



The effects of Abemaciclib on cell cycle and apoptosis regulation in anaplastic thyroid cancer cells

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Abstract

Background: Anaplastic thyroid cancer (ATC) is an aggressive subtype of thyroid cancer, accounting for 1 to 2% of all cases. Deregulations of cell cycle regulatory genes including cyclins, cyclin-dependent kinases (CDKs), and endogenous inhibitors of CDKs (CKIs) are hallmarks of cancer cells and hence, studies indicate the inhibition of CDK4/6 kinases and cell cycle progression as potent therapeutic strategies. In this study, we investigated the anti-tumor activity of Abemaciclib, a CDK4 and CDK6 inhibitor, in ATC cell lines.

Methods and results: The ATC cell lines C643 and SW1736 were selected to study the antiproliferative effects of Abemaciclib using a cell proliferation assay and crystal violet staining assay. Annexin V/PI staining and cell cycle analysis by flow cytometry were also performed to examine the effects on apoptosis induction and cell cycle arrest. Wound healing assay and zymography analysis examined the effects of the drug on invasive abilities of ATC cells and Western blot analyses were applied to further study the anti-tumor mechanism of Abemaciclib, in addition to combination treatment with alpelisib. Our data demonstrated that Abemaciclib significantly inhibited cell proliferation and increased cellular apoptosis and cell cycle arrest in ATC cell lines, while considerably reducing cell migration and colony formation. The mechanism seemed to involve the PI3K pathway.

Conclusion: Our preclinical data highlight CDK4/6 as interesting therapeutic targets in ATC and suggest CDK4/6-blockade therapies as promising strategies in this malignancy.

Keywords Anaplastic thyroid cancer (ATC) · Chemoresistance · CDK4/6 · Abemaciclib

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Introduction

Thyroid cancer (TC) is the most common endocrine malignancy with an increasing incidence rate, expected to become the fourth most common cancer by 2030 [1, 2]. Anaplastic thyroid cancer (ATC) is an aggressive subtype of this disease accounting for 1 to 2% of all TCs. However, it is associated with a fatal outcome, contributing to 14–50% of TC related-deaths [3]. Most patients with ATC are clinically presented with a rapidly-enlarging neck mass often with symptoms of hoarseness, respiratory obstruction, and dysphagia [4]. Moreover, 40% of the patients exhibit a highly invasive feature with lymph node metastases while distant metastases to the lung, bones, brain and other parts of the body are observed in the remaining cases.

Thyroidectomy is a surgical treatment aimed to remove the primary tumor and stop the spread of the tumor into adjacent organs. However, in most cases, tumor dissemination renders the surgery ineffective [5]. Other treatment modalities include radioiodine therapy, radiotherapy, chemotherapy and a combination of these regimens [3], but side effects of chemotherapy and therapy resistance are the major therapeutic hurdles in the patients [5, 6]. Therefore, despite the development of new therapeutic modalities, the median survival for ATC is about 6 months [7, 8], indicating the urgent need to identify more effective therapeutic approaches for this lethal malignancy.

Although the mechanisms underlying the formation of ATC have not been comprehensively clarified, current evidence indicates that aberrations in genes involved in cell proliferation may play key roles [9, 10]. Deregulation of cell cycle regulatory genes including cyclins, cyclin-dependent kinases (CDKs) and endogenous inhibitors of CDKs (CKIs) is a hallmark of cancer cells [11]. CDKs are serine/threonine kinases that play indispensable roles in cell proliferation [12]; mitogenic signals induce the binding of D-type cyclins to CDK4 and 6 to initiate phosphorylation of the tumor suppressor retinoblastoma (Rb) protein. This event leads to the inhibition of the E2F family of transcription factors which are responsible for S-phase and mitotic progression [13]. The cyclin D-CDK4/6-p16-Rb pathway is altered in most human cancers, leading to abnormal cell proliferation and thereby tumor initiation and malignancy progression [13, 14]. Accordingly, many studies indicate that there is a significant overexpression of cyclin D1 in ATC compared to other thyroid carcinomas which suggests a possible role of Cyclin D1 expression in ATC pathogenesis [15].

Abemaciclib (Verzenio®) is a small molecule inhibitor of CDK4 and CDK6 that is structurally different from other dual inhibitors, palbociclib and ribociclib, with greater selectivity for CDK4. Abemaciclib impedes phosphorylation of Rb protein and thus induces cell cycle arrest in

the G1 phase [16]. Anti-tumor activities of Abemaciclib, both as monotherapy and in combination therapies, have been reported in multiple neoplasms including melanoma, glioblastoma, non-small cell lung cancer and mantle cell lymphoma [17]. Notably, Abemaciclib has been approved in combination with an aromatase inhibitor as initial endocrine-based therapy for postmenopausal women with hormone receptor-positive (HR⁺), human epidermal growth factor receptor 2-negative (EGFR⁻) advanced or metastatic breast cancer [12, 18]. It should be noted that the activation of other signaling pathways in cancer cells may interfere with the therapeutic efficacy of CDK inhibitors as suggested by different studies [19]. Therefore, in this study, we investigated the anti-tumor activity of Abemaciclib in the ATC cell lines C643 and SW1736 and suggested a combination therapeutic approach to improve the outcome.

