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The effects of Sulindac on cell viability, cell cycle and angiogenesis in pharyngeal cancer cells

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Abstract

Objective: The present study aimed to investigate the effects of Sulindac on cell viability, cell cycle and angiogenesis in pharyngeal cancer cell lines (FaDu).

Methods: FaDu cells were incubated in a medium in a 5% CO2 incubator at 37°C, after which they were proliferated and passaged. IC50 concentration was used to determine the Sulindac dose. Cells were analyzed for cell viability, cell count and cell cycle after Sulindac administration. Immunohistochemistry (vascular endothelial growth factor receptor 2) and western blot (A disintegrin and metalloproteinase with thrombospondin-like motifs 1-ADAMTS1) analyses were used for angiogenesis assessment.

Results: Cell viability decreased in pharyngeal cancer cells after Sulindac administration. In addition, FaDU cells were arrested in the G2/M phase. Sulindac was found to slightly increase vascular endothelial growth factor receptor 2 (VEGF-R2) and decrease ADAMTS1 levels in pharyngeal cancer cells.

Conclusion: Sulindac showed positive results on cell proliferation in the treatment of pharyngeal cancer cells. However, it could cause a slight increase in angiogenesis.

Keywords: Sulindac, cell cycle, cell viability, angiogenesis, pharyngeal cancer.

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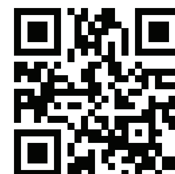

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Introduction

Head and neck cancers are the third most common cancer in the world, with 500,000 newly identified cases each year.[1] Surgery, radiotherapy, chemotherapy and targeted treatment options are available for patients with locally limited tumors.[2] However, relapse of the disease reduces the effectiveness of these treatments.[3] The combination of surgery, radiotherapy and chemotherapy often leads to severe and permanent dysfunctions that harm patients' quality of life. In patients with metastatic tumors, survival is limited to 7-10 years.[4] Therefore, new treatment options are needed for head and neck cancers.

Sulindac is a nonsteroidal anti-inflammatory drug (NSAID) that non-selectively blocks cyclooxygenase enzymes (COX-1 and COX-2). The link between cyclooxygenase and cancer is mainly through the synthesis of COX-2 and prostaglandins. COX-2 and synthesis of PGE_2 stimulate the proliferation and invasion of cancer cells while suppressing apoptosis or stimulating apoptosis. However, when NSAIDs suppress COX-2, either selectively or non-selectively, the opposite of the above occurs in cancer cells through transcription factors, growth factors and cytokines.[5] The in vitro anticancer effects of NSAIDs on various types of cancer have been known for several years.[6-8] In recent studies, using Sulindac in nude mice has been shown to inhibit tumor growth by increasing apoptosis in lung, stomach and colorectal cancers.[9-14] Sulindac has been found to have a synergistic effect with other chemotherapeutic agents, such as cisplatin, paclitaxel and docetaxel.[14-21] Sulindac has been found to exhibit a synergistic activity with anticancer drugs.[16] Sulindac has also been shown to suppress invasion, apoptosis and angiogenesis of different tumors.[22,23] Although Sulindac acts through the inhibition of prostaglandins, its exact mechanism of cancer prevention is still unknown.[1]

Angiogenesis plays a crucial role in tumor growth and metastasis.[24,25] The VEGF family of receptors and ligands are the most critical factors involved in angiogenesis and lymphangiogenesis.[25] Among these, VEGF-A is one factor supporting the angiogenesis phenotype of tumors and is related to immunosuppression in head and neck cancers.[25-27] Over-expression of VEGF in head and neck cancers is associated with drug resistance and poor prognosis.[28-34] In one study, VEGF expression of 1002 patients affected by oral cavity, pharynx and larynx cancer were evaluated, and it was found that high expression caused higher mortality in the second year of the disease.[35] A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) is a member of the ADAM protease family and a vital angiogenesis inhibitor.[36] The ADAMTS1 gene encodes a multi-domain protein containing a metalloproteinase domain, a disintegrin domain and thrombospondin (TSP) type I motifs.[37] This enzyme inhibits in vitro endothelial cell proliferation, fibroblast growth factor-2-induced vascularization and VEGF-induced angiogenesis.[38] ADAMTS1 directly binds VEGF and suppresses endothelial cell proliferation by blocking VEGFR2 phosphorylation.[39]

