

EVALUATION OF THE ANTICARCINOGENIC EFFECT OF TAK-242, A TLR4 INHIBITOR, IN ORAL SQUAMOUS CELL CARCINOMA (IN VITRO STUDY)

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ABSTRACT

INTRODUCTION: Cancer immunotherapy has been recently presented as one of the most efficient treatment approaches for many types of cancer. Toll-like receptor 4 (TLR4) upregulation is involved in the proliferation, migration, and metastasis of many tumors. TAK-242 (also known as Resatorvid) is a TLR4 antagonist which specifically binds to the intracellular domain of the receptor and inhibits all TLR4 interplays with its downstream signaling molecules suggesting a new promising anticarcinogenic strategy.

OBJECTIVES: The present research aimed to estimate the antitumorigenic effects of TAK-242 on oral squamous cell carcinoma-4 (OSCC-4) cell line.

METHODOLOGY: Verification of TLR4 expression in OSCC-4 cells was established using immunocytochemistry to proof the presence of the studied receptor in the cell line. Next, the cytotoxic effect of TAK-242 was evaluated using MTT assay. The sublethal IC50 dose was calculated then apoptotic induction ability of the drug was assessed using Annexin V/PI assay.

RESULTS: In the present research, it was concluded that TLR4 was significantly expressed in OSCC-4 cells. In addition, its inhibitor (TAK-242) halted cell proliferation by inducing apoptosis of OSCC-4 cells.

CONCLUSIONS: TAK-242 has a significant anticarcinogenic effect on OSCC-4 cells and could be further studied experimentally.

KEYWORDS: Innate immunity, TLR4, Oral squamous cell carcinoma, TAK-242.

RUNNING TITLE: Detection of apoptotic properties of TAK-242 in OSCC-4.

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INTRODUCTION

Toll-like receptors (TLRs), a unique group of transmembrane receptors, perform significant functions in the innate immunity through recognition of pathogen-associated molecular pattern (PAMP) and danger associated molecular pattern (DAMP) (1). More than 11 different members of TLRs are expressed in human body cells, each one is activated by different ligands (2). TLR4, the most explored within all recognized TLRs, is stimulated by several PAMPs such as lipopolysaccharide of Gram-negative bacteria and DAMPs such as High mobility group box 1 protein (3). Toll-like receptor 4 is involved in the pathogenesis of several types of cancer (4-5). Following activation and

Dimerization of TLR4, two different signaling pathways are initiated: Myeloid Differentiation 88 dependent and Myeloid Differentiation 88 independent. As a result, Nuclear Factor-kappa is translocated to the nucleus and down streaming inflammatory mediators and cytokines are produced (6). These immune molecules, particularly IL-6, assist in cell survival, immune escape, invasiveness, and metastasis of the malignant cells (7).

Considering the significant role of TLR4 in tumorigenesis and its over expression noted in different types of cancer, a great attention has been given to the

blockage of TLR4 signaling as a novel anticancer modality (8-9).

For example, some experimental studies proved that TLR4 silencing can decrease cancer cell proliferation, enhance the results of chemotherapy, prevail over drug resistance, and increase overall survival (6).

Despite the fact that there are many antagonists for TLR4, TAK-242 (resatorvid) is a small molecule inhibitor of TLR4 which specifically and selectively links to the intracellular domain of the receptor and inhibits all TLR4 interplays with its downstream signaling molecules (10). In consequence, the activation of Nuclear Factor-kappa and the correlated cytokines is prevented (11). Furthermore, many researches have shown that TAK-242 has no effect on TLR1, 2, 3, 5, 6, 7, 9, nor does it interfere with TLR4 dimerization (2-12).

Oral squamous cell carcinoma (OSCC) is considered the most prevalent cancer hitting the oral cavity (13-14). Despite recent advances in the diagnostic and therapeutic techniques, the outcomes and the prognosis of the disease are still unsatisfactory (15). Therefore, new lines of treatment targeting the molecules involved in development and progression of OSCC are needed.