Materials and methods

Antibodies and chemicals

Antibodies were purchased as follows: FOXM1 (G-5, diluted 1:500), cyclin E1 (HE12, diluted 1:500), cyclin D1 (clone A-12, diluted 1:500), survivin (clone FL-142, diluted 1:500) and β -actin (clone C4, diluted 1:5000) (Santa Cruz Biotechnology). Abemaciclib and alpelisib were purchased from Adooq Bioscience and dissolved in DMSO; the final concentration of DMSO did not exceed 0.1% [v/v] in all the treatments. Cisplatin (a platinum-based DNA-damaging agent), carboplatin (a platinum-based DNA-damaging agent), paclitaxel (a taxane inhibitor of microtubule disassembly) and doxorubicin (an inhibitor of topoisomerase II) were acquired from the Pharmacy of Shariati Hospital (Tehran, Iran).

Cell culture

The human anaplastic thyroid cancer cell lines C643 and SW1736 were obtained from the National Cell Bank of Iran (NCBI, Tehran, Iran) and were authenticated by STR profiling using the Cell ID™ system (Promega). Cells were maintained at 37 °C and 5% CO₂ in a humidified incubator and cultured according to the provider's recommendations. Cells were regularly tested for mycoplasma contamination.

Cell viability test

The cells were seeded (2×10^3 cells/well) into 96-well plates. After 24 h, the cultures were treated with increasing concentrations of Abemaciclib for 48 h and the percentage

of viable cells was determined using an MTT assay. DMSO-treated cells were used as the control group.

Crystal violet staining assay

The cells were plated in 6-well plates (6×10^4 cells/well) and treated with the drugs for 48 h. The cultures were then washed with PBS, fixed with ice-cold methanol and stained with crystal violet (0.5% w/v). The images were acquired with an inverted microscope.

Apoptosis assay

Annexin V staining was carried out using an Annexin V-FITC apoptosis detection kit (Invitrogen) according to the manufacturer's protocol. In apoptotic cells, phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a phospholipid-binding protein that binds to PS and is detected using flow cytometry. The results were analyzed using a Partec PAS III flow cytometer (Partec) and Windows™ FloMax® software (Partec).

Scratch assay

Approximately 3×10^5 cells were seeded into 6-well plates. When the cells were confluent, a linear wound was made using a pipette tip. The cultures were washed with phosphate buffer saline (PBS) and then treated with Abemaciclib for 48 h. The migration area was scanned at different time intervals by an inverted microscope.

Zymography

A zymography assay was conducted to evaluate the activities of matrix metalloproteinase-2 (MMP-2) and MMP-9. Equal amounts of protein from the cell culture supernatants after 48 h treatment were electrophoresed by polyacrylamide gels plus gelatin A (for MMP-2) or gelatin B (for MMP-9). After electrophoresis, the gels were soaked in reactivation buffer for 12 h and then stained with Coomassie Brilliant Blue. The enzymatic activities were evaluated by the size of the clear band on the gel [20] and results were quantified using GelQuant software.

Western blot

Whole-cell lysates were prepared in ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors (Roche Molecular Biochemicals). The proteins were separated by SDS-PAGE

and transferred to PVDF membranes. The membranes were blocked in 5% skim milk for 1 h at room temperature and then probed with primary and secondary antibodies (Sigma). β -actin was used as the loading control and the proteins were detected using a BM chemiluminescence detection kit (Roche Molecular Biochemicals).

Statistical analysis

All data were evaluated in triplicate against the untreated control cells and collected from three independent experiments. Data were graphed and analyzed using GraphPad Prism Software 7.0 using one-way ANOVA. All data are presented as mean \pm standard deviation (SD). *P* values < 0.05 were considered statistically significant.

Results

ATC cell lines show chemoresistance to different drugs

Chemoresponsiveness of the ATC cell lines SW1736 and C643 to cisplatin, paclitaxel, carboplatin, and doxorubicin was measured using MTT assays and are presented in Fig. 1. The data demonstrated that the ATC cell lines were considerably resistant to cisplatin, carboplatin, and doxorubicin.

Abemaciclib suppresses cell proliferation and clonal growth

An MTT assay was conducted to evaluate the antiproliferative effects of Abemaciclib on the ATC cells (Fig. 2A) and the results showed decreased cell proliferation in a concentration-dependent manner in both cell lines. The effect of Abemaciclib on cell viability was further confirmed using crystal violet staining (Fig. 2B).

