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Abemaciclib (CDK4/6 Inhibitor) Blockade Induces Cytotoxicity in Human Anaplastic Thyroid Carcinoma Cells

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Abstract

Background: Thyroid cancer is the most prevalent endocrine malignancies globally. Anaplastic thyroid carcinoma (ATC) accounts for 1-3% of all Thyroid cancer. The evidence showed that ATC is a highly invasive solid tumor with poor prognosis. Despite conventional chemotherapy treatments, a considerable number of patients show developing resistance to therapeutic agents and tumor relapse. The aim of this study was the investigation anti-tumor effect of Abemaciclib (novel targeted cancer therapy drug) on Anaplastic Thyroid carcinoma SW1736 and C643 cell lines.

Methods: SW1736 and C643 cell lines were treated by desire concentrations of Abemaciclib (0, 1, 2.5, 5, 10, and 20 μM) and cell viability was measured by MTT assay. Also, Anoikis resistance assay was conducted for non-adherent the cells in the exposure of Abemaciclib. The gene expression of apoptotic and anti-apoptotic genes was conducted by quantitative Real-time PCR.

Results: Abemaciclib at the concentration of 10 and 20 μM effectively reduced cell proliferation and growth of the ATC cells compared to the control (p=0.000). Furthermore, we showed that 10 and 20 μM doses of the Abemaciclib inhibited the non-adherent ATC cells which were resistant to Anoikis death significantly (p=0.001). Moreover, we demonstrated this targeted therapy significantly reduced anti-apoptotic gene expression levels (*BCL2* and *CMYC*) (p<0.05) and increased apoptotic gene expressions such as *P21* and *BAX* (p<0.05).

Conclusions: Our data suggested that Abemaciclib can be utilized as a novel therapeutic agent in ATC cancer. Further *in vivo* and *in vitro* investigations are needed to evaluate molecular and clinical mechanisms of Abemaciclib.

Keywords: Abemaciclib, Anaplastic Thyroid Carcinoma, CDK4/6 inhibitor.

Introduction

Thyroid cancer is the most prevalent endocrine malignancies which was originated from thyroid follicular cells (1, 2). It is estimated that up to 2030, it will be forth cancer-related death worldwide (3). The incidence of thyroid cancer varies according to environmental factors such as age, sex and geographic regions (4). Prevalence of Thyroid cancer is more frequent in women than men (5). There are several types of thyroid cancer such as papillary (PTC), follicular (FTC), medullary (MTC) and anaplastic thyroid cancer (ATC) (6, 7). Anaplastic thyroid cancer is the most aggressive thyroid malignancies which accounts for 1- 3% of all cases with thyroid cancer diagnosis (8). The evidence showed that ATC is an extremely aggressive solid tumor and fatal between in malignancies (9). The characteristics of ATC included a large mass, necrosis, and hemorrhage, which usually attacks broadly to the thyroid gland parenchyma and penetrate to the surrounding soft

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neck tissues (9, 10). It can disseminate and invade to distant such as lung, lymph nodes (LNS) and bone (9). The median survival of the patients is approximately 3 to 5 months, which mainly due to distance metastasis (8, 11). Current treatments for ATC patients include surgery, radioactive iodine therapy, radiotherapy, chemotherapy, and hormone therapy (12-14). Despite conventional chemotherapy treatments, a large number of the patients show developing resistance to therapeutic agents and treatment approaches (15, 16). Therefore, there is an urgent need for finding novel therapeutic strategies in ATC patients (16, 17). Recently, using targeted molecular markers as new approaches to the treatment of ATC have been developed to overcome the resistance (6, 18). Previous studies demonstrated that several genetic aberrations such as mutations, rearrangement, copy number, and chromosomal gain or loss could drive ATC (19, 20). Furthermore large body of evidence depicted the putative roles of signaling pathways including Src signaling, JAK/STAT, MAPK, PI3K/Akt, NF-κB, TSHR, Wnt-β-catenin and Notch signaling pathways (21). These signaling pathways have significantly contributed to the development of thyroid cancer particularly ATC (21). It has been shown that the expression of the cyclin-dependent kinase (CDK) is remarkably increased in ATC which stimulated cell proliferation (22). Cyclin D via activation of CDK4/6 leads to initiating G1 phase of the cellular cycle (23). Currently, several novels targeted small molecule therapies were developed which target CDKs in the cell cycle (24). Recent studies showed that Palbociclib, Ribociclib, and Abemaciclib significantly inhibited CDK4/6 and reduced cell proliferation (25, 26). Abemaciclib (LY2835219) is a novel orally bioavailable drug that selectively inhibits CDK4 /6 and suppresses phosphorylation of the Rb that leads to G1 arrest (25, 27-30). Therefore, arresting the cell cycle in the G1 phase caused suppressing DNA synthesis and inhibiting cancer cell growth (24). In this study, we studied the effect of Abemaciclib on Anaplastic Thyroid Carcinoma cells.

