Potent Inhibition of Thyroid Cancer Cells by the MEK Inhibitor PD0325901 and Its Potentiation by Suppression of the PI3K and NF- κ B Pathways

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Background: We recently demonstrated inhibition of thyroid cancer cells by the MEK inhibitor CI-1040. The objective of this study was to use a potent new-generation MEK inhibitor PD0325901 to further investigate the therapeutic potential of specifically targeting MEK in the MAP kinase pathway for thyroid cancer.

Methods: We examined the effects of PD0325901 on a variety of cellular and molecular activities of thyroid cancer cell lines with distinct genotypes.

Results: PD0325901 remarkably inhibited MAP kinase pathway signaling in the thyroid cancer cells tested. It potently inhibited cell proliferation ($IC_{50} = 0.059\text{--}0.783\ \mu\text{M}$) and arrested cell cycle at the G0/G1 phase of cells harboring *BRAF* or *RAS* mutations but not cells harboring wild-type alleles or the *RET/PTC1* rearrangement. Synergistic inhibitory effects were observed when PD0325901 was combined with phosphatidylinositol 3-kinase (PI3K) or NF- κ B pathway inhibitors in most cells, including the *RET/PTC1*-harboring cells. PD0325901 could inhibit invasion and anchorage-independent growth of thyroid cancer cells independently of the type of genetic alterations. This compound did not seem to have significant proapoptotic effects, however.

Conclusions: The MEK inhibitor PD0325901 has a wide range of potent inhibitory effects on thyroid cancer cells, some of which seemed to be genotype-selective, consistent with the results previously observed with an early-generation MEK inhibitor, CI-1040. The data provide further evidence that targeted inhibition of MEK may be therapeutically effective for thyroid cancer, particularly if the PI3K and NF- κ B pathways are concurrently inhibited.

Introduction

THE RET $\rightarrow$ RAS $\rightarrow$ RAF $\rightarrow$ MEK $\rightarrow$ MAPK/ERK signaling pathway (MAPK pathway) regulates a multitude of cellular functions, including cell proliferation, division, differentiation, and motility (1,2). Constitutive activation of the MAPK pathway through genetic alterations, including *RAS* and *B-type RAF (BRAF)* mutations, is common in human cancers and is associated with cell malignant transformation and aggressiveness, implicating that targeted inhibition of the MAPK pathway may potentially be an effective therapy for human cancers (3,4). A wide spectrum of inhibitors against the components (mainly RAF and MEK) of MAPK pathway have been discovered, which showed anticancer potential by suppressing tumor cell growth both *in vitro* and *in vivo* (5). The fact that ERK is the only known substrate of MEK has fueled strong interest in developing pharmacological inhibitors of MEK as a means to block ERK activation. Currently, several MEK inhibitors, including CI-1040, AZD6244, and PD0325901, which all are orally active, have entered

clinical trials on human cancers (5). These MEK inhibitors dually inhibit MEK1 and MEK2 and are noncompetitive with ATP, making them strictly selective for MEK1/2 versus other kinases (5). The PD0325901 compound is a CI-1040-derived MEK inhibitor, which has a 50-fold increase in potency against MEK1/2, improved bioavailability, and longer duration of target suppression than CI-1040 (6). Tumor xenograft model study showed remarkable suppression of melanoma and colon cancer cells harboring the V600E *BRAF* mutant by PD0325901 (7). A recent phase I/II clinical trial in patients with breast, colon, nonsmall-cell lung cancer, or melanoma showed that PD0325901 was well tolerated, phosphorylation of ERK (p-ERK) in the tumors was suppressed, and a significant number of patients achieved partial response or disease stabilization (5).

Follicular epithelial-derived thyroid cancer is the most common endocrine malignancy with a rapidly rising incidence in recent years (8–11). This cancer is histologically classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC). ATC,

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although uncommon, is a deadly and aggressive cancer. Although PTC and FTC are differentiated and highly treatable, they can become incurable when they have lost differentiation and responses to radioiodine treatment. These patients impose a major therapeutic challenge currently. Genetic alterations that drive thyroid tumorigenesis and progression through aberrant activation of the MAPK pathway are frequently found in thyroid cancers, including rearrangements of the *RET* proto-oncogene (12), *RAS* mutation (13), and the T1799A *BRAF* mutation (14). We recently demonstrated inhibition of thyroid cancer cells by the MEK inhibitor CI-1040 (15). The inhibition of thyroid cancer cells by CI-1040 may be expectable also for the newer generation of this inhibitor, but this possibility remains undetermined. In the present study, we tested the effects of the second-generation CI-1040-derived MEK inhibitor PD0325901 on thyroid cancer cell lines with various genotypes to further investigate the therapeutic potential of targeting MEK for thyroid cancer.