Abemaciclib induces cell cycle arrest and apoptosis

Due to the antiproliferative effects of Abemaciclib, we sought whether this anti-tumor agent impeded cell cycle progression. After treating C643 cells with concentrations of 0.5, 1, 2.5, 5, 10, and 20 μ M, the percentage of cells at the S phase increased; the two highest concentrations showed increased cell count at the sub-G1 phase as well. Similar results were observed in SW1736 cells, although this cell line was less responsive, especially to lower concentrations (Fig. 3A). Next, we investigated the role of Abemaciclib in inducing ATC cell apoptosis. During apoptosis, phosphatidylserine in the inner side of the plasma membrane translocates to the surface [21]; staining for phosphatidylserine-binding

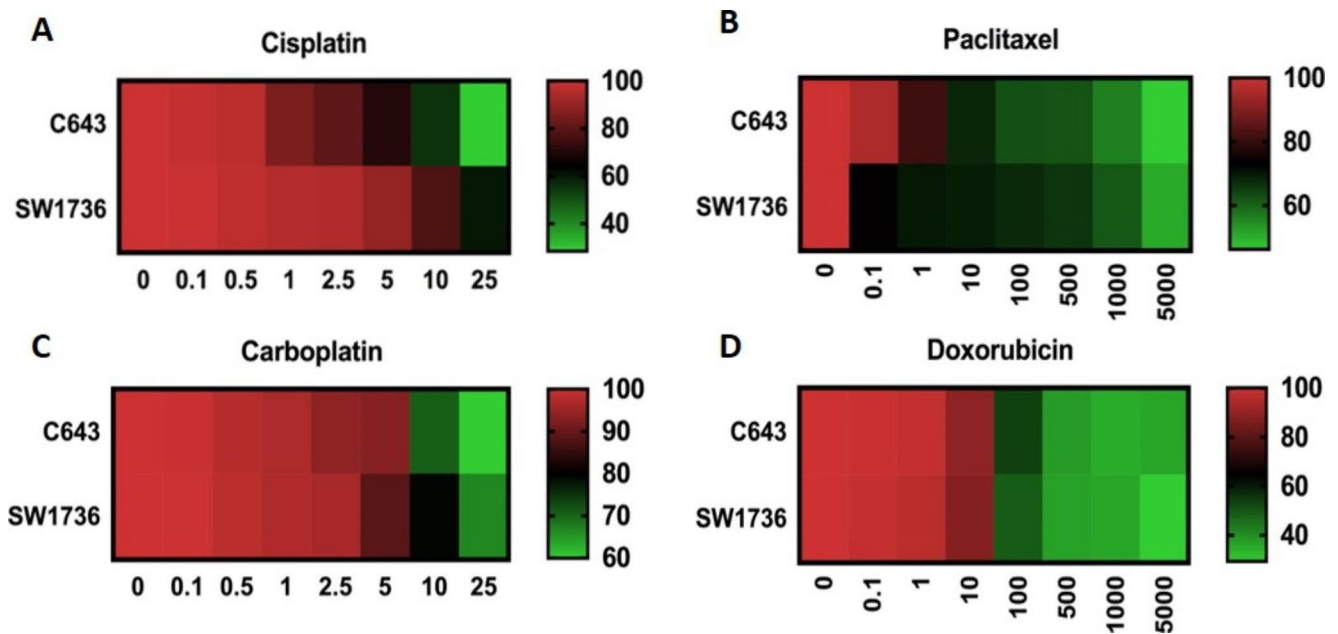
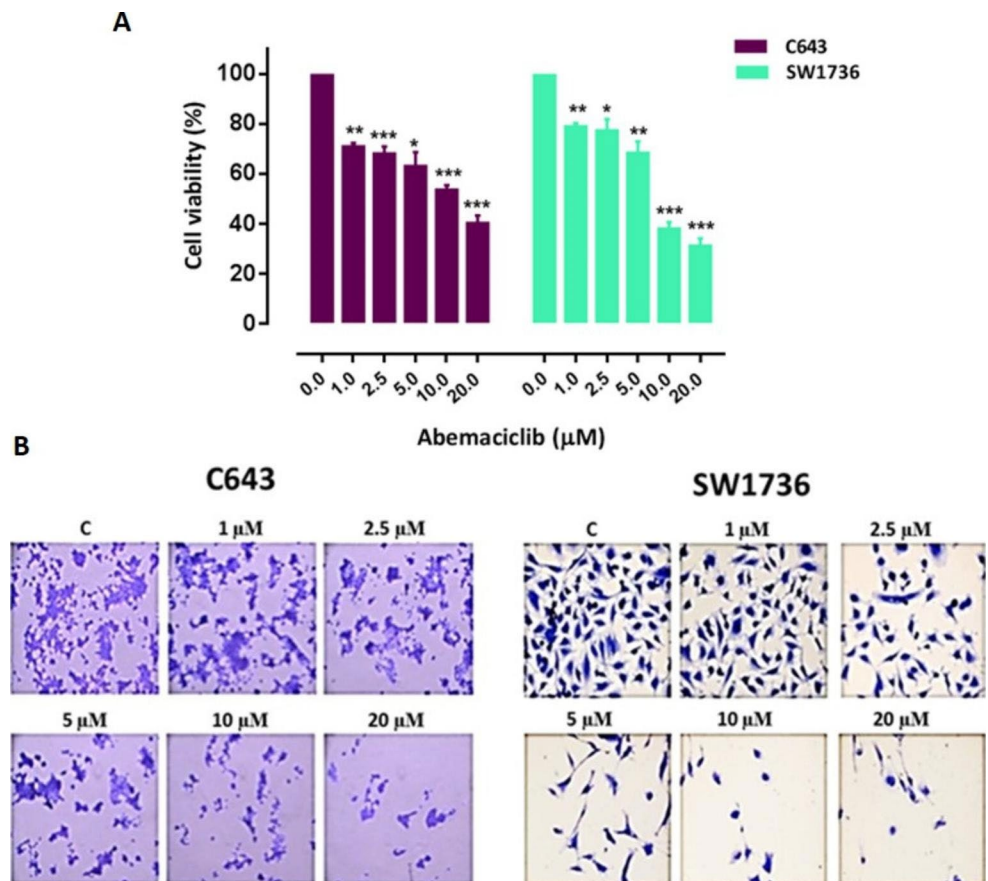


