The Akt-Specific Inhibitor MK2206 Selectively Inhibits Thyroid Cancer Cells Harboring Mutations That Can Activate the PI3K/Akt Pathway

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Context: The phosphoinositide 3-kinase (PI3K)/Akt pathway is widely postulated to be an effective therapeutic target in thyroid cancer.

Objective: The aim of the study was to test the therapeutic potential of the novel Akt inhibitor MK2206 for thyroid cancer.

Design: We examined the effects of MK2206 on thyroid cancer cells with respect to the genotypes of the PI3K/Akt pathway.

Results: Proliferation of thyroid cancer cells OCUT1, K1, FTC133, C643, Hth7, and TPC1, which harbored PIK3CA, PTEN, Ras, or RET/PTC mutations that could activate the PI3K/Akt pathway, was potently inhibited by MK2206 with IC50 values mostly below or around 0.5 μM. In contrast, no potent inhibition by MK2206 was seen in most of the Hth74, KAT18, SW1736, WRO, and TAD2 cells that did not harbor mutations in the PI3K/Akt pathway. The inhibition efficacy was also genetically selective. Specifically, the average inhibition efficacies were 59.2 ± 11.3 vs. 36.4 ± 8.8% (P = 0.005) at 1 μM MK2206 and 64.4 ± 11.5 vs. 38.5 ± 18.9% (P = 0.02) at 3 μM MK2206 for cells with mutations vs. cells without. The SW1736 cell, lacking mutations in the PI3K/Akt pathway, had minimal response to MK2206, but transfection with exogenous PIK3CA mutants, PIK3CA H1047R and E545K, significantly increased its sensitivity to MK2206. MK2206 also completely overcame the feedback activation of Akt from temsirolimus-induced mammalian target of rapamycin suppression, and the two inhibitors synergistically inhibited thyroid cancer cell growth.

Conclusions: Our study demonstrates a genetic selectivity of MK2206 in inhibiting thyroid cancer cells by targeting the PI3K/Akt pathway, supporting a clinical trial in thyroid cancer. (J Clin Endocrinol Metab 96: E577–E585, 2011)
Considerable progress has occurred in understanding the derangements of molecular signaling pathways in thyroid cancer. Among them is the phosphoinositide 3-kinase (PI3K)/Akt pathway, which, driven by genetic alterations, plays a fundamental role in the tumorigenesis and aggressiveness of human cancers (8–11). This signaling pathway regulates cell proliferation, division, differentiation, and apoptosis and, when deranged, promotes tumorigenesis. In the activation process of the PI3K/Akt pathway, signals from cell membrane receptors, such as receptor tyrosine kinases, cause the activation of class IA PI3K, in which the PI3KCA is a major catalytic subunit that catalyzes the conversion of phosphatidylinositol-4, 5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate (PIP₃). The latter recruits the serine-threonine protein kinase Akt to the cell membrane where Akt is phosphorylated and activated by phosphoinositide-dependent kinases. Activated Akt plays a critical role in amplifying the signaling of this pathway by phosphorylating a wide array of protein substrates, thereby regulating various cellular functions. Other signaling molecules that can activate the PI3K/Akt pathway include Ras and RET/PTC (12, 13). PTEN, a phosphatase of PIP₃, plays a fundamental role in the degradation of PIP₃ and hence termination of the signaling of the PI3K/Akt pathway (14, 15).

Among the signaling events downstream of Akt in the PI3K/Akt pathway, activation of the mammalian target of rapamycin (mTOR) is a major one (16, 17). mTOR exists in two complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR and raptor (rapamycin-sensitive) and, upon activation, phosphorylates effectors such as ribosomal protein S6 kinase (p70S6K). Activated p70S6K regulates protein synthesis, cell proliferation, and other molecular and cellular functions (18). mTORC2 consists of mTOR and rictor (rapamycin-insensitive) and, interestingly, among its substrates to phosphorylate is the upstream Akt. Suppression of mTORC1 by rapamycin could also cause Akt phosphorylation through a feedback mechanism involving the induction of upstream receptor tyrosine kinase signaling (19, 20). Such rapamycin-initiated Akt signaling activates the rapamycin-insensitive mTORC2, which, in turn, can further phosphorylate Akt, thus creating a self-enhancement mechanism for Akt when under the influence of rapamycin. This may be an important mechanism for cancer cell resistance to mTOR inhibitors, which may require combination treatments to overcome.

Genetic alterations are common in many of the components of the PI3K/Akt pathway in thyroid cancer, which is the basis of the widely observed aberrant activation of this pathway in this cancer (10, 11, 21). Thus, the PI3K/Akt pathway is an attractive therapeutic target for thyroid cancer (22–27).