Materials and Methods

Cell culture

Thyroid tumor cell lines KAK1, KAT5, KAT7, KAT10, and KAT18 were provided by Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY); the PTC cell line NPA, FTC cell lines MRO and WRO, and the ATC cell line DRO were from Dr. Guy J.F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA); the ATC cell lines C643, SW1736, and HTh7 were from Dr. N.E. Heldin (University of Uppsala, Uppsala, Sweden); the TPC1 cell line was provided by Dr. Alan P. Dackiw (Johns Hopkins University, Baltimore, MD); the PTC cell line BCPAP was from Dr. Massimo Santoro (University Federico II, Naples, Italy); and the FTC cell line FTC133 was from Georg Brabant (Christie Hospital, Manchester, UK). Per genotyping analysis, KAK1, KAT5, KAT7, and KAT10 cells were all from an ATC cell line, ARO cell, which was originally from Guy J.F. Juillard (per communication by Dr. James A. Fagin, Memorial Sloan-Kettering Cancer Center, New York, NY). After many years of independent culture and passaging, these cells presumably evolved into different subclones of ARO cells with some different properties (data not shown). The cell lines were cultured in RPMI 1640 medium supplemented with 10% calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and penicillin-streptomycin in a 37°C humidified incubator with 5% CO₂. For some of the experiments, cells were treated with PD0325901 (Pfizer Global Research and Development, Ann Arbor, MI), LY294002 (Sigma, St. Louis, MO), or PS1145 (Sigma) with the indicated concentrations and time, and the medium and agents were replenished every 24 hour.

Western blotting analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer as described previously (16). After resolution on denaturing polyacrylamide gels, total cellular proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and blotted with primary antibodies from Santa Cruz (Santa Cruz, CA), including antiphospho-ERK (Sc-7383), anti-ERK1 (Sc-94), or anticyclin D1 (sc718), followed by incubation with second antibodies, including HRP-conjugated anti-rabbit (Sc-2004) or anti-mouse (Sc-2005)

immunoglobulin G (IgG) antibodies. The antigen-antibody complexes were visualized using the ECL detection system (Amersham Pharmacia Biotech).

Cell proliferation assay

MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay was used to evaluate cell proliferation as we described previously (16). Briefly, cells (800/well) were seeded into a 96-well plate, and after 5 days of treatment with the indicated concentrations of PD0325901, 10 μ L of 5 mg/mL MTT (Sigma) was added to cell culture, followed by addition of 100 μ L of 10% sodium dodecyl sulfate (SDS) solution 4 hours later. After incubation for another 12 hours, the plates were read on a microplate reader at a test wavelength of 570 nm and a reference wavelength of 670 nm. IC₅₀ values were calculated using the Reed-Muench method (17).

Cell cycle analysis

Propidium iodide (PI) staining was used for cell cycle analysis. Briefly, after an exposure to 0.1 μ M PD0325901 for 48 hours, 10⁶ cells were suspended in 1 mL of phosphate-buffered saline (PBS) and fixed with 70% ethanol overnight. Following washing with PBS twice, cells were resuspended in 0.5 mL of PBS, combined with 0.5 mL of a DNA extraction buffer (0.192 M Na₂HPO₄, 0.008% Triton X-100 (v/v), and pH 7.8), and incubated at room temperature for 5 minutes. After centrifugation to remove the supernatant, cells were resuspended in 1 mL of a DNA-staining solution (PBS with 0.002% PI and 0.02% DNase-free RNase) and incubated for 30 minutes at room temperature in the dark. Cells were then analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS, CA), and analysis of the multivariate data was performed with CELL Quest software (BDIS) at a Johns Hopkins Flow Cytometry core facility.

