodecyl sulfate solution. This led to the creation of purple crystals in the mitochondria of cells, which were then dissolved in the medium. Spectrophotometry was performed to measure the absorbance at the test wavelength of 570 nm and the reference wavelength of 670 nm. Relative cell growth was reflected by the intensity of the purple color. IC₅₀ values of PLX4032 were calculated for each cell line using a computer program based on the classical Reed-Muench method [12].

3. Results

3.1. *BRAF*^{T1799A}-dependent inhibition of the MAP kinase pathway signaling by the *BRAF*^{V600E} inhibitor PLX4032

Phosphorylation of ERK is a key step down stream of *BRAF* and MEK in the signaling of the MAP kinase pathway and is widely used as a measure of the signaling activity of this pathway. We therefore examined the effect of the *BRAF*^{V600E} inhibitor PLX4032 on the phosphorylation of ERK (p-ERK) in thyroid cancer cell lines. As shown in Fig 1A in both OCUT1 and K1 cells, which harbored the *BRAF*^{T1799A} mutation, PLX4032 potently inhibited p-ERK; significant inhibition was seen at 0.1 μ M and nearly complete inhibition was seen at 0.5 μ M. This effect of PLX4032, as shown in Fig 1B, was quick; complete inhibition occurred within 1 h after the addition of the inhibitor and significant inhibition remained at 24 h. As shown in Fig 1C, p-ERK in the *BRAF* mutation-harboring SW1736 and BCPAP cells was also inhibited by PLX4032. In the *BRAF* mutation-negative cells, including FTC133, KAT18, Hth74, and WRO cells, PLX4032 did not show significant effect on p-ERK. PLX4032 paradoxically had a stimulatory effect on p-ERK in C643 and Hth7 cells that harbored *Ras* mutations (Fig 1C). This paradoxical activation of MAP kinase pathway by PLX4032 was consistent with the finding of similar stimulatory effects of *BRAF*^{V600E} inhibitors on the MAP kinase pathway in other cancer cells harboring *Ras* mutations, which was hypothesized to be due to Ras activity-facilitated transactivation of RAF dimers [13].

3.2. *BRAF*^{T1799A}-dependent inhibition of the proliferation of thyroid cancer cells by the *BRAF*^{V600E} inhibitor PLX4032

As the *BRAF*^{V600E} inhibitor PLX4032 could effectively inhibit the signaling of the MAP kinase pathway in *BRAF*^{T1799A} cells, as presented above, we next examined the effects of this drug on the proliferation of thyroid cancer cells. As shown in Fig 2, thyroid cancer cell lines OCUT1, SW1736, K1, and BCPAP that harbored the *BRAF*^{T1799A} mutation were effectively inhibited by PLX4032 in a dose-dependent manner. This inhibition was very potent as reflected by low IC₅₀ values, ranging from 0.1149 to 1.156 μ M (Table 1). For comparison, we tested the thyroid cancer cells that did not have *BRAF*^{T1799A} mutation, including FTC133, KAT18, Hth74, and WRO cells. As shown in Fig 2, these cells resisted the inhibition by PLX4032. This resistance was well reflected by high IC₅₀ values in these cells, ranging from 56.674 to 1349.788 μ M (Table 1). We also tested C643 and Hth7 cells that did not harbor the *BRAF*^{T1799A} mutation but harbored *Ras* mutations. As shown in Fig 2, these two cells showed much better responses to PLX4032 than the cells (FTC133, KAT18, and WRO) that did not harbor *BRAF* or *Ras* mutations. This was also reflected by the much lower IC₅₀ values in the former cells than the latter cells (Table 1). The mechanism for the differential effects of PLX4032 on cell proliferation (Fig 2 and Table 1) and ERK phosphorylation (Fig 1) in *Ras* mutation-harboring cells is not clear. However, the responses of the two *Ras* mutation-harboring cells to PLX4032 were relatively modest compared with the cells harboring the *BRAF*^{T1799A} mutation, with IC₅₀ values in the former being several times to 25 times higher than the latter (Table 1).

