MK-2206, an Allosteric Akt Inhibitor, Enhances Antitumor Efficacy by Standard Chemotherapeutic Agents or Molecular Targeted Drugs In vitro and In vivo

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Abstract
The serine/threonine kinase Akt lies at a critical signaling node downstream of phosphatidylinositol-3-kinase (PI3K) and is important in promoting cell survival and inhibiting apoptosis. An Akt inhibitor may be particularly useful for cancers in which increased Akt signaling is associated with reduced sensitivity to cytotoxic agents or receptor tyrosine kinase inhibitors. We evaluated the effect of a novel allosteric Akt inhibitor, MK-2206, in combination with several anticancer agents. In vitro, MK-2206 synergistically inhibited cell proliferation of human cancer cell lines in combination with molecular targeted agents such as erlotinib (an epidermal growth factor receptor inhibitor) or lapatinib (a dual epidermal growth factor receptor/human epidermal growth factor receptor 2 inhibitor). Complementary inhibition of erlotinib-insensitive Akt phosphorylation by MK-2206 was one mechanism of synergism, and a synergistic effect was found even in erlotinib-insensitive cell lines. MK-2206 also showed synergistic responses in combination with cytotoxic agents such as topoisomerase inhibitors (doxorubicin, camptothecin), antimitabolites (gemcitabine, 5-fluorouracil), anti-microtubule agents (docetaxel), and DNA cross-linkers (carboplatin) in lung NCI-H460 or ovarian A2780 tumor cells. The synergy with docetaxel depended on the treatment sequence; a schedule of MK-2206 dosed before docetaxel was not effective. MK-2206 suppressed the Akt phosphorylation that is induced by carboplatin and gemcitabine. In vivo, MK-2206 in combination with these agents exerted significantly more potent tumor inhibitory activities than each agent in the monotherapy setting. These findings suggest that Akt inhibition may augment the efficacy of existing cancer therapeutics; thus, MK-2206 is a promising agent to treat cancer patients who receive these cytotoxic and/or molecular targeted agents. Mol Cancer Ther; 9(7): 1956–67. ©2010 AACR.

Introduction
Abnormal activation of phosphatidylinositol-3-kinase (PI3K) has been validated as an essential step in the initiation and maintenance of human tumors. Akt is a serine-threonine kinase activated by growth factors or survival factors through PI3K to promote cell growth and survival (1–3). Constitutive pathway activation can result from distinct and/or complementary biological events including (a) constitutively activating mutations or amplification of receptor tyrosine kinases (RTK); (b) amplification of PI3K; (c) activating mutations in the PIK3CA gene encoding the p110α catalytic subunit; (d) overexpression of the downstream kinase Akt; (e) loss or inactivating mutations of the tumor suppressor gene phosphatase and tensin homolog (PTEN), an endogenous negative regulator of the PI3K pathway; or (f) constitutive recruitment and activation by mutant forms of the RAS oncogene (4–7). One important function of activated PI3K in cells is the inhibition of apoptosis, and Akt is a good candidate for mediating these PI3K-dependent cell survival responses. Akt is an antiapoptotic factor in many different cell death stimuli, including the withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation and treatment with chemotherapeutic drugs, and ischemic shock (8–11). Indeed, Akt activation and overexpression is often associated with resistance to chemotherapy or radiotherapy (12–14). Reversal of drug resistance has been shown by PI3K inhibitors and PTEN overexpression in PTEN-null cells (14–17). Dominant-negative mutants of Akt enhance the cytotoxicity of chemotherapeutic agents, suggesting an important role of Akt in drug resistance (16, 18). Thus, clinically suitable small-molecule inhibitors of Akt have great potential in cancer treatment (19–22). Moreover, the identification
MK-2206 is an orally active allosteric Akt inhibitor that is under development for the treatment of solid tumors. MK-2206 is a highly potent and selective Akt inhibitor. It is equally potent toward purified recombinant human Akt1 (IC_{50}, 5 nmol/L) and Akt2 enzyme (IC_{50}, 12 nmol/L) and approximately 5-fold less potent against human Akt3 (IC_{50}, 65 nmol/L; ref. 24). In the present report, we describe the combination effects of MK-2206 with various anticancer drugs, including docetaxel, carboplatin, gemcitabine, 5-fluorouracil (5-FU), doxorubicin, camptothecin, and RTK inhibitors such as lapatinib and erlotinib.

Materials and Methods

Drugs and chemicals

The chemical name of MK-2206 is 8-[4-(1-amino-cyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]napthyridin-3(2H)-one hydrochloride [1:1]. Its chemical structure is shown in Supplementary Fig. S1. For in vitro experiments, the stock solutions of MK-2206, erlotinib (Roche), lapatinib (GlaxoSmithKline), docetaxel (Sigma), and camptothecin (Sigma) were formulated in DMSO, carboplatin, and doxorubicin (Sigma) in sterile distilled water, gemcitabine (Eli Lilly), and 5-FU (Sigma) in PBS (pH 7.4).