In the present study, we tested the therapeutic potential of a novel allosteric Akt inhibitor, MK2206, its genetic dependency, and its combinational effect with the mTOR inhibitor, temsirolimus, in thyroid cancer cells.

### Materials and Methods

#### Thyroid cancer cell lines and reagents

C643, Hth7, Hh74, and SW1736 human cancer cell lines were originally from Dr. N. E. Heldin (University of Uppsala, Uppsala, Sweden); the TPC1 cell line was from Dr. Alan P. Dackiw (Johns Hopkins University, Baltimore, MD); FTC133 was from Dr. Geor Grub (University of Manchester, Manchester, UK); OCUT1 was from Dr. Naoyoshi Onoda (Osaka City University Graduate School of Medicine, Osaka, Japan); K1 was from Dr. David Wyndford-Thomas (University of Wales College of Medicine, Cardiff, UK); KAT18 was from Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY); and WRO-82-1 was from Dr. G. J. F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA). The normal thyroid cell line TAD2 was from Dr. Mario Vitale (Università Federico II, Naples, Italy). All the cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in 5% CO₂ at 37 °C, except for FTC133, which was cultured in DMEM/Ham’s F-12 medium. MK2206, purchased from ChemiTek (Indianapolis, IN), and temsirolimus, from Wyeth Pharmaceuticals (Madison, NJ), were dissolved in dimethylsulfoxide and ethanol, respectively, at 10 mM in stock and stored at −20 °C. The drugs were used at the indicated final concentrations in culture medium containing 5% fetal bovine serum and replenished every 24 h.

#### Western blotting

Cells were washed with PBS and lysed in RIPA buffer with 1% phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). The protein samples were loaded onto 10% SDS-PAGE and electronically transferred into polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 for 1 h and then blotted with the following primary antibodies: anti-phospho-Akt1/2/3 (Ser 473) (sc-7985-R), anti-Akt1/2 (sc-8312), anti-Actin (sc-1616-R) (all from Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho-p70S6K (9205) (Cell Signaling Technology, Beverly, MA). The membranes were then washed with PBS with 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated antirabbit (sc-2004) or antimouse (sc-2005) secondary antibodies. The signals were visualized with the chemiluminescent enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

#### Proliferation assay

Cell proliferation assay after drug treatments was performed using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylnitrazole bromide (MTT) assay. In brief, cells were plated in 96-well plates in triplicate and treated with drugs at the indicated concentrations for 5 d. The drug was replenished every 24 h. At the end of culture, 10 μl of 5 mg/ml MTT agent (Invitrogen, Carlsbad, CA) was added to each well and incubated for 4 h.
4 h, followed by overnight incubation with 100 μl of 10% sodium dodecyl sulfate solution. Absorbance was measured on a microplate reader at the test wavelength of 570 nm and the reference wavelength of 670 nm. IC50 values were calculated using the Reed-Muench method (28).

Analysis of combined drug effects
Based on the median-effect method of Chou and Talalay (29), we used the CalcuSyn software (Biosoft, Great Shelford, Cambridge, UK) to calculate the combination index (CI) and isobologram to quantitatively determine drug interactions, as described previously (27). CI values below 1, equal to 1, and above 1 represent synergism, additivity, and antagonism, respectively. The isobologram is formed by plotting the concentrations of each drug required for 50% inhibition (ED50) on x- and y-axes, respectively, and connecting them to draw a line segment, which is ED50 isobologram. Combination data points that fall on, below, and above the line segment represent additivity, synergy, and antagonism, respectively.

Statistical analysis of data
Data presented here represent at least three similar experiments. Relative cell growth graphic and cell proliferation inhibition rates were presented as mean ± SD. Comparison of cell proliferation rates of MK2206 between the mutation-positive cell group and the mutation-negative cell group was performed using unpaired two-sample t test.

Results
Potent inhibition of the Akt phosphorylation in thyroid cancer cells by MK2206
We first examined the dose- and time-dependent effects of the Akt inhibitor MK2206 on the signaling of the PI3K/Akt pathway in thyroid cancer cells by treating two selected cells, OCUT1 and FTC133, at various concentrations of the drug and different time points. As shown in Fig. 1A, MK2206 inhibited Akt phosphorylation (p-Akt) in a dose-dependent manner; at 0.01 μM of MK2206, an inhibition of Akt phosphorylation was already visible, and a complete inhibition was observed at 0.5 μM. Significant drug effects started as early as 1 h and lasted for at least 24 h (Fig. 1B). We also tested the effect of MK2206 in the remaining thyroid cancer cell lines and a normal human thyroid cell line TAD2 at 0.5 μM for 24 h and observed a complete or near-complete inhibition of Akt phosphorylation in nearly all these cells (Fig. 1C).