Fig. 1 The ATC cell lines were highly resistant to chemotherapeutic drugs. Chemoresponsiveness of the C643 and SW1736 cell lines to (A) cisplatin ($\mu\text{g/ml}$), (B) paclitaxel (ng/ml), (C) carboplatin ($\mu\text{g/ml}$), and

(D) doxorubicin (ng/ml) was assessed after 48 h treatment using MTT assays. The percentages of proliferation (vertical numbers) after treatment with various doses of drugs (horizontal numbers) are presented

Fig. 2 Abemaciclib suppresses cell proliferation. (A) The effect of Abemaciclib on the proliferation of SW1736 and C643 cell lines after 48 h of treatment was assessed using an MTT assay. (B) Staining of SW1736 and C643 cell lines by crystal violet after 48 h of Abemaciclib treatment further demonstrated reduced viability in the ATC cells in a concentration-dependent manner. The scale bar is 200 μm



protein annexin V and flowcytometry analysis indicated that Abemaciclib mostly exerted its cytotoxic effect by inducing

apoptosis in ATC cells. After treatment with a concentration

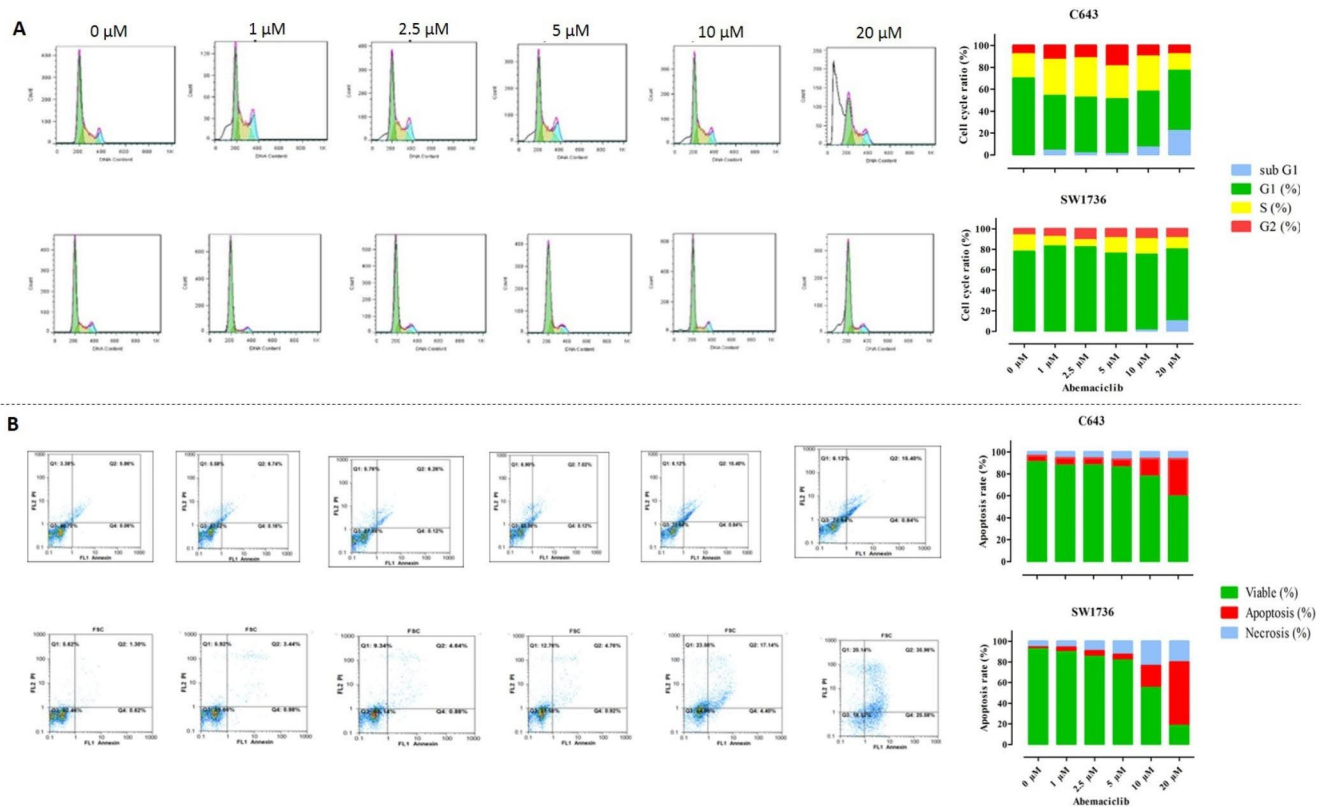


Fig. 3 Abemaciclib induces cell cycle arrest and apoptosis. **(A)** Following the treatment for 48 h, Abemaciclib induced cell cycle arrest in the C643 cell line and altered the percentage of cells at different phases. However, no significant effect was observed in SW1736 cells. **(B)** Apoptotic cell death was measured by annexin V staining

of 20 μM , 61% of SW1736 cells, as well as 34% of C643 cells, underwent apoptosis (Fig. 3B).

Abemaciclib attenuates cell motility

Migration and invasion of tumor cells lead to metastasis which is a hallmark of malignancy [22]. We investigated the migration ability of ATC cells upon treatment with concentrations 0.5, 1, 2.5, 5, 10, and 20 μM of Abemaciclib. Scratch assay showed that the mobility of SW1736 and C643 cells was attenuated after the treatment (Fig. 4A). Furthermore, tumor invasion and migration