Combination of an Allosteric Akt Inhibitor MK-2206 with Etoposide or Rapamycin Enhances the Antitumor Growth Effect in Neuroblastoma

Zhijie Li, Shuang Yan, Navid Attayan, Sridevi Ramalingam, and Carol J. Thiele

Abstract

Purpose: Activation of Akt is a marker of decreased event-free or overall survival in neuroblastoma patients. MK-2206, a novel allosteric Akt inhibitor, is now tested in clinical trials in adult cancers. In this study, effect of MK-2206 on tumor growth and murine survival, alone or in combination, with etoposide or rapamycin was evaluated.

Experimental Design: The anticell proliferation effect of MK-2206 was tested in eight neuroblastoma cell lines by MTS assay. Caspase-3/7 activity, cell-cycle analysis, and reactive oxygen species (ROS) production were determined. Effect of MK-2206 combined with etoposide or rapamycin was evaluated in vitro and in vivo. Akt phosphorylation was measured by Western blotting in neuroblastoma cells and tumors.

Results: In vitro, MK-2206 treatment inhibited neuroblastoma cell proliferation, which was accompanied by a cell line selective G1 arrest of cell cycle or production of ROS. A synergistic effect between MK-2206 and etoposide was detected in four tested neuroblastoma cell lines via caspase-dependent apoptosis, whereas increased inhibition of cell growth induced by combination of MK-2206 and rapamycin was mediated by ROS production. In vivo, MK-2206 alone decreased tumor growth and increased murine survival at dose that inhibited Akt phosphorylation in tumors. MK-2206, in combination with etoposide or rapamycin, caused a significant decrease in tumor growth and increase of murine survival compared with MK-2206 alone.

Conclusion: Akt inhibition by MK-2206 increased the efficacy of etoposide or rapamycin. Our study supports future clinical evaluation of MK-2206 in combination with conventional cytotoxic therapy or with rapamycin in high-risk neuroblastoma patients. Clin Cancer Res; 18(13); 3603–15. ©2012 AACR.

Introduction

Neuroblastoma is the most common extracranial solid tumor in childhood (1). It accounts for more than 7% of malignancies in patients younger than 15 years and causes 10% of all pediatric oncology deaths (2). Approximately half of all neuroblastoma patients are diagnosed with high-risk disease, and despite aggressive treatment strategies, such as high-dose chemotherapy, radiotherapy, and bone marrow transplantation, the overall survival rate of these patients is dismal (3). The recent inclusion of anti-GD2 immunotherapy significantly increased the survival of high-risk patients compared with standard therapy (4). In spite of these improvements, therapies are still inadequate for some 40% of patients with high-risk disease. Therefore, development of new treatment approaches is still a challenge to improve treatment efficacy in high-risk neuroblastoma patients.

Targeting key mediators of tumor survival signaling pathways is one approach to improve therapies, and we have shown that activated Akt mediates resistance to cytotoxic agents used in neuroblastoma treatment (5). Activated Akt is a downstream target of BDNF/TrkB signaling pathway (5, 6) and ALK pathway (7–9) in neuroblastoma cells. Both pathways have been implicated in pathogenesis of neuroblastoma tumors (6–10). Cellular processes regulated by Akt include cell proliferation, growth and survival, metabolism, angiogenesis, and tissue invasion. All these processes represent the hallmarks of cancer, and a burgeoning literature has defined the importance of alterations of Akt activity in human cancer and experimental models of tumorigenesis (11).

Recently we showed that an Akt inhibitor perifosine had antitumor growth effect alone or in combination with chemotherapy in neuroblastoma, both in vitro and in vivo (12, 13). As perifosine works not only on Akt but also on other molecular targets or pathways (14, 15), we sought to evaluate how a specific Akt inhibitor affects the sensitivity of
neuroblastoma cells to cytotoxic agents. MK-2206, an allosteric Akt inhibitor, binds to the Akt protein at a site in the pleckstrin homology domain that causes a conformational change in the protein that prevents its localization to the plasma membrane, thus inhibiting its subsequent activation (16). As an anticancer agent, MK-2206 is being tested both in vitro and in vivo in adult tumors (17–23) and in a spectrum of pediatric tumors (24). Recently, in a clinical trial, stable disease was observed in patients with advanced solid tumors (25). The activation of Akt in neuroblastoma and its association with biologic characteristics of poor prognosis makes MK-2206 a potential approach to improve therapeutic efficacy for neuroblastoma tumors. In this study, we evaluated the antitumor growth effect of MK-2206 as a single agent, and in combination with a cytotoxic drug, etoposide, or rapamycin. This study provides the basis for the combined use of molecular targeted drug of Akt with other treatment regimens in neuroblastoma, or other cancers with aberrant Akt activation, and has important clinical implications.

