4-Hydroxybutenolide impairs cell migration, and invasion of human oral cancer SCC-4 cells via the inhibition of NF-κB and MAPK signaling pathways

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Abstract. 4-Hydroxybutenolide (K87), a synthetic compound from furfuryl alcohol via photooxidation, was used to investigate whether it can inhibit mobility, migration and invasion of SCC-4 human oral cancer cells in vitro. Cell viability was measured by flow cytometric assay, the enzymatic activities of MMP-2/9 were assayed by gelatin zymography analysis, the protein levels were assayed by western blotting, confocal laser microscopy and EMSA assay, and the gene expression of MMP-2/-7, FAK and ROCK1 mRNA were assayed by PCR. K87 decreased the percentage of viable cells in dose-dependent manner. K87 suppressed cell mobility, migration and invasion of SCC-4 cells dose-dependently. K87 inhibited the enzymatic activities of MMP-2/9 of SCC-4 cells. Western blot analysis revealed that K87 decreased the protein levels in NF-κBp65, COX-2, ROCK1 and Rho A, MMP-1, -2,- 7, -9, VEGF, GRB2, SOS1, PI3K, PKC, PERK, p-PERK, FAK, MEKK3, MKK7, ERK1/2, JNK1/2, p-p38, p38, p-c-Jun, AKT, TIMP2, but increased the protein levels of iNOS, Ras, IRE-1a, p-c-JNK, p-AKT(308), p-AKT(473) and TIMP1. Results from PCR indicated that K87 inhibited the gene expression of MMP-2/-7, FAK and ROCK1 mRNA. Furthermore, confocal laser microscopy was used to confirm that K87 inhibited the translocation of RHOA and ROCK1 in SCC-4 cells. EMSA assay also show that K87 suppressed the nuclear activation of NF-κB and these effects are time-dependent. Western blotting assay indicated that expression of NF-κBp105, NF-κBp50 and NF-κBp65 proteins were decreased and these effects are time-dependent. Based on these observations, we suggest that K87 may be used as a potential agent for anticancer metastasis of human oral cancer in the future.

Introduction

Head and neck cancer is the sixth most frequent cancer worldwide (1) and over 90% of head and neck cancer occurs in the oral cavity (2). Oral squamous cell carcinoma (OSCC) is a disease in the epithelial neoplasm with increased prevalence responsible for most malignant lesions in the head and neck. This disease represents only ~3% of all malignancies of the human body (3), it can lead to invasion and metastasis to cause adverse complication for the prognosis and treatment with chemotherapy of patients. The inhibition of cancer cell metastasis is one of the critical steps to therapy and research in human cancer (4). The identification of molecular pathways involved in cancer cell metastasis plays an important role for anticancer function of pharmacologic compounds (5).

Tumor metastasis is one of the major causes of morbidity and mortality in cancer patients. Molecular mechanisms of cancer cell metastasis are involved a series of molecular events such as the tumor cell-induced extracellular matrix (ECM) which provides biochemical and mechanical barriers (6), ECM component binding and angiogenesis are involved in cancer cell metastatic processes, degradation of basement membrane of ECM is a critical step in the processes of tumor metastasis (7,8). Matrix metalloproteinases (MMPs) play a critical role in ECM degradation and these enzymes are considered to be essential factors involved in tumor metastasis such as MMP-2 and -9 (8,9) and MMP-9 is considered to be one of the critical MMPs involved in cancer invasion in breast cancer (10,11). The inhibition of MMP-2 and/or MMP-9 expression, or their upstream regulatory pathways could be one of the treatment options for patients with cancer.

Nuclear factor-kB (NF-κB) signaling pathway plays an important role in cancer cell escape from anticancer drug
induced cell apoptosis and resistance of chemotherapy in many human cancer cells including head and neck cancer (12-16). NF-κB is restricted by the inhibitory protein IκB and after IκB is phosphorylated, NF-κB is liberated which enter the nucleus to regulate gene expression involved in cell proliferation, cell survival and apoptosis (17,18). NF-κB is associated with cancer cell invasion and metastasis in various cancers (19-22).