occur when the basement membranes and extracellular matrix (ECM) are dissolved by matrix metalloproteinases (MMPs) [23, 24]. MMPs are a group of zinc-dependent endopeptidases that work towards ECM turnover; MMP-2 and MMP-9 are two MMPs that may be distinguished by their extreme efficiency in the degradation of gelatin A and B, respectively. Previous studies have shown MMP-2 and MMP-9 are involved in metastasis and disease progression in ATC patients [25]. Accordingly, we carried out zymography assays and found that in the C643 cell line, the activity of MMP2 at most

after 48 h of treatment with different concentrations of Abemaciclib. Annexin V-positive cells are considered as early apoptotic, whereas PI uptake indicates necrosis. Cells positive for both dyes are considered as late apoptotic. Abemaciclib induced both early and late apoptosis in ATC cells

concentrations (0.5, 1, and 20 μM) and MMP9 at almost all concentrations was decreased. However, the SW1736 cells showed decreased activities of both MMPs at concentrations higher than 2.5 μM (Fig. 4B).

Abemaciclib modulates the cell cycle- and apoptosis-regulatory proteins

To figure out the mechanisms underlying Abemaciclib-induced cell death, we examined the effects of the drug on modulators of cell cycle progression and apoptosis induction. According to the results of Western blot, Abemaciclib down-regulated cell cycle-regulatory proteins (FOXM1, Cyclin E1 and Cyclin D1) as well as survivin that is involved in apoptosis regulation (Fig. 5). Concentrations 10 and 20 μM caused the most significant effect.

FOXM1 is a major transcription factor that acts downstream of the PI3K/Akt pathway. This protein is expressed in highly-proliferative tissues as well as tumor cells and its dysregulation is associated with many hallmarks of cancer progression [26]. Cyclin D1 forms a complex with CDK4 and CDK6 which helps the cell transition from the G1 to