Translational Relevance
Recently, aberrant Akt activation was identified as a novel indicator of poor prognosis in neuroblastoma. In this study, we investigated whether a novel allosteric Akt inhibitor MK-2206 increased the sensitivity of neuroblastoma to etoposide or rapamycin. Here we showed that in vitro, a synergistic effect was detected in combination of MK-2206 with etoposide through apoptosis, and MK-2206 enhanced the sensitivity to rapamycin via reactive oxygen species; in vivo, a significant increased antitumor growth effect and murine survival advantage were observed in the combination of MK-2206 with etoposide or rapamycin. This study provides the basis for the combined use of molecular targeted drug of Akt with other treatment regimens in neuroblastoma, or other cancers with aberrant Akt activation, and has important clinical implications.

Cell lines and cell culture
Eight human neuroblastoma cell lines—SK-N-AS (AS), SK-N-BE2(BE2), NGP, SY5Y, SMS-KCNR(KCNR), SKN-FL, SKN-DZ, LAN-5, and a normal human retinal pigment epithelial cell line (ARPE-19) (refs. 6, 12, 26–28; Supplementary Table S1) were used in this study. All tumor cell lines have been authenticated by single nucleotide polymorphism analyses and are genetically homogeneous (S.I. Chanock, Division of Cancer Genetics and Epidemiology, National Cancer Institute, Bethesda, MD). Cells were cultured in RPMI-1640 medium (Mediatech Inc.) containing 10% FBS (Gemini Bio-Products) as described before (4).

Reagents and antibodies
MK-2206 was obtained from Merk & Co., Inc. through Cancer Therapy Evaluation Program (CTEP). Etoposide was obtained from Bedford Laboratories. Z-VAD-fmk was purchased from R&D Systems, Inc., N-acetyl-l-cysteine (NAC) and α-tocopherol were obtained from Sigma-Aldrich. Chloromethyl-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA) was purchased from Invitrogen. Anti-Akt antibody, anti-phospho-Akt (P-Akt, Ser473) antibody, anti-S6 antibody (T-S6), anti-phospho-S6 (P-S6, Ser235/236) antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody, anti-cleaved caspase-3 antibody as well as rapamycin were purchased from Cell Signaling Technology.

Cell treatment and cell proliferation assay
To detect cell proliferation, neuroblastoma cells were treated with MK-2206 at concentrations ranging from 0.001 to 20 μmol/L, or etoposide ranging from 0.05 to 50 μg/mL, or rapamycin ranging from 0.1 to 100 nmol/L for 48 hours. For combination of MK-2206 and etoposide, MK-2206 was administrated 2 hours before etoposide. For combination of MK-2206 and rapamycin, rapamycin was given 2 hours before MK-2206. To assess mechanisms of cell death, cells were pretreated with Z-VAD-fmk or NAC for 2 hours before other drugs were administrated. At end of the experiment, cell proliferation was measured using MTS assay (inner salt assay) according to manufacturer's specification (Promega Corporation). The percentage of cell proliferation was calculated by dividing absorbance value of treated neuroblastoma cells by absorbance value of control cells within each group. All experiments were conducted 2 to 3 times.

Reactive oxygen species measurements
Neuroblastoma cells were treated with MK-2206 for 24 hours, cultured with CM-H2DCF-DA (5 μg/mL) at 37°C for 30 minutes, and harvested and washed with PBS twice. The fluorescence intensity of DCF was determined by flow cytometry.

Assay of caspase-3 and caspase-7 activity
Neuroblastoma cells were treated with reagents either alone or in a combination for 24 hours. The activity of caspase-3/7 was evaluated using Caspase-Clo 3/7 Assay Kit (Promega Corporation) according to manufacturer's instruction. Luminescence was detected by a luminometer, Victor 3 (PerkinElmer Life and Analytical Sciences).

Western blotting
To evaluate Akt and mTOR signaling pathways after drug treatment, protein lysates were extracted from neuroblastoma cells (5 minutes on ice) or neuroblastoma tumor tissues (sonication tumor tissues for 15 minutes) using a Mammalian Protein Prep Kit (Qiagen). Immunoblotting was carried out to detect P-Akt, P-S6, T-Akt, and T-S6 as described previously (4).

Cell-cycle analysis
Neuroblastoma cells were treated with MK-2206 for 48 hours, washed with PBS, and incubated with RNase A (Roche) at 100 μg/mL and propidium iodide (Sigma-
Aldrich Corp) at 50 μg/mL for 30 minutes at room temperature. The stained cells were analyzed for DNA content by Flow cytometry (Becton Dickinson & Co.). FlowJo software (BD Biosciences) was used to quantify the percentage of cells in different stages of the cell cycle.