Akt is involved in the progression of metastasis through various signaling mechanisms such as NF-κB, which is a direct target of Akt (23). Activator protein-1 (AP-1), NF-κB via mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/Akt pathways led to regulation of the expression of the MMPs gene (24,25). MMPs and their regulatory pathways were the targets for anticancer drugs and chemotherapeutic agents (26). The aim of the present study was to investigate the molecular mechanisms of K87 suppressing the migration and invasion of human oral cancer SCC-4 cells. This is the first study that the anti-metastatic activity of K87 involves NF-κB and MMP-2/9 inhibition in human oral cancer SCC-4 cells in vitro.

Materials and methods

Chemicals and reagents. K87, a synthetic compound from furfuryl alcohol by photoxidation (27) was obtained from the laboratory of Dr Y.-H. Kuo, and the chemical structure is shown in Fig. 1. Dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection system was obtained from Amersham Life Science, Inc. (Arlington Heights, IL, USA).

Cell culture. Human oral cancer SCC-4 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in DMEM:F12 medium (Gibco-Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (Sigma-Aldrich) on a 75-cm² tissue culture flasks in a humidified atmosphere of 5% CO₂ at 37°C (28,29).

Cell viability assays. SCC-4 cells (1x10⁵ cells/well) were placed in a 12-well plate for 24 h and were incubated with K87 (0, 1, 2.5, 5, 10, 15 and 20 µM) or 0.5% DMSO as a vehicle control for 48 h. Cells were stained with PI (5 µg/ml) and then viability was immediately measured using flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) assay as previously described (28).

Wound healing assay. Cells (1.5x10⁵ cells/well) were placed in a 6-well plate and incubated at 37°C for 24 h. The confluent cells were scratched with a 200 µl pipette tip, followed with PBS washing, and then treated with K87 (0, 1 and 2.5 µM) in a serum-free medium for 12 and 24 h. Cells were examined and photographed under a fluorescence microscope (Axio Observer A1; Carl Zeiss, Oberkochen, Germany). The total number of cells migrated into the scratched area was calculated (30).

Cell migration and invasion assays. For cell migration assay, Transwell (BD Biosciences, Franklin Lakes, NJ, USA) cell culture chambers (8 mm pore size; Millipore, Billerica, MA, USA) were coated with collagen and SCC-4 cells (5x10⁴ cells/well) were maintained in serum-free 24 and 48 h. The migrating cells in the lower surface of the filter were fixed with 4% formaldehyde in PBS and were stained with 2% crystal violet. Cell number were counted and photographed under a light microscope at x200. Cell invasion assay was done as the cell migration assay except the filter membrane was coated with Matrigel from a BioCoat Matrigel invasion kit not for collagen as previously described (30,31).

Gelatin zymography assay. SCC-4 cells (1.5x10⁵ cells/well) were seeded in 6-well plate for 24 h and then were incubated with K87 (0, 0.25, 0.5, 1 and 2.5 µM) in serum-free DMEM:F12 medium for 24 and 48 h and the conditioned medium was collected and the total proteins measured. A total of 50 µg of protein was separated on 10% SDS-PAGE containing 0.2% gelatin by electrophoresis. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated in a reaction buffer (50 mM Tris-HCl, pH 7.5, 0.02% NaN₃, 150 mM NaCl, 10 mM CaCl₂, 1 µM ZnCl₂) for
18 h at 37°C while shaking. After incubation, the gels were stained with 0.2% Coomassie blue in 10% acetic acid and 50% methanol (31-33). Both MMP-2/9 gelatinolytic activities were visualized as the presence of clear bands with a blue (negative staining) background.

Western blotting assay. SCC-4 cells (1.5x10^6 cells) in 10-cm dishes were incubated with K87 (0 and 2.5 µM) for 6, 12, 24 and 48 h. Cells were collected, washed, centrifuged and lysis buffer [40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40] was added to the cell pellet for 30 min. In each sample protein was quantitated as previously described (32,34). A total of 30 µg of protein was loaded on a gel [10% sodium dodecyl sulphate (SDS)/polyacrylamide] for western blot analysis. The gel was transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by electro-blotting at 300 mA for 90 min. After blocking with 5% non-fat skim milk, the membrane was probed with primary antibodies against NF-κBp65, iNOS, COX-2, ROCK1, Rho A, MMP-1, -2, -7, -9, VEGF, GRB2, Ras, SOS1, PI3K, PKC, PERK, p-PERK, IRE-1α, FAK, MEK3, MKK7, ERK1/2, JNK1/2, p-c-JNK, p-p38, p-chemokine, p-AKT, p- Akt(308), p-AKT(473), TIMP2, TIMP1 and β-actin. After incubation, membrane was stained for secondary antibody for enhanced chemiluminescence (Amersham Life Science) as previously described (32,34). The antibody binding was detected by enhanced chemiluminescence (ECL) procedures according to the manufacturer’s recommendation.