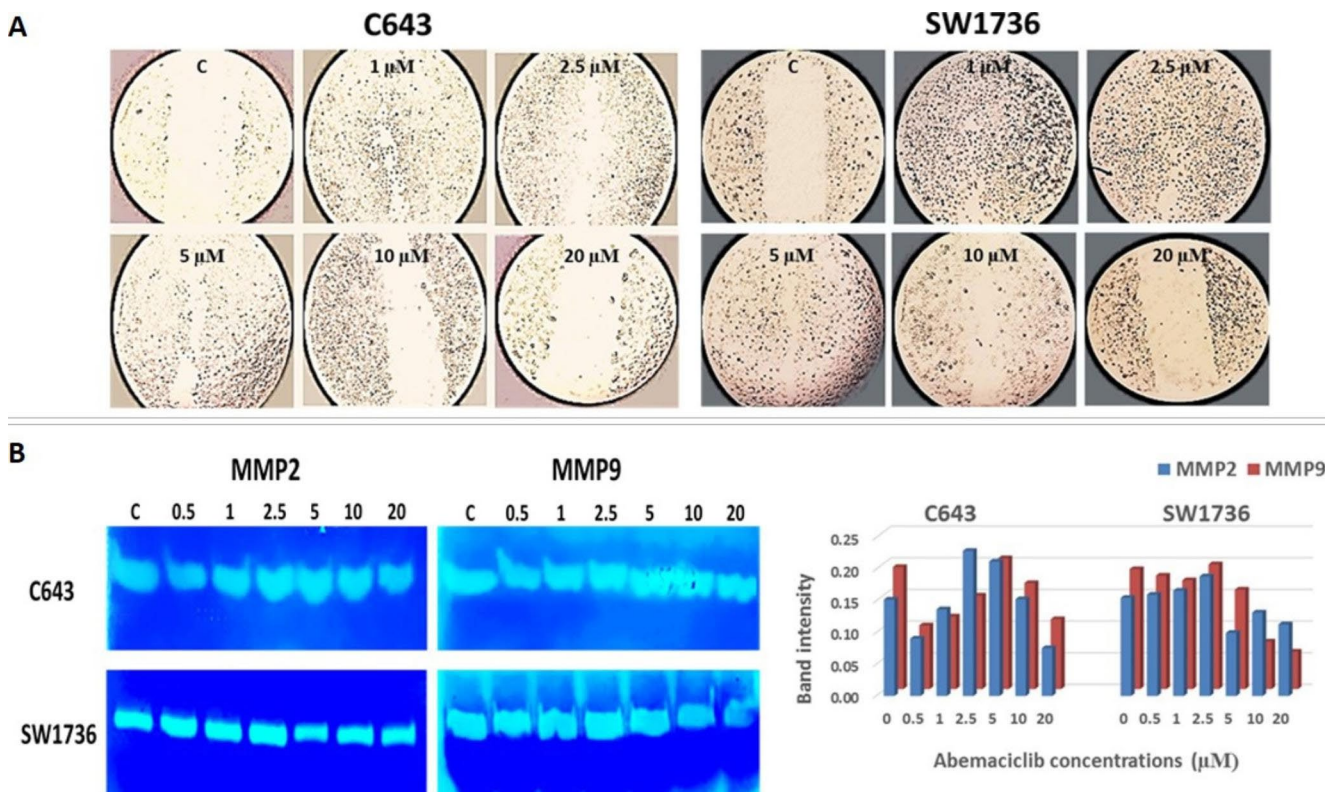


Fig. 4 Abemaciclib attenuates cell motility and invasion ability. **(A)** Abemaciclib inhibited migration of ATC cells as demonstrated by reduced width in scratch assays in a dose-dependent manner. **(B)** As

the results of the zymogram present, Abemaciclib inhibited the enzymatic levels of MMPs at different concentrations in both cell lines

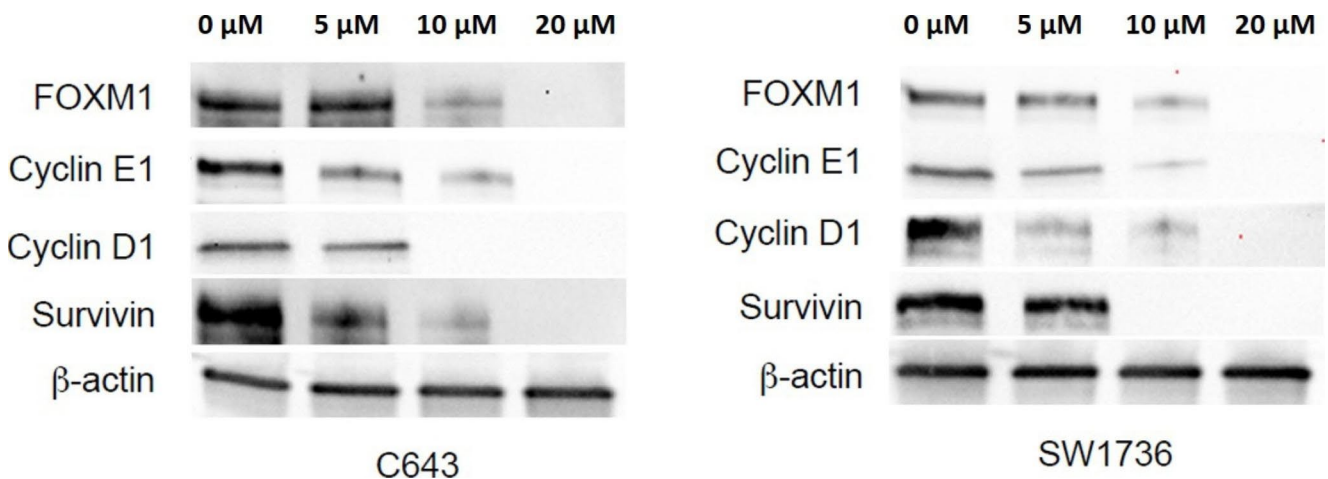


Fig. 5 Abemaciclib modulates cell cycle- and apoptosis-regulatory proteins. The Effects of the different concentrations of Abemaciclib on the expression of pro-apoptotic proteins were assessed by Western