The present study aimed to investigate the effects of Sulindac on cell viability, cell cycle and angiogenesis in pharyngeal cancer cell lines (FaDU).

Materials and Methods

Cell Culture

FaDU cells were obtained from the American Type Culture Collection (American Type Culture Collection (ATCC; Manassas, VA, USA). FaDU were passaged in Dulbeccos Modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin in an incubator containing 5% CO2. Sulindac was prepared by dissolving in 1% of dimethylsulfoxide (DMSO). The study was designed with two groups: group 1: FADU cells and group 2: Sulindac treated FaDU cells. Dose trials were made three times to adjust the Sulindac dose and an IC50 test was performed for dose trials. IC50 (half-maximal (50%) inhibitory concentration) experiments were carried out for the dose determination of Sulindac. FaDU cells were seeded in wells

(6000/well) and incubated at 37° C. Then, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 400 µM, 600 µM, 800 µM and 1000 µM doses of Sulindac were applied to the cells and incubated for 48 hours. After this, tetrazolium 3-(4, 5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H (MTS) and 5-methyl-phenazinium methyl sulfate (PMS) (20/1) mixture was added, incubated for 1-2 hours and cell proliferation measured with a spectrophotometer (490 nm). The blank mean was subtracted from the mean absorption for each dose for IC50 calculation. Graphs were prepared with non-treated NT accepted as 100% and the 50% resultant dose was accepted as the IC50 dose.

Cell counting and viability test

$1x10^5$ cells were transferred to each tube for cell viability and count. 450 µl Count Viability Reagent (MCH100102, Millipore Co, Billerica, MA, USA) was added to the cell suspension (50 µl) to prepare a diluted staining sample, and the Muse™ Cell Analyzer (Millipore Co., Hayward, CA, USA) was used to perform measurements.

Cell cycle

$1x10^5$ cells were transferred to each tube to assess the cell cycle. 70% ethanol was added to the tubes, and they were incubated for 3 hours at -20°C. Then, 200 µl Cell Cycle Reagent (MCH100106, Millipore Co, Billerica, MA, USA) was added, the tubes were incubated for 30 min at room temperature in the dark and measured by a cell analyzer. After the determination of DNA content, index and distribution of peaks of the cell cycle phases, the percentage of cells in G0/G1, S and G2/M phases was calculated with this analyzer. G0, G1, G2 and S phases are the preparation phases of the pre-mitosis cell cycle. Cell division occurs during the M phase.

Immunohistochemistry

For immunohistochemical analysis, cells were seeded in a 24-well plate before drugs were added. Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then washed, incubated with vascular endothelial growth factor receptor 2 (VEGF-R2) antibody (9698S, Cell Signaling Technology, MA, USA) and stained with 3.3 diaminobenzidine (DAB).

Western Blot

Pharyngeal cancer cells were seeded in T-25 flasks at a density of 2.5-5 x 10^5 cells/flask in 4 ml medium. They were incubated at 37°C for 24 hours. Following adhesion to the surface, cells were treated with 200 µM Sulindac and then incubated for 72 h. After this step, the medium in the flasks was placed individually into falcon tubes, and the protein was isolated. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein samples were transferred to nitrocellulose membrane and blocking was performed. Cells were incubated in ADAMTS1 (12897S, Cell Signaling Technology, MA, USA) primary antibody and anti-rabbit secondary antibody overnight at 4°C for immunologic detection. Enhanced chemiluminescence (ECL) kit (34095, Thermo Fisher Scientific, MA, USA) and an imaging system (Fusion Solo, Vilber Lourmat, Paris, France) were used for chemiluminescent detection. A membrane imaging software (Image J, NIH, Bethesda, MD, USA) calculated band densities.

