Differential Responses of Human Papillary Thyroid Cancer Cell Lines Carrying the RET/PTC1 Rearrangement or a BRAF Mutation to MEK1/2 Inhibitors

Ying C. Henderson, MD, PhD; Mitchell J. Fredrick, PhD; Gary L. Clayman, DDS, MD

Objectives: To examine the effects of 2 mitogen-activated protein kinase kinase (MEK1/2) inhibitors on papillary thyroid carcinoma (PTC) cell lines carrying the RET/PTC1 rearrangement or a BRAF mutation. In PTC, RET/PTC1 rearrangement or BRAF mutations results in constitutional activation of RET kinase or BRAF, respectively. Along the RET or BRAF signaling cascades, the activated RET kinase or BRAF activates MEK1/2, and then mitogen-activated protein kinases (extracellular signal-related kinase 1/2 [ERK1/2]) is activated. Activated ERK1/2 enters the nucleus and phosphorylates a variety of transcription factors, resulting in cancer cell proliferation. The MEK1/2 inhibitors, PD98059 and U0126, have been shown to inhibit cell growth in other cancers.

Design: In vitro study.

Subjects: Papillary thyroid carcinoma cell lines carrying the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16).

Intervention: We treated PTC cells carrying the RET/PTC1 rearrangement or a BRAF mutation with 2 MEK1/2 inhibitors (PD98059 and U0126).

Main Outcome Measures: Using Western blot analysis, we detected the expression of phosphorylated ERK1/2 and expression of cleaved poly(ADP-ribose) polymerase (PARP) in cells after treatment with either inhibitors. Growth inhibition was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: Using Western blot analysis, we detected the dephosphorylation of ERK1/2 in PTC cells carrying the RET/PTC1 rearrangement or a BRAF mutation after treating the cells with 2 MEK1/2 inhibitors (PD98059 and U0126). In addition, both PD98059 and U0126 completely inhibited the growth of the PTC cells carrying a BRAF mutation but partially inhibited the growth of the PTC cells carrying the RET/PTC1 rearrangement. Finally, we observed PARP cleavage only in cells with a BRAF mutation in the Western blot analysis.

Conclusion: These data suggested that treatment with MEK1/2 inhibitors can be used as tools for inhibiting the growth of PTC cells.
on exon 15. The negative charge introduced by glutamic acid at position 600 mimics the effect of phosphorylation at an adjacent site when BRAF is activated and results in constitutive activation of BRAF. Others have detected the V600E mutation of BRAF in various human cancers, including melanoma (up to 66%), ovarian carcinoma (up to 40%), and colorectal carcinoma (up to 20%). In PTC, the incidence of RET/PTC rearrangements ranges from 2.5% to 67.0%, and the incidence of BRAF mutations ranges from 29% to 83% depending on the cohort being studied. No reports of an overlap of BRAF mutation and RET/PTC rearrangement have been found in the literature, indicating that only 1 of these 2 events is likely sufficient to induce successful tumor cell proliferation.

At the end of the RET/RAS/RAF/MEK/ERK signal transduction pathway, the ERK1/2 protein enters the nucleus and phosphorylates a variety of transcription factors that regulates cellular proliferation, differentiation, and apoptosis. There are 2 MEK1/2 inhibitors available commercially, PD98059 and U0126. Both are specific and noncompetitive inhibitors of dual MEKs (MEK1 and MEK2). However, U0126 is more potent than PD98059 in terms of its inhibition of MEK1/2. Previous studies have shown that MEK1/2 inhibitors PD98059 and U0126 inhibit various types of cancer cell growth including breast carcinoma, melanoma, and colon cancer.

In the study described herein, we examined the effects of these 2 MEK1/2 inhibitors on PTC cell lines carrying the RET/PTC1 rearrangement or a BRAF mutation. We found that both inhibitors were able to inhibit PTC cell proliferation. However, we observed poly(ADP-ribose) polymerase (PARP) cleavage only in PTC cells carrying a BRAF mutation.

### METHODS

#### CELL CULTURE

The PTC cell line carrying the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16) was kindly provided by Jerome Hershman, MD (VA Greater Los Angeles Healthcare System, Los Angeles, California). The cells were maintained in RPMI1640 medium containing 10% fetal bovine serum, nonessential amino acid mixture, 1mM sodium pyruvate, and 2mM L-glutamine in a 37°C incubator supplied with 95% air and 5% carbon dioxide.

#### CHEMICALS

PD98059 and U0126 were purchased from VWR (West Chester, Pennsylvania). PD98059 was dissolved in dimethyl sulfoxide (DMSO) as a 50mM stock solution and stored in the dark at −20°C. Fresh U0126 was made in DMSO as a 10mM solution before each use.

#### POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Protein extracts from PTC cells were prepared in lysis buffer containing 20mM Tris–hydrochloric acid, pH 7.4, 1% octyl phe-noxy polyethoxethanol (Triton X-100; Sigma–Aldrich, St Louis, Missouri), 300mM sodium chloride, 1mM phenylmethlysulfonl fluoride, 50mM sodium fluoride, 1mM sodium vanadate, and proteinase inhibitor cocktail III (Calbiochem, San Diego, California). Total protein concentrations were estimated using the Bradford assay (Bio-Rad, Hercules, California) with bovine serum albumin as a standard. For Western blot analysis, proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels using a miniprotein II electrophoresis system (Bio-Rad). The proteins were then transferred to Hybond-ECL membranes (Amersham Bioscience, Piscataway, New Jersey) using a mini-transblot electrophoretic transfer cell (Bio-Rad) at 80 V for 1 hour at room temperature. After transfer, the membranes were blocked and probed with antibodies at 4°C overnight as indicated by the manufacturer. Phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, and PARP antibodies (Cell Signaling Technology, Danvers, Massachusetts) were used at a dilution of 1:1000 and a monoclonal antibody against actin (Sigma-Aldrich) was used at a dilution of 1:2000.

#### CELL PROLIFERATION ASSAY

BHP2-7 and BHP5-16 cells (×10^4) were plated in 24-well plates (Costar, Cambridge, Massachusetts) with 1mL of medium in triplicate for 5 days. MEK1/2 inhibitors were added to the cells on day 0 and day 2. For MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in phosphate-buffered saline at 5 mg/mL was added to each well (0.2 mL) every day, and the cells were incubated at 37°C for 3 hours. The liquid was then aspirated from the wells and discarded. Stained cells were dissolved in 0.5 mL of DMSO and counted for absorption at 570 nm using a Synergy HT multidetection microplate reader (BioTek Instruments, Winooski, Vermont). Statistical analysis was performed using the Tukey honest significant difference test for post hoc analysis, and P<.05 was considered statistically significant.

### RESULTS

#### MEKI/2 INHIBITORS DECREASE THE PHOSPHORYLATION OF ERK1/2

To determine the optimal concentration of MEK1/2 inhibitors (PD98059 and U0126) for the dephosphorylation of PTC cells, we treated cells with 50µM to 150µM PD98059 or 1µM to 50µM U0126 for 24 hours. Protein extracts were prepared after these treatments and p-ERK1/2 was examined using Western blot analysis (Figure 1A). Treatment with 50µM PD98059 or 10µM U0126 significantly decreased the expression of p-ERK1/2 whereas it did not affect the expression of total ERK1/2 (Figure 1A). We selected 75µM PD98059 and 10µM U0126 in the ensuing studies to observe the maximum effects of these inhibitors.

We performed a time course study to determine the duration of ERK1/2 inactivity in the cells. The PTC cells were treated with 75µM PD98059 or 10µM U0126 for 1 hour to 72 hours, and the p-ERK1/2 proteins were detected using Western blot analysis (Figure 1B). The amount of p-ERK1/2 decreased after treatment with PD98059 for 1 hour compared with that in untreated cells, and the expression of p-ERK1/2 returned to normal at 6 hours after treatment. Also, the expression of p-ERK1/2 decreased dramatically after only 1 hour of treatment with U0126 and remained low for up to 48 hours; expression...
of p-ERK1/2 began to be detectable again 48 to 72 hours after the treatment (Figure 1B). Although both PD98059 and U0126 dephosphorylated ERK1/2, this dephosphorylation was clearly reversible. The recovery of p-ERK1/2 expression in these cells following removal of both PD98059 and U0126 from medium after treatment for 24 hours appeared to be the similar to that in untreated cells (Figure 1B). The level of total ERK1/2 protein expression remained unchanged during treatment with either PD98059 or U0126.

After establishing the optimal dose of PD98059 and U0126 and performing the time course study, we sought to determine whether ERK1/2 is deactivated in PTC cells based on the presence of the RET/PTC1 rearrangement or a BRAF mutation. We treated PTC cells carrying the RET/PTC1 rearrangement or a BRAF mutation with 75µM PD98059 or 10µM U0126 for 1 hour and evaluated the expression of the p-ERK1/2 using Western blot analysis (Figure 1C). The expression of p-ERK1/2 decreased in both types of PTC cells after treatment with either PD98059 or U0126. The expression of total ERK1/2 proteins remained unchanged during the treatment.