Cell lines and cell culture

Cell lines were purchased from the American Type Culture Collection, except for A2780, which was obtained from the European Collection of Cell Culture. Cells were cultured in RPMI 1640 containing heat-inactivated 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified incubator at 37°C in 5% CO_{2}.

Cell proliferation assay and determination of combination index

Cells were seeded at a density of 2 to 3 × 10^5 per well in 96-well plates. Twenty-four hours after plating, varying concentrations of the drug, either as a single agent or in combination, were added to the wells. Cell proliferation was determined by using the CellTiter-Glo assay (Promega) according to the manufacturer’s recommended protocol. The soluble protein concentration was determined by using a detergent-compatible protein assay kit (Pierce). Proteins were resolved on SDS-PAGE, transferred onto a nitrocellulose filter, and probed with the following antibodies: phosphor-Akt (Ser473), total Akt, phosphor-PRAS40 (Thr244), and total S6. The protein-antibody complexes were detected by using an enhanced chemiluminescence kit (GE Healthcare) according to the manufacturer’s instructions.

Flow cytometry

Cells treated with various reagents were harvested by exposure to trypsin, fixed, and stained with propidium iodide by using CycleTEST plus DNA reagent kit (Becton Dickinson). At least 1 × 10^6 cells were analyzed for DNA content with the use of a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson).

Induction of phosphor-Akt by carboplatin or gemcitabine

Cells were seeded at a density of 5 × 10^4 per well in 96-well plates. Twenty-four hours after plating, carboplatin or gemcitabine was added to the cell culture at an IC_{50} that was independently determined in a cell proliferation assay. The concentrations of carboplatin used for each cell line were 185 μmol/L (UMC-11), 200 μmol/L (NCI-H441), 112 μmol/L (NCI-H1703), 89 μmol/L (NCI-H661), 103 μmol/L (NCI-H1993), and 19 μmol/L (NCI-H460). The concentrations of gemcitabine used for each cell line were 185 μmol/L (UMC-11), 200 μmol/L (NCI-H441), 0.7 nmol/L (NCI-H1703), 10 nmol/L (NCI-H661), 28 nmol/L (NCI-H1993), and 5 nmol/L (NCI-H460). After a 4-, 8-, or 24-hour exposure, the cellular amount of pAkt was determined with a quantitative pAkt (serine 473)/total Akt MSD assay kit (Meso Scale Discovery) according to the manufacturer’s recommended protocol.

Efficacy studies in mouse xenograft models

All animal experiments were done according to an Institutional Animal Care and Use Committee-approved protocol.
Institutional guidelines for the proper and humane use of animals in research were followed. The facility has been approved by Association for Assessment and Accreditation of Laboratory Animal Care. Animals were used between the ages of 6 and 7 weeks and purchased from Charles River Japan and CLEA Japan Co. Ltd. Human tumor cells were suspended in 50% Matrigel (BD) and 50% PBS and were injected s.c. into the left flank of the mice.

When the mean tumor size reached 0.13 cm³ for the SK-OV-3 or 0.2 cm³ for the NCI-H292, HCC70, PC-3, and NCI-H460 models, the mice were randomized into control and treatment groups with approximately equivalent ranges of tumor volume between groups (n = 5 animals per group). The following vehicles were used to dose the compounds: 30% Captisol (Cydex) for MK-2206; 0.5% methylcellulose + 0.1% Tween 80 for erlotinib; distilled water for lapatinib; 0.73% ethanol in saline for docetaxel (Aventis); and saline for carboplatin (Bristol-Myers Squibb) and gemcitabine (Eli-Lilly). The control group received vehicle only. Tumor volume was measured with calipers twice a week. Animal body weight and physical signs were monitored during the experiments. Tumor volume was calculated, taking length to be the longest diameter across the tumor and width to be the perpendicular diameter, by using the following formula: (length × width)² × 0.5. Relative tumor volume was assessed by dividing the tumor volume on different observation days with the starting tumor volume. Statistical significance was evaluated by using the two-way repeated ANOVA test followed by Dunnett’s test or an unpaired t test.

**Pharmacodynamic study in mouse xenograft models**

When the mean tumor size reached 0.2 cm³, mice were randomized into control and treatment groups (n = 3). The tumors were collected from animals, snap frozen in liquid nitrogen, and stored at −80°C for pharmacodynamic analysis. Tumor tissue was lysed in lysis buffer and processed for Western blot analysis as described in the Western blot section.