Invasion assay

Cell invasion was assayed using Matrigel-coated Transwell cell culture chambers (6.5-mm diameter, 8- μ m pore size; BD Biosciences, NJ) according to the instructions of the manufacturer. Briefly, cells (5 $\times$ 10⁴ cells/well) suspended in serum-free medium were placed in the upper chamber of the Transwell insert, and RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber. PD0325901, at a final concentration of 0.05 μ M, was added to the culture medium in the treatment group, and dimethyl sulfoxide (DMSO) was used as a control. Following incubation for 22 hours at 37°C with 5% CO₂, noninvasive cells in the upper chamber were removed and invasive cells were fixed in 100% methanol and stained with 0.5% crystal violet in 2% ethanol. The numbers of invasive cells were counted after photography. Experiments were carried out in triplicate.

Anchorage-independent growth assays

Briefly, 4000 cells/well were seeded in six-well plates in 0.3% agar over a bottom layer of 0.6% agar as described previously (16). After 12 days of culture in the presence or absence of 0.05 μ M PD0325901, cell colony number was counted under a microscope, and photography was taken.

Results

Rapid and potent inhibition of the MAPK pathway signaling by PD0325901 in thyroid tumor cell lines

As summarized in Table 1, thyroid cancer cell lines with various genotypes in the MAPK pathway were used in this study, including KAK1, KAT5, KAT7, and KAT10 that harbored a heterozygous T1799A *BRAF* mutation, DRO and NPA that harbored a homozygous T1799A *BRAF* mutation, TPC1 that harbored the *RET/PTC1* rearrangement, and C643 that harbored an activating G37C homozygous *HRAS* mutation in codon 13 with a G13R amino acid substitute (18). Two thyroid cancer cell lines, MRO and WRO, harbored no known mutation (wild-type alleles) in the MAPK pathway. To determine the effect of the MEK inhibitor PD0325901 on the MAPK pathway signaling in thyroid cancer cells, we first examined the concentration response and time course of the effects of PD0325901 on ERK phosphorylation in two selected cell lines, KAT5 and DRO cells, which harbored heterozygous and homozygous *BRAF* mutation, respectively. As shown in Figure 1A, inhibition of the MAPK pathway, in terms of suppression of ERK phosphorylation, occurred in both cell lines in a concentration-dependent manner, being remarkable at only 0.005 μ M in both cells and complete at 0.01 μ M in KAT5 and 0.02 μ M in DRO cells. The inhibition of the MAPK pathway occurred rapidly; complete inhibition with 0.1 μ M PD0325901 was seen at 0.5 hours after addition of the inhibitor to the cell culture. This inhibition remained for at least 24 hours (Fig. 1B). Similar inhibition of the MAPK pathway by PD0325901 was also achieved and remained for at least 24 hours in other eight thyroid tumor cell lines regardless of their genotypes although a low level of residual ERK phosphorylation was observed in KAK1 and TPC1 cells after treatment (Fig. 1C). Correspondingly, expression of cyclin D1, a key cell cycle regulatory molecule that is upregulated by the MAPK pathway signaling (19,20), was also inhibited by PD0325901 in these cells except for the MRO cell in which no significant expression of cyclin D1 was detectable under these experimental conditions (Fig. 1C).

PD0325901 preferentially inhibited proliferation of thyroid tumor cells harboring BRAF or RAS mutations

Given the potent inhibitory effect of PD0325901 on MAPK pathway signaling and cyclin D1 expression in thyroid tumor cells, we investigated the effect of this MEK inhibitor on the proliferation of these cells. We found that PD0325901 significantly inhibited proliferation of the seven cell lines that harbored *BRAF* or *RAS* mutations—KAK1, KAT5, KAT7, KAT10, NPA, DRO, and C643 cells (Fig. 2). This inhibition occurred in a concentration-dependent manner, all with a high potency as reflected by the low IC_{50} values, ranging from 0.059 to 0.783 μ M (Table 1). In contrast, MRO and WRO cells, which harbored no known genetic alterations in the MAPK pathway, were resistant to PD0325901, with IC_{50} values >30 μ M (Table 1 and Fig. 2). Interestingly, PD0325901 had also only a minimal effect on the proliferation of TPC1 cells, which harbored *RET/PTC1* rearrangement, with an IC_{50} of 13.029 μ M (Fig. 2 and Table 1). Thus, similar to the MEK inhibitor CI-1040 (15), PD0325901, a new-generation MEK inhibitor derived from CI-1040, also seemed to preferentially inhibit proliferation of thyroid cancer cells harboring *BRAF* or *RAS* mutations.

