Cytostatic Activity of Adenosine Triphosphate-Competitive Kinase Inhibitors in *BRAF* Mutant Thyroid Carcinoma Cells

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Context: The V600E mutation accounts for the vast majority of thyroid carcinoma-associated BRAF mutations.

Objective: The aim was to study the effects of the two BRAF V600E ATP-competitive kinase inhibitors, PLX4032 and PLX4720, in thyroid carcinoma cell lines.

Experimental Design: We examined the activity of PLX4032 and PLX4720 in thyroid carcinoma cell lines harboring BRAF V600E (8505C, BCPAP, SW1736, BHT101), NRAS Q61R (HTH7), KRAS G12R (CAL62), HRAS G13R (C643), or RET/PTC1 (TPC-1) oncogenes. Normal thyrocytes (PC Cl 3) were used as control.

Results: Both compounds inhibited the proliferation of BRAF mutant cell lines, but not normal thyrocytes, with a half maximal effective concentration (EC_{50}) ranging from 78–113 nM for PLX4720 and from 29–97 nM for PLX4032. Doses equal to or higher than 500 nM were required to achieve a similar effect in BRAF wild-type cancer cells. Phosphorylation of ERK 1/2 and MAPK kinase (MEK) 1/2 decreased upon PLX4032 and PLX4720 treatment in BRAF mutant thyroid carcinoma cells but not in normal thyroid cells or in cell lines harboring mutations of RAS or RET/PTC1 rearrangements. PLX4032 and PLX4720 treatment induced a G_1 block and altered expression of genes involved in the control of G_1-S cell-cycle transition. 8505C cell tumor xenografts were smaller in nude mice treated with PLX4032 than in control mice. This inhibition was associated with reduction of phospho-ERK and phospho-MEK levels.

Conclusions: This study provides additional evidence of the promising nature of mutant BRAF as a molecular target for thyroid carcinoma cells. (***J Clin Endocrinol Metab*** **95: 450–455, 2010)**

There is no effective systemic treatment for papillary thyroid carcinoma patients if their disease does not concentrate radioiodine (1). Anaplastic thyroid carcinoma is a very rare tumor with a dismal prognosis (1). BRAF mutation leading to V600E amino acid substitution has emerged as the most frequent genetic lesion in thyroid cancer, and it occurs particularly in patients with aggressive disease (2–4). Several small-molecule RAF [or RAF pathway, *e.g.* MEK (MAPK kinase)] kinase inhibitors are being developed (5–12). Importantly, BRAF mutation was a molecular determinant of cancer cell sensitivity to BRAF/MEK blockade (8–11). A 7-azaindole, named PLX4720,

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For editorial see page 60

Abbreviations: DUSP/MKP, Dual specific ERK phosphatase; MEK, MAPK kinase; pERK, phospho-ERK; RAS, rat sarcoma viral oncogene; RET/PTC1, rearranged during Transfection/Papillary Thyroid Carcinoma.

inhibited BRAF V600E with a half maximal inhibitory concentration (IC_{50}) of 13 nM. It also inhibited mutant (Y340D and Y341D) CRAF with an IC_{50} of 6.7 nM (13). The compound inhibited wild-type BRAF at a 10-fold higher concentration than BRAF V600E and was selective against a panel of 70 kinases. A related compound, PLX4032, also selectively targeted BRAF V600E (IC_{50} = 44 nM); besides BRAF, only BRK showed PLX4032 inhibition with an IC_{50} = 240 nM out of a series of 65 different kinases (13). PLX4032 had modest antiproliferative effects (IC_{50} >10,000 nM) in BRAF wild-type and RET/PTC1-positive TPC-1 thyroid cancer cells (14). Here, we have extended these studies by assessing the efficacy of both PLX4720 and PLX4032 in a panel of thyroid carcinoma cell lines harboring BRAF, rat sarcoma viral oncogene (RAS), or rearranged during Transfection/Papillary Thyroid Carcinoma (RET/PTC1) oncogenes.