**Results**

**Concurrent Akt and EGFR inhibition is synergistic in human non–small-cell lung cancer lines**

We evaluated the antiproliferative effects of MK-2206 and the EGFR inhibitor erlotinib in combination on seven non–small-cell lung cancer (NSCLC) and skin epidermoid cell lines. We selected NSCLC cell lines because erlotinib is used to treat patients who have locally advanced or metastatic NSCLC. The A431 epidermoid cell line was used because of its EGFR amplification. Table 1A summarizes the IC₅₀ values of each agent and the CI values for eight cell lines. MK-2206 by itself causes growth inhibition of the eight cell lines with an IC₅₀ that ranges between 3.4 and 28.6 μmol/L. MK-2206 alone more potently inhibited the cell growth of Ras wild-type (WT) cell lines (A431, HCC827, and NCI-H292; IC₅₀s of 5.5, 4.3, and 5.2 μmol/L, respectively) as compared with Ras-mutant cell lines (NCI-H358, NCI-H23, NCI-H1299, and Calu-6; IC₅₀s of 13.5, 14.1, 27.0, and 28.6 μmol/L, respectively), with the exception of NCI-H460, which has a PIK3CA E545K

<table>
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<th>Combination index</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ED₅₀</td>
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<tr>
<td>(A) Erlotinib</td>
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<td></td>
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<tr>
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(B) Lapatinib

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<td>SK-OV-3</td>
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<td>NCI-N87</td>
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(Continued on the following page)
mutation (IC50, 3.4 μmol/L). These results are consistent with previous findings that cell lines with Ras/mitogen-activated protein kinase pathway activation are resistant to PI3K inhibitor (26). Erlotinib alone inhibits cell growth with an IC50 ranging from 0.02 to 35.6 μmol/L. HCC827, which contains EGFR amplification and deletion (E746–750), was the most erlotinib-sensitive cell line among the evaluated lines (IC50, 0.02 μmol/L). To determine the combination effects, cells were treated with both agents at fixed ratios spanning the IC50 of each agent. CI values were calculated on the basis of parameters derived from median-effect plots of MK-2206 alone, erlotinib alone, and the combination of two agents at fixed ratios. CI values were determined at different dose-effect levels: at ED50 (effective dose of 50% response), ED75 (that of 75% response), and ED90 (that of 90% response). A CI <1 is evidence for synergy, whereas a CI >1 indicates antagonism. A CI value close to 1 indicates additivity.

The combination of MK-2206 and erlotinib induces apoptosis by caspase activation

To confirm the synergistic interaction of MK-2206 with erlotinib, we evaluated the induction of apoptotic cell death in NCI-H292 cells. MK-2206 and erlotinib alone at concentrations up to 3 and 20 μmol/L, respectively, did not induce a significant increase in activated caspase-3/7 (Fig. 1A). Erlotinib alone did not activate caspase-3/7 up to 40-fold higher dose than IC50 (0.5 μmol/L) in the cell proliferation assay (Table 1A), suggesting that apoptosis induction did not contribute much in inhibition of cell proliferation by erlotinib. The combination of both agents induced activated caspase-3/7 in a dose-dependent manner. Caspase activation became apparent at 2.5 μmol/L erlotinib and 3 μmol/L MK-2206 or higher. These results suggest that the combination of these agents enhances cell death and that this enhancement leads, at least in part, to the synergistic inhibition of cell proliferation.

Table 1. Synergistic inhibition of cell proliferation by MK-2206 combined with erlotinib or lapatinib in 14 tumor cell lines (Cont’d)

<table>
<thead>
<tr>
<th>IC50 (μmol/L)</th>
<th>Genotypes</th>
<th>MK-2206</th>
<th>Erlotinib/Lapatinib</th>
<th>PI3K</th>
<th>PTEN</th>
<th>Ras</th>
<th>EGFR/ErbB2</th>
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<td>1.9</td>
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<td>WT</td>
<td>EGFR amp</td>
</tr>
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<td>0.02</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>EGFR E746–750 del</td>
</tr>
<tr>
<td>5.2</td>
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<td>13.5</td>
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<td>K-Ras G12C</td>
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<tr>
<td>14.1</td>
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<tr>
<td>27.0</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>N-Ras Q61K</td>
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<tr>
<td>28.6</td>
<td>35.6</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>K-Ras Q61K</td>
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<tr>
<td>3.4</td>
<td>19.9</td>
<td>PIK3CA E545K</td>
<td>WT</td>
<td>K-Ras Q61H</td>
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<tr>
<td>0.3</td>
<td>3.2</td>
<td>WT</td>
<td>F90 fs</td>
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<td>2.6</td>
<td>4.5</td>
<td>WT</td>
<td>A72 fs</td>
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<tr>
<td>0.1</td>
<td>0.03</td>
<td>WT</td>
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<tr>
<td>2.9</td>
<td>5.5</td>
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<td>5.1</td>
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<td>1.6</td>
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<td>ErbB2 amp</td>
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</table>

NOTE: Cells were simultaneously treated for 72 h with MK-2206 and erlotinib or lapatinib at constant concentration ratios spanning the IC50 dose of each agent. The evaluation of cell growth inhibitory effects was done by monitoring the cellular ATP concentration as described in Materials and Methods. CI values were calculated by the Chou and Talalay method for drug interactions using CalcuSyn software for the different fractions affected (the CI values at ED50, ED75, and ED90 were summarized). Mutation data were from the COSMIC database or reference article (47).