Materials and methods

Cell Culture and drug

Human ATC cell lines (SW1736and C643) were obtained from Stem Cell Research Center and cultured in RPMI 1640 (Gibco, USA) with 10% Fetal Bovine Serum (Gibco, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin. All cells incubated at 37°C with 5% CO2 and 95% humidity. Abemaciclib (Adooq Bioscience LLC, USA) was purchased and prepared by diluting the powder in Dimethyl sulfoxide (DMSO, Sigma, USA). The stock solution (10 mM) was prepared and stored at -20 C.

Cell Viability Assay

SW1736 and C643 cells lines (2,000 cells) were seeded in 96-well plates in 100 µL media for 24 hours. After 24 hours of incubation, the cells were treated with desired concentrations of Abemaciclib (0, 1, 2.5, 5, 10 and 20 µM) for 48 hours. To increase the accuracy of the experiments, all the treatments were done in triplicate. After 48 hours, cell proliferation was measured by MTT assay. 100µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to the each well and incubated at 37°C for 4 hours. After incubation, the medium was removed and 100µl DMSO was added to each the well, then the absorbance of each well was measured at 570 nm by an ELISA 96-well plate reader.

Anoikis resistance assay

Anoikis resistance assay measured the cell death or apoptosis in anchorage-dependent cells when they lost attachment from the extracellular matrix (ECM) or other cells. 96-well plate was precoated by poly-HEMA. Ten thousand cells were seeded in each well. The cells were treated with different concentrations of the Abemaciclib and incubated for 48h. Finally, live cells measured via a microscope and quantified by MTT assay.

RNA Extraction and cDNA Synthesis

After treatment of SW1736 and C643 cell lines with different concentrations of the Abemaciclib for 48 hours, RNA was extracted using RNX-Plus Solution for total RNA isolation (Cinagene, Iran) according to the manufacturer's instrument. The quantity and quality of the extracted RNA were investigated by the 2% gel electrophoresis method. Then RNA concentration was measured by Nanodrop (ThermoScientific, USA). cDNA was prepared by Takara Bio PrimeScript TM RT reagent (Japan) according to manual instruction. The total volume for this reaction was 20 µl that included 2 µg total RNA, 4 µl 5X buffer, 1 µl dNTP, 1 µl RNase Inhibitor, 2 µl random hexamers, 1 µl Reverse Transcriptase (M-MuLV) and DEPC water.

Gene expressions Analysis

Gene expression levels were evaluated via Quantitative Real-time PCR on light cycler grade 96-well plates (Roche Life Science, Germany). The total volume was 20µL including 10µL SYBR Green, 1µL primer and 2µL cDNA and DEPC water. The primers used are listed in table 1. *HPRT* was considered as a reference gene. All samples were run in triplicate.