PD0325901 preferentially induced G0/G1 cell cycle arrest of thyroid cancer cells harboring BRAF or RAS mutations

To investigate whether the inhibition of cell proliferation by PD0325901 resulted from cell cycle arrest, we analyzed cell cycle changes after PD0325901 treatment in selected four cell lines, KAT10, C643, TPC1, and MRO, which harbor *BRAF* mutation, *RAS* mutation, *RET/PTC1* rearrangement, and wild-type allele in the MAPK pathway, respectively. As shown in Figure 3, in *BRAF* mutation-harboring KAT10 cells, PD0325901 caused a 31% and 41% reduction in S and G2/M fractions, respectively, with a corresponding increase of 17% in the G0/G1 fraction. Similarly, in *RAS* mutation-harboring C643 cells, PD0325901 caused a 35% and 60% reduction in

TABLE 1. GENETIC ALTERATIONS IN VARIOUS THYROID CANCER CELL LINES AND THEIR SENSITIVITY TO PD0325901

Cell line	Derived from	Genetic alteration	IC_{50} of PD0325901 (μ M) for inhibition of proliferation
KAK1	ATC	<i>BRAF</i> T1799A (heterozygous)	0.783
KAT5	ATC	<i>BRAF</i> T1799A (heterozygous)	0.066
KAT7	ATC	<i>BRAF</i> T1799A (heterozygous)	0.059
KAT10	ATC	<i>BRAF</i> T1799A (heterozygous)	0.364
DRO	ATC	<i>BRAF</i> T1799A (homozygous)	0.085
BCPAP	PTC	<i>BRAF</i> T1799A (homozygous)	0.131
SW1736	ATC	<i>BRAF</i> T1799A (heterozygous)	0.017
C643	ATC	<i>HRAS</i> G37C (homozygous)	0.090
NPA	PTC	<i>BRAF</i> T1799A (homozygous)	0.140
TPC1	PTC	<i>RET/PTC1</i> rearrangement	13.029
MRO	FTC	Wild-type ^a	55.055
WRO	FTC	Wild-type ^a	31.746
FTC133	FTC	Wild-type ^a	22.630
KAT18	ATC	Wild-type ^a	8.615
HTH7	ATC	Wild-type ^a	7.043

^aNo *BRAF* mutation, *RAS* mutation, or *RET/PTC1* rearrangement was detected in these cell lines. IC_{50} was calculated by the Reed–Muench method using the averaged inhibition rates at seven PD0325901 concentration points from four experiments as shown in Figure 2.

ATC, anaplastic thyroid cancer; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer.

FIG. 1. PD0325901 inhibited the MAPK pathway signaling in thyroid tumor cells. Cells were lysed for Western blotting after PD0325901 treatment. The MAPK pathway activity was reflected by the level of phosphorylated ERK detected with specific antiphosphorylated ERK (p-ERK) antibody. Total ERK (t-ERK) was used for quantity control of proteins. (A) Concentration response of the inhibitory effects of PD0325901 on MAPK pathway in two *B-type RAF* (*BRAF*) mutation-harboring thyroid cancer cell lines KAT5 and DRO. Cells were treated with the indicated concentrations of PD0325901 for 2 hours. (B) Time course of the inhibitory effects of PD0325901 on MAPK pathway in KAT5 and DRO cells. Cells were treated with 0.1 μ M PD0325901 for the indicated times. (C) Inhibitory effects of PD0325901 on MAPK pathway in various thyroid tumor cells. Cells were treated with 0.1 μ M PD0325901 (+) or vehicle DMSO (–) for 24 hours.

S and G2/M fractions, respectively, accompanied by a 22% increases in the G0/G1 fraction. These results demonstrate G0/G1 cell cycle arrest by PD0325901 in these cells. In contrast, PD0325901 treatment resulted in no significant changes in G0/G1, S, and G2/M fractions in TPC1 and MRO cells, which harbored *RET/PTC1* and wild-type alleles in the MAPK pathway, respectively (Fig. 3). No increase in the sub-G0/G1 nuclear fraction, which is indicative of apoptosis, was seen after PD0325901 treatment in these cell lines.