Real-time polymerase chain reaction (PCR). SCC-4 cells (1.5x10^5 cells/well) were seeded onto 6-well culture plates and incubated with K87 (0 and 2.5 µM) for 24 h and were collected and total RNA was extracted using the QiaGen RNeasy Mini kit (Qiagen, Valencia, CA, USA) as previously described (35,36). Total RNA was reverse-transcribed with High Capacity cDNA Reverse Transcription kit at 42°C for 30 min according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA, USA). All samples for quantitative PCR were done under the following conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C, 1 min at 60°C using 1 ml of the reverse-transcribed cDNA, 2X SYBR-Green PCR Master Mix (Applied Biosystems) and 200 nM of forward (F) and reverse (R) primers, including MMP-2: F-CCCCAGACAGGTTATCTTGAC and R-GCTTGCCTTGCTTTT; ROCK1-F-ATTCATTCCTACCCTCTACC and R-TGTTGGGACTTAACGGCATCT; glycer-aldehyde 3-phosphate dehydrogenase (GAPDH): F-ACACCACCTCTCCACCTT and R-TAGCCCAAATTCGTTGTC, forward (F) and reverse (R) primers, including MMP-2: F-CCCCAGACAGGTTATCTTGAC and R-GCTTGCCTTGCTTTT; ROCK1-F-ATTCATTCCTACCCTCTACC and R-TGTTGGGACTTAACGGCATCT; glycer-aldehyde 3-phosphate dehydrogenase (GAPDH): F-ACACCACCTCTCCACCTT and R-TAGCCCAAATTCGTTGTC.

Confocal laser scanning microscopy. SCC-4 cells (1.5x10^5 cells/well) were maintained in 6-well chamber slides and were incubated with K87 (0 and 2.5 µM) for 24 h. All samples were fixed for 15 min with 3% formaldehyde in PBS and labeled for immunofluorescence. Primary antibodies against RHO A and ROCK1 were diluted 1:100 with blocking buffer. They were washed with PBS, and were stained with secondary FITC-conjugated goat anti-mouse IgG at 1:200 dilutions (green fluorescence). Cell nuclei were counterstained with PI (red fluorescence) and then were examined and photomicrographed using a Leica TCS SP2 confocal spectral microscope as previously described (32,37).

Electrophoretic mobility shift assay (EMSA). SCC-4 cells (1.5x10^6 cells/10-cm dish) were treated with K87 (0 and 2.5 µM) for 0, 2, 4, 8 h. After incubation, NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) was used to perform nuclear extracts as previously described (38,39) and protein concentration was measured and biotin end-labeled oligonucleotide sequences were 50-Biotin-GATCCAGGGGACTTTCCCTAGC-30 corresponding to the consensus site of NF-κB. A total of 5 µg of nuclear extract protein was used for EMSA with LightShift Chemiluminescent EMSA kit based on the manufacturer's protocol. The nuclear extracts was incubated with Biotin end-labeled duplex DNA and the reaction mixture was separated on 6.0% polyacrylamide gel electrophoresis, and transferred onto nylon membranes which were subjected to ultraviolet (UV) light cross-link for 1 min. Membrane was incubated with blocking buffer containing stabilized streptavidin-horseradish peroxidase conjugate (1:2,000) incubated with the substrates of the ECL kit for 15 min. The NF-κB signals on the membranes were detected using Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology).

Preparation of cytosolic and nuclear extracts. SCC-4 cells were treated with K87 (0 and 2.5 µM) for 0, 2, 4, 6 and 8 h. Cells were washed twice with ice-cold PBS and then immediately were suspended with the lysis buffer (0.1 mM EDTA, 1 mM DTT, 0.1% Nonide P-40) containing stabilized streptavidin-horseradish peroxidase conjugate (1:2,000) incubated with the substrates of the ECL kit for 15 min. The NF-κB signals on the membranes were detected using Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology).

Statistical analysis. All assays were performed in triplicate and data are expressed as means ± SD. Statistically significant differences between K87 treated and untreated (control) groups were tested by the Student’s t-test. A P<0.05 was considered to indicate significant differences.