Abbreviations: NA, not available; amp, amplification; del, deletion; fs, frame shift.

Indeed, the IC50s of the erlotinib-insensitive cells such as NCI-H23 and NCI-H1299 were shifted 2.4-fold (from 10.6 to 4.4 μmol/L) and >7.9-fold (from >30 to 3.8 μmol/L), respectively, in combination with 1 μmol/L MK-2206 (data not shown).
Concurrent inhibition of Akt and EGFR suppresses the signaling of both Akt and Erk

The effects of MK-2206 and erlotinib on the Akt and Erk signaling pathways were evaluated in A431 cells. Phosphorylations of Akt and EGFR were activated at detectable levels under normal culture conditions (Fig. 1B). Downstream signaling molecules, such as Erk, GSK3α/β, PRAS40, or ribosomal S6 protein, were also activated. Erlotinib alone was sufficient to inhibit EGFR phosphorylation and the Ras/Erk pathway represented by phosphorylation of Erk (threonine 202/tyrosine 204). However, erlotinib alone did not inhibit PI3K pathway signaling molecules such as phospho-Akt and phospho-PRAS40 (Fig. 1B, lanes 2–4). MK-2206 alone (Fig. 1B, lanes 5) or in combination with erlotinib (Fig. 1B, lanes 6–8) decreased the phosphorylation of Akt on serine 473 and the phosphorylation of direct substrate PRA540 on threonine 246. Thus, MK-2206 in combination with erlotinib suppressed both the Ras/Erk and PI3K pathways, and this combination enabled more profound inhibition of phosphorylation of GSK3α/β (on serine 21/serine 9) and ribosomal S6 protein (on serine 240/serine 244), which are further downstream signaling molecules that correlate with cell growth and survival. A similar effect was confirmed in HCC827 cells with the same sets of antibodies (data not shown). These results suggest that dephosphorylation of parallel key signaling molecules in both the Akt and Erk pathways may potentiate the cytotoxic effect of erlotinib in combination with MK-2206.

Combined treatment of MK-2206 with erlotinib leads to in vivo tumor regression

The pharmacodynamic and antitumor effects of erlotinib and MK-2206 in combination were tested in a mouse xenograft model bearing NCI-H292 tumors. MK-2206 (120 mg/kg) was orally administered 2 hours after erlotinib (50 mg/kg), and tumors were isolated 14 hours after erlotinib administration. Inhibition of phospho-Akt for the PI3K pathway and phospho-Erk for the Ras/Erk pathway in tumors was determined by Western blot analysis (Fig. 2A). MK-2206 alone moderately inhibited phospho-Akt (53.1 ± 6.2%) and only slightly inhibited phospho-Erk (27.7 ± 5.9%). Erlotinib alone only moderately inhibited phospho-Erk, whereas it only slightly, if at all, inhibited phospho-Akt in tumor (data not shown). Cotreatment potentiated the inhibition of phospho-Akt (79.9 ± 3.1%) and phospho-Erk (53.5 ± 5.8%). Inhibition of Akt activity in tumor was confirmed by immunoprecipitation-kinase assay using cell lysates prepared from xenograft tumors (data not shown). These results suggest that the combination of erlotinib and MK-2206 enhanced the inhibition of both pathways.

To evaluate antitumor efficacy, tumor-bearing mice were treated with two different MK-2206 dosing regimens: 120 mg/kg, orally, three times a week for 2 weeks, or 360 mg/kg, orally, once a week for 2 weeks in the presence or absence of erlotinib at 50 mg/kg, orally, once a day for 5 days (Fig. 2B). Monotherapy with MK-2206 in a three-times-a-week schedule was not efficacious, and the once-a-week regimen mediated only moderate antitumor efficacy. Although erlotinib alone mediated significant tumor growth suppression, cotreatment with MK-2206 dramatically enhanced its antitumor efficacy, including tumor regression (Fig. 2B). Both once-a-week and three-times-a-week dosing regimens of MK-2206 enhanced the antitumor efficacy to the same extent. All treatments were well tolerated, and no animals died during the course of the treatment. Animals who received cotreatment experienced weight loss during the treatment period, but they returned to their normal weight after the end of the treatment period (day 16). Thus, the combination of MK-2206 and erlotinib exerted significantly greater in vivo antitumor effects than either agent alone.