In vivo studies

Neuroblastoma cells (AS, NGP, BE2, and SY5Y) were harvested, washed with Hanks balanced salt solution (HBSS; Invitrogen), and resuspended in HBSS and Matrigel (Trevigen). AS, NGP, or BE2 (2 x 10^6 cells) or SY5Y (4 x 10^6 cells) cells in 100 μL were inoculated into subcutaneous tissue of right flank of 5- to 6-week-old female athymic nude mice (Frederick, MD). When tumors reached 100 to 200 mm^3, treatment began. MK-2206 (100 or 200 mg/kg) was administered by oral gavage, and etoposide (100 mg/kg) and rapamycin (5 mg/kg) were given by intraperitoneal injection. MK-2206 and etoposide were given 3 times a week on Monday, Wednesday, and Friday. Rapamycin was given 5 days a week from Monday to Friday. Dimensions of tumors were measured 3 times a week, and tumor volume (mm^3) was calculated as (L x W^2)/4, in which L indicates length in millimeters and W indicates width in millimeters. To determine mice survival, we counted the days from the date treatment was administered to the time when tumors reached a length of 20 mm, or to the end of the experiment in treatment groups. Mice bearing AS, NGP, or BE2 (2 x 10^6 cells) or SY5Y (4 x 10^6 cells) tumors were treated with MK-2206 at 200 mg/kg for 3 times to evaluate P-Akt levels in tumors; Mice bearing NGP tumors were treated with rapamycin at 2.5 or 5 mg/kg for 2 weeks to evaluate P-S6 levels.

All xenograft studies were approved by the Animal Care and Use Committee of the National Cancer Institute, and all mouse treatments, including their housing, were in accordance with the institutional guidelines (PB-023).

Statistical analysis

The synergistic effects between 2 agents in in vitro experiments were evaluated through combination index (CI) values obtained with the CompuSyn software bought from CompuSyn, Inc. (www.CompuSyn.com; ref. 29). Other statistical analysis was carried out with the Graphpad Prism software (GraphPad Software, Inc., version 3.0). Comparisons between 2 groups were carried out using unpaired Student t test; comparisons among 3 or more groups were carried out using one-way ANOVA with Bonferroni correction. Mice survival was evaluated by Kaplan–Meier method. All P values less than 0.05 were considered statistically significant.

Results

MK-2206 inhibits cell proliferation and Akt phosphorylation in neuroblastoma cells

To study the effect of MK-2206 on neuroblastoma cell proliferation, 8 neuroblastoma cell lines (AS, NGP, BE2, SY5Y, KCNR, SKN-FI, LAN-5, and SKN-D2) and ARPE-19 cells were selected and treated with MK-2206 for 48 hours. MK-2206 treatment induced a concentration-dependent inhibition of cell proliferation in these neuroblastoma cell lines, with the IC_{50} ranging from 0.6 μmol/L in NGP cells to 16.5 μmol/L in AS cells (Supplementary Table S1). However, high concentrations of MK-2206 (15 and 20 μmol/L) also inhibited proliferation of the nontransformed cell line ARPE-19, which had an IC_{50} of 59 μmol/L (Fig. 1A, left).

To assess whether MK-2206–induced inhibition of cell proliferation was mediated via a caspase-dependent apoptotic pathway, caspase-3/7 activity was evaluated after MK-2206 treatment. There was not a statistically significant increase in caspase-3/7 activity in neuroblastoma cells treated with MK-2206 at concentrations of 10 μmol/L or less. Only cells treated with high (15 or 20 μmol/L) concentrations of MK-2206 showed increased caspase-3/7 activity (Fig. 1A, right). Pretreatment with pan-caspase inhibitor Z-VAD-fmk before MK-2206 did not protect cells from MK-2206–induced inhibition of cell proliferation at concentrations of 10 μmol/L or less, and only partially protected cells treated with MK-2206 at 15 or 20 μmol/L (data not shown).

To evaluate whether MK-2206 inhibits cell proliferation via regulation of cell-cycle arrest, cell-cycle analysis was carried out by flow cytometry in SY5Y, NGP, BE2, and AS cells exposed to MK-2206 for 48 hours (Fig. 1B). Increased concentrations of MK-2206 (2.5–10 μmol/L) caused an initial accumulation of cells in G1 phase of the cell cycle in SY5Y and BE2 cells (up to 18% increase), followed by a reduced percentage of cells in S-G2-M phase. Increase of cells in G1 phase was not significant in NGP and AS cells. At concentration of 15 μmol/L, an increase of cells in Sub-G1 phase was detected in all the tested 4 neuroblastoma cell lines, indicating the presence of apoptosis (Fig. 1B).