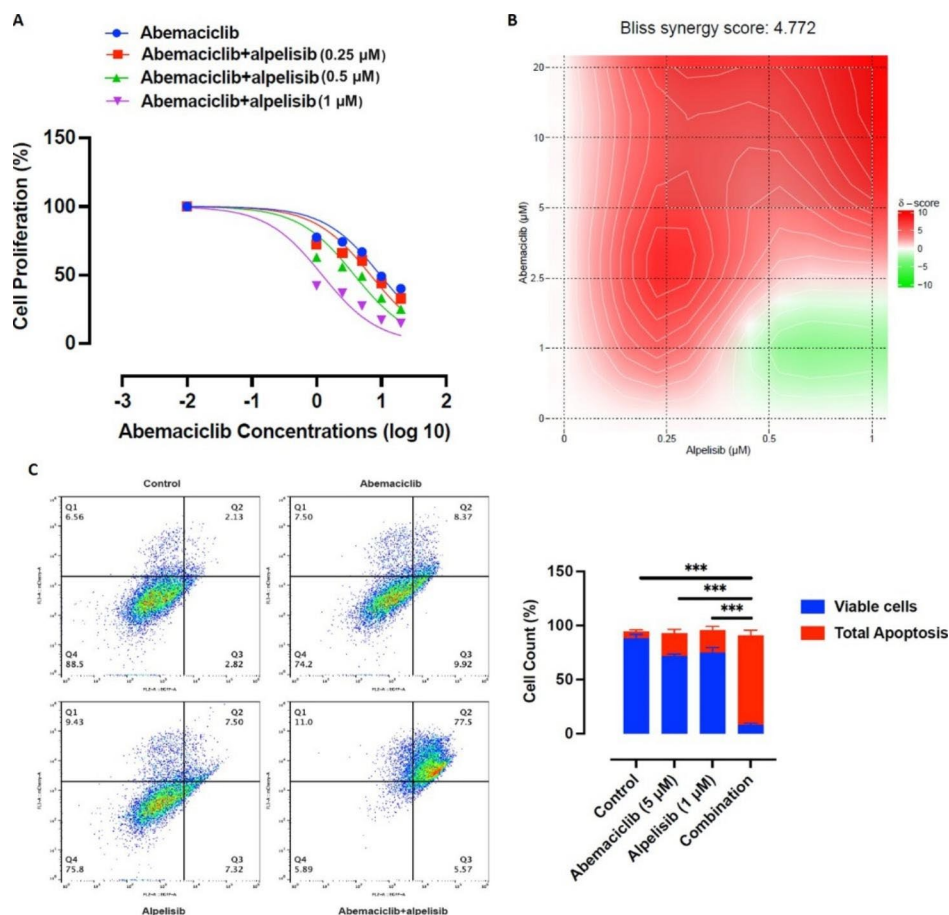
Blot analysis. As shown, FOXM1, Cyclin E1, and Cyclin D1 were significantly down-regulated after treatment in both cell lines to regulate cell cycle, as well as decreased levels of surviving that affect apoptosis

S phase of the cell cycle; cyclin E1 forms a similar complex with CDK2 to facilitate the same process. Both these proteins are known to be over-expressed in various tumor types [27]. Moreover, survivin is a member of the inhibitor of apoptosis (IAP) protein family that inhibits caspases and blocks cell death; survivin expression is significantly up-regulated in ATC cases [28].

PI3K inhibition enhances the efficiency of Abemaciclib

Considering the effect of Abemaciclib on FOXM1 inhibition and the fact that this protein is an effective transcription factor of the PI3K-AKT-FOXO signaling cascade [29], we

Fig. 6 PI3K inhibition enhances the outcome of treatment with Abemaciclib in the C643 cell line. **(A)** Based on MTT results, the combination of Abemaciclib and the PI3K-inhibitor alpelisib resulted in a significant shift in the EC_{50} values of the drugs. **(B)** Bliss SynergyFinder confirmed the synergistic effect of the drugs in most concentrations (marked in red in the 2D graph). **(C)** An annexin V/PI apoptosis assay showed that after the combination treatment of Abemaciclib (5 μ M) and alpelisib (1 μ M), the level of apoptosis significantly increased from about 18 to more than 83%



used alpelisib, an FDA-approved inhibitor of the PI3K/AKT pathway, in combination with Abemaciclib as a potentially effective method for improving the outcome of therapy. This molecule targets the p110 α subunit of PI3K and has been suggested for the treatment of advanced breast cancer [30]. The MTT results of various combination treatments of Abemaciclib (concentrations 1, 2.5, 5, 10, and 20 μ M) and Alpelisib (0.25, 0.5, and 1 μ M) indicated that the co-treatment of these drugs resulted in more effective inhibition of cell proliferation in the C643 cell line (Fig. 6A) and they have a synergistic effect (Fig. 6B).

To confirm the results, a combination treatment of Abemaciclib (5 μ M) and Alpelisib (1 μ M) showed that this approach significantly increased the amount of drug-induced apoptosis in the C643 cell line compared to treating with either drug individually (Fig. 6C).

Discussion

ATC is one of the most lethal subtypes of thyroid cancer with a dismal prognosis and considerable clinical challenges [5]. Despite current standard therapies, the treatment of ATC is a debated clinical issue and faces many barriers.

