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Disruption of the AKT Pathway Inhibits Metastasis in an Orthotopic Model of Head and Neck Squamous Cell Carcinoma

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Abstract

Introduction—Merck's MK-2206 is an orally active, allosteric inhibitor of AKT; a component of the phosphatidylinositol-3 kinase (PI3K) pathway. The PI3K-AKT pathway is a downstream signaling pathway that has recently been found to play an important role in head and neck squamous cell carcinoma (HNSCC). Here we examine the role AKT inhibitors may play in preventing metastasis in HNSCC.

Methods—Cell migration after 24 hour treatment with sub-therapeutic doses of MK-2206 was assessed using an ELISA assay in 4 HNSCC cell lines: CAL27, FaDu, SCC-1 and SCC-5). *In vitro* effect of MK-2206 on cell migration was assessed by making linear scratches in culture plates after cell lines were grown to confluency. Images were taken at 8, 16 and 24 hours. *In vivo* analysis was performed on Nude mice with human SCC1-orthotopic tongue tumors. After tumors were allowed to grow for 7 days, mice were treated with oral dosing of 120 mg/kg of MK-2206 every other day for 2 weeks. Tumor size was assessed after each treatment using a pair of digital calipers. At the end of the treatment period, mice were sacrificed and cervical lymph nodes were assessed for metastasis using fluorescent imaging of tumor cell markers.

Results—Sub-therapeutic doses of MK-2206 was sufficient to significantly reduce cell migration in FaDu, SCC-1 and SCC-5 cell lines ($p < 0.001$) but not in Cal27 ($p = 0.09$). *In vitro* scratch test results in SCC-1 cells yielded significant reduction in cell movement at 8, 16 and 14 hours ($p < 0.001$). *In vivo* orthotopic model yielded significant reduction in primary tumor size ($p = 0.04$) and reduction in positive cervical lymph nodes ($p = 0.01$) between treatment and control mice. In addition we found 100% survival of MK-2206 treated mice after two weeks of treatment compared with 70 % survival in our control group ($p = 0.03$).

Conclusions—Treatment with MK-2206 is sufficient to inhibit HNSCC chemotaxis and migration *in vitro*. In an orthotopic model, treatment with MK-2206 reduces primary tumor size and cervical metastasis while improving survival. MK-2206 currently being used in phase II clinical trials for combination treatment of metastatic solid tumors and may be useful for treating HNSCC as well.

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Statement: Inhibition of AKT phosphorylation results in reduced proliferation and increased apoptosis of head and neck squamous cell carcinoma *in vitro* and *in vivo*. In two murine models, and in an ex vivo model using patient tumor samples, suppression of AKT phosphorylation with MK-2206 resulted inhibition of signaling pathways involved in protein synthesis, cell migration and rescue from apoptosis. Combination of MK-2206 with anti-EGFR targeted therapy demonstrated an additive effect on restricting tumor growth and activation of downstream AKT signaling components.

No potential conflicts of interest were disclosed.

Keywords

MK-2206; head and neck squamous cell carcinoma; AKT; EGFR

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy world-wide and accounts for 3.3% of all cancer diagnoses in the United States [1]. Most patients with early stage HNSCC can be effectively treated with single modality treatment using local radiation or surgical intervention. However, more than half of the estimated 36,000 Americans developing HNSCC in 2010 will present with locoregionally advanced stage disease, and have a five-year survival of less than 50% [2]. The use of platinum-based chemotherapy as a radiosensitizing agent has been shown to improve treatment outcomes, but results in significant morbidity and mortality. As a result, exploring new treatments that target specific head and neck cancer cell pathways are of critical importance.

The discovery that epithelial tumors overexpress the epidermal growth factor receptor (EGFR) led to the development targeted therapies in HNSCC. EGFR stimulation results in cell proliferation, circumvention of apoptosis, and increased tumor cell metastasis. In HNSCC, these events have been shown to be mediated downstream by the protein kinase B (AKT) signaling pathway. In addition, increasing levels of AKT activation correlates with an increase in the malignant potential of HNSCC [3]. AKT activation occurs by phosphorylation at the T308 and S473 residues and has been validated as a critical step in the initiation and maintenance of metastatic tumors [4]. However, it is unclear if there is a therapeutic benefit of AKT inhibition in head and neck cancer. To this end, we evaluate a novel AKT inhibitor currently in clinical trials to determine its potential in the treatment of head and neck cancers.