Concurrent Akt and EGFR/human EGFR-2 inhibition is synergistic in human breast cancer lines

We evaluated the combination effects of MK-2206 with lapatinib, an EGFR/human EGFR-2 (Her2) dual inhibitor used to treat breast cancer. Human breast cancer cell lines with PI3K pathway activation or ovarian and gastric tumor cells with HER2 gene amplification were used. Table 1B summarizes the IC50 values for each agent and the CI values for six cell lines when both agents were added simultaneously. The combination of MK-2206 and lapatinib showed synergistic tumor growth inhibition in all tested cell lines, irrespective of mutations in PI3K or PTEN, with a CI range between 0.07 and 0.69 (see Table 1B, column labeled combination index). We further evaluated the sequence dependency of the synergistic interaction by determining the CI values with three different treatment schedules: (a) 24-hour pretreatment with MK-2206 followed by 72-hour exposure with lapatinib, (b) 24-hour pretreatment with lapatinib followed by 72-hour exposure with MK-2206, and (c) simultaneous treatment for 96 hours with both MK-2206 and lapatinib. Interestingly, all three treatment schedules showed similar CI values that ranged from 0.18 to 0.47 (data not shown). These results suggest that the treatment schedules may not limit the synergistic effect of MK-2206 with lapatinib in tumor growth inhibition.

The combination of MK-2206 with lapatinib enhances cell death by caspase activation

Similar to erlotinib, MK-2206 enhanced cell death induction by lapatinib in the HCC70 breast cancer line. Lapatinib alone induced only a small amount of activated caspase-3/7 (~2-fold at 10 μmol/L), but the addition of 3 μmol/L MK-2206 induced nearly a 7-fold increase in caspase-3/7 activation (Fig. 1C). These results support the idea that distal inhibition of both EGFR/Her2 and Akt kinase induces potent apoptotic cell death in a variety of cancer types in vitro.
Concurrent inhibition of Akt and EGFR/Her2 enhances the suppression of phosphorylation of S6 protein

The mechanisms of synergistic cell growth inhibition and enhanced apoptosis after treatment with MK-2206 and lapatinib seemed to be similar to the mechanisms induced by the erlotinib combination. Lapatinib (10 μmol/L) alone did not alter the phosphorylation of Akt on serine 473 in the HCC70 cell line at any exposure time up to 24 hours (Fig. 1D, lanes 1–5). The phosphorylation of Akt (serine 473) was inhibited after a 3-hour exposure to MK-2206, and this inhibition was maintained for at least 24 hours. MK-2206 also suppressed the phosphorylation of S6 ribosomal protein (serine 240/244) in a time-dependent manner (Fig. 1D, lanes 6–10). This was enhanced in the presence of both lapatinib and MK-2206, such that the phosphorylation of S6 protein was barely detectable in combination-treated cells, especially after a 24-hour exposure, with minimal change in the amount of total ribosomal S6 protein (Fig. 1D, lanes 11–14).

Combination of MK-2206 and lapatinib in the HCC70 and SK-OV-3 xenograft models

In vitro combination effects were tested in the human breast cancer HCC70 and the ovarian cancer SK-OV-3 models. Lapatinib alone was not active in the HCC70 model, whereas MK-2206 alone showed only moderate efficacy with statistical significance (P < 0.0001; Fig. 2C). Consistent with the in vitro results, the combination of agents yielded a significantly greater inhibition of HCC70 tumor growth than either agent alone (P < 0.0001). Although lapatinib or MK-2206 alone moderately inhibited SK-OV-3 tumor growth, the combination of...
agents yielded a significantly greater antitumor effect than either agent alone, and in a dose-dependent manner ($P < 0.0001$; Fig. 2D). The combination of lapatinib and MK-2206 showed a safe profile in mice; no mortality, body weight loss, or any toxic signs were observed during the experiment.

**MK-2206 enhances the growth inhibitory effects of docetaxel in a sequence-dependent manner**

We determined the effect of MK-2206 in combination with seven chemotherapeutic agents commonly used to treat cancer patients. First, we assessed the combination of MK-2206 and docetaxel in three different treatment...
sequences. Twenty-four hours of exposure to docetaxel followed by a 72-hour exposure to MK-2206 resulted in a significant synergistic antiproliferative effect in the BT-474 breast cancer cell line, with a CI ranging from 0.3 to 0.8 (Table 2A, iii; Supplementary Fig. S2). On the other hand, the reverse sequence (MK-2206 followed by docetaxel) showed an antagonistic effect, with a CI ranging from 1.5 to 1.6 (Table 2A, i). The concurrent treatment schedule was slightly synergistic or additive with a CI ranging from 0.7 to 1.0 (Table 2A, ii). These data show a sequence-dependent synergistic cell growth inhibition by docetaxel when combined with MK-2206. Table 2B summarizes the CI values for the eight breast cancer cell lines. Synergism between the two agents occurred in six cell lines, and additivity occurred in the remaining two cell lines (MDA-MB-453 and BT-20). These results suggest that MK-2206 may significantly enhance the antitumor efficacy of docetaxel against breast tumors when administered in a stepwise schedule.