Inhibition of proliferation of thyroid cancer cells by MK2206
We next examined the inhibitory effects of MK2206 on the proliferation of thyroid cancer cells with various mutations in the PI3K/Akt pathway (presented in Table 1). Thyroid cancer cells OCUT1, K1, FTC133, C643, Hth7, and TPC1 harbored PIK3CA, PTEN, Ras, or RET/PTC mutations that could activate the PI3K/Akt pathway. MK2206 inhibited the proliferation of all these cells with IC50 values in the low micromolar range, mostly below or around 0.5 μM (Fig. 2 and Table 1). Thus, all the cells that harbored mutations that could activate the PI3K/Akt pathway could be potently inhibited by MK2206. In the group of cells that did not harbor known mutations in the PI3K/Akt pathway, MK2206 displayed a minimal effect or no inhibitory effect on SW1736, WRO, and TAD2 cells; these cells, particularly WRO and TAD2 cells, were resistant to MK2206 with IC50 values above 1000 μM (Fig. 2 and Table 1).
TABLE 1. Genotypes of thyroid cell lines and their sensitivities to MK2206

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origins</th>
<th>Genetic alterations</th>
<th>IC50 of MK2206 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCUT1</td>
<td>ATC</td>
<td>PIK3CA (H1047R)</td>
<td>0.14</td>
</tr>
<tr>
<td>K1</td>
<td>PTC</td>
<td>PIK3CA (E545K)</td>
<td>0.52</td>
</tr>
<tr>
<td>FTC133</td>
<td>FTC</td>
<td>PTEN (allele deletion and R130*)</td>
<td>0.18</td>
</tr>
<tr>
<td>C643</td>
<td>ATC</td>
<td>HRAS (G13R)</td>
<td>0.27</td>
</tr>
<tr>
<td>Hth7</td>
<td>ATC</td>
<td>NRAS (Q61R)</td>
<td>4.5</td>
</tr>
<tr>
<td>TPC1</td>
<td>PTC</td>
<td>RET/PTC1 rearrangement</td>
<td>0.59</td>
</tr>
<tr>
<td>Hth74</td>
<td>ATC</td>
<td></td>
<td>2.19</td>
</tr>
<tr>
<td>KAT18</td>
<td>ATC</td>
<td></td>
<td>4.62</td>
</tr>
<tr>
<td>SW1736</td>
<td>ATC</td>
<td></td>
<td>47.56</td>
</tr>
<tr>
<td>WRO</td>
<td>FTC</td>
<td></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>TAD2</td>
<td>Normal</td>
<td></td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

+/-, Heterozygous mutation; +/-, homozygous mutation; *, stop codon.

cancer cells, we examined the sensitivities of SW1736 cells transfected with two PIK3CA mutants, H1047R and E545K, as described previously (25), to MK2206. SW1736 cells had no detectable genetic mutations in the PI3K/Akt pathway and were relatively resistant to MK2206 (Fig. 2 and Table 1). As shown in Fig. 4A, SW1736 cells transfected with PIK3CA H1047R or E545K showed a higher level of p-Akt than cells transfected with the vector, demonstrating activation of the PI3K/Akt pathway by the mutants. Interestingly, SW1736 cells transfected with the two PIK3CA mutants were more potently inhibited by MK2206 than cells transfected with the vector (Fig. 4B), with IC50 values of 1.76, 2, and 33 μM, respectively (Fig. 4C). The PIK3CA mutants also significantly increased the inhibition rates of SW1736 cells (Fig. 4D). Specifically, the cell inhibition rates at 1 μM MK2206 were 31 ± 3.3, 40 ± 3.3, and 47.4 ± 3.7% for cells transfected with vector, H1047R, and E545K, respectively (P = 0.03 and 0.005, respectively, for comparison of each mutant with the vector). The cell inhibition rates at 3 μM MK2206 were 30.2 ± 2.7, 58.4 ± 4, and 47.8 ± 2.6% for cells transfected with vector, H1047R, and E545K, respectively (P = 0.006 and 0.001, respectively, for comparison of each mutant with the vector). These results provided further evidence that genetic alterations in the PI3K/Akt pathway potentiated the inhibition of thyroid cancer cells by MK2206.