Materials and Methods

Reagents

PLX4720 and PLX4032 were provided by Plexxikon Inc. (Berkeley, CA). A list of the other reagents is available as supplemental data (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Cell cultures

The human thyroid carcinoma cell lines were grown in DMEM (Invitrogen, Groningen, The Netherlands) containing 10% fetal bovine serum. The Fischer rat-derived differentiated thyroid follicular cell line PC Cl 3 (hereafter referred to as "PC") was grown in Coon's modified Ham F12 medium supplemented with 5% calf serum and a mixture of six hormones (Sigma Chemical Co., St. Louis, MO). Cell sources and genotyping are described in the supplemental data (15).

Miscellaneous methods

Cell proliferation, cell cycle analysis, protein studies, RNA extraction, RT-PCR, animal studies, and statistical analysis were performed according to standard procedures. A description is available in the supplemental data.

Results

PLX4032 and PLX4720 inhibition of BRAF mutant thyroid carcinoma cells growth

Cell lines harboring BRAF V600E, RET/PTC1, or RAS mutations were used together with normal PC thyrocytes as control. To confirm the identity of the thyroid cancer cell lines used in this study, DNA profiling using short tandem repeat was performed (Supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), and mRNA expression of thyroid differentiation markers was determined (Supplemental Fig. S1). SW1736, BCPAP, TPC-1, 8505C, and CAL62 expressed at least one thyroid marker; instead, C643 and HTH7 were negative for all the markers. Cells were plated in 2.5% serum and treated with different concentrations of PLX4720, PLX4032, or vehicle (NT) and counted daily for 4 d (Fig. 1A). Treatment with PLX4720 and PLX4032 caused a proliferation inhibition of BRAF mutant cells with an EC_{50} in the nanomolar range (Supplemental Table S2). Importantly, at 1000 nM of both compounds, the EC_{50} for proliferation inhibition of normal PC cells was not reached; EC_{50} for thyroid carcinoma cell lines harboring RET/PTC1 or RAS mutations was equal or higher than 500 nM (Fig. 1A and Supplemental Table S2).

PLX4032 and PLX4720 inhibit ERK phosphorylation in BRAF mutant thyroid carcinoma cells

We treated with PLX4032 BRAF-, RAS-, or RET/PTC1-positive cells for 2 h and determined ERK pathway activity by immunoblot with phospho-ERK 1/2 and -MEK1/2 antibodies. At the dose of 100 nM, PLX4032 inhibited ERK and MEK phosphorylation in BRAF mutant cells. In contrast, phosphorylation of ERK and MEK did not decrease (and in some cases even increased) upon treatment with PLX4032 (100 or 1000 nM) of cell lines harboring RET/PTC1 or RAS mutations. No ERK inhibition was observed in normal thyroid cells (Fig. 1B). A detailed dose-response for ERK and MEK phosphorylation for PLX4720 and PLX4032 showed that both inhibited ERK phosphorylation with an IC_{50} ranging from 50–100 nM in BRAF mutant but not in PC cells (Supplemental Fig. S2).

However, ERK phosphorylation was not totally inhibited at the dose of up to 1000 nM in BRAF mutant cells (Fig. 1B); even a higher dose of PLX4032 (2500 nM) did not further inhibit ERK phosphorylation both in 8505C (BRAF V600E/V600E) and SW1736 (BRAF WT/V600E) cells (Supplemental Fig. S3A). DUSP/MKP (dual specific ERK phosphatases) are transcriptional targets of the ERK pathway, their induction by ERK representing a negative feedback circuit that is able to terminate ERK signal (16). PLX4032 and PLX4720 treatment of SW1736 evoked a rapid (DUSP5) and sustained (DUSP6/MKP3) decrease of mRNA abundance of both phosphatases (Supplemental Fig. S3B), thereby likely causing a relaxation of the negative feedback and therefore an incomplete ERK dephosphorylation. BRAF mutant cell proliferation was not completely inhibited even at 1000 nM PLX4032 or PLX4720 (Fig. 1A). Either disabling of negative feedbacks or pathways other than the ERK one may be responsible for such incomplete growth inhibition.