Synergistic inhibitory effects on cell proliferation of PD0325901 when combined with inhibitors of the PI3K or NF- κ B pathways

The phosphatidylinositol 3-kinase (PI3K) and NF- κ B signaling pathways were previously shown to play a role in the proliferation of thyroid cancer cells (21–24). To investigate whether interference with these pathways may increase the sensitivity of thyroid cancer cells to the inhibition by PD0325901, we tested the effects of combined use of PD0325901 with the PI3K pathway inhibitor LY294002 or with the NF- κ B pathway inhibitor PS1145 on thyroid cancer cells. As shown in Figure 4A, treatment with LY294002 alone caused $\geq 40\%$ inhibition of proliferation in KAK1, KAT5,

KAT7, KAT10, and TPC1 cells, and treatment with PS1145 alone caused $\geq 40\%$ inhibition in KAK1 and KAT10 cells, suggesting that the PI3K and NF- κ B pathways were partially involved in the proliferation of some of these thyroid cancer cells. Combination with PD0325901 resulted in a further 25–95% inhibition (compared with LY294002 or PS1145 treatment alone) of proliferation in seven cells that harbor *BRAF* mutation or *RET/PTC1* rearrangement (Fig. 4A, B)—KAK1, KAT5, KAT7, KAT10, NPA, DRO, and TPC1 cells. Therefore, in most of these cells, a synergistic or additive effect of PD0325901 combined with LY294002 or PS1145 was observed. As a specific example, PD0325901 at 0.02 μ M induced a much more pronounced inhibition when combined with LY294002 or PS1145 than when used alone in these cells (Fig. 4B). This was particularly obvious for KAK1, KAT5, KAT7, KAT10, NPA, DRO, and TPC1 cells. Of particular interest was the TPC1 cells, which were not sensitive to PD0325901 alone at all the concentrations tested (Fig. 2) but showed a dramatic synergistic inhibition by LY294002 or PS1145 combined with PD0325901 (Fig. 4A, B). For example, compared with LY294002 or PS1145 alone, PD0325901 at 0.02 μ M, which alone did not have effect, resulted in a further inhibition by 50% and 43% when combined with LY294002 or PS1145, respectively. This inhibition became more pronounced with increasing concentrations of

FIG. 2. PD0325901 inhibited the proliferation of thyroid tumor cells harboring *B-type RAF* (*BRAF*) or *RAS* mutations. Cells were treated with the indicated concentrations of PD0325901 for 5 days, and MTT assay was then performed to evaluate cell proliferation. IC₅₀ values of PD0325901 for each cell line were calculated as described in the "Materials and Methods" section and are shown in Table 1. BRAF mutation +/-, heterozygous *BRAF* mutation; BRAF mutation +/+, homozygous *BRAF* mutation; HRAS mutation +/+, homozygous *HRAS* mutation; and RAS/BRAF-WT, wild-type *RAS* and *BRAF* alleles. The data represent average $\pm$ standard deviation of four experiments.

PD0325901 (Fig. 4A), in contrast to the minimal effects of PD0325901 alone on the proliferation of TPC1 cells (Figs. 2 and 4). An overall inhibition of 87% and 75% of TPC1 cell proliferation was achieved at 0.5 μ M PD0325901 combined with LY294002 or PS1145, respectively (Fig. 4). Only a minimal further effect of PD0325901 on cell proliferation when combined with LY294002 or PS1145 was observed in WRO and MRO cells that lacked genetic alterations in the MAPK pathway. Surprisingly, the synergistic effects of combination of PD0325901 with LY294002 or PS1145 were not observed in C643 cells (Fig. 4A, B). Although C643 harbors *HRAS* mutation, no Akt activation was observed in this cell line (25), which may explain the absence of synergistic effects of PD0325901 in combination with LY294002.

PD0325901 inhibited the invasion of thyroid cancer cells harboring genetic alteration in the MAPK pathway

Previous studies showed a role of mutant *RAS*, *BRAF*, and *RET/PTC* in the invasion and metastasis of thyroid cancer through activating MAPK pathways (26). To investigate whether PD0325901 could suppress invasion of thyroid can-

cer cells with various genotypes, we performed Matrigel invasion assay on four cell lines, NPA, C643, TPC1, and MRO cells, which harbored *BRAF* mutation, *RAS* mutation, *RET/TPC1* rearrangement, and wild-type allele in the MAPK pathway, respectively. Figure 5 shows a representative experiment of cell invasion after treatment with and without PD0325901. The number of invading cells was decreased by 93% and 84% in NPA and TPC1 cells, respectively, after treatment with PD0325901. Although natural invasion of the C643 cell was modest after a 24-hour culture (Fig. 5), PD0325901 further inhibited this invasion by 80%. In contrast, PD0325901 only had a minimal inhibitory effect on the invasion of MRO cells, which did not harbor genetic alterations in the MAPK pathway.