4. Discussion

Genetic-based cancer therapy targeting specific mutations has become an increasingly recognized effective therapeutic strategy for many human cancers [14–16], including thyroid cancer [17]. Given the common occurrence of the *BRAF*^{T1799A} mutation and its important oncogenic role in thyroid cancer pathogenesis and aggressiveness [4,5], in this study, we tested the hypothesis that inhibitors of *BRAF*^{V600E}, the resulting *BRAF* mutant of the *BRAF*^{T1799A} mutation, might be particularly effective in inhibiting thyroid cancer cells harboring the *BRAF*^{T1799A} mutation and may thus be used for *BRAF* mutation-targeted therapy for thyroid cancer. We found that, in comparison with thyroid cancer cells harboring the wild-type *BRAF* gene, thyroid cancer cells harboring the *BRAF*^{T1799A} mutation responded far more effectively to the *BRAF*^{V600E} inhibitor PLX4032 in the inhibition of the signaling of the MAP kinase pathway. Correspondingly, proliferation of thyroid cancer cells harboring the *BRAF* mutation was more potently and efficaciously inhibited by PLX4032 with much lower IC₅₀ values, in nano molar to low micro molar ranges, in striking contrast with cells harboring no mutations. These data sufficiently and effectively demonstrated a high *BRAF*^{T1799A} mutation-selective sensitivity of thyroid cancer cells to PLX4032, consistent with its expected function as a *BRAF*^{V600E}-selective *BRAF* inhibitor. Our results are very similar to the findings reported recently on the effects of *BRAF*^{V600E} inhibitors on melanoma cells [8,9]. The results in this study on the *BRAF*^{T1799A}-dependent effects of PLX4032 are also consistent with the results in a recent study by Salerno et al. on PLX4032 in thyroid cancer cells [18]. This study included a number of different thyroid cancer cell lines than the Salerno et al. study [18] and still showed the *BRAF*^{T1799A} dependency of PLX4032, further demonstrating the *BRAF*^{T1799A}-dependent therapeutic potential of *BRAF*^{V600E} inhibitors for thyroid cancer.

In this study, the two *Ras* mutation-harboring cells, C643 and Hth7, showed less sensitivity to PLX4032 than *BRAF*^{T1799A} mutation-harboring cells (OCUT1, SW1736, K1, and BCPAP). But the response was more potent than the wild-type cells – cells without *BRAF* mutation or *Ras* mutations (FTC133, KAT18, Hth74, and WRO). This seems to be somewhat different than the conclusion on PLX4032 in these two cells in the Salerno et al. study [18], in which the authors stated that these *Ras* mutation-harboring cells did not respond to PLX4032. On closer examination of the Salerno et al. study [18], however, it is clear that at a concentration of around 0.5 μ M at four days of treatment, PLX4032 did show a visible inhibitory effect on the *Ras* mutation-harboring cells; at the highest concentration –1.0 μ M used in the Salerno et al. study [18], PLX4032 had a further inhibitory effect on these cells. These results, in fact, seemed to be consistent with the findings in this study at similar drug concentrations (Fig 2). In this study, at the additional concentration point of 3.0 μ M, PLX4032 caused a further inhibition, with relatively low IC₅₀ values calculated for these two *Ras* mutation-harboring cells (Table 1). The inhibition in these cells by PLX4032 did not seem to be attributable to a non-specific effect as this inhibition was not as significant in the wild-type cells (FTC133, KAT18, Hth74, and WRO) at the same drug concentrations, particularly at 0.30 and 1.00 μ M (Fig 2). It is noticeable that the treatment duration of cells with PLX4032 in the Salerno et al. study [18] was 4 days, while our drug treatment duration was longer (5 days). This may be a reason that we observed a more

remarkable inhibition of the two *Ras* mutation-harboring cells by PLX4032, particularly at concentration points 0.30, 1.00, and 3.00 μ M (Fig 2). It has been reported that melanoma cells and other cancer cells harboring *Ras* mutations did not respond to BRAF^{V600E} inhibitors [9,13]. It would be interesting to investigate the molecular mechanisms to explain why proliferation of some of the thyroid cancer cells harboring *Ras* mutations could be inhibited by PLX4032, albeit relatively modestly.