Results

K87 decreased the cell viability of human oral cancer SCC-4 cells. SCC-4 cells were treated with K87 (0, 1, 2.5, 5, 10, 15 and 20 µM) for 48 h and total percentage of viable cells were measured and the results are shown in Fig. 1B.
Figure 2. K87 suppressed the mobility, migration and invasion and activities of MMP-2/-9 of SCC-4 cells. Cells (1.5x10^5 cells/well) were placed in a 6-well plate and incubated at 37°C for 12 and 24 h. The confluent cells were scratched with a 200-µl pipette tip and then treated with 0, 1 and 2.5 µM in a serum-free medium for 12 and 24 h. After treatment, the cells were examined and photographed under a fluorescence microscope (A). The total number of cells migrated into the scratched area was calculated (B). Cells (5x10^4 cells/well) were placed on filter which is coated with collagen and were incubated with K87 (0, 1.0 and 2.5 µM) for 24 and 48 h. Then they penetrated through to the lower surface of the filter which were stained with crystal violet and were photographed under a light microscope at x200 (C) and the cells were counted and percentage of inhibition were calculated (D). Cells penetrated through with the Matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at x200 (E).
K87 inhibited cell mobility, migration and invasion and activities of MMP-2/-9 of SCC-4 cells. Results from Fig. 1B indicated that K87 at >5 µM decreased over 25% cell viability, thus, we selected 1 and 2.5 µM for wound healing assay and results are shown in Fig. 2A and B. After treatment with 2.5 µM K87 for 12 and 24 h, cells remained creviced, while untreated wounds healed better. At higher concentrations and longer time of treatment of K87 both have higher inhibition of cell mobility (Fig. 2A and B). Transwell cell migration and invasion assays were used for investigating the inhibition of K87 on cell migration and invasion and results are present in Fig. 2C and D. K87 significantly inhibited cell migration by 52 and 64% at 24 h and 58 and 76% at 48 h both for 1.0 and 2.5 µM of K87 treatment (Fig. 2C and D). K87 significantly inhibited cell invasion by 24 and 47% for 24 h, and 42 and 67% for 48 h both for 1.0 and 2.5 µM of K87 treatment (Fig. 2E and F). Inhibition of migration and invasion in SCC-4 cells was concentration- and time-dependent. Gelatin zymography was performed to detect the gelatinolytic activity in conditioned media of SCC-4 cells treated by K87 and results are shown in Fig. 2G. K87 was markedly effective in inhibiting the gelatinolytic activity of MMP-2/-9. MMP-2/-9 activities were observed to be decreased in a dose- and time-dependent manner.

K87 affects the expression of migration and invasion associated proteins in SCC-4 cells. We have found that K87 suppressed cell mobility, migration and invasion, thus, we evaluated the expression of these molecules in SCC-4 cells. Western blot analysis revealed that K87 decreased the protein levels in NF-κBp65, COX-2, ROCK1 and Rho A (Fig. 3A), MMP-1, -2, -7, -9 and VEGF (Fig. 3B), GRB2, SOS1, PI3K, PKC, PERK and p-PERK (Fig. 3C), FAK, MEKK3, MKK7, ERK1/2, JNK1/2, p-p38 and p38 (Fig. 3D), p-c-Jun, AKT and TIMP2 (Fig. 3E), but increased the protein levels of iNOS (Fig. 3A), Ras and IRE-1α (Fig. 3C), p-c-JNK (Fig. 3D), p-FAK (308),p-AKT(473) and TIMP1 (Fig. 3E). These findings indicated that K87 affected cell mobility, migration, invasion, angiogenesis and metastasis associated protein in SCC-4 cells. The expressions of majority of proteins were downregulated by K87 in a time-dependent manner in SCC-4 cells.

K87 downregulates the mRNA expression of MMP-2 and -7, FAK and ROCK1 in SCC-4 cells. RT-PCR was performed for an inhibitory effect of K87 on the protein expression of MMP-2 and -7, FAK and ROCK1 via the depressed the levels of MMP-2, and -7, FAK and ROCK1 mRNAs expression and results are shown in Fig. 4. K87 (2.5 µM) significantly downregulated MMP-2 and -7, FAK and ROCK1 mRNA expressions by 25, 91, 72 and 17%, respectively, in SCC-4 cells. The highest inhibition of mRNA expression was in MMP-7. We suggested that K87 downregulated expression of MMP-2 and -7, FAK and ROCK1 at the transcriptional level.