To verify whether the reduced phosphorylation of ERK was indeed mediated by BRAF inhibition, we performed

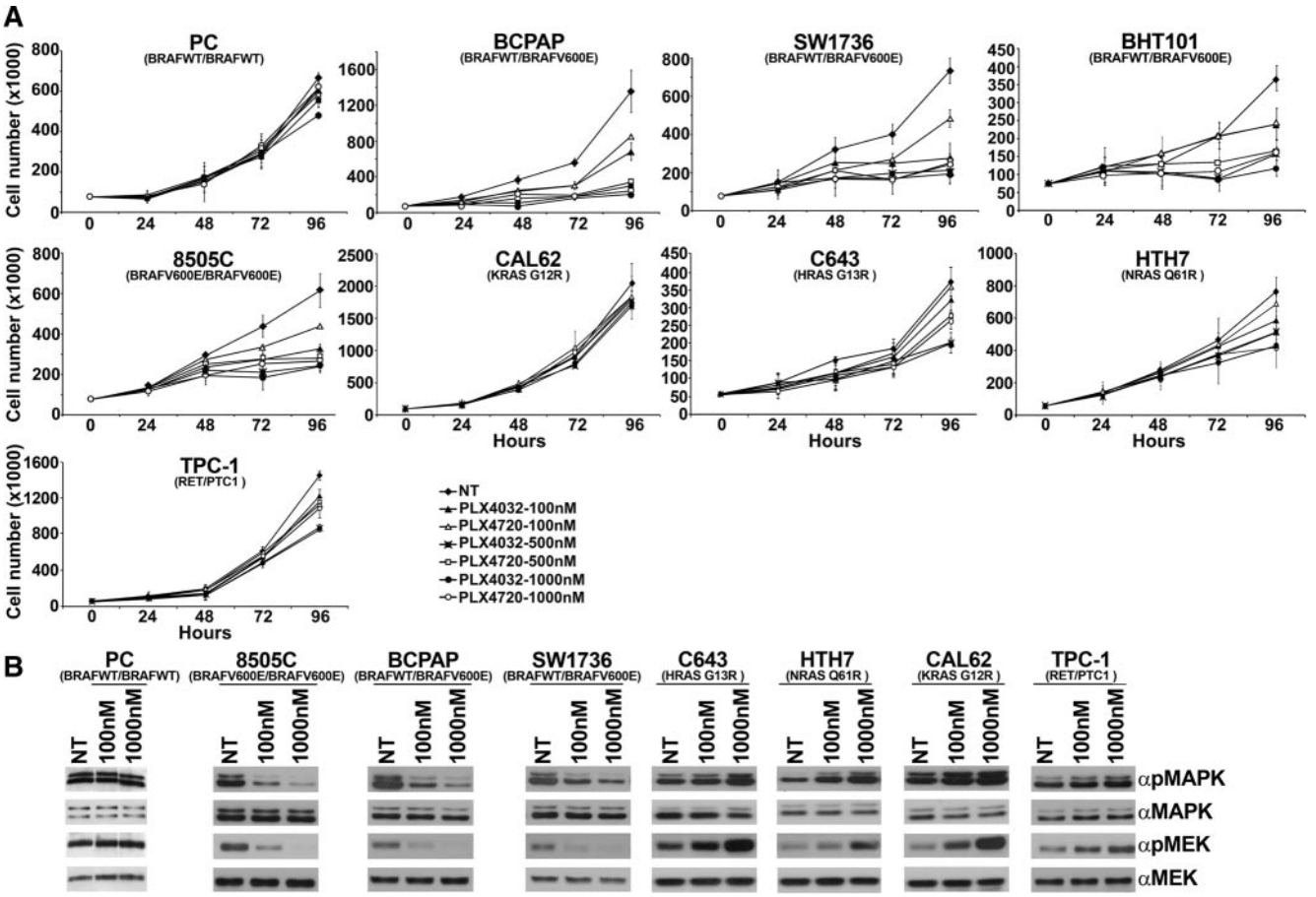

FIG. 1. *PLX4720* and *PLX4032* in thyroid carcinoma cells. A, Cells (5×10^4) were plated in triplicate in 35-mm dishes in 2.5% serum. One day later, different concentrations of PLX4720, PLX4032, or vehicle (NT) were added, and cells were counted at different time points. Each point represents the mean value of triplicates, and error bars represent 95% confidence intervals. B, Cell lines were kept in 2.5% serum and treated with increasing concentrations of PLX4032. Two hours later, cells were lysed and analyzed by Western blotting. NT, Not treated.

an *in vitro* BRAF kinase assay. As shown in Supplemental Fig. S4, at the dose of 50–500 nM, PLX4032 inhibited BRAF enzymatic activity in the SW1736 and 8505C cells, whereas up to 500 nM PLX4032 did not inhibit the wild-type BRAF kinase in normal thyroid cells.

Effects of serum on PLX4032 activity

Growth factors present in the serum could affect sensitivity to ERK pathway inhibition (12). Thus, we treated cells with PLX4032 in 0.1, 2.5, and 10% serum. Inhibition of phospho-ERK (pERK) and -MEK in BCPAP and 8505C but not in SW1736 cells was more profound in 0.1% serum; however, growth inhibition sensitivity did not increase, rather it even decreased, in low serum conditions (Supplemental Fig. S5).