Merck compound MK-2206 has been shown to be an orally active, allosteric inhibitor of AKT that does not bind to the active site of AKT phosphorylation directly, but instead alters the shape of AKT so that ATP binding at the T308 and S473 residues is prevented. MK-2206 has been assessed for treatment of breast and colon cancer, but its role in head and neck cancer has not been established. When AKT is activated the result is an increase in downstream signal transduction pathways involved in cell migration, proliferation and metastasis [5]. Although AKT is known to be constitutively activated in a large percent of HNSCC, inhibition of AKT to prevent metastatic spread has not been studied in this patient population.

Targeted therapy in the form of monoclonal antibody to EGFR is becoming increasingly important in treating HNSCC. EGFR is activated by ligand binding followed by homo- or hetero-dimerization with another member of the EGFR superfamily leading to increased recruitment of phosphoinositol-3-kinase (PI3K) and persistently high levels of AKT in the plasma membrane. The most commonly used targeted agent, cetuximab, has been shown to demonstrate a survival advantage compared with radiation alone in the treatment of patients with locoregionally advanced HNSCC [6]. In patients with recurrent/metastatic disease however, overall survival was found to be only improved by 1–2 months [7]. The modest success of cetuximab highlights the need for new compounds that more effectively target the EGFR signaling pathway in HNSCC. In the present study, we investigate inhibition of the AKT pathway using MK-2206 as a viable strategy for preventing growth and metastasis of HNSCC *in vitro* and *in vivo* models.

METHODS

Drugs and chemicals

The chemical name of MK-2206 (Merck Research Laboratories, Rahway, NJ) is 8-[4-(1-aminocyclobutyl) phenyl]-9-phenyl-1,2,4-triazolo[3,4-f] [1,6]naphthyridin- 3(2H)-one hydrochloride. For *in vitro* and *in vivo* experiments, the stock solutions of MK-2206 and cetuximab (Imclone, New York, NY) were formulated in drug delivery vehicle of 10% DMSO.

Cell culture

The head and neck cancer cell lines SCC-1, SCC-5, FaDu and Cal27 (ATTC) were maintained in DMEM and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. All cells were cultured to 70%–90% confluence before passage. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Normal dermal fibroblast isolation from primary culture was described previously.

Cell Transformation

SCC-1 cells were plated at a density of 0.5×10^5 in a 24-well plate, 24 hours prior to viral infection. Cells were cultured in 0.5 ml of complete optimal medium (with serum and antibiotics) and incubated at 37°C with 5% CO₂ overnight. Lentivirus was thawed in a 37°C water bath and prepared in a mixture of complete medium with Polybrene. Media was aspirated from plate wells and replaced with 0.5 ml of this prewarmed Polybrene/media mixture per well (for 24-well plate). Cells were infected by adding 10 μ l of viral stock. The infected target cells were analyzed for transient expression or selected for stable expression using puromycin. Luciferase; Luc2 expression was screened for by imaging cells in 24 well plates with 150 μ g/ml D-luciferin in the culture medium. Puromycin selection pressure was used to generate stable SCC-1 cell lines.

Flow Cytometry

Human cancer cell lines; SCC-1, SCC-5, FaDu and Cal27 were aliquoted (1×10^5 cells/tube) and stained with calcein AM for cell viability assays (BD Pharmingen). Calcein was utilized as an early marker of apoptosis. Following appropriate washes, cells were analyzed for fluorescent counts (50k event minimum) using an Accuri C6 flow cytometer (Accuri Cytometers inc., Ann Arbor, MI). All experimental groups were analyzed in triplicate.

Immunohistochemistry

Serial sections of 5 μ m thickness were cut from the formalin fixed, paraffin embedded tissue blocks and floated onto charged glass slides (Super-Frost Plus, Fisher Scientific) and dried overnight at 60°C oven. A minimum of ten serial H&E stained sections were obtained from each tissue block and assessed for presence of tumor pathology. All sections subject to immunohistochemistry were de-paraffinized and rehydrated prior to antigen retrieval. The tissue sections were heat treated with 0.01M Tris-1mM EDTA buffer (pH 9) using a pressure cooker (CEPC 800, cook's essentials®, China) for 5 mins at maximum pressure (15 lb/in²). Following antigen retrieval, all sections were gently washed in deionized water, and then transferred to TBST (0.05M Tris-based solution in 0.15M NaCl with 0.1% v/v Triton-X-100, pH 7.6). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. To further reduce non-specific background staining, slides were incubated with 3% normal goat or horse serum for 20 min (Sigma, St. Louis, MO) according to the host where primary antibodies were produced. All slides were then incubated at 4°C overnight; Rabbit anti-TUNEL (1:1000, AbCam), or Rabbit anti-Ki67 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were achieved by eliminating the primary antibodies

from the diluents. Following washing with TBST, peroxidase-conjugated Goat anti-Rabbit IgG (1:5000, Jackson ImmunoResearch, West Grove, PA) were applied to the sections for 30 min, at room temperature. Diaminobenzidine (DAB, ScyTek Laboratories, Logan, UT) was utilized as the chromagen, and Hematoxylin (7211, Richard-Allen Scientific, Kalamazoo, MI) as the counterstain.