K87 alters translocation of RHOA and ROCK1 in SCC-4 cells. RHOA and ROCK1 nuclear translocation was examined with confocal microscopy immunofluorescent imaging technique (Fig. 5). K87 treated SCC-4 cells were significantly decreased in the presence of RHOA (Fig. 5A) and ROCK1 (Fig. 5B) in the nuclei. The functional consequence and the underlying mechanism of the nuclear RHOA and ROCK1 translocation in cancer cells needs further investigations.

K87 affects NF-κB DNA binding activity and protein expressions in SCC-4 cells. Western blotting showed that K87 inhibited the expression of NF-κB in SCC-4 cells (Fig. 3A). Thus, for further understanding the NF-κB binding, we used EMSA assay and the results are shown in Fig. 6A. K87 suppressed the nuclear activation of NF-κB and these effects are time-dependent. Cells were treated with K87 (2.5 µM) for 2, 4 and 8 h, then harvested for cytosolic and nuclear extracts for western blotting assay and results are shown in Fig. 6B and C. IKK, p-IKK and IκBα (Fig. 6B), NF-κBp65, NF-κBp50 and NF-κBp105 protein expression (Fig. 6C) was decreased in agreement with the results from EMSA assay which shown that K87 suppressed the nuclear activation of NF-κB (Fig. 6A).
Discussion

Oral cancer is highly prevalent among the human population and is one of the most common cancers in males in Taiwan. Surgery, radiotherapy and chemotherapy has improved survival rates in the early stage of this disease, but the local recurrences and distant metastases are still a serious problem for oral cancer patients. Currently, novel therapeutic strategies and new drugs for defeating oral cancer cell metastases are essential. Cancer metastasis is a multistep process including cell mobility, migration, invasion, intravasation, entry into blood or lymphatic vessel, extravasation, and developing new tumors in other organs (41) that make it more difficult to treat patient, and account for the main causes of death in cancer patients with treatment failure.

We investigated the pharmacological activity of K87 on SCC-4 cell mobility, migration and invasion. Our findings demonstrated that: i) K87 decreased total cell viability (Fig. 1B), suppressed cell mobility (Fig. 2A and B), migration and invasion (Fig. 2C and D) and inhibited MMP-2 and -9 activities (Fig. 2G); ii) K87 inhibited cell migration and invasion associated protein expression (Fig. 3E); iii) K87 downregulates the
mRNA expression of MMP-2 and -7, FAK and ROCK1 (Fig. 4); iv) K87 alters translocation of RHOA and ROCK1 in SCC-4 cells (Fig. 5); v) K87 affects NF-κB DNA binding activity in SCC-4 cells in vitro (Fig. 6A) and inhibited the expression of NF-κB proteins (Fig. 6B and C) in SCC-4 cells.

K87 is a synthesized chemical and not known to exhibit anticancer property by inhibiting cancer cell growth, migration and invasion. Our studies investigated that K87 significantly suppressed the mobility, migration and invasion of SCC-4 cells, which did not result from cell growth arrest, because we selected low concentrations of K87 (0-2.5 µM) throughout the experiments. Our findings indicated that K87 treatment strongly attenuated the cell migration and invasion of SCC-4 cells through downregulation of MMP-2, -7, FAK and ROCK1 mRNA (Fig. 4) and protein levels and MMP-2
Figure 7. The possible signaling pathways for K87-inhibited migration and invasion of SCC-4 human oral cancer cells.

expression of NF-κBp105, NF-κBp50 and NF-κBp65 (Fig. 6B and C) and NF-κB binding DNA (Fig. 6A) in SCC-4 cells. MMPs are regulated primarily through NF-κB at the level of transcription through PI3K/Akt pathway (60,61). NF-κB is constitutively activated through a PI3K-dependent activation of IKK (62,63). The K87-mediated suppression of NF-κB in SCC-4 cells probably offers a molecular basis for its ability to inhibit cell migration and invasion.

The present study showed that K87 inhibited cell mobility, invasion and migration of SCC-4 cells by regulating the activities of MMP-2 and -9. K87 inhibited the MAPK (p38, ERK and JNK) signaling pathway by reducing AKT/PI3K, and NF-κBp65 leading to MMP-2/-9 downregulation as summarized in Fig. 7. Based on these observations, we suggested that K87 inhibited the migration and invasion of SCC-4 cells via the inhibition of MAPK and NF-κB signaling pathways.

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