In summary, this study demonstrates that the BRAF^{V600E}-selective inhibitor PLX4032 can selectively inhibit thyroid cancer cells that harbor the BRAF^{T1799A} mutation. Both qualitative and quantitative data support the high effectiveness and potency of PLX4032 in inhibiting thyroid cancer cells harboring the BRAF^{T1799A} mutation. Thus, the BRAF^{T1799A} mutation confers thyroid cancer cells a significant sensitivity to PLX4032. Together with previous studies, this study provides strong implications for a therapeutic opportunity for PLX4032 as an effective drug for currently incurable types of PTC and ATC that harbor the BRAF^{T1799A} mutation. Given the recent strong clinical data that demonstrated remarkable therapeutic effects of BRAF^{V600E} inhibitors in melanoma patients [10,11], the present preclinical study supports the pursuit of a clinical trial on PLX4032 in thyroid cancer patients.

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Fig. 1.

Effects of the BRAF^{V600E} inhibitor PLX4032 on ERK phosphorylation in thyroid cancer cells. (A) Concentration-dependent responses of PLX4032 in OCUT1 and K1 cells. Cells were treated with PLX4032 at the indicated concentrations for 6 h in culture medium containing 5% FBS. Cell lysates were analyzed by Western blotting with antibodies specific to phospho-ERK (p-ERK), ERK1, and β-actin. ERK1 and β-actin were analyzed simultaneously for quality control of proteins. (B) Time-dependent response of the effects of PLX4032 in OCUT1 and K1 cells. Cells were treated with PLX4032 at 1 μM for the indicated times and Western blotting assay was performed as in Fig 1 A. (C) Effects of PLX4032 on p-ERK in the remaining thyroid cancer cells. Cells were treated with PLX4032 at 1 μM for 24 h. Western blotting assay was performed as in Fig 1A.

Fig. 2. *BRAF*^{T1799A} mutation-dependent inhibition of thyroid cancer cells by the *BRAF*^{V600E} inhibitor PLX4032. Cells were treated with the indicated concentrations of PLX4032 for 5 days with the drug replenished daily by changing the culture medium containing 5% FBS. MTT assay of cell proliferation was performed at the end of the 5-day treatment. Cells in the upper row harbor the *BRAF*^{T1799A} mutation, cells in the lower row harbor *Ras* mutations, and cells in the middle row are wild-type, harboring neither *BRAF* nor *Ras* mutations. The values on the y-axis represent the relative cell growth rate, with control growth (in the absence of the drug) being defined as 1.

Table 1

Mutation status of thyroid cancer cell lines and their sensitivities to the BRAF^{V600E} inhibitor PLX4032 – IC₅₀ Values.

Cell Lines	Genetic Alterations	IC ₅₀ (μM)
OCUT1	<i>BRAF</i> (V600E ^{+/-})	0.410
SW1736	<i>BRAF</i> (V600E ^{+/-})	0.115
K1	<i>BRAF</i> (V600E ^{+/-})	0.827
BCPAP	<i>BRAF</i> (V600E ^{+/+})	1.156
FTC133	–	56.674
KAT18	–	541.660
Hth74	–	1349.788
WRO	–	943.728
C643	<i>HRAS</i> (G13R ^{+/-})	2.896
Hth7	<i>NRAS</i> (Q61R ^{+/-})	0.973

+/-, heterozygous mutation; +/+, homozygous mutation.