Statistical data, including mean values, proportions, and standard deviations, were calculated using Microsoft Excel 2010. Pre- and post-treatment data were considered for the percentage change.

Results

Drug Dose Trials

After incubation with serial doses of Sulindac (from 30 µM to 1000 µM), a dose of 200 µM was determined as the IC50 for FaDU cells (Figure 1). This dose was used for cell viability, cell cycle, immunohistochemistry and western blot analysis.

Cell Viability Testing

Cell viability was determined with a cell analyzer. Cell viability of FaDU cell cultures exhibited a decrease (36.58%) following Sulindac use (Table 1).

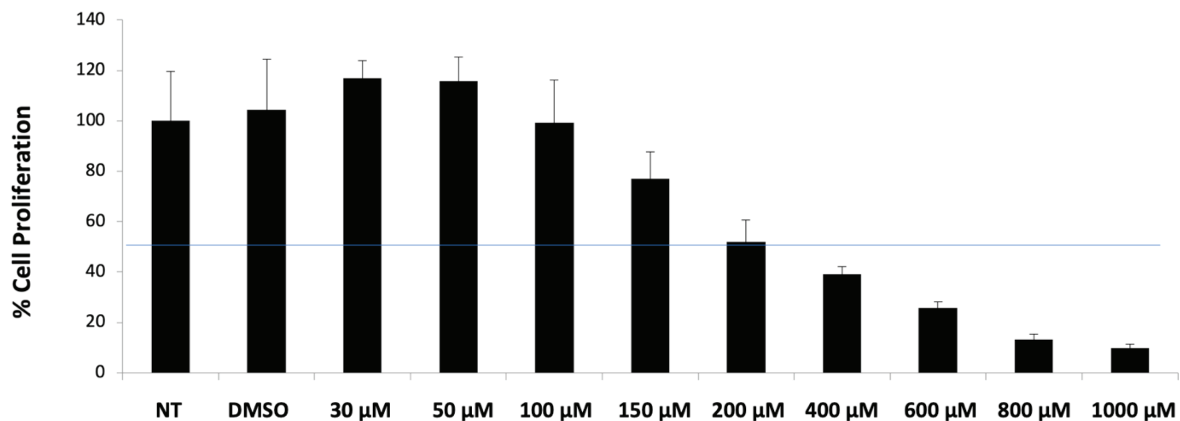

Figure 1. Cell proliferation results with different doses of Sulindac (30-1000 µM). IC50 dose was determined as 200 µM (NT: non-treated, DMSO: dimethyl sulfoxide, µM: micromolar).

Table 1. Cell viability results of FADU (Group I) and Sulindac treated FADU cells (Group II)

Group/Cell viability	Live cells	Dead cells
Group I (FaDU)	96.9%	3.1%
Group II (FaDU+Sulindac)	61.2%	38.8%

FaDu: pharyngeal cancer cell lines

Cell Cycle

The effect of Sulindac on cell cycle distribution was examined to assess whether growth inhibition of Sulindac-treated cells is mediated through changes in the cell cycle. Incubation with Sulindac (200 µM) resulted in increased number of cells in the G2/M phase (Table 2).

Table 2. Cell cycle results (the percentage of cells in G0/G1, S and G2/M phases) of both groups.

Cell cycle phases	FaDU (Group I)	FaDU+Sulindac (Group II)
G0/G1 phase	56.7%	49.1%
S phase	17.1%	13%
G2/M phase	24.7%	36.6%

FaDu: pharyngeal cancer cell lines

Immunohistochemistry

FaDU pharyngeal cancer cells treated with Sulindac showed a higher VEGFR-2 expression level compared to non-treated cells (Figure 2 a-b).