We confirmed the in vivo combination effect of MK-2206 with docetaxel in a PC-3 xenograft model (human prostate cancer cell line). Docetaxel or MK-2206 alone showed moderate antitumor efficacy (P = 0.0002 for docetaxel, P < 0.0001 for MK-2206; Fig. 3A). Cotreatment with docetaxel followed by MK-2206 exhibited greater tumor growth inhibition than each agent alone (P = 0.0001 for docetaxel, P < 0.0001 for MK-2206). Mice who received docetaxel alone, MK-2206 alone, or both agents lost body weight (4%, 8%, and 9%, respectively) during the treatment period; however, their weight returned to the pretreatment level after the end of treatments (day 15).

The combination of MK-2206 and chemotherapeutic agents induces synergistic cell growth inhibition

We explored the effect of MK-2206 when combined with chemotherapeutic agents having different modes of action: doxorubicin and camptothecin (topoisomerase inhibitors), gemcitabine and 5-FU (antimetabolites), and carboplatin (DNA cross-linkers) in lung NCI-H460 or ovarian A2780 tumor cell lines. Significant synergy was observed between MK-2206 and these antitumor agents in both NCI-H460 and A2780 cells. The CI values at ED_{50}, ED_{75}, and ED_{90} were 0.17 to 0.66 and 0.27 to 0.70, respectively (Table 3). These results indicate that MK-2206 synergistically enhanced cell growth inhibition induced by chemotherapeutic agents.

We further evaluated the induction of apoptotic cell death by monitoring caspase-3/7 induction. MK-2206 enhanced caspase-3/7 induction by carboplatin (at a fixed concentration of 8 μmol/L) in a sequence-dependent manner (Supplementary Fig. S3A). Simultaneous treatment or pretreatment with carboplatin prior to MK-2206 induced A2780 cell death in a dose-dependent manner, whereas pretreatment with MK-2206 prior to carboplatin did not enhance cell death. Cell death induction was confirmed

<p>| Table 2. Synergistic inhibition of cell proliferation by the combination of MK-2206 and docetaxel in breast cancer cell lines |
|-----------------|-------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>ED_{50}</th>
<th>ED_{75}</th>
<th>ED_{90}</th>
<th>PI3K</th>
<th>PTEN</th>
<th>Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Sequence dependency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT-474</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) MK-2206 followed by docetaxel</td>
<td>1.6</td>
<td>1.5</td>
<td>1.6</td>
<td>PIK3CA K111N</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>(ii) Simultaneous</td>
<td>1.0</td>
<td>0.8</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii) Docetaxel followed by MK-2206</td>
<td>0.8</td>
<td>0.5</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B) Other cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>WT</td>
<td>L108R</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>1.2</td>
<td>0.6</td>
<td>0.3</td>
<td>PIK3CA H1047R</td>
<td>E307K</td>
<td>WT</td>
</tr>
<tr>
<td>BT-20</td>
<td>1.0</td>
<td>0.7</td>
<td>0.5</td>
<td>PIK3CA H1047R</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MCF7</td>
<td>0.7</td>
<td>0.5</td>
<td>0.3</td>
<td>PIK3CA E545K</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC70</td>
<td>0.9</td>
<td>0.7</td>
<td>0.5</td>
<td>WT</td>
<td>F90 fs</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.9</td>
<td>0.7</td>
<td>0.7</td>
<td>WT</td>
<td>WT</td>
<td>B-Raf G464V, K-Ras G13D</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
<td>WT</td>
<td>A72 fs</td>
<td>WT</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
<td>WT</td>
<td>NA</td>
<td>WT</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with MK-2206 and docetaxel at constant concentration ratios spanning the IC_{50} dose of each agent. The evaluation of cell growth inhibitory effects was done by monitoring the cellular ATP concentration as described in Materials and Methods. CI values were calculated by the Chou and Talalay method for drug interactions using CalcuSyn software for the different fractions affected (the CI values at ED_{50}, ED_{75}, and ED_{90} were summarized). Mutation data were from the COSMIC database or reference article (47).
by sub-G1 fluorescence-activated cell sorting assay. Flow cytometric analysis revealed that MK-2206 (0.12–3 μmol/L) in combination with carboplatin increased the cell population with a fractional DNA content (cells in a sub-G1 phase) to >40% after 72 hours (Supplementary Fig. S3B). These results indicate that a specific blockade of Akt by MK-2206 enhanced the induction of cell death by carboplatin via activation of the caspase cascade. Combination of MK-2206 with doxorubicin, camptothecin, gemcitabine, and 5-FU also yielded a dose-dependent enhancement of the sub-G1 population in A2780 cells (data not shown). These results were consistent with the synergistic interaction of the combination for cell growth inhibition. MK-2206 enhanced the apoptotic cell death induced by six chemotherapeutic agents with different modes of action.