MK2206 suppressed the mTOR inhibitor temsirolimus-induced Akt activation and synergistically inhibited the proliferation of thyroid cancer cells when combined with temsirolimus

The feedback of mTOR on Akt found in other cancer cells predicted Akt activation with the use of mTOR inhibitors (19, 20), which may represent a mechanism for the low therapeutic efficacy of mTOR inhibitors used alone in some clinical studies on human cancers (30, 31). We thus tested whether in thyroid cancer cells, the currently widely clinically tried mTOR inhibitor temsirolimus could also induce activation of Akt and, if yes, whether the novel Akt inhibitor MK2206 could be used to overcome it. We selected two thyroid cancer cells, OCUT1 and K1, both of which harbored PIK3CA mutations, for this test. As shown in Fig. 5A, phosphorylation of the mTOR downstream target p70S6K was inhibited by temsirolimus at 10 nM as expected, suggesting that this mTOR inhibitor exerted the expected target effect. At the same time, Akt phosphorylation was dramatically increased by temsirolimus. This feedback effect on Akt was already significant at 1 h after the treatment of cells, which lasted for at least 8 h. Remarkably, when combined with MK2206, the temsirolimus-induced Akt activation was completely suppressed (Fig. 5B). Thus, the novel Akt inhibitor MK2206 could completely over-
come the activating feedback of mTOR inhibition on Akt, demonstrating its potential utility in combination therapy with temsirolimus for thyroid cancer. Both temsirolimus and MK2206 could substantially inhibit the phosphorylation of p70S6K (Fig. 5B), confirming their expected targeting of the PI3K/Akt pathway.

Given the key roles of Akt and mTOR in the PI3K/Akt pathway signaling and their independent functions as well as the feedback effect of the latter on the former, we next explored possible synergistic effects of targeting both of them on thyroid cancer cell proliferation. To this end, we selected treatment of OCUT1 and K1 cells with MK2206 and temsirolimus, individually or in combinations, with constant ratios of the two at the indicated concentrations for 5 d. Consistent with the results in Fig. 2, OCUT1 and K1 cells both showed a high sensitivity to MK2206; similarly, temsirolimus also potently inhibited proliferation of the two cells (Fig. 5C, upper panel), consistent with our previous findings (25, 27). Combination of the two drugs markedly potentiated the inhibitory effect of each other on cell proliferation (Fig. 5C, upper panel). Using the Chou-Talalay method (29), we calculated the CI and isobologram to further examine the interactions between the two inhibitors. As shown in the middle panel of Fig. 5C, all the CI values, at different combinations of the indicated concentrations of the two inhibitors, were less than 1 in the two cell lines, with virtually all the points less than 0.5. These CI values suggest a strong synergism between the...
two inhibitors. In the isobologram analysis, with a line drawn to connect the ED50 points of MK2206 and temsirolimus on the x- and y-axes, respectively, the combination data points fell below the line in both cells (Fig. 5C, lower panel), which again demonstrated the strong synergism of MK2206 and temsirolimus in the inhibition of thyroid cancer cell proliferation. We also performed apoptosis assay under the MK2206 treatment conditions and did not find cell apoptosis. This was the case even when MK2206 was used in combination with temsirolimus (data not shown). Therefore, the MTT results of these drugs in thyroid cancer cells reflected inhibition of cell proliferation.

**Discussion**

MK2206 is a recently developed novel allosteric Akt inhibitor that showed great promise in phase I clinical trials on patients with advanced solid tumors (32). In the present study, we tested the therapeutic potential of MK2206 for thyroid cancer using various thyroid cancer cells with known genotypes in the PI3K/Akt pathway. We demonstrated its potent and efficacious inhibition of proliferation of thyroid cancer cells that harbored mutations in the PI3K/Akt pathway. This was particularly the case with the cells harboring classical *PTEN* and *PIK3CA* mutations, consistent with the powerful role of these mutations in the activation of PI3K/Akt pathway in human cancers (14, 33). Significant preferentiality of the effects of MK2206 was also seen in cells that harbored Ras and RET/PTC mutations, which could also activate the PI3K/Akt pathway (12, 13). The effect of MK2206 was minimal, in general, in cells that did not harbor mutations in the PI3K/Akt pathway. This genetic dependency of MK2206 in thyroid cancer cells was reflected both by lower IC50 values and higher inhibition rates in cells harboring mutations in the PI3K/Akt pathway. Moreover, exogenous expression of PIK3CA mutants in thyroid cancer cells that normally harbored no detectable mutations in the PI3K/Akt pathway significantly increased the sensitivity of cells to MK2206, which further supported the genetic-dependent effect of MK2206. It is noted that MK2206 displayed dependence on genetic alterations activating the PI3K/Akt pathway in its inhibition of cell growth, but it inhibited p-Akt in virtually all cells irrespective of their genotypes albeit with variations in extent. MAPK kinase inhibitors could also generally inhibit p-ERK irrespective of the *BRAF* mutation status but only inhibited the growth of cells harboring the *BRAF* mutation (23, 27). This has been interpreted as showing that cells with *BRAF* mutation have become addicted to and dependent on the overdriving of the MAPK pathway by *BRAF* mutation for survival. Our results of the Akt inhibitor MK2206 could be explained by the same mechanism—cells with genetic alterations activating the PI3K/Akt pathway have become addicted to and dependent on the overdriving of the PI3K/Akt pathway by these genetic alterations.