Despite the fact that several studies investigated the antitumorigenic properties of TAK-242 in different types of cancer both *in vivo* and *in vitro*. To the best of our knowledge, none of these researches has examined the therapeutic capacity of this inhibitor in OSCC. Given this, the present study was conducted to assess the anticarcinogenic effect of TAK-242, TLR4 antagonist, on OSCC cells.

The null hypothesis of the current study was that TAK-242 would exhibit no significant anticarcinogenic effect on OSCC-4 cells.

MATERIALS AND METHODS

1- Cell lines and treatment

Oral squamous cell carcinoma-4 cell line was ordered from ATCC (American Type Culture Collection, USA), cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose. 10% Fetal-bovine serum (FBS) and 1% Penicillin-Streptomycin were added to the culture media. Cultured cells were kept in 5% CO₂ at 37 °C. OSCC-4 cell line was frequently screened for Mycoplasma and fungal infection.

TAK-242 was ordered from TOCRIS Bioscience (Minneapolis, USA). 5 mg of TAK-242 was dissolved in 0.553 ml of dimethyl sulfoxide (DMSO) to obtain 553.1 ul stock solution of the drug. It was preserved in -20 °C. Upon application, the solution was diluted with the culture media in order to reach the intended concentrations.

Verification of TLR4 expression in OSCC-4 cells

The cultured cells were trypsinized 24 hours before fixation, centrifugated, and resuspended in complete media. 200µl of the suspension was aspirated (using an automatic pipette) and placed on a positively-charged slide. The slides were left to air-dry for 5 minutes in a dust free environment.

Cells were fixed in 95% ethanol for 10 minutes. The next step was permeabilization with 0.25% Triton X-100, and blocking in 0.3% H₂O₂ for 10 minutes. Then, TLR4 antibody (ThermoFisher, USA) was added and the cells were incubated for 60 minutes at 37 °C. After washing in PBS, the secondary antibody was applied. After 30 minutes, Strept-Avidin-Peroxidase conjugate was incubated, and the cells were washed in PBS.

The 0.02% diaminobenzidine hydrochloride (DAB substrate) including 0.03% H₂O₂ was mixed with the DAB chromogen 20 minutes before the application (20 µl DAB chromogen per 1 ml DAB substrate). The DAB mixture was then applied to the cells, incubated for 5-10 minutes in the dark at room temperature then washed in PBS in the same manner. This step was done to visualize the peroxidase activity and show the color of the antibody staining.

The cells were washed in water and counterstained by Mayer's Haematoxylin for 1-4 minutes. Finally, the slides were allowed to dehydrate in 4 different concentrations of alcohol (95%, 95%, 100%, and 100% respectively) 5 minutes each and the mounting the cover slips was established using a mounting DPX media.

2- MTT Cytotoxicity assay

Oral squamous cell carcinoma-4 cells were seeded in 96-well plate (about 5 × 10³ seeding density), after 1 day the media was discarded and replaced with new media containing 11 different concentrations of TAK-242 (40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 uM).

After 48 hours, the cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for four hours. Following, the media was replaced by 100 µl DMSO/well in order to vanish the crystals of formazan. The percentages of cell viability was detected using ELISA plate reader (Tecan, Infinite F50).

3- Annexin/ propidium iodide (PI) apoptosis assay using flow cytometry

The capability of TAK-242 to induce apoptosis in OSCC-4 cells was determined using annexin V-FITC and PI assay by flow cytometer. About 3 × 10⁵ cells were seeded for 24 hours in 6-well plates. After that, 120 uM of the TLR4 inhibitor was added.

After 48 hours, cells were trypsinized, centrifuged, and resuspended in 1ml of Annexin V Binding Buffer and centrifuged again for 10 minutes. All the supernatant cells were discarded and 5 ul of PI stain was added together with 10 µL of Annexin V-FITC. Then the cells were kept in dark for 30 minutes. The percentages of viable, necrotic, and apoptotic cells were calculated by using BD FACS Calibur flow cytometer and Cell Quest™ software following the instructions supplied by the Annexin V-FITC kit.