Western Blot

ADAMTS1 levels were analyzed in the two groups by western blot. Following the administration of Sulindac, ADAMTS1 showed a decrease in FaDU cells (Figure 3). Band density of the surface area for ADAMTS1 protein expression determined with Image J software was 3.897 and 1.418 for groups 1 and 2, respectively. An approximately 2.75 fold decrease was determined in the band density after Sulindac application.

Discussion

Scheper et al. [1] found that Sulindac at a dose of 150 µM repressed cell proliferation and increased apoptosis. These effects were statistically significant compared to other COX inhibitors. Like these results, our study found that cell viability was reduced in pharyngeal cancer cells by Sulindac. However, we found an effective dose of Sulindac to be 200 µM after IC50 trials. Besides this, we did not test any other NSAIDs for comparison of efficacy.

G0, G1, G2 and S phases are the preparation phases of

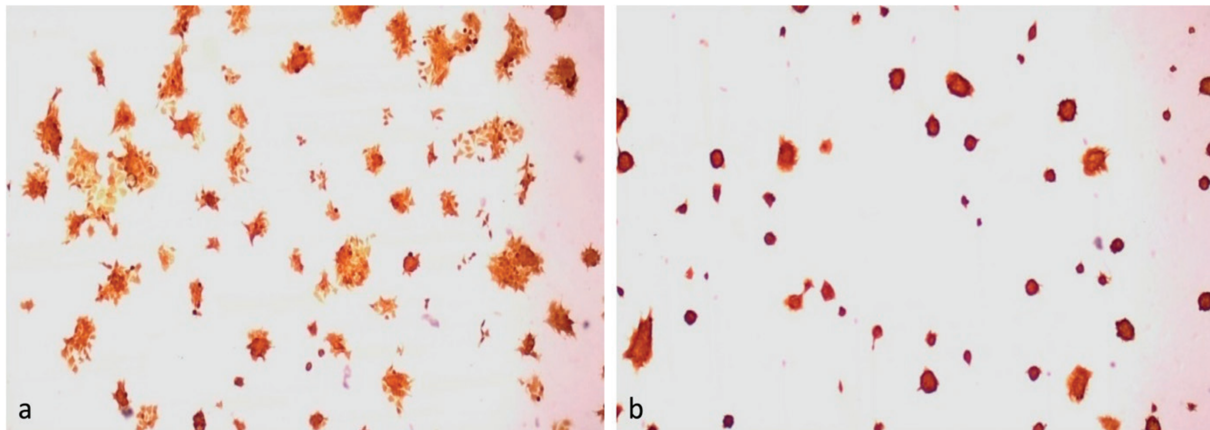

Figure 2. VEGFR-2 expression, a. FaDU cells (Group I) b. FaDU cells +Sulindac (Group II)

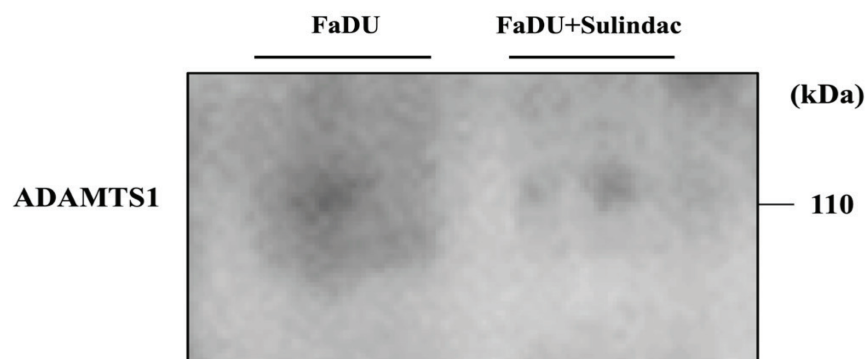

Figure 3. ADAMTS1 expression in group I (FaDU cells) and group II (FaDU cells +Sulindac). The molecular weight of ADAMTS1 protein is 110 kDa.