We previously reported that perifosine, an older-generation Akt inhibitor, also preferentially inhibited thyroid cancer cells that harbored genetic alterations in the PI3K/Akt pathway (25). Perifosine is an alkyl phospholipid, which structurally resembles naturally occurring phospholipids. The mechanism in its inhibition of PI3K/Akt signaling seemed to involve the disturbing of the membrane localization of Akt and hence its phosphorylation (34). Therefore, perifosine does not directly affect Akt. Moreover, due to its phospholipid nature, perifosine perturbs multiple other pathways related to phospholipids, in
addition to the PI3K/Akt pathway. Nevertheless, it is interesting, and important as well from a therapeutic point of view, to see the preferentiality of perifosine-induced cell inhibition in thyroid cancer cells that harbored genetic alterations in the PI3K/Akt pathway (25). However, given the potential promiscuous effects of perifosine, more specific agents are needed to definitely test the concept of genetic-based targeting of the PI3K/Akt pathway as an effective therapeutic strategy for thyroid cancer. We reasoned in the present study that using MK2206 could help fulfill this mission because, as a potent non-ATP competitive allosteric Akt inhibitor (35), MK2206 could specifically inhibit the Akt kinases. It is noted that the Hth74 cell had resistance to perifosine (25), whereas it was sensitive to MK2206 in the present study. This probably reflects the fact that perifosine is less specific for the PI3K/Akt pathway and, by disturbing multiple signaling pathways, may result in unexpected net outcomes.

The strong response of Hth74 cell to MK2206 may, however, suggest the existence of an unidentified mutation that could activate the PI3K/Akt pathway in this cell. The present results provide further evidence to support the advocacy for a genetic-based approach in designing therapies for thyroid cancer using novel agents (36). Because genetic alterations in the PI3K/Akt pathway are particularly common in FTC and ATC (11, 21), PI3K/Akt genetic-based targeting can be expected to be particularly useful for these thyroid cancers, whereas therapeutic genetic targeting in the MAPK pathway may be particularly effective for PTC (36).

The mTOR inhibitor temsirolimus has been widely tested in clinical trials for human cancers and is a highly promising new anticancer drug. Our recent preclinical studies also demonstrated the promises of this drug for thyroid cancer, but its inhibition rate or efficacy in thyroid cancer cells was relatively modest (25, 27). In clinical trials on human cancers, the response rates of temsirolimus varied among different studies and cancers, often with limited responses (30, 31, 37, 38). One likely explanation is the phosphorylation and activation of Akt through the feedback mechanisms initiated by temsirolimus-induced inhibition of mTORC1 (17, 19, 39). In the present study, we demonstrated an increase in the phosphorylation of Akt upon inhibition of mTOR by temsirolimus in thyroid cancer cells. This represents an important mechanism that could potentially limit the efficacy of temsirolimus as a future treatment of thyroid cancer. We demonstrated that this activating effect of mTOR suppression by temsirolimus on Akt could be completely abolished by MK2206. We also demonstrated a strong synergism of the two inhibitors in their inhibition of the proliferation of thyroid cancer cells, similar to the enhanced inhibition of other cancer cells by dually suppressing Akt and mTOR using other inhibitors (39, 40).
Therefore, the combined use of MK2206 and temsirolimus, when guided by the genotypes of the PI3K/Akt pathway, may prove to be a particularly effective future treatment for thyroid cancer.

In summary, the present study demonstrates a promising genetic-dependent therapeutic potential of MK2206 for thyroid cancer. The data provide further evidence that mutation-based targeting of the PI3K/Akt pathway using Akt inhibitors is an attractive therapeutic strategy for thyroid cancer. In this context, genetic-guided combinational use of MK2206 and temsirolimus may prove to be a particularly effective treatment for this cancer, which warrants a clinical trial.

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