4- Statistical Analysis

Two-way ANOVA test was used to analyze the data. All the graphs and illustrations are designed by GraphPad Prism Software 7.04. The same program was used for P value calculation. All data are presented as mean ± standard deviation (SD).

RESULTS

Verification of TLR4 expression in OSCC-4 cells
 The OSCC-4 cells showed positive immunosignals to the TLR4 antibody. Evident cytoplasmic reaction was detected in all cells with lacking of the reaction in the nuclei. The cells showed malignant criteria such as pleomorphism, increased nucleo-cytoplasmic ratio, abundant mitotic figures, and apoptosis (Figure 1).
 Cell Viability Assay

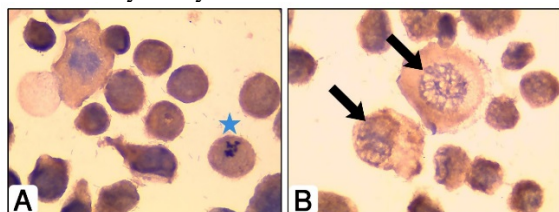


Figure 1: Light photomicrographs of OSCC-4 cells showing positive intense diffuse brownish cytoplasmic TLR4 immunosignals in all cells (a & b). Lacking of the reaction in the nuclei is evident. The cells are pleomorphic with large nuclei. Note the abnormal mitotic figures (*) and the disintegrated nuclei (black arrows).

MTT cytotoxicity assay was conducted in order to estimate the cytotoxic capability of the TLR4 antagonist in OSCC-4 cells. The findings showed that TAK-242 lowered the percentage of cell viability in treated cells, with IC50 value equals 120 μ M and R squared = 0.9115 (Figure 2).

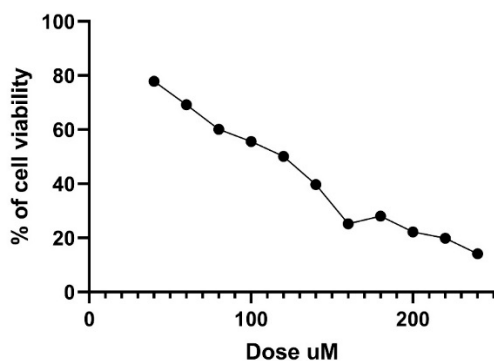


Figure 2: Line graph showing the viable cell percentage of TAK-242 different doses, the IC50 value was detected 48 hours after drug application. Dose 120 μ M represents the least toxic dose.

Assessment of Apoptosis using Flow Cytometry
 Annexin/PI assay was performed to assess the percentage of apoptosis and necrosis. The conducted assay revealed that treated OSCC-4 cells showed significant increase in the percentages of apoptosis (21.38%) and necrosis (11.4%) when compared to the untreated cells; apoptosis (5.9 %) and necrosis (2.63 %). In this assay Two- way ANOVA test was carried out. (Figures 3, 4).

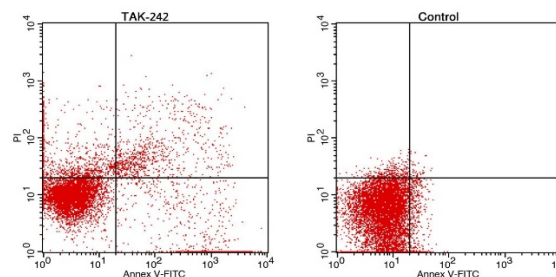


Figure 3: Scatter Plots for Annexin/PI apoptosis detection assay using flow cytometry. The percentages of living cells, early apoptosis, late apoptosis, and necrosis were presented in the lower left, lower right, upper right, and upper left quadrant respectively.