Cell Migration Assays

In vitro scratch assay was performed according to previously described methods [8]. Briefly, FaDu and SCC-1 cells were incubated for 12 hours in 6-well culture plates containing DMEM and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Once 100% confluence was observed, a 1 mm linear “scratch” was made in the adherent cell monolayer with a 200 μ L pipette tip. The scratch area to be used for assay was marked with a permanent marker on the underside of the 6-well plate. Debris was removed from wells by washing the cells once with 1 ml of the growth medium and then replaced with 3 ml of medium specific for the *in vitro* scratch assay. Assay medium contained 1% FBS with 100 nM MK-2206 was added to 3 wells in treatment group and 1% DMSO vehicle to 3 wells in control group. Linear distance between cells on either side of the scratch was measured in 10 locations for each well at 0, 8, 16 and 24 hours.

FaDu and SCC-1 chemotaxis was assessed using a QCM cell migration assay (Millipore, Billerica, MA). Cells were incubated in a 96 well plate containing serum free media. Each well contains a microporous polycarbonate membrane with 8 μ M pore size. Cells of interest are pipetted onto the top of the insert and incubated with 1% FBS. Migratory cells move through the pores of the membrane and cling to the bottom of the polycarbonate membrane in response to a DMEM media with 10% FBS loaded into the bottom feeder tray. Cells that migrate through the membrane are then lysed and total protein is quantified using an ELISA plate reader. The IC₅₀ of MK-2206 was found to be 1 μ M, we treated cells in both the scratch assay and the invasion assay with 100 nM to mitigate the anti-proliferation effect of MK-2206 on migration assays.

Orothotopic Tongue Tumor Model

Athymic Nude female mice aged 4 to 6 weeks were injected with Luciferase expressing SCC-1 Cells (2.5×10^5) in the anterior portion of their tongues. 7 days after tumors were implanted mice began treatment with 120 mg/kg MK-2206 (n=10 in each treatment group) or vehicle alone. Tongue tumors were measured before treatments QOD. Luciferase expression of SCC-1/Luc cells was assessed using a GE explore optics IVIS photon counter with a saturating dose of luciferin given 10 minutes before imaging by IP injection once each week. At the end of treatment, mice were injected with cetuximab conjugated to Cy5.5 near infrared dye. After 48 hours, neck dissections were performed and cervical metastasis was assessed using the Pearl Imuplse (LiCor). Pathology samples were taken from positive and negative lymph nodes and histological analysis was performed to confirm optical imaging response (data not shown).

In Vivo Dosing with MK-2206

Treatment with MK-2206 was performed by oral gavage. MK-2206 was provided by Merck in powder form, and the agent was dissolved in DMSO and then diluted to 120 mg/kg with sterile water. A neonatal feeding tube was inserted into the stomach of anesthetized mice and dosed three times a week. The 120 mg/kg dose of MK-2206 has been previously established by others to be effective *in vivo*. In a western blot analysis of HNSCC tumors post treatment, we found that a 120 mg/kg dose of MK-2206 was able to inhibit AKT kinase phosphorylation for 24 hours, without resulting weight loss observed at higher concentrations (data not shown).

RESULTS

MK-2206 causes apoptosis of SCC-1, SCC-5, FaDu, OSC-19 and Cal27 cells *in vitro*

The *in vitro* effect of logarithmic dose of MK-2206 on SCC-1, SCC-5, FaDu, OSC-19 and Cal27 cells was assessed using flow cytometry with calcein AM as a marker of cell viability (Figure 1a). Of the cancer cell lines, SCC-1 was the most sensitive with an $IC_{50} = 1.02 \pm 0.42 \mu M$ while OSC-19 were the most resistant $IC_{50} = 4.02 \pm 2.23 \mu M$ (Figure 1b). Normal dermal fibroblast (NDF) cells had a $IC_{50} = 18.67 \pm 3.97$ which was 4.5 to 18 fold greater compared to HNSCC cell lines. SCC-1 and FaDu cells were found provide the greatest therapeutic index and were selected for use in our animal experiments. The effect of MK-2206 on phosphorylation of AKT, and phosphorylation of three direct substrates of AKT (GSK3b, BAD and PRAS40) was assessed by Western blotting (Figure 1c). We found that monotherapy with 1, 10 or 100 μM MK-2206 was sufficient to reduce expression of p-GSK3b, p-BAD or p-PRAS40 *in vitro*. Reducing AKT phosphorylation corresponded with a decrease in cellular metabolism as measured by an ATP ELISA assay (Figure 1d). Total AKT cellular protein remained consistent with micromolar MK-2206 treatment, but expression of p-AKT was inhibited with MK-2206 at concentrations greater than 1 μM .