As it is reported that reactive oxygen species (ROS) induction inhibits cell growth without an increase of caspase activity (30), we assessed whether there is any change of ROS production during MK-2206 treatment. In Fig. 1C, MK-2206 treatment induced a right shift of histogram at FL-1 (DCF) in BE2 and NGP cells, an upshift in AS cells, whereas SY5Y is not affected. This indicated that MK-2206 induces an increase in ROS in BE2 and NGP cells, but not in AS and SY5Y cells. Pretreatment with ROS inhibitor NAC partially blocked the MK-2206–induced inhibition of cell proliferation in BE2 and NGP cells (data not shown).

To study the inhibition effect of MK-2206 on Akt signaling pathway, neuroblastoma cells were treated with MK-2206 for 2 hours. A decrease of phosphorylated Akt (P-Akt) levels (from 40%–70%) was detected in SY5Y, BE2, and NGP cells at 0.1 μmol/L, whereas a 60% decrease of P-Akt was observed at 0.01 μmol/L in AS cells. The levels of phosphorylated S6 (P-S6), a downstream target of Akt/mTOR, were reduced by MK-2206 at 1 μmol/L or higher in SY5Y, NGP, and BE2 cells, but no reduction of P-S6 was detected in AS cells. Total Akt and total S6 were not influenced by MK-2206 treatment (Fig. 1D).

These data indicated that MK-2206 treatment induced inhibition of neuroblastoma cell proliferation and Akt
phosphorylation in neuroblastoma cells. Cell-cycle arrest and ROS production may mediate the reduced proliferation seen in cells treated with MK-2206. However, at concentrations necessary to inhibit Akt, caspase-dependent apoptosis is not induced.

**In vivo effects of MK-2206 as a single agent on tumor growth and mice survival**

To determine whether MK-2206 affects neuroblastoma tumor growth in vivo, mice bearing AS, BE2, SY5Y, and NGP tumors were treated with MK-2206 at 100 or 200 mg/kg.
High-dose MK-2206 (200 mg/kg) showed antitumor growth effect in all the 4 neuroblastoma tumors with varying efficacy (Fig. 2A). The tumor growth inhibition was 22% in AS tumors ($P = 0.037$), 30% in BE2 tumors ($P = 0.014$), 44% in SY5Y tumors ($P = 0.004$), and 48% in NGP tumors ($P = 0.007$), whereas low-dose MK-2206 (100 mg/kg) only significantly inhibited the growth of NGP tumors ($P = 0.002$). There was a 27% inhibition of tumor growth in NGP tumors ($P = 0.002$).

Figure 2. In vivo effects of MK-2206 as a single agent on tumor growth and mice survival. AS, BE2, SY5Y, and NGP cells were injected into mice. Mice with tumors were treated with vehicle or MK-2206 (100 or 200 mg/kg/d). A, tumor volumes from control and treated groups were compared when the last mouse from each control group was euthanized. Bar, SE. /C3/C3, $P < 0.05$; /C3/C3, $P < 0.01$, treated group versus control group. B, survival curve was plotted by Kaplan–Meier analysis. /, $P < 0.05$; ##, $P < 0.01$ treated group versus control group. C, neuroblastoma tumors were treated with 200 mg/kg/d MK-2206 for 3 times, total protein was extracted for analysis of P-Akt, T-Akt, P-S6, T-S6, and GAPDH.
growth in SY5Y tumors at the low dose, although this did not reach statistical significance ($P = 0.06$).

There was a statistically significant survival advantage in MK-2206-treated mice bearing BE2, SY5Y, and NGP xenograft tumors at 200 mg/kg but not 100 mg/kg (Fig. 2B). The median survival time for control versus MK-2206 was 16 days versus 19 days ($P = 0.018$) in mice bearing BE2 tumors, 31 days versus 44 days ($P = 0.009$) in mice bearing SY5Y tumors, 30 days versus 43 days ($P = 0.005$) in mice bearing NGP tumors. MK-2206 did not significantly affect the mice survival bearing AS xenograft tumors (Fig. 2B).

To determine whether MK-2206 treatments inhibited the target, we assessed the level of P-Akt and P-S6 in tumors from control and MK-2206–treated mice (3 doses at 200 mg/kg/dose). The level of P-Akt was inhibited in MK-2206–treated tumors in all the tested 4 neuroblastoma cell lines, yet inhibition of P-S6 was not detected in neuroblastoma tumor xenografts (Fig. 2C). These data indicated that, as a single agent, MK-2206 showed a significant inhibition of tumor growth in mice bearing AS, NGP, BE2, and SY5Y xenograft tumors (4 of 4) and a statistically significant increase in the survival of mice bearing NGP, BE2, and SY5Y tumors (3 of 4). These effects were accompanied with inhibition of P-Akt in tumor tissues.