The ability of MK-2206 to potentiate the in vivo antitumor efficacy of carboplatin and gemcitabine was investigated in a NCI-H460 xenograft model (human NSCLC).
As shown in Fig. 3B, although the antitumor efficacy of carboplatin (P = 0.0268) or MK-2206 (P = 0.0066) alone was moderate, the combination of both agents produced a statistically significant increase in the antitumor response as compared with monotherapy (P < 0.0001). Similarly, MK-2206 also potentiated gemcitabine activity in a NCI-H460 mouse xenograft model (Fig. 3C). Gemcitabine (P = 0.0107) or MK-2206 (P = 0.0022) as a single agent inhibited tumor growth only moderately. The binary combination produced a statistically significant increase in the antitumor response as compared with gemcitabine alone (P = 0.0236). All treatments were well tolerated. Very slight (7–9%) body weight loss occurred during the treatment period, but the mice returned to their pretreatment weight by day 14 to day 16.

Discussion

Our objective was to identify suitable classes of antitumor agents that could be sensitized by an allosteric Akt inhibitor, MK-2206, in preclinical models to guide the strategy in combination with other antineoplastic agents. The PI3K/Akt pathway transduces signals from the upstream RTKs such as EGFR or Her2. Downregulation of this pathway seems to be correlated with the sensitivity to these RTK inhibitors in tumor growth inhibition (27–29). However, we observed that erlotinib or lapatinib alone inhibited the phosphorylation of Akt, but not the phosphorylation of Akt and its downstream substrate PRA540. The PI3K/Akt pathway may be activated independently of the activation status of EGFR or Her2 in these cells. Even in these cases, cotreatment with MK-2206 blocked the Akt pathway in the presence of EGFR/Her2 inhibitors. Modulation of both the Akt and Erk pathways is proposed to be one mechanism for the combination effects.

Molecular-targeted agents acting on EGFR and/or Her2 have clinical benefits to patients whose tumors harbor addiction to these RTKs (30–32). However, the efficacy of erlotinib or lapatinib as a monotherapy is limited by the development of therapeutic resistance in NSCLC or breast cancers, respectively (33–36). Thus, strategies are needed to overcome such resistance. Mechanisms leading to resistance may include mutations in EGFR (such as T790M), PI3K/Akt pathway activation via loss of PTEN or oncogenic PIK3CA, crosstalk with other RTKs, and activation of other RTKs such c-met or insulin-like growth factor I receptor via amplification or overexpression (33–35, 37, 38). MK-2206 is expected to block the nodes of the signaling cascade related to these resistance pathways, and the combination therapy with MK-2206 may provide an efficacious therapy for these patients.

The combination effect of EGFR inhibitor with PI3K inhibitor (LY294002) was also reported by another group (17). Our data confirmed their findings by using two structurally different EGFR inhibitors and MK-2206, which inhibits Akt, a downstream regulator of PI3K. Moreover, we observed the synergy on multiple tumor cell lines from various origins such as lung and breast with both WT and mutant Ras and PI3K pathway regulators. These results would be helpful to guide strategies of clinical trials on the combination of EGFR or EGFR/Her2 inhibitor with Akt inhibitor. They found that combined inhibition of the PI3K/AKT and Ras/mitogen-activated protein kinase/Erk kinase/Erk pathways integrated death signals at the level of BAD phosphorylation. It would be interesting to determine whether a similar mechanism was operative in the case of MK-2206.

MK-2206 alone showed more potent cell growth inhibition of Ras WT cells as compared with Ras-mutant cells. In combination with Ras pathway inhibitors such as mitogen-activated protein kinase/Erk kinase, RAF inhibitors may potentiate the antiproliferation activity of MK-2206 on Ras-mutant cells. Indeed, effective uses of PI3K and mitogen-activated protein kinase/Erk kinase inhibitors to treat Ras-mutant cells in combination are reported by other groups (39, 40).