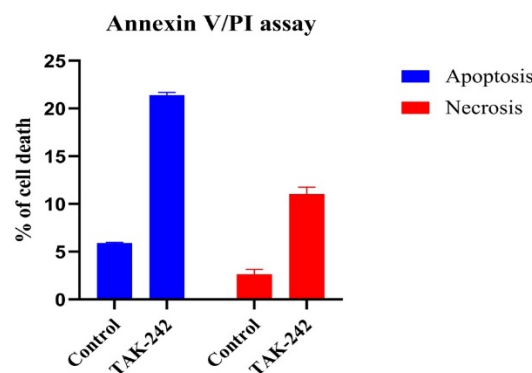


Figure 4: The results of annexin V/PI test revealed that TAK-242 induced apoptosis (21.3%) and necrosis (11.4%) of the treated cells. Statistically significant value of ****P \leq 0.0001.

DISCUSSION

As a consequence of the new advances in understanding the molecular biology, immunotherapy has recently emerged as one of the most exciting and evolving treatment approaches for many types of cancer (16).

Toll-like receptors are transmembrane sensors that mainly expressed in the innate immune cells, dendritic cells, and epithelial cells (17). Marked over expression of TLRs has been detected in many tumors, indicating that they could be involved in the pathophysiology of cancer (18). Various researches have proved that dysregulated TLR4 signaling enhances the proliferation, migration and metastasis of cancer cells (19).

Toll-like receptor 4 activation has been linked to the proliferation and immune surveillance of malignant cells in human head and neck squamous cell carcinoma (20). Additionally, esophageal squamous cell carcinoma (ESCC) invasion and metastasis were both reported to be significantly impacted by TLR4 upregulation (21). Based on these details, the present study was conducted to explore the anticarcinogenic properties of TLR4 inhibition using TAK-242 in OSCC cells.

The present study proved that OSCC-4 cells expressed TLR4. Evident cytoplasmic reaction was detected in all cells with lacking of the reaction in the nuclei. These results are consistent with findings of an earlier study

revealed that the immune signals of this receptor were clearly detected in human OSCC cells and its activation was involved in development of resistance to cisplatin (22). A study conducted in 2018 by Omar et al (23) assessing the immunoreaction of different grades of OSCC to anti-TLR4 antibody in the Egyptian population. This study concluded that moderately and well differentiated OSCC showed significant immunoreaction to the antibody. Additionally, Sato et al (24) stated that there is significant expression of TLR4 not only on the cell membrane but also in the cytoplasm in ESCC cells. In the present study, it has been reported that TAK-242 has a significant cytotoxic effect on OSCC-4 cells. In consistence, Kashani et al (7) stated that TLR4 antagonist, TAK-242, was cytotoxic when applied to ovarian cancer cell line. Furthermore, a recent study revealed that breast cancer cells' viability was significantly reduced by TLR4 inhibition. The cells' relative sensitivity to TAK-242 varied, though (19). In the current work, it has been proved that the percentages of apoptotic cells and necrotic cells after treatment are significantly increased compared to the control group. Consistently, latest researches demonstrated that TAK-242 prevented skin cancer cells' proliferation in mice through the downregulation of TLR4 (25-26). In addition, caspase dependent apoptosis in colon cancer cells was significantly improved after the treatment with TAK-242 (27). All in all, TAK-242 has a significant anticarcinogenic effect on OSCC cells. However, it should be considered that this work was conducted on only one cell line and combination with the conventional chemotherapeutic agents was not applied. Thus, additional *in vitro* and *in vivo* research would be requested to support the findings of the present work and to open the door to potential clinical trials in the near future. Considering limitations of this work, the null hypothesis was rejected.

CONCLUSION

TAK-242, TLR4 inhibitor, has been demonstrated to be a novel therapeutic modality in OSCC.

CONFLICT OF INTEREST

The authors state that they have no conflicts of interest.

FUNDING

This research was generously funded by Science, Technology & Innovation Funding Authority (STDF) Id (45030).

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