Effects of MEK inhibition

We used the MEK inhibitor PD0325901 as an alternative tool for ERK pathway inhibition. As shown in Supplemental Fig. S6A, PD0325901 efficiently inhibited ERK phosphorylation both in SW1736 and PC cells. Treatment with the compound caused a dose-dependent proliferation inhibition of SW1736 cells ($EC_{50} = 0.4$ nM), whereas 50% of inhibition was observed in normal thyrocytes only at concentrations of at least 50 nM (Supplemental Fig. S6B). These findings indicate that BRAF mutant cancer cells are more strictly dependent on ERK pathway than normal thyrocytes.

Characterization of PLX4032 and PLX4720-mediated cell cycle effects in thyroid cells

SW1736 underwent a marked G_1 arrest, starting at 24 h (Fig. 2A) and lasting up to 48 h (data not shown), upon PLX4032 and PLX4720 (500 nM) treatment. No increase in the sub G_1 fraction was detectable, indicating that the treatment was cytostatic rather than cytotoxic, as reported also with other agents targeting the BRAF/MEK pathway (6–11). Importantly, PLX4032 and PLX4720 treatment (500 nM) did not modify the cell cycle profile of normal control cells (Fig. 2A). To better characterize the G_1-S transition block, we analyzed by quantitative RT-PCR the expression levels of cell cycle-related mRNAs. PLX4720 and PLX4032 induced a reduction of the mRNA levels of positive regulators of the G_1/S transition starting at 6 h and