Various cytotoxic agents, irradiation, or molecular targeted drugs such as mammalian target of rapamycin

Table 3. Synergistic inhibition of cell proliferation by MK-2206 combined with five chemotherapeutic agents in A2780 ovarian and NCI-H460 NSCLC cell lines

<table>
<thead>
<tr>
<th>Chemotherapeutic agents</th>
<th>Mode of action</th>
<th>A2780 ovarian cells</th>
<th>NCI-H460 NSCLC cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ED50</td>
<td>ED75</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Topo inhibitors</td>
<td>0.70</td>
<td>0.43</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Topo inhibitors</td>
<td>0.56</td>
<td>0.40</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>Anti-metabolites</td>
<td>0.57</td>
<td>0.43</td>
</tr>
<tr>
<td>5-FU</td>
<td>Anti-metabolites</td>
<td>0.68</td>
<td>0.54</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>DNA cross-linkers</td>
<td>0.49</td>
<td>0.43</td>
</tr>
</tbody>
</table>

NOTE: Cells were simultaneously treated for 72 h with MK-2206 and chemotherapeutic agents at constant concentration ratios spanning the IC50 dose of each agent. The CI values were determined by the same procedure described in Table 1. A2780 has WT Ras and PTEN deletion (K128-R130), whereas NCI-H460 has PIK3CA mutation (E545K) and K-Ras activation mutation (Q61H).
inhibitors activate the PI3K/Akt pathway in cells (11, 14, 41–44). Akt activation is also associated with resistance to chemotherapy. Ectopic expression of constitutively activated Akt1 in human NSCLC NCI-H460 cells causes partial resistance toward chemotherapeutics (45). Furthermore, downregulation of Akt1 by RNA interference or inhibition of Akt by overexpression of PTEN increases the sensitivity of NSCLC cells or ovarian cancer cells to cisplatin (15, 46). In our experiments, carboplatin and gemcitabine induced a transient increase in Akt phosphorylation in four of six cell lines with a peak at 8 hours (NCI-H1703, NCI-H661, NCI-H1993, and NCI-H460; Supplementary Fig. S4A and B). Cotreatment with 3 μmol/L MK-2206 completely inhibited the Akt phosphorylation induced by carboplatin or gemcitabine in NCI-H661 and NCI-H1993 cell lines (data not shown). Interestingly, these four cell lines exhibited synergistic inhibition of cell growth and enhancement of cell death (Supplementary Table S1). Thus, downregulation of the phospho-Akt induction by carboplatin or gemcitabine, which may result in suppression of the cell survival pathway, partly explains the synergistic cell growth inhibition by MK-2206 with these antitumor agents. On the other hand, Akt phosphorylation was not activated by either agent in UMC-11 and NCI-H441 cells. However, MK-2206 enhanced carboplatin-induced cytotoxicity in NCI-H441 cells and gemcitabine-induced cytotoxicity in both cell lines. Other mechanisms responsible for the synergism remain to be elucidated.

The identification of optimal dosing regimens and schedules is important for the successful clinical evaluation of cancer therapeutics, especially when therapies are combined. Preliminary results are useful to guide the design of clinical study protocols. In our experiments, the sequential treatment with docetaxel followed by MK-2206 or simultaneous treatment with the two agents caused synergistic cell growth inhibition. In contrast, treatment with MK-2206 followed by docetaxel antagonized the efficacy of docetaxel. She et al. reported that an Akt inhibitor induced a G1 cell cycle arrest in a subset of human tumor cells (27). We also confirmed that MK-2206 treatment resulted in the accumulation of G1-phase cells. Docetaxel is known to target M-phase cells. Induction of G1 arrest by MK-2206 before docetaxel may limit the activity of phase-specific cytotoxic agents. On the other hand, the efficacy of MK-2206 combined with erlotinib or lapatinib was not influenced by the treatment schedule.

We used in vivo efficacy studies to compare different MK-2206 dosing schedules with erlotinib. Interestingly, the antitumor efficacy of MK-2206 with intermittent dosing (once a week at 360 mg/kg) was quite similar to the efficacy of more frequent dosing (three times a week at 120 mg/kg) when MK-2206 was combined with erlotinib. This result suggests that continuous exposure with MK-2206 might not be necessary for the combination efficacy with erlotinib in the preclinical model. It seems that more flexible dosing schedules could be used in clinical combination trials, especially if the combination was not well tolerated with continuous dosing or exposure.

In the complex signaling networks within genetically heterogenous human cancers, inhibition of one axis (one targeted molecule or one pathway) may be insufficient because other components often compensate for the targeted axis. In fact, the activation of the Akt survival pathway via several mechanisms impedes the therapeutic efficacy of cytotoxic drugs and molecular targeted agents. Our results show that the combination of MK-2206 with these agents clearly results in an enhanced antitumor efficacy over that seen with monotherapy. Our results provide a rationale for the clinical application of these combinations across multiple types of cancers. MK-2206 is now in phase I clinical trials (24). Clinical evaluation of MK-2206 combination therapies is planned based on the results of the preclinical studies described in this report. These findings will hopefully translate into effective therapies for human cancers.

Disclosure of Potential Conflicts of Interest

All authors are or were employees of Merck Co. Ltd. or Banyu Pharmaceutical Co. Ltd., a subsidiary of Merck. No other potential conflicts of interest were disclosed.

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References


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