Inhibition of AKT prevents cell migration and chemotaxis *in vitro*

The effect of MK-2206 on cell migration was assessed *in vitro* with a wound assay and analysis of cell invasion (Figure 3). SCC-1 and FaDu cells were grown to confluence and a wound assay was performed in the presence or absence of 0.1 μM MK-2206. Images of cell migration for MK-2206 treated culture plates (Figure 3a) show delayed migration of SCC-1 cells at 8 ($p=0.04$), 16 ($p<0.001$) and 24 hours ($p<0.001$) (Figure 3b). An *in vitro* assay was carried out to measure invasion of cells treated with MK-2206 towards a chemotactic agent through a thin matrigel barrier. In Figure 3c, FaDu and SCC-1 cells were shown to migrate significantly less when treated with MK-2206 compared to controls ($p=0.04$ and $p=0.02$, respectively).

Inhibition of AKT by MK-2206 prevents regional metastasis and improves survival

SCC-1 tumor cells were implanted in the oral tongue of immunocompromised mice and were found to have significantly lower total bioluminescence ($p=0.029$) after 14 days of treatment compared to controls (Figure 4a). Tumor size was smaller measured by bioluminescence (Figure 4b, $p<0.001$) and by digital calipers (Figure 4c, $p=0.047$). Histological sections processed for H&E of the tongue tumors shows smaller tumor with area of central necrosis in MK-2206 treated samples (Figure 4c). Measurement of regional lymphadenopathy in the neck was performed using fluorescently labeled cetuximab after two weeks of treatment as previously demonstrated [9]. The rates of cervical metastasis were lower (75% vs. 25%) and the average number of positive nodes was significantly greater in control than in treated mice (Figure 4d, $p=0.011$). Furthermore, MK-2206 treated animals showed improved survival (Figure 4e, $p=0.03$) compared to control.

DISCUSSION

Treatment of advanced stage head and neck squamous cell carcinoma has progressed from surgery alone towards a multi-modality approach that favors development of targeted anti-tumor agents with non-overlapping toxicities to decrease patient morbidity and mortality. Toxicity associated with conventional chemoradiation has slowed advances in treatment dosing rates and thus survival [10]. Anti-EGFR targeted monoclonal antibody therapy has shown survival benefits across a range of treatment settings but is currently the only targeted therapy approved for head and neck squamous cell carcinoma. We and others have shown that the AKT pathway is constitutively activated in HNSCC cells and is likely an important

target for future therapies. And as a molecular signaling molecule distal to EGFR, AKT is a likely candidate for therapeutic intervention.

In the current study, *in vitro* dose response curves show that different HNSCC cell lines have consistent susceptibility to MK-2206 although with a range of IC₅₀ concentrations between 89 and 145 nM. HNSCC cell lines with greater IC₅₀ values did not have a reduction of phosphorylation at nanomolar doses of MK-2206 (data not shown). The *in vivo* sensitivities of SCC-1 cell lines mirrored their relative sensitivity identified *in vitro*. MK-2206 monotherapy had reduced growth compared to untreated controls for both cell lines tested in the orthotopic tongue model. Histopathological parameters demonstrated both a reduction in proliferation and an increase in apoptosis as predicted by *in vitro* data. The Ki67 antigen positive cells are regarded as G1, S, G2 or M phase cells, while G0 phase cells are Ki-67 negative. SCC-1 tongue tumors yielded significant reductions in Ki67 in tumors (data not shown) treated with 120 mg/kg MK-2206 delivered three times per week by gavage. Inhibition of AKT in HNSCC has been previously demonstrated *in vitro*; but has not been shown *in vivo* for an orally active agent like MK-2206.