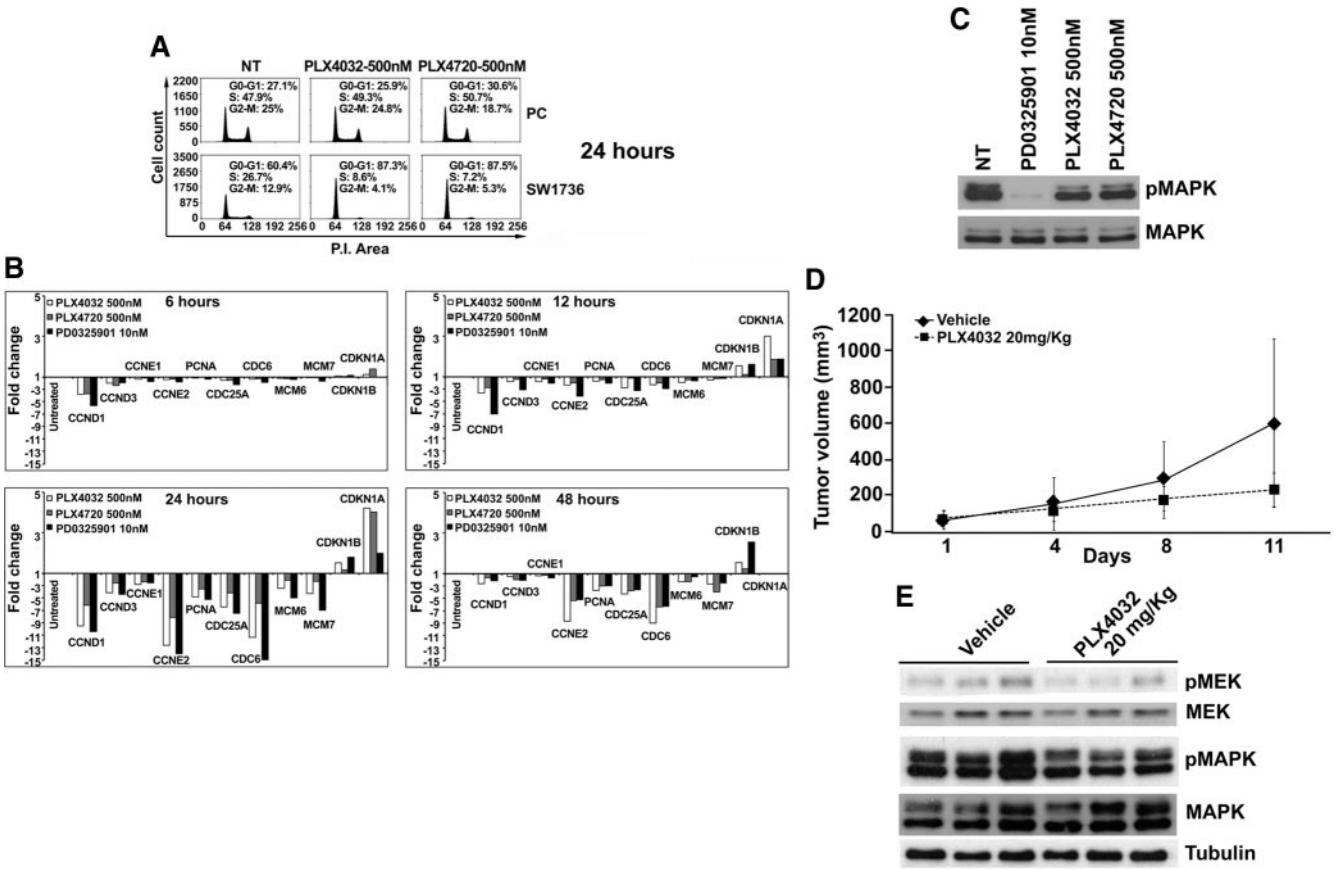

FIG. 2. Cell cycle effects of PLX4032 and PLX4720 in thyroid cancer cells. A, SW1736 and PC cells were incubated with PLX4032 (500 nM) and PLX4720 (500 nM) or vehicle (NT) at the indicated time points, and cell cycle distribution was determined by flow cytometry. B, SW1736 cells were incubated with PLX4032 (500 nM), PLX4720 (500 nM), PD0325901 (10 nM), or vehicle (NT) at different time points; RNA was extracted, and mRNA levels of the indicated genes were measured by quantitative RT-PCR and reported as fold change with respect to vehicle-treated cells. C, SW1736 cells were incubated with PLX4032 (500 nM), PLX4720 (500 nM), PD0325901 (10 nM), or vehicle (NT) for 2 h, lysed, and immunoblotted with the indicated antibodies. D, 8505C cells (2×10^7 per mouse) were inoculated sc into the right dorsal portion of BALB/c nude mice. When tumors (~100 mm^3) appeared, animals were randomly assigned to two groups (eight mice per group) to receive PLX4032 (20 mg/kg) or vehicle by oral gavage. Treatment was administered for 5 consecutive days per week for 2 wk (d 1 is the treatment-starting day). Tumor diameters were measured with calipers, and tumor volumes were calculated. *Error bars* represent 95% confidence intervals. E, Proteins (40 μg), extracted from representative tumors 3 h after the last dose of compound, were immunoblotted with the indicated antibodies. NT, Not treated.