INHIBITION OF CELL GROWTH BY PD98059 AND U0126

We performed MTT assays on PTC cells with the RET/PTC1 rearrangement or with a BRAF mutation using 75µM PD98059 or 10µM U0126 (Figure 2). After treatment for 4 days, we observed dramatic inhibition of growth of cells with a BRAF mutation (88% reduction in cell growth, P<.001) compared with that of the control cells on day 4. We detected a more modest inhibition of growth of cells with the RET/PTC1 rearrangement (61% and 68% for PD98059 and U0126, respectively; P<.001).

INHIBITION OF CELL GROWTH BY PD98059 OR U0126 IS CAUSED BY PARP CLEAVAGE IN PTC CELLS WITH A BRAF MUTATION

To determine the mechanism of cell-growth inhibition by PD98059 and U0126, we evaluated the expression of
Figure 3. Detection of apoptosis only in cells with a BRAF mutation. A, Cells with the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16) were treated with 75µM PD98059 (+) for 4 days and 1 to 4 days, respectively. B, Cells with the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16) were treated with 10µM U0126 (+) for 4 days and up to 2 days, respectively. The cleaved poly(adenosine diphosphate–ribose) polymerase (PARP) protein was detected in cells with a BRAF mutation when treated with PD98059 for 1 to 4 days or with U0126 for 1 to 2 days using Western blot analysis. The PARP protein was detected in cells with either the RET/PTC1 rearrangement or a BRAF mutation. Actin was used as a loading control and cells treated with dimethyl sulfoxide only were used as positive controls (-).

COMMENT

In the present study, we evaluated the commercially available MEK1/2 inhibitors PD98059 and U0126 for their effects on PTC cells with the RET/PTC1 rearrangement or with a BRAF mutation. Both inhibitors were able to decrease the phosphorylation of ERK1/2. Also, they both inhibited the growth of PTC cells, although to a different extent in cells with the RET/PTC1 rearrangement and cells with a BRAF mutation. Cleaved PARP appeared to be the mechanism of growth inhibition in cells with a BRAF mutation only after treatment with PD98059 or U0126. The mechanism of growth inhibition in cells with the RET/PTC1 rearrangement remains to be determined.

Different types of cancer cells exhibit different levels of sensitivity to PD98059 and U0126 in terms of the concentration necessary to dephosphorylate ERK1/2. For example, in studies of breast carcinoma cell lines treated with PD98059 at 10µM8,21 and with U0126 at 2µM,31,32 the dephosphorylation of ERK1/2 was observed using Western blot analysis. Investigators also treated melanoma cells with 40µM PD98059 and 3µM U0126,29,55 and colon cancer cells with 50µM PD98059 and 20µM U0126,29 the ERK1/2 was dephosphorylated using Western blot analysis. Namba et al36 treated PTC cells with 5µM U0126 for 24 hours and found that it inhibited cell growth by 40% in NPA cells (a PTC cell line with a BRAF mutation) but not in TPC-1 (a PTC cell line with the RET/PTC1 rearrangement). In addition, Specht et al37 treated PTC cells with 1-25µM of U0126 for 3 days and found that inhibition of the growth of NPA cells was statistically significant at 10µM. Our data confirmed the results obtained by Namba et al36 and Specht et al37. When we treated PTC cells with 1µM to 5µM U0126 for 24 hours, the p-ERK1/2 was still detectable using Western blot analysis but was undetectable with 10µM U0126. When we treated PTC cells with 10µM U0126 for up to 4 days, we observed greater than 90% inhibition of growth of PTC cells with a BRAF mutation. The increased growth inhibition in our study compared with that in the study by Namba et al36 (90% vs 40%) was probably a result of our use of a higher concentration of U0126 (10µM vs 5µM).

For PD98059, Ouyang et al31 treated rat thyroid cells carrying a BRAF mutation with 75µM PD98059 and found dephosphorylation of ERK1/2 using Western blot analysis. No information on the concentration of PD98059 used to treat human PTC cells was available in the literature. In most of our experiments, we treated cells with 75µM PD98059, which is the highest concentration that PTC cells can tolerate before crystallization and cytotoxic effects occur. Although treatment with 50µM PD98059 resulted in a decrease in the expression of p-ERK1/2, our time-course study showed that even with treatment at 75µM, the dephosphorylation of ERK1/2 was only transitory (up to 6 hours for p-ERK2 and 24 hours for p-ERK1).