Although AKT inhibition has not previously been shown to inhibit metastasis in HNSCC, its multi-factorial involvement in cellular signaling makes it a rational candidate to inhibit tumor invasion and metastasis. Because the *in vitro* IC₅₀ of MK-2206 was 100 nM, we chose this dose for the *in vitro* migration assays. That a low dose of MK-2206 (100 nM) was able to inhibit cell migration *in vitro* suggests a potentially large therapeutic window. In the present study, nanomolar doses of MK-2206 significantly inhibited *in vitro* migration in both two-dimensional and three-dimensional assay of tumor cell migration. This is consistent with other cancer studies showing inhibition of AKT phosphorylation reduces cell invasion [11]. Although the mechanism of this phenomenon is unclear, some have shown a decrease in MMP expression after exposure to AKT inhibitor. Orthotopic tongue tumors had a significant reduction in both tumor size and number of cervical lymph node metastases. *In vivo* inhibition of the AKT pathway has been shown to inhibit tumor growth and metastasis by blocking angiogenesis [12]. When treated with MK-2206, the Kaplan-Meier plot of their survival yielded a significant difference in survival between control and treatment populations ($p=0.03$). A transgenic thyroid cancer model has shown that increased AKT expression is associated with metastasis [13]. The mechanism by which this is accomplished appears to be multi-faceted, but includes reduction in glucose uptake, decreased cell motility, reduction in proliferation and induction of apoptosis as mediated by p-BAD, p-GSK3 β and p-PRAS40. In the case of GSK-3 β these effects are mediated through p-AKT phosphorylation causing its proteolytic removal. This results in decreased transcription of the transmembrane protein E-cadherin that forms adhesions between adjacent cells, thus leading to cell detachment [14].

In the current study, we present novel findings that have not previously been demonstrated including the anti-proliferative effect of AKT inhibition in cervical lymph node metastasis and in human tumors *in vivo*. MK-2206 is currently in phase II clinical trials for patients suffering from locally advanced or metastatic solid tumors [15]. In studies using erlotinib to treat gliomas, immunohistochemical analysis of phosphorylated AKT successfully predicted response to treatment. In these studies it was also shown that patients with elevated levels of phosphorylated AKT had higher response rates and longer time to progression than patients with low levels of phosphorylated-AKT. The search for molecular predictors of sensitivity to EGFR-directed treatment should therefore be extended to markers of PI3-K/AKT activation and MK-2206 may have great potential in patients' refractory to anti-EGFR therapies.

CONCLUSIONS

This pre-clinical study provides evidence that a novel small molecule AKT inhibitor, MK-2206, may have promise as a single agent to reduce tumor growth and metastasis. MK-2206 was shown to inhibit growth in multiple murine models of HNSCC, as well as reduce lymph node metastasis. Based on our results MK-2206 should be considered for further investigation as a promising treatment modality for HNSCC.

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Figure 1. Response of HNSCC cell lines to MK-2206

Dose response curves of four HNSCC cell lines (SCC-1, FaDu, SCC-5, Cal27) after logarithmic dosing of MK-2206. IC₅₀ values were: SCC-5 = 127 nM, FaDu = 89 nM, SCC-1 = 101 nM, Cal27 = 148 nM.

Figure 2. *In vitro* assessment of MK-2206 treatment of HNSCC cell migration and invasion
(a) Brightfield images (200×) of confluent SCC-1 cells showing re-growth following *in vitro* scratch test. Distance between edges of linear scratch following treatment with 0.1μM MK-2206 were significantly greater ($p < 0.001$) at 8, 16 and 24 hours compared to control
(b). Cell invasion assay showed significantly less cellular chemotaxis following 24 hour treatment with MK-2206 in both FaDu ($p = 0.04$) and SCC-1 ($p = 0.02$) cells **(c).** (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 3. Affect of MK-2206 on orthotopic tongue tumors and cervical metastasis

(a) Photon counts of SCC-1 tongue tumors treated with MK-2206 treated mice ($n = 10$) showed tumor-specific bioluminescence signal, that dropped significantly on day 14 ($P = 0.029$). Bars = S.E.M. (b) Tumor size measured by bioluminescence. (c) MK-2206 treatment resulted in significant inhibition of tumor growth ($P = 0.047$), when compared with digital caliper measurements of untreated control tumor, prior to harvest on day 15. H&E staining of tongue tumors showed noticeable necrosis in MK-2206 treated tongue tumors and decreased normal tongue muscle in control tumors.

Figure 4. MK-2206 inhibits cervical metastasis and improves survival *in vivo*

(a) Neck dissections on Day 15, yielded significantly more metastases in controls than in treatment group ($p = 0.011$). Anti-EGFR antibody labeled with near infrared fluorescent dye (Cy5.5) demonstrate the presence of tumor metastases in control and MK-2206 treated mice. (b) Kaplan-Meier survival curve for orthotopic tongue tumor model. Treated mice (dashed line) had significantly better percent survival than control mice ($p=0.03$). (*, $p < 0.05$; **, $p < 0.01$).