The synergistic inhibition of cell proliferation with combination treatments of MK-2206 and etoposide

Etoposide is an active chemotherapeutic drug used in neuroblastoma treatment. To evaluate whether MK-2206 increases the sensitivity of neuroblastoma cells to etoposide, we first assessed the responses of neuroblastoma cells to etoposide. Etoposide treatment induced a dose-dependent decrease in cell proliferation in all 4 neuroblastoma cell lines tested (Fig. 3A). An unbiased ratio, concentrations of MK-2206 and etoposide spanning the $IC_{50}$ for each cell line were selected for combination study by calculating CI value with ComboSyn software (Fig. 3B). A CI $< 1$ indicates synergism, a CI = 1 reflects an additive effect, and a CI $> 1$ indicates drug antagonism. A synergistic effect between MK-2206 and etoposide was observed in the 4 neuroblastoma cell lines as the CI values were smaller than 1 (Fig. 3B). Similar results were found using a combination of cisplatin and MK-2206 (Supplementary Fig. S1).

To study whether the synergistic inhibition of cell proliferation caused by MK-2206 and etoposide was modulated via enhanced caspase-dependent apoptosis, we evaluated caspase-3/7 activity. MK-2206 did not increase the caspase-3/7 activity in NGP, BE2, and SY5Y cells although there was a (30%) increase in AS cells. However, the addition of MK-2206 to etoposide induced higher caspase-3/7 activity than etoposide alone. The activation of caspase-3/7 activity for etoposide versus etoposide + MK-2206 was 1.9-fold versus 4.2-fold in AS cells, 1.4-fold versus 2-fold in NGP cells, 1.6-fold versus 3.0-fold in BE2 cells, and 2.2-fold versus 5.0-fold in SY5Y cells (Fig. 3C). Pretreatment of neuroblastoma cells with pan-caspase inhibitor Z-VAD-fmk before administration of MK-2206 and/or etoposide showed that Z-VAD-fmk blocked the inhibition of cell proliferation induced by etoposide or etoposide + MK-2206 in AS, SY5Y, NGP, and BE2 cells (Fig. 3D). Consistent with the lack of caspase-3/7 activity induced by MK-2206 alone, Z-VAD-fmk did not alter MK-2206 effects. These data indicated that MK-2206 increased the sensitivity of neuroblastoma cells to etoposide by enhancing the caspase-dependent apoptosis.

The combination effects of MK-2206 and etoposide on tumor growth and mice survival in vivo

To evaluate the efficacy of MK-2206 and etoposide combination against the tumor growth and mice survival in vivo, mice bearing AS, SY5Y, and NGP tumors were treated with etoposide (10 mg/kg) and MK-2206 (200 mg/kg) alone or in combination. In mice bearing AS tumors, there was a 52% inhibition of tumor growth in etoposide + MK-2206 combination group, which was significantly higher than 24% inhibition of growth in etoposide-treated group ($P = 0.05$) or 22% growth inhibition in MK-2206–treated group ($P = 0.046$; Fig. 4A). The survival of mice was also significantly increased in combination group with median survival time of 36 days, compared with 29 days in etoposide-treated group ($P = 0.0024$) or 25 days in MK-2206–treated group ($P = 0.0064$; Fig. 4B). In mice bearing NGP tumors, inhibition of tumor growth in etoposide + MK-2206 combination group was 66%, which was significantly higher than 28% in etoposide-treated group ($P = 0.01$) or 40% in MK-2206–treated group ($P = 0.008$; Fig. 4A). The survival of mice was also significantly increased in combination group with median survival time of 39 days, compared with 32 days in etoposide-treated group ($P = 0.035$) or 29 days in MK-2206–treated group ($P = 0.035$; Fig. 4B). In mice bearing SY5Y tumors, inhibition of tumor growth in etoposide + MK-2206 combination group was 62%, which was higher than 40% in etoposide-treated group ($P = 0.045$) or 44% in MK-2206–treated group ($P = 0.05$; Fig. 4A). The survival of mice was also significantly increased in combination group with an unreached median survival time during the experiment, compared with 40 days in the etoposide-treated group ($P = 0.048$) or 44 days in MK-2206–treated group ($P = 0.049$; Fig. 4B). Furthermore, we evaluated the effect of the combination of MK-2206 and etoposide on P-Akt levels and cell death. Inhibition of P-Akt was detected in MK-2206 and MK-2206/etoposide treated AS, NGP, and SY5Y tumors. P-Akt levels decreased in SY5Y xenografts from mice treated with etoposide but not in etoposide-treated mice bearing AS and NGP tumors. An increase in cleaved caspase-3 was detected in AS, NGP, and SY5Y xenografts from mice treated with etoposide alone or with the combination of MK-2206 and etoposide (Fig. 4C). These data indicated that by combining MK-2206 and etoposide, the antitumor growth effect and mice survival advantage were increased compared with either agent alone.

