ABSTRACT

Background and objectives: Activation of adenosine A2a receptor has been shown to induce the growth and metastasis of cancer cells. The role of this receptor in esophageal cancer has not yet been determined. The present study aimed to investigate effects of an adenosine A2a receptor antagonist (3, 7-dimethyl-1-propargylxanthine) on growth of esophageal cancer cells.

Methods: Real-time polymerase chain reaction was performed to evaluate mRNA expression of the A2a adenosine receptor in KYSE-30 and YM-1 esophageal cancer cell lines. Effects of the antagonist on viability of the cells were evaluated by MTT assay.

Results: At low concentrations, the antagonist had no effect on cell viability. However, at concentrations ≥200 μM, the antagonist significantly reduced viability of both cell lines (p<0.05).

Conclusion: The results of this study indicate that the adenosine A2a receptor antagonist exerts inhibitory effects on KYSE30 and YM-1 esophageal cancer cell lines. Therefore, the use of this antagonist can be exploited as a therapeutic target for the treatment of esophageal cancer.

Keywords: Cell Death, 3,7-dimethyl-1-propargylxanthine, Esophageal Neoplasms.
INTRODUCTION
Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer deaths. The incidence of this disease has increased significantly in recent decades due to its rapid progression and poor prognosis (1). The Golestan Province (northeastern Iran) is one of the hotspots for esophageal cancer (2). Despite recent advances, current anti-cancer therapies are often unable to eradicate cancer (3). In recent years, the discovery of the signaling pathways involved in cancer has led to the identification of molecular targets for cancer therapy. Different experimental studies have shown that targeting the intracellular pathways can eliminate cancer cells (4), and several studies have been performed on purine receptors. There are basically two main families of purine receptors: adenosine receptor or P1-purinergic receptor whose endogenous ligand is adenosine and P2-purinergic receptor which tend to bind uridine diphosphate, uridine triphosphate, adenosine diphosphate, and adenosine triphosphate (5). Adenosine receptors belong to a large family of G-protein-dependent receptors, which are divided into four subgroups: A1, A2a, A2b, and A3. This classification is based on pharmacological findings, biochemical structure, and intracellular signaling (6). Activation of adenosine A1 and A3 receptors inhibits adenylyl cyclase and decreases the intracellular level of cyclic adenosine monophosphate, but adenosine A2a and A2b receptors stimulate the adenylyl cyclase and increase intracellular cyclic adenosine monophosphate content (7). Up to now, altered expression of the adenosine receptors has been reported in various cancers (8-10).

The development of selective and potent antagonists and agonists of adenosine receptors has been the subject of various pharmacological studies (11). One of the unique effects of adenosine receptors is their involvement in cell growth and cell death, particularly apoptosis (12). The A2a receptor is involved in endothelial cell proliferation. Adenosine has been shown to exert its mitogenic effects by regulating the expression, synthesis, and secretion of growth factors such as fibroblast growth factor and vascular endothelial growth factor. As a result, the migration and proliferation of capillary epithelial cells to a new location and the formation of new blood vessels occur (13).

MATERIALS AND METHODS
The KYSE-30 cell line was purchased from the Pasteur Institute of Iran. The YM-1 esophageal cancer cell line was obtained from the Biochemistry Department of Golestan University of Medical Sciences (Gorgan, Iran). The study received approval from the Ethics Committee of Golestan University of Medical Sciences (ethical code: IR.GOUUMS.REC.1398.364). The cells were cultured in Dulbecco’s Modified Eagle Medium (Bioidea, Iran) with 10% fetal bovine serum (Gibco, Germany), 100 μg/ml streptomycin (Bioidea, Iran), and 100 U/ml penicillin (Bioidea, Iran). The cells were kept at 37 °C in a humidified atmosphere with 5% CO2. After trypsinization, the cells were seeded onto T25 flasks (SPL, South Korea) and cultured until reaching >80% confluency. Total RNA extraction was performed by using the RNX-Plus kit according to the manufacturer’s instructions (Sinaclon, Iran). After treatment with DNase I (Thermo fisher, USA), the extracted RNA (100 ng) was used for cDNA synthesis by using the RevertAid™First Strand cDNA Synthesis kit (Yekta Tajhiz Azma, Iran) based on the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) was carried out using the SYBR Green Master Mix (Yekta Tajhiz Azma, Iran). Primer sequences for the A2a adenosine receptor were derived from PrimerBank. Primer sequences for GAPDH were derived from a previous study (8) (Table 1). The real-time PCR reactions were done in the StepOnePlus™ System (Applied Biosystem, USA). The cycling conditions were as follows: initial denaturation for 10 minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 seconds, and annealing and extension for 1 min at 60 °C. The obtained cycle thresholds were calculated by using the 2−(ΔΔCT) method. Relative gene expression levels were calculated by defining a ratio between the amount of gene expression and the endogenous control. An analysis of the melting curve (60 °C to 95 °C increments of 0.3°C) was also performed to determine the melting temperature of specific amplicons and primer dimmers. All tests were performed in triplicate.