Inactivation of the ERK1/2 by PD98059 and U0126 appeared to be a rapid and reversible process as shown in Western blot analysis. After only 1 hour of treatment with either inhibitors, expression of p-ERK1/2 decreased dramatically in cells with the RET/PTC1 rearrangement and cells with a BRAF mutation. The duration of suppressed p-ERK1/2 expression appeared to be longer with treatment with U0126 than with treatment with PD98059. Nevertheless, both PD98059 and U0126 totally inhibited the growth of cells with a BRAF mutation but only partially inhibited the growth of cells with the RET/PTC1 rearrangement.

The apoptosis pathway is mediated by a cascade of caspases. First, procaspase 9 is activated and results in the cleavage of procaspase 9. Then the cleaved caspase 9 activates caspase 3.38 The substrate of caspase 3 is PARP,
and activation of PARP results in the cleavage of PARP. Cleaved PARP is known to be involved in DNA cleavage and apoptosis execution. Others have reported induction of apoptosis by PD98059 and U0126 using PARP cleavage as an indicator in breast cancer cells, melanoma cells, colon cancer cells, and luteinized granulose cells. In the present study, we also detected PARP cleavage in cells with a BRAF mutation as early as 1 day after exposure to either PD98059 or U0126. The PARP cleavage in cells with a BRAF mutation was detected up to 4 days after treatment with PD98059 or U0126 (data not shown). We did not detect PARP cleavage in cells with the RET/PTC1 rearrangement after treatment with either inhibitor. These results suggest that cells with a BRAF mutation may be more sensitive to some MEK1/2 inhibitors than cells with the RET/PTC1 rearrangement in terms of induction of apoptosis. Furthermore, cells with the RET/PTC1 rearrangement may use pathways other than those used by cells with a BRAF mutation to obtain cellular growth inhibition by these same inhibitors. Despite the inhibitory effects of these inhibitors to PTC cells, both PD98059 and U0126 were used for in vitro study only due to the poor solubility of PD98059 and inactivity of U0126 in vivo. Several new inhibitors have been developed and approved for human use. BAY 43-9006, developed by Bayer Pharmaceuticals Corp, West Haven, Connecticut, is a multifunction kinase inhibitor inhibiting both BRAF and RET kinase activity. CI-1040 (PD0184352), developed by Pfizer Inc, New York, New York, is a specific inhibitor of MEK1/2 and has been shown to inhibit tumor growth in other type of tumors as well. These new and less toxic therapies are clearly needed for PTC, a disease that is increasing in incidence and prevalence of MEK activity. Future studies should examine these new inhibitors that specifically inhibit the BRAF/MEK/ERK pathway in patients with PTC.

Submitted for Publication: November 15, 2006; final revision received February 2, 2007; accepted April 18, 2007.

Correspondence: Gary L. Clayman, DDS, MD, Department of Head and Neck Surgery, Unit 441, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (gclayman@mdanderson.org).

Author Contributions: Dr Clayman had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Henderson, Fredrick, and Clayman. Acquisition of data: Henderson and Clayman. Analysis and interpretation of data: Henderson, Fredrick, and Clayman. Drafting of the manuscript: Henderson and Clayman. Critical revision of the manuscript for important intellectual content: Henderson, Fredrick, and Clayman. Obtained funding: Clayman. Administrative, technical, and material support: Henderson. Study supervision: Fredrick and Clayman.

Financial Disclosure: None reported.

Funding/Support: This work was partially supported by National Institutes of Health Independent Award R01 DE-13954, National Institutes of Health Specialized Program of Research Excellence in Head and Neck Cancer grant 1P50-CA-97007, M. D. Anderson Cancer Center Support grant 5P30-CA-16672, the Michael A. O'Bannon Endowment for Cancer Research, the Betty Berry Cancer Research Fund, the State of Texas Tobacco Settlement Funds, and National Cancer Institute Cancer Center Support (CORE) grant CA-16672 for media production.

Additional Contributions: Jerome Hershman, MD, from VA Greater Los Angeles Healthcare System (Los Angeles, California) provided all of the PTC cell lines; Dianna Roberts, PhD, performed statistical analysis; Donald Norwood critically reviewed and edited the manuscript; Ya’an Kang, PhD, Chandrani Chattopadhyay, PhD, Abhijit Mazumdar, PhD, Arumugam Jayakumar, PhD, Mary Wang, MS, Katrina Briggs, Paula Holton, MS, and Kelli Cottingham provided technical support; and Sonia Perez helped prepare the manuscript.

REFERENCES


32. Ripple MO, Kalmadi S, Eastman A. Inhibition of either phosphatidylinositol 3-kinase/ Akt or the mitogen/extracellular-regulated kinase, MEK/ERK, signaling pathways suppress growth of breast cancer cell lines, but MEK/ERK signaling is critical for cell survival. *Breast Cancer Res Treat*. 2005;93(2):177-188.