Combination of MK-2206 and rapamycin enhanced the inhibition effect on cell proliferation via ROS

To study whether cell growth inhibition could be enhanced by targeting one more node in the Akt signaling pathway, rapamycin was added to the combination of MK-2206 and etoposide. MK-2206 and etoposide combination enhanced the antitumor growth effect and mice survival advantage were increased compared with either agent alone. Combination of MK-2206 and rapamycin enhanced the inhibition effect on cell proliferation via ROS.
Figure 3. The synergistic inhibition of cell proliferation with combination treatment of MK-2206 and etoposide. A, AS, BE2, NGP, and SY5Y cells were treated with etoposide for 48 hours, cell proliferation was evaluated. B, AS, BE2, NGP, and SY5Y cells were treated with MK-2206 and etoposide either alone or in combination for 48 hours. The concentrations of each agent spanned the IC50 dose in each cell line. Cell proliferation and CI were evaluated. C, caspase-3/7 activity was detected in AS, BE2, NGP, and SY5Y cells treated with MK-2206 [NGP and SY5Y (5 μmol/L), AS and BE2 (7.5 μmol/L)] and etoposide [AS (20 μg/mL), BE2 (1 μg/mL), NGP (0.1 μg/mL), and SY5Y (0.1 μg/mL)] either alone or in combination for 24 hours. Bar, SD. ##, P < 0.01: MK-2206/etoposide-treated cells versus MK-2206-treated cells; C, #P < 0.01: MK-2206/etoposide-treated cells versus etoposide-treated cells.

D, AS, SY5Y, NGP, and BE2 cells were pretreated with Z-VAD-fmk (100 μmol/L) for 2 hours followed by treatment of MK-2206 (7.5 μmol/L in AS, BE2 cells, 5 μmol/L in SY5Y and NGP cells) and etoposide [AS (20 μg/mL), SY5Y (0.1 μg/mL), NGP (0.1 μg/mL), and BE2 (1 μg/mL)] either alone or in combination. Cell proliferation was evaluated 48 hours later. Bar, SD. #, P < 0.05, ##, P < 0.01: Z-VAD-fmk/etoposide-treated cells versus etoposide-treated cells; **, P < 0.01: Z-VAD-fmk/etoposide/MK-2206–treated cells versus etoposide/MK-2206–treated cells.
pathway, the response of neuroblastoma cells to rapamycin, a mTOR inhibitor, was evaluated. Neuroblastoma cells were treated with rapamycin for 48 hours. Rapamycin-induced inhibition of cell proliferation was observed at concentrations of 1 nmol/L, whereas no further inhibition was observed by increasing concentrations of rapamycin from

Figure 4. In vivo effects of MK-2206 and etoposide on tumor growth and mice survival. Mice bearing AS, NGP, and SY5Y tumors were treated with MK-2206 (200 mg/kg) and etoposide (10 mg/kg) either alone or in combination. A, tumor volumes from control and treated groups were compared when the last mouse in each control group was euthanized. Bar, SE. B, mice survival was compared through Kaplan–Meier curves. #, P < 0.05; ##, P < 0.01: MK-2206/etoposide-treated group versus MK-2206-treated group. /C3, P < 0.05; /C3/C3, P < 0.01: MK-2206/etoposide-treated group versus etoposide-treated group. C, neuroblastoma tumors were harvested at end points, total protein was extracted for analysis of P-Akt, T-Akt, cleaved caspase-3, and GAPDH.
Figure 5. The combination effect of MK-2206 and rapamycin in vitro. AS, BE2, NGP, and SY5Y cells were used. A, neuroblastoma cells were treated with rapamycin for 48 hours and cell proliferation was evaluated. Bar, SD. B, neuroblastoma cells were treated with rapamycin (10 nmol/L) for 2 hours followed by MK-2206 (7.5 μmol/L) treatment for another 2 hours, either alone or in combination. Total protein was extracted to detect P-S6, T-S6, P-Akt, T-Akt, and GAPDH levels. C, neuroblastoma cells were treated with rapamycin (10 nmol/L) for 2 hours followed by MK-2206 (7.5 μmol/L) treatment and cell proliferation was evaluated after 48 hours (left) or caspase-3/7 activity was detected after 24 hours (right). Bar, SD. #, P < 0.05; ##, P < 0.01: MK-2206/rapamycin-treated cells versus MK-2206-treated cells; *, P < 0.05; **, P < 0.01: MK-2206/rapamycin-treated cells versus rapamycin-treated cells. NS: no statistical significance. D, NGP, BE2, AS, and SY5Y cells were pretreated with NAC (100 mmol/L) for 2 hours followed by MK-2206 (7.5 μmol/L) and rapamycin (10 nmol/L) treatment, either alone or in combination for 48 hours. Cell proliferation was detected. Bar, SD. #, P < 0.05: NAC/MK-2206–treated cells versus MK-2206–treated cells; @, P < 0.05: NAC/rapamycin-treated cells versus rapamycin-treated cells; *, P < 0.05; **, P < 0.01; NAC/rapamycin/MK-2206–treated cells versus rapamycin/MK-2206–treated cells.
10 to 100 nmol/L (Fig. 5A). To confirm the inhibition of mTOR pathway by rapamycin, neuroblastoma cells were treated with MK-2206 and rapamycin alone or in combination. The levels of P-Akt and the downstream target of mTOR, P-S6, were detected. Rapamycin alone or in combination with MK-2206 completely inhibited P-S6 in the 4 neuroblastoma cell lines. Within the time points we tested, rapamycin did not affect P-Akt levels (Fig. 5B). Neuroblastoma cells were treated with the same concentrations of MK-2206 and rapamycin for 48 hours. A combination of MK-2206 and rapamycin significantly enhanced inhibition of cell proliferation in BE2 and NGP cells compared with either alone. There was little to no effect of the combination in AS or SY5Y cells (Fig. 5C, left).