Cells (10^4 cells per well) were seeded in 96-well plates. After 24 hours, the cells were treated with 20 μl of 3, 7-dimethyl-1-propargylxanthine (DMPX, Sigma, USA) as
the antagonist of A2A adenosine receptor in various concentrations (0.1, 1, 10, 50, 100, 200, and 500 μM) for 48 hours. Cells treated with 20 μl of dimethyl sulfoxide (DMSO) were considered as negative controls. Next, 20 μl (0.5 mg/ml) of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (Sigma-Aldrich, USA) were added to each well. After 4 hours, the culture medium was carefully removed, and 150 μl of DMSO solution were added to each well. Absorbance of each well versus the control was read at 570 nm using an enzyme-linked immunosorbent assay reader. The percent of cells stained by MTT was determined by comparison of the optical density of each sample with that of the control group. Results are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey post-hoc test was used to identify statistically significant differences. All analysis was carried out in SPSS (version 22) at significance of 0.05.

**RESULTS**

Expression level of the A2a adenosine receptor in YM-1 cells was slightly higher than in KYSE30 cells (p>0.05) (Figure 1). The effect of DMPX on the viability of cells was also examined. For this purpose, YM-1 and KYSE30 esophagus cancer cells were treated with various concentrations of DMPX (0.1-500 μM) for 48 hours, and the inhibition of cell proliferation was measured by MTT assay. Based on the results, the antagonist had no effect on cell viability at low concentrations. However, at concentrations ≥200 μM, DMPX significantly inhibited cell proliferation in a dose-dependent manner (Figures 2 and 3). The half maximal inhibitory concentration (IC50) for YM-1 and KYSE-30 cells was 767.5 μM and 682.8 μM, respectively.

![Figure 1- Expression level of A2a adenosine receptor in YM-1 and KYSE30 cells](image1.png)

**Figure 1- Expression level of A2a adenosine receptor in YM-1 and KYSE30 cells**

![Figure 2-Effect of the adenosine A2a receptor antagonist on the growth of YM-1 cells. * indicates statistically significant difference compared to control cells.](image2.png)

**Figure 2-Effect of the adenosine A2a receptor antagonist on the growth of YM-1 cells. * indicates statistically significant difference compared to control cells.**
the previous study, this study reported that extracellular adenosine activates caspases 3 and 9 by disrupting mitochondrial membrane potential, thereby inducing apoptosis by A2a receptor activation. According to this study, the adenosine A2a receptor antag, DMPX, reverses the effect of adenosine, and the adenosine A2a receptor agonist, CGS21680, was able to mimic the effect of adenosine and reduce cell viability.

In 2018, a study reported that adenosine A2a receptor agonists induced cell growth in squamous cell carcinoma, by a mechanism dependent to the ERK1/2 pathway. In contrast, some studies reported that activation of the A2a receptor had anti-cancer effects. As it was illustrated by Tamura et al., the adenosine A2a receptor agonist induced apoptosis in liver cancer cells by decreasing Bcl-2 expression and increasing Bad expression. These results indicate that the effects of this receptor may vary depending on the type of cancer.

**CONCLUSION**

The results of this study indicate that the adenosine A2a receptor antagonist exerts inhibitory effects on KYSE30 and YM-1 esophageal cancer cells in a dose-dependent manner. Therefore, the use of this antagonist can be exploited as a therapeutic target for the treatment of esophageal cancer.

**ACKNOWLEDGMENTS**

The results of this study have been extracted from a MSc thesis authored by Parisa Zeinali, which was supported by the Golestan University of Medical Sciences, Iran.
DECLARATIONS
FUNDING
This study was financially supported by the Golestan University of Medical Sciences (grant number: 111191).

Ethics approvals and consent to participate
The study received approval from the Ethics Committee of Golestan University of Medical Sciences (ethical code: IR.GOUMS.REC.1398.364).

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding publication of this article.

REFERENCES


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