Table 1. The list of the primers

Gene	Accession Number	Forward Primer	Reverse Primer	Size (bp)
P21	NM_000389	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT	130
BAX	NM_138761	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT	155
BCL-2	NM_000633	CGGTGGGGTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC	90
HPRT	NM_000194	GGACAGTACGGGAGATCACAG	GCACTAATTTCCTTCAGGGATCG	111
CMYC	NM_002467	GTCAAGAGGCGAACACACAAC	TTGGACGGACAGGATGTATGC	162

Statistical Analysis

The results were analyzed by GraphPad Prism5 software. Student's t-test and One-way ANOVA were used to mean comparisons between the groups. The quantitative Real-time PCR data were analyzed using the formula: Gene dosage ratio=$2^{-\Delta\Delta Ct}$. The data were expressed as Mean ± SD. P-values <0.05 were considered statistically significant.

Results

Cell viability assay

To determine the cytotoxicity of the Abemaciclib, MTT assay was conducted for the SW1736 and C643 cell lines in different concentrations of the Abemaciclib for 48 hours (Fig. 1). The results showed that Abemaciclib in 10 and 20 µM reduced cell viability significantly in both cell lines (p=0.000).

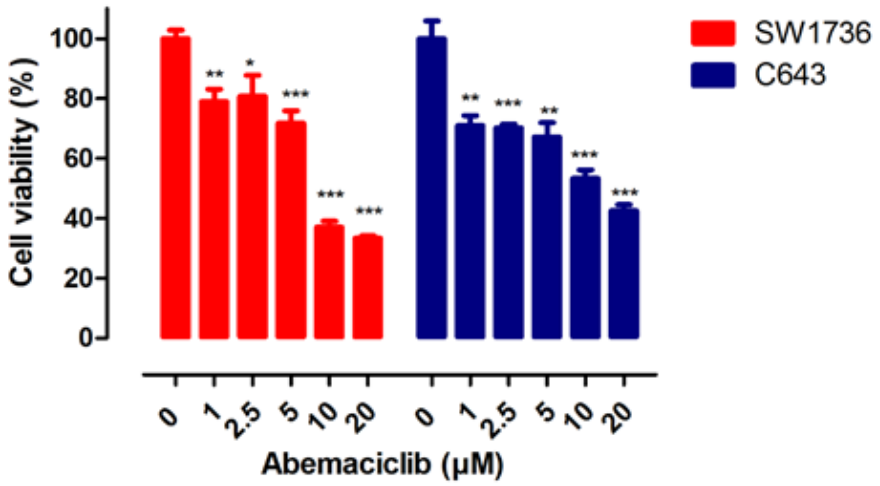

Fig. 1. Cytotoxicity effect of the Abemaciclib in the SW1736 and C643 cell lines. The cells were treated with different concentrations of Abemaciclib for 48h, and then metabolic activity was measured by MTT assays.

Anoikis resistance Assay Results

Anoikis resistance assay was conducted with poly-HEMA–coated culture palates. The SW1736 and C643 cell lines were treated in different concentrations of the Abemaciclib for 48 h. The proportion of viable cells were measured by MTT assay (Fig. 2). The results showed that Abemaciclib in 10 and 20 µM reduced the cell viability of the non-adherent cells significantly (p=0.001)

Abemaciclib induces pro-apoptotic gene expressions

To evaluate the molecular mechanisms of cytotoxic effects of Abemaciclib on the ATC cells, we studied the effects of Abemaciclib on the gene expression of pro-apoptotic and anti-apoptotic genes. To investigate this, the SW1736 and C643 cells were treated to Abemaciclib for 48h. Abemaciclib reduced mRNA levels of anti-apoptotic genes (*BCL2* and *cMyc*) (p<0.05). Furthermore, we have observed an increase in mRNA levels of pro-apoptotic genes (*P21* and *BAX)* (p<0.05) (Fig. 3).