the pre-mitosis cell cycle, and cell division occurs during the M phase. Sulindac treated cells showed an increase in the G2/M phase of the cell cycle. This finding demonstrated that Sulindac could slow down the progression of cells into mitosis by holding them in the preparation phase of the cell cycle for pharyngeal cancer cells. Lee et al. [40] used indomethacin and NS-398, selective COX-2 inhibitors, in head and neck cancer. They showed that COX-2 was increased in cancer cells and found that COX-2 inhibitors inhibited cell growth in a dose-dependent manner, with an increased cell count in the G0/G1 phase and a decrease of cells in the S phase, increasing apoptosis. Rahman et al. [41] used Sulindac at a dose of 100 µM and its oxidative metabolite in hepatocellular carcinoma cell culture. Sulindac-treated cells showed an arrest in the G0/G1 phase that was not time-dependent. They also found a decrease in the percentage of cells in the S and G2/M phase. In contrast, we found that Sulindac paused the cell cycle of FaDU cells in the G2/M phase. The reason for this result might be because we have used a different and higher dose of Sulindac. We anticipated that after the application of Sulindac, the progression of cells into mitosis for pharyngeal cancer cells might be slowed down.

Sulindac treated pharyngeal cancer cells showed a higher VEGFR expression level compared to non-treated cells, although this increase was not very significant. In addition,

following the administration of Sulindac, a decrease of ADAMTS1 was reported for FaDU cells. This result suggests that angiogenesis may also increase following Sulindac in pharyngeal cancers. Also, antiangiogenic mechanisms may also be partially inhibited. Gallo et al. [42] examined COX-2, PGE2, and VEGF in head and neck cancers using histological and immunohistochemical analyses and observed increased COX-2, PGE2 and VEGF in tumor tissue along with increased angiogenesis in the surrounding tissue. They showed that NS-398 (selective COX-2 inhibitor) inhibited COX-2 and decrease PGE2 and VEGF, suppressing angiogenesis. Tsuji et al. [43] found that over-expression of COX-2 in colorectal cancer cells stimulated the production of angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF). They demonstrated that NSAIDs, including NS-398 (selective COX-2 inhibitor) and aspirin (non-selective COX inhibitor), inhibited these angiogenic factors, inhibiting proliferation, migration and angiogenesis. Despite this, we have observed that Sulindac increases VEGFR slightly in pharyngeal cancer cells and suppresses ADAMTS1. Unlike previous studies that described an antiangiogenic effect on head and neck cancer cells, Sulindac did not show these effects in our study. This may be due to different and higher doses of this drug in the present study.

In our study, Sulindac reduced the proliferation of pharyngeal cancer cells and kept them at certain stages of the cell cycle. The level of ADAMTS1, which is important for cell regulation and protects the cell from damage [44], may have decreased after Sulindac. Therefore, Sulindac might have exhibited its main effect on ADAMTS1 since the increase in VEGFR after Sulindac application was very low. Due to the inhibition of ADAMTS1, which has an anti-angiogenesis effect, a slight increase in VEGFR may have occurred in pharyngeal cancer cells, in contrast to previous studies.

Conclusion

This study showed that positive outcomes for cell proliferation could be achieved in the treatment of pharyngeal cancer cells using Sulindac. However, we concluded that Sulindac, as an NSAID, could potentially increase angiogenesis. Further studies should be conducted to elucidate the exact mechanisms of NSAIDs in pharyngeal cancers.

Acknowledgement: None.

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the Adnan Menderes University (Project Number: TPF-15073).

Informed Consent: Informed consent was not obtained due to the nature of this study.

Author Contributions: Designing the study – F.A., A.E., E.G.Y.; Collecting the data – K.E., E.G.Y.; Analysing the data – A.E.; Writing the manuscript – F.A.; Confirming the accuracy of the data and the analyses – A.E.

Conflict of Interest: The authors have no conflicts of interest to declare.

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