lasting up to 48 h upon treatment. Moreover, increased mRNA levels of the cyclin-dependent kinase inhibitors CDKN1A and CDKN1B were observed after PLX4720 and PLX4032 treatment. Similar results were obtained using the MEK inhibitor PD0325901 (Fig. 2B). The extent of pERK inhibition is shown in Fig. 2C.

Inhibition of 8505C-induced tumor formation in nude mice by PLX4032

Nude mice bearing tumors induced by 8505C cells were treated *per os* with PLX4032 or with vehicle (eight for each group). After 11 d of treatment, mice treated with PLX4032 had smaller tumors than control mice, although the difference did not reach statistical significance at the ANOVA test ($P = 0.123$) (Fig. 2D). Tumor-growth inhibition was associated with a reduction of ERK (~2-fold) and MEK (~4-fold) *in vivo* phosphorylation levels (Fig. 2E). ERK dephosphorylation, however, was not complete; this may have been caused either by the limited dose of the compound or the rapid relaxation of negative feedbacks as discussed above.

Discussion

Here, we show that PLX4032 and PLX4720 inhibited ERK pathway phosphorylation and proliferation of thyroid cancer cells harboring the BRAF mutation. The refractoriness of normal thyroid cells to PLX4032 and PLX4720 may be explained by both the reduced activity of the compounds on wild-type BRAF—as demonstrated by BRAF kinase assay—and the lack of dependence of normal cells on BRAF pathway for their proliferation—as demonstrated by the experiments with the MEK inhibitor. Being involved in the transmission of signals downstream RET/PTC and RAS oncoproteins, BRAF targeting may

have efficacy also in thyroid cancer cells negative for BRAF (17–19). However, RET/PTC1- and RAS mutation-positive cells were quite resistant to PLX4032 and PLX4720. This is consistent with the reduced activity of the compound on wild-type BRAF with respect to BRAF V600E (12). Other studies showed that MEK inhibitors are also more active in BRAF-mutant than BRAF-wild-type thyroid cancer cells, suggesting a different degree of addiction to the ERK pathway (8–12). The reason for this differential sensitivity is still unknown. Pratilas *et al.* (20) have suggested that BRAF wild-type cells are resistant to MEK inhibition because ERK signaling "output" is not as elevated as in BRAF mutant cells. Also in the thyroid system, pMEK levels were more markedly elevated in BRAF mutant compared with BRAF wild-type cells (Refs. 8 and 12 and our unpublished observations). However, also in BRAF mutant cells, ERK inhibition was not complete. ERK inhibition was in general more intense in low serum. It is possible that either serum or disabled negative feedbacks (reduced levels of ERK phosphatases) contribute to such residual pERK levels. Instead, complete pERK inhibition was seen by using 10 nM PD0325901; it is possible that the higher efficacy of the MEK inhibitor compared with the BRAF one depends on the level of the signaling cascade the two kinases act. For instance, inhibition of BRAF (being upstream of MEK) may lead to relaxation of a higher number of feedbacks, compared with inhibition of MEK. Alternatively, this phenomenon may simply reflect the specific activity of each compound on the respective target kinase.

Studies with cultured cancer cell lines may reveal only the cell autonomous responses and can overlook the role played by cancer stem cells. Moreover, it is unlikely that in a clinical setting blockade of one single signaling pathway will be sufficient, particularly in tumors as aggressive as anaplastic thyroid carcinomas. Accordingly, PLX4032 treatment did not cause a complete regression of 8505C cell tumors at the dose tested. With these limitations, our study provides additional evidence of the promising nature of mutant BRAF as a molecular target for BRAF V600E-positive iodine-refractory thyroid cancer patients.

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