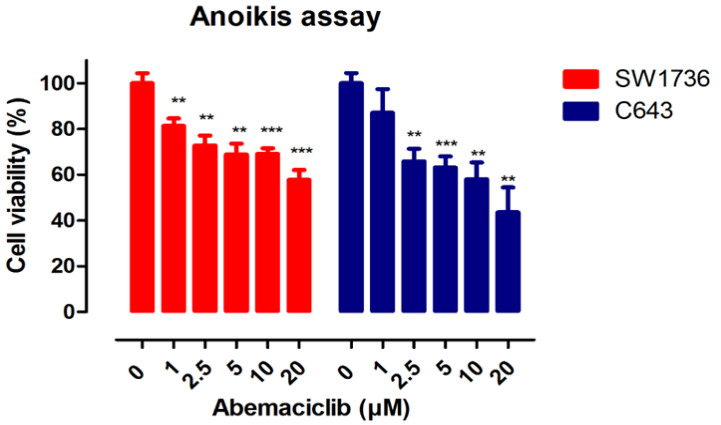

Fig. 2. Anoikis resistance assay results for ATC cells. The proportions of the viable cells were measured by MTT assay. Our data showed that Abemaciclib at the concentration of 10 & 20 µM reduced cell proliferation significantly in comparison to controls.

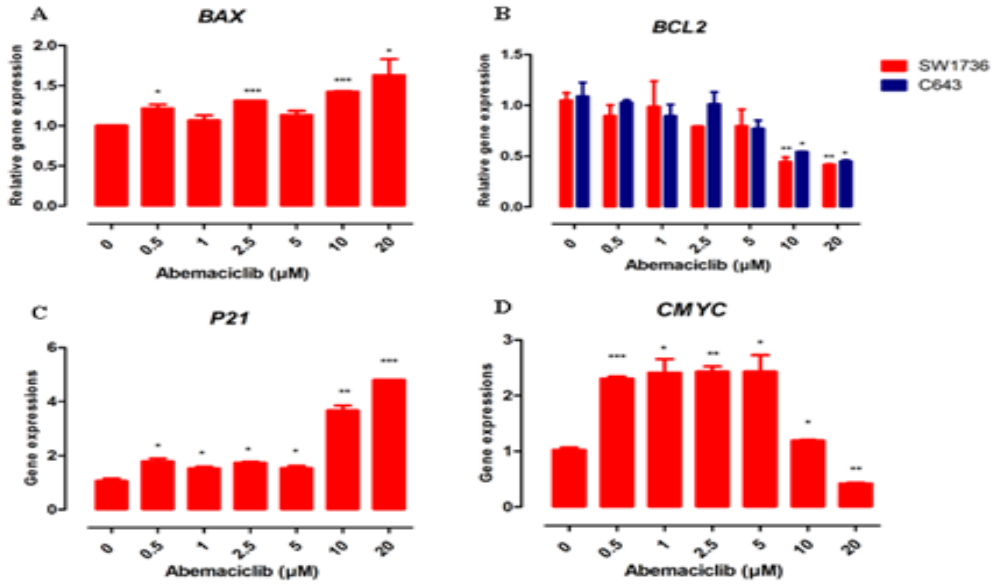

Fig. 3. The gene expression in the exposure of Abemaciclib in the cells. **A)** *BAX* expression was significantly increased at 10 & 20 µM concentrations of Abemaciclib after 48 hours' treatment only in the SW1736 cells. **B)** Abemaciclib reduced *BCL2* expression in the cell lines after 48 hours significantly at 10 & 20 µM concentrations in comparison to control. **C)** Abemaciclib increased *P21* expression just in the SW1736 cell line after 48 hours significantly at 10 & 20 µM concentrations in comparison to control. **D)** Reduction of *CMYC* gene expression at a concentration of 20 µM Abemaciclib, after 48 hours in SW1736 cells was significant. However, the Abemaciclib has not any significant impact on the expression levels of the *CMYC* gene in the C643 cells.