PD0325901 abolished anchorage-independent growth of thyroid tumor cells

Anchorage-independent cell growth is a hallmark of the neoplastic phenotype (27). We examined the effects of PD0325901 on anchorage-independent growth of thyroid-tumor cells. As shown in Figure 6, although the cells examined

FIG. 3. Effects of PD0325901 on cell cycle of thyroid cancer cells. After treatment with $0.1 \mu\text{M}$ PD0325901 for 48 hours, cells were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry. The percentages of cells in G0/G1, S, and G2/M phases are indicated for each cell line. Cells treated with vehicle DMSO were used as control.

all formed colonies in soft agar with different anchorage-independent growth ability, treatment with PD0325901 nearly completely abolished colony formation of all these cells regardless of their genotypes, including TPC1 and MRO cells whose proliferation and cell cycle were not sensitive to this compound. Interestingly, TPC1 and MRO cells, which lacked *BRAF* and *RAS* mutations, showed the lowest ability to form colonies on soft agar. The complete inhibition of cell colony formation by PD0325901 was consistent with the complete or nearly complete inhibition by this compound of the MAPK pathway signaling in these cells (Fig. 1).

Effects of PD0325901 alone or in combination with PI3K or NF- κ B pathway inhibitors on cell proliferation in additional thyroid cancer cell lines

While this manuscript was in revision, Leboeuf *et al.* (28) reported similar data of PD0325901 on thyroid cancer cell lines. In their studies, while thyroid cancer cells with *BRAF* mutation were preferentially sensitive to PD0325901, cell lines that lack *BRAF* and *RAS* mutations and *RET/TPC1* rearrangement had more variable responses to the compound, with IC_{50} differing more than 300-fold (28), which is apparently different from the results in the present study and may reflect variable properties of different cells. To confirm the consistency of the genotype-dependent effects of PD0325901 observed in the present study, we expanded the study to test five additional thyroid cancer cell lines—the *BRAF* mutation-harboring BCPAP and SW1736 cells and the wild-type FTC133, KAT18, and HTh7 cells that harbored no *BRAF* mutation, *RAS* mutation, or *RET/TPC1* rearrangement (Table 1).

Like the wild-type WRO and MRO cells tested early in this study (Fig. 2), wild-type FTC133, KAT18, and HTh7 cells were also insensitive to PD0325901, with IC_{50} ranging from 7.04 to $22.63 \mu\text{M}$. In contrast, proliferation of *BRAF* mutation-harboring BCPAP and SW1736 cells was potently inhibited by PD0325901, with IC_{50} ranging from 0.017 to $0.131 \mu\text{M}$ (Fig. 7A and Table 1).

We also tested the effects of combined use of PD0325901 with LY294002 or PS1145 on these five thyroid cancer cell lines (Fig. 7B, C). The effect of PD0325901 was moderately potentiated by LY294002 in the *BRAF* mutation-harboring BCPAP and SW1736 cells but not in the wild-type cells (Fig. 7B, C). The PS1145 compound showed some potentiating effects in some of the wild-type cells (Fig. 7B, C).

Discussion

Although thyroid cancer is usually curable with conventional surgery and radioiodine ablation, it can be aggressive and incurable in some patients, particularly in those with extensive disease that has lost radioiodine avidity (11,29,30). The recently demonstrated importance of the MAPK pathway in tumorigenesis and progression of thyroid cancer suggests that this pathway may be a novel effective therapeutic target for thyroid cancer (14,31). Given its tight substrate selectivity (only ERK1 and ERK2) and its unique ability to act as a dual specificity kinase, MEK1/2 plays a central role in the integration of signals into the MAPK pathway and is an attractive target for pharmacologic intervention of MAPK pathway in cancer (5,6). Previously, we showed that inhibition of MAPK pathway by an early-generation MEK inhibitor CI-1040