To study whether the enhanced effect in BE2 and NGP cells was through caspase-dependent apoptosis, caspase-3/7 activity was evaluated. No significant increase in caspase-3/7 activity was observed in MK-2206 and rapamycin-treated cells, alone or in combination, in any of the 4 neuroblastoma cell lines tested (Fig. 5C, right). As MK-2206 treatment induced an increase of ROS in BE2 and NGP cells (Fig. 1C), we pretreated the cells with ROS inhibitor NAC before MK-2206 and rapamycin treatment to study whether ROS is involved in the combination effect of MK-2206 and rapamycin. Figure 5D shows that pretreatment with NAC blocked the enhanced inhibition of cell proliferation induced by combination of MK-2206 and rapamycin in NGP and BE2 cells, although it had much less effect or no effect on either of the individual agents. Compared with NGP and BE2 cells, NAC had much less effect in AS cells or no effect on SY5Y cells (Fig. 5D). Another ROS inhibitor a-Tocopherol showed a similar effect in NGP and BE2 cells (Supplementary Fig. S2). Also, an increase in ROS production was detected in the combination of MK-2206 and rapamycin, compared with MK-2206 treatment alone (Supplementary Fig. S3). These data indicated that the anticell proliferation effect induced by the combination of MK-2206 and rapamycin was mediated in large part through the generation of ROS.

The combination effect of MK-2206 and rapamycin on tumor growth and mice survival in vivo

To assess the efficacy of MK-2206 and rapamycin combination against tumor growth and mice survival in vivo, mice bearing NGP tumors were first treated with rapamycin at either 2.5 or 5 mg/kg for 2 weeks to determine a dose that effectively inhibited mTOR pathway in vivo. Although either the 2.5 or 5 mg/kg dosing of rapamycin significantly inhibited P-S6 levels in tumor xenografts, P-Akt levels were elevated at 2.5 mg/kg, but not 5 mg/kg dosing schedule (Fig. 6A). Thus to test the combination therapy, we treated mice with 5 mg/kg rapamycin and 200 mg/kg MK-2206.

There was a 76% inhibition of NGP tumor xenograft growth in the cohort receiving MK-2206 + rapamycin, which is significantly higher than 40% inhibition of tumor growth in MK-2206–treated group (P = 0.0001) or 56% in rapamycin-treated group (P = 0.03; Fig. 6B). The survival of mice was also significantly increased in combination group with median survival time of 44.5 days, compared with 29 days in MK-2206–treated group (P = 0.002) or 37.5 days in rapamycin cohort (P = 0.025; Fig. 6C). Furthermore, we evaluated the combination effect of MK-2206 and rapamycin on P-Akt and P-S6 at end points. Although rapamycin at 5 mg/kg did not affect P-Akt at 2 weeks (Fig 6A), a longer
time treatment increased P-Akt levels (Fig. 6D). Also a longer time treatment of MK-2206 increased P-S6 level, whereas a combination of MK-2206 and rapamycin induced an inhibition of both P-Akt and P-S6 (Fig. 6D). These data showed that combination of MK-2206 and rapamycin increased the antitumor growth effect and enhanced the mice survival compared with either agent alone.

Discussion

In this study, an enhanced anticell proliferation effect was found in combination of MK-2206 and etoposide via apoptosis, or in combination of MK-2206 with rapamycin via ROS production in vitro. In vivo, the antitumor growth effect and mice survival were increased when MK-2206 was combined with etoposide or rapamycin. Inhibition of Akt phosphorylation was detected in neuroblastoma cells in vitro and in vivo.