Discussion

Thyroid cancer is considered as the most prevalent endocrine malignancies globally (4). ATC accounts for 1-3% of all Thyroid cancer (31). The evidence showed that ATC is a highly invasive solid tumor with poor prognosis (10). Distance metastasis of ATC usually occurs on lymph nodes, lung, and bones (9, 32). Distance metastasis remarkably reduces the median survival of the patients (33, 34). Current treatments for ATC patients include surgery, radioactive iodine therapy, radiotherapy, chemotherapy, and hormone therapy (5, 9). Doxorubicin and cisplatin are the most commonly administered chemotherapy agents for ATC patients (35). Doxorubicin imposes its anti-tumor effects via inhibition of histone deacetylases which remodeling the chromatins (36, 37). Cisplatin is also an anti-tumor agent, interacts with DNA and leads to DNA breakage which significantly kills proliferative cells (38). The most prevalent side effects of these therapeutic agents are myelosuppression and cardiotoxicity (33). Despite conventional chemotherapy treatments, a huge number of patients show developing resistance to therapeutic agents and tumor relapse (39). Therefore, there is an urgent need to find novel therapeutic approaches in ATC patients (40). Recently, Targeted cancer therapies medications can inhibit the growth and progression of the cancer cells by direct inhibition of specific pivotal targets (16, 41). Target therapies effectively inhibit cell proliferation and invasion in comparison to conventional chemotherapies (13). It can be used alone or in combination with other chemotherapies to increase the effectiveness of cancer treatments (13). Recently, investigations have been showed that CDK inhibitors have potential therapeutic effects for various cancers, including, hepatocellular carcinoma, liposarcoma, melanoma, breast cancer, lung adenocarcinoma, glioma and renal cancer (23, 42). The industrial production of CDK4, CDK6 inhibitors have been synthesized explicitly with the aim of anticancer medications (43). Our investigation revealed Abemaciclib reduced cell proliferation and growth of the ATC cells effectively. Furthermore, we showed that Abemaciclib inhibited the non-adherent ATC cells which were resistant to Anoikis death. Moreover, we demonstrated this targeted therapy significantly reduced anti-apoptotic gene expression levels (*BCL2* and *CMYC*) and increased apoptotic gene expressions such as *P21* and *BAX*. Generally, the Abemaciclib is administrated as a targeted cancer drug with an anti-cancer effect which exerts its function through induction of the cell cycle arrest in the G1 and apoptosis in cancer cells (29). In fact, this drug can inhibit the growth and proliferation of cancer cells through inhibiting cell cycle components such as CDK4 and CKD6 (44). Currently, Abemaciclib has been introduced into clinical trials due to its high specificity and appropriate function (43). Multiple studies have been conducted on this inhibitor and different impacts have been observed and reported (45-47). In a study, the anti-tumor activity of Abemaciclib has been investigated among Japanese cancerous patients (lung, breast, colon, intestinal, glioblastoma and melanoma cancer). the patients continuously used 150-200 mg of the Abemaciclib orally every 12 hours for 28 days. The results showed that Abemaciclib has a safety profile and anti-tumor effects in a maximum dose of 200mg when taken orally every 12 hours (48). In another survey, the effect of Abemaciclib along and in combination with Fulvestrant in patients with Hormone receptor-positive breast cancer was investigated, which showed all the patients had tumor reduction and increasing survival rate (29, 49). Also, Abemaciclib had a significant impact on KRAS positive mutant non-small cell lung cancer (NSCLC) (50). The best of our knowledge, this is the first investigation of Abemaciclib on thyroid cancer. Altogether, our findings demonstrated that Abemaciclib could effectively reduce cell proliferation of SW1736 and C643 cells lines. Also, we showed that Abemaciclib reduced anti-apoptotic gene expression mRNA levels whereas increased apoptotic gene expression mRNA levels via inhibiting cell cycle components such as CDK4 and CKD6.

Our data suggested that Abemaciclib can be utilized as a therapeutic agent in ATC cancer.

Further in vivo and in vitro investigations are needed to evaluate molecular and clinical mechanisms of Abemaciclib.

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The authors declare that there is no conflict of interest.

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