FIG. 4. Effects of concurrent inhibition of the MAPK pathway and the PI3K pathway or the NF- κ B pathway on proliferation of thyroid cancer cells. **(A)** MTT assay was performed to evaluate cell proliferation after cells were treated for 5 days with 10 μ M LY294002, a PI3K pathway inhibitor, or 10 μ M PS1145, a NF- κ B pathway inhibitor, with or without the MEK inhibitor PD0325901 at the indicated concentrations. **(B)** Comparison of the inhibition rates of cell proliferation by 0.02 μ M PD0325901 with/without the presence of 10 μ M LY294002 or PS1145. For treatment with PD0325901 alone, the inhibition rate at 0.02 μ M PD0325901 was calculated by the formula $1 - (N_p/N_0)$; for treatment with PD0325901 + LY294002 or PS1145, the inhibition rate was calculated with the formula $1 - (N_{com}/N_{L/P})$. N_p : relative cell number after treatment with 0.02 μ M PD0325901; N_0 : relative cell number of control cells without treatment; these data are extracted from Figure 2. N_{com} : relative cell number after treatment with combination of 0.02 μ M PD0325901 and 10 μ M LY294002 or PS1145; $N_{L/P}$: relative cell number after treatment with 10 μ M LY294002 or PS1145 alone.

FIG. 5. Effects of PD0325901 on the invasion of thyroid cancer cells. **(A)** Representative results of invasive cells (magnification: $\times 10$). **(B)** Bar graph, corresponding to **(A)**, of the average numbers of invasive cells from three experiments. Log scale is used for Y-axis. The average numbers are represented by the columns, and the standard deviations by the bars. Cell invasion was assayed using Matrigel-coated Transwell cell culture chambers. Cells were treated with or without $0.05 \mu\text{M}$ PD0325901 for 22 hours, followed by examination and quantification of the invasive cells as described in the "Materials and Methods" section.

effectively suppressed a number of activities of thyroid cancer cells (15). In the present study, we further investigated the therapeutic potential of targeting MEK in the MAPK pathway in thyroid cancer cells by examining the effects of a more potent newer-generation MEK inhibitor PD0325901 (derived from CI-1040) on extended cellular and molecular properties.

Although the MAPK pathway signaling was potentially inhibited by PD0325901 in all the thyroid tumor cell lines examined, this MEK inhibitor selectively inhibited proliferation of thyroid tumor cells with *BRAF* or *RAS* mutations but not cells with *RET/PTC1* rearrangement or wild-type alleles (Fig. 2). Similar genotype-selective effects of PD0325901 were seen on cell cycle arrest at G0/G1 (Fig. 3), consistent with the *BRAF* or *RAS* mutation-selective effects of this MEK inhibitor on cell proliferation (Fig. 2). We cannot rule out the possibility that these differential effects of PD0325901 on different cells reflected tumor-type selectivity of the inhibitor as many of these cells were originally derived from different types of thyroid tumors. However, the genotype selectivity of the effects of PD0325901 on cell proliferation and cell cycle arrest apparently at least applies to *BRAF* mutation and *RAS* mutations as these genetic alterations are the driving force for thyroid tumorigenesis. As genetic heterogeneity likely exists in these cells, based on the current data, we are not able to conclude whether the apparent *BRAF* or *HRAS* mutation-

dependent effects of PD0325901 were exclusively attributable to these mutations. Also, although the results of PD0325901 with the G37C mutation in codon 13 of the *HRAS* gene are likely applicable to other *HRAS* mutations, such as those common mutations in codon 12 and 61, this remains to be tested. Interestingly, although there was virtually no inhibition of the proliferation of *RET/PTC1*-harboring cells by PD0325901 itself (Fig. 2), an inhibition by this MEK inhibitor could be induced or significantly potentiated by concurrent inhibition of the PI3K or the NF- κ B pathway (Fig. 4), consistent with the fact that *RET/PTC* rearrangements are coupled to these multiple signaling pathways that are important for proliferation of cancer cells (32,33). Therefore, it may not be surprising that MEK inhibitor alone did not show significant inhibition on the proliferation and some other cellular events of *RET/PTC1*-harboring thyroid cancer cells. It remains to be examined how the PD0325901 compound affects thyroid cancer cells harboring other *RET/PTC* rearrangements, such as *RET/PTC3*.