MK-2206 works as an allosteric Akt inhibitor and shows greater than 100-fold selectivity for Akt compared with 256 other kinases (25). So it is considered as a Akt inhibitor with high specificity compared with other Akt inhibitors (31), and few off-target effects have been reported (25). As an anticancer agent, inhibition of MK-2206 on tumor cell growth in vitro or in vivo has been found in adult cancers (17, 18, 22, 23). Recently, a clinical trial of MK-2206 showed stable disease in 6 of 33 patients with advanced solid tumors, and it was well tolerated at biologically active doses that inhibit Akt signaling (25). Reducing cytotoxicity and increasing treatment efficacy is the ideal goal for tumor treatment, so rational combination of different agents is a commonly used strategy. In vitro studies found that MK-2206 has synergistic antitumor cell growth effect when combined with different molecules targeted inhibitors (18, 22, 23, 32) or cytotoxic agents (17). Similar results were found in in vivo studies (17, 23). Clinical trials assessing combination of MK-2206 with molecules targeted inhibitors or with chemotherapy are ongoing. A recent study in pediatric cancer patients reported the effect of MK-2206 as a single agent. ALL is the most sensitive to MK-2206, whereas neuroblastoma is less sensitive. But the biologic inhibition of Akt by MK-2206 and the combination of MK-2206 with other therapies, which is important for cancer treatment especially when one agent is not effective, were not investigated (24). In contrast, we addressed these issues in our study. To our knowledge, the evaluation of MK-2206 effect on pediatric tumors in combination with either chemotherapeutic drugs or mTOR inhibitor rapamycin was not reported before. Our data will provide evidence for clinical trials in neuroblastoma patients and for evaluation of MK-2206 in other pediatric tumors that have activation of Akt.

The mechanisms underlying antitumor cell growth effect of MK-2206 were investigated. As MK-2206 at concentrations of 15 μmol/L or more inhibited cell growth not only in neuroblastoma cells but also in ARPE-19 control cells, we focused on concentrations 15 μmol/L or less for our analysis. Our data indicated that caspase-dependent apoptosis is not substantially involved in MK-2206 effect, which is consistent with results in lung cancer cells (17). We observed ROS production in NGP and BE2 cells, which was blocked by NAC, and a G1 phase arrest in SY5Y and BE2 cells. Cell-cycle arrest or ROS production has not been reported as mechanisms of MK-2206–induced inhibition of cell proliferation before. But they do not seem to be the common mechanisms in the 4 neuroblastoma cells lines tested. So it is possible that cell type or cell line–dependent mechanisms are involved. Given the heterogeneity of neuroblastoma cell lines, this is not unusual. We did find that neuroblastoma cell lines with p53 mutations were less sensitive to MK-2206 than those with wild-type p53. In contrast, ALK mutation, MYCN amplification, or 1pLOH could not distinguish neuroblastoma cell lines with different sensitivity to MK-2206. This suggests that p53 mutations maybe a potential indicator of MK-2206 resistance.

Further investigation on the mechanisms underlying combination effect of MK-2206 and etoposide showed that etoposide-induced caspase-dependent apoptosis in neuroblastoma cells was enhanced when combined with MK-2206, implying that neuroblastoma cells with low level of activated Akt are sensitive to etoposide treatment. Similar results were found when MK-2206 was combined with EGFR receptor (EGFR) inhibitor erlotinib or the dual EGFR/her2 inhibitor lapatinib (17).

In contrast to the increased apoptosis seen in combination of MK-2206 with etoposide, cell death induced by MK-2206 and rapamycin combination was blocked by pretreatment with NAC but not Z-VAD-fmk, indicating a role for generation of ROS. The ROS-mediated cell growth inhibition without changes in caspase activity has been detected in thymoquinone-treated prostate cancer cells (30). Recently, it is reported that acute increases in intracellular concentrations of ROS induced cell death in lung cancer cells via inhibiting pyruvate kinase M2 (PKM2; refs. 33, 34), so it may be possible that MK-2206 may inhibit PKM2 by inducing ROS, then inhibit glucose metabolism to induce cell growth inhibition or cell death.

In conclusion, our data provide in vitro and preclinical evidence for clinical trials of MK-2206 in combination with conventional therapy to improve treatment efficacy in patients with high-risk neuroblastoma. The potential mechanisms of MK-2206 will help for selection of different combination agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Z. Li, C.J. Thiele
Development of methodology: C.J. Thiele
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Li, S. Yan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Li, N. Attayan, S. Ramalingam, C.J. Thiele
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Writing, review, and/or revision of the manuscript: C.J. Thiele
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Attayan, S. Ramalingam

Study supervision: C.J. Thiele

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Combination of an Allosteric Akt Inhibitor MK-2206 with Etoposide or Rapamycin Enhances the Antitumor Growth Effect in Neuroblastoma

Zhijie Li, Shuang Yan, Navid Attayan, et al.