The significant effects of PI3K and NF- κ B inhibitors on proliferation of several other thyroid cancer cells suggest an important role of these pathways in thyroid cancer tumorigenesis. Their additive or synergistic effects with PD0325901 suggest a strong therapeutic potential of targeting multiple pathways in thyroid cancer. This may be an important therapeutic strategy for PD0325901 particularly given its lack of

FIG. 6. Effects of PD0325901 on anchorage-independent colony formation of thyroid tumor cells in soft agar. Cells were seeded in 0.3% agar over a bottom layer of 0.6% agar. After 12 days of culture with/without 0.05 μ M PD0325901, cell colony number was counted under a microscope, and cell colonies were photographed. **(A)** Representative results of colony formation of thyroid tumor cells treated with PD0325901 (+) or vehicle DMSO (–). **(B)** Bar graph, corresponding to **(A)**, of the colony numbers from three experiments for each cell type. The average numbers were represented by the columns, and the standard deviations by the bars. Only cell colonies containing greater than 50 cells were counted.

proapoptotic effect. The differential effects of PD0325901 on cell proliferation in the different thyroid cancer cell lines tested in the present study were different from the patterns of PD0325901-induced inhibition of cell invasion and anchorage-independent colony formation; these latter cellular events were all inhibited by PD0325901 in thyroid tumor cells harboring *BRAF* or *RAS* mutation or *RET/PTC1* rearrangement (Figs. 5 and 6). In fact, colony formation was inhibited even in cells with wild-type alleles (Fig. 6), consistent with the recent demonstration that MAPK pathway is required for cancer cell transformation (34). Although PD0325901 had different inhibitory effects on various cellular events or behaviors of thyroid tumor cells, it showed uniformly potent inhibition on the MAPK pathway signaling as reflected by inhibition of p-ERK and cyclin D1 expression in all these cells (Fig. 1). Therefore, the dependence of different cellular events or behaviors of thyroid tumor cells on MAPK pathway and its

genetic alterations varies, apparently with *BRAF* and *RAS* mutation being most depended upon, particularly for cell proliferation, cycling, and invasion.

Preferential inhibition of *BRAF* mutation-harboring thyroid tumor cells over *RET/PTC1*-harboring cells was also shown for the MEK inhibitors PD098059 and U0126 (35), although these inhibitors may not be clinically useful due to their pharmaceutical limitations (6). Similar to the action of PD0325901, the *BRAF* mutation- or *RAS* mutation-selective inhibition of thyroid tumor cell proliferation was recently demonstrated for the MEK inhibitors CI-1040 (15) and AZD6244 (28,36). A *BRAF* mutation- or *RAS* mutation-selective growth inhibition by MEK inhibitors was also reported in other human cancer cells (37,38), consistent with the notion of MAPK pathway addiction by which the activating mutations in this pathway sensitize tumors to the effects of corresponding inhibitors (39,40).

FIG. 7. Effects of PD0325901 alone or combined with inhibitors of PI3K and NF- κ B pathway on the proliferation of additional thyroid cancer cell lines. **(A)** Effects of PD0325901 on cell proliferation. Cells were treated with PD0325901 at the indicated concentrations for 5 days, and MTT assay was performed to evaluate cell proliferation. IC₅₀ values of PD0325901 for each cell line were calculated as described in the “Materials and Methods” section and are shown in Table 1. **(B)** Effects of PD0325901 in combination with the PI3K pathway inhibitor LY294002 or NF- κ B pathway inhibitor PS1145 on cell proliferation. MTT assay was performed to evaluate cell proliferation after cells were treated for 5 days with 10 μ M LY294002 or 10 μ M PS1145 with or without PD0325901 at the indicated concentrations. **(C)** Comparison of the inhibition rates of cell proliferation by 0.02 μ M PD0325901 in the absence or presence of 10 μ M LY294002 or PS1145. The inhibition rates were calculated as described in the legend to Figure 4. The data for relative cell number with or without treatment with 0.02 μ M PD0325901 are extracted from **(A)**.

Note Added in Proofs

After acceptance of this paper, the origin of NPA and ARO cells has become seriously questioned. The conclusions in the paper, however, are supported by the results with other thyroid cancer cells used and remain as stated.

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