Current investigations have demonstrated that aberrant CDKs' activities have a major contribution to tumor progression and invasion [31] since activation of CDKs promotes proliferation and cell cycle progression [32]. For instance, overexpression of D-type cyclins, mutation or amplification of CDK4/6, loss of cyclin D-CDK4/6 negative regulators such as p16^{INK4A}, deletion of CDKN2A (which encodes p16^{INK4A}), and CCND1 (which encodes cyclin D1) amplification are considered as common events in multiple human cancers [33].

A large body of evidence has indicated the inhibition of CDKs as a novel potential therapeutic approach in cancer [34, 35]. Recently, the three highly selective oral CDK4/6 inhibitors palbociclib (PD0332991), ribociclib (LEE011), and Abemaciclib (LY2835219) were shown to inhibit Rb phosphorylation in breast cancer and colon carcinoma cells [36, 37]. Abemaciclib (VerzenioTM) is an effective, highly selective, and orally administrated small-molecule inhibitor of CDK4/6 which is largely used as a monotherapy in endocrine refractory disease [38, 39]. In 2016, for the first time, Patnaik et al. demonstrated the efficacy and safety profile of a single-agent therapy of Abemaciclib for patients with breast cancer, non-small cell lung cancer, and other solid tumors [17] and since then, several studies have considered

the effects of Abemaciclib on advanced breast cancer patients. In phase II of the MONARCH 1 clinical trial study on the refractory hormone receptor-positive (HR⁺), human epidermal growth factor receptor 2-negative (HER2⁻) metastatic breast cancer patients, it was shown that Abemaciclib was well-tolerated and demonstrated desirable clinical outcome (median progression-free survival rate: 6.0 months, and median overall survival rate: 17.7 months) [40]. In the MONARCH 2 randomized clinical trial study, Abemaciclib plus fulvestrant promoted the overall survival rate to 9.4 months for patients with HR⁺/HER2⁻ advanced breast cancer [41]. Moreover, in the recent study at the MONARCH 3 randomized clinical trial it was shown that Abemaciclib plus a nonsteroidal aromatase inhibitor increased progression-free survival and objective response rate (ORR), along with promising safety in HR⁺/ERBB2⁻ advanced breast cancer cases [42].

Regarding the mechanism of action, preclinical investigations showed that Abemaciclib therapy led to a depletion in phosphorylation of Rb, cell cycle arrest, and cell proliferation inhibition. Moreover, Abemaciclib also decreased TopoII α expression and DNA synthesis by blocking CDK4/6 in the estrogen receptor-positive (ER⁺) breast cancer cells [43]. In our previous study, we showed that Abemaciclib significantly decreased cell proliferation, inhibited non-adherent cells, and induced apoptotic gene expression in ATC cell lines [44]. In consistence, the current study showed that Abemaciclib inhibited cell cycle progression and induced apoptosis in ATC cells by down-regulating cyclin D1, cyclin E1 and FOXM1. Similarly, a study by Bellelli showed that FOXM1 is highly expressed in ATC and that its inhibition using RNA interference methods could significantly reduce cell invasion; drugs that target this protein also reduced tumor burden in a mouse model of the disease [45]. Moreover, the correlation between FoxM1 expression and MMP-9 in papillary thyroid carcinoma (PTC) and their association with cancer progression has been suggested [46] which could be in accordance with the results of Western blot and zymography in our study. Besides Abemaciclib, a variety of other CDK4/6 inhibitors have been broadly surveyed in ATC. For example, it has been demonstrated that palbociclib (a CDK4/6 inhibitor) inhibited ATC cell growth; however, it conveyed resistance in the cells promptly by increasing the levels of cyclin D1 and D3. Moreover, palbociclib plus omipalisib (a PI3K/mTOR inhibitor) synergistically inhibited cell proliferation and growth of ATC cells [47].

While various studies have focused on identifying molecular pathways involved in ATC progression and different signaling molecules have been investigated (e.g. MAPK and RAF/ERK), the PI3K pathway has been constantly suggested as one of the major signaling pathways

involved in malignancies of the thyroid. Considering its role in the self-renewal of cancer cells and the expression of sodium/iodide symporter (NIS), which is essential for normal thyroid function, the PI3K/Akt/mTOR signaling has become a well-known target for ATC cancer therapies [48]. Gillis et al., for instance, introduced PI3K/Akt/mTOR as one of the drivers of tumorigenesis in thyroid and showed that using inhibitors of this pathway such as buparlisib or alpelisib could significantly improve therapeutic outcomes in ATC cell lines [49]. The involvement of this pathway in papillary thyroid carcinoma, another form of malignancy in the thyroid, has also been investigated [50]. Based on the noticeable number of studies that suggested the effect of the PI3K pathway in suppressing carcinogenesis in the thyroid and its positive effect on CDK-targeting drugs [51], we used a well-known PI3K inhibitor, alpelisib, which showed a significant synergistic effect with Abemaciclib. In consistence with the aforementioned studies, our therapeutic approach resulted in enhanced apoptosis induction and demonstrated promising anti-tumor effects of combined CDK4/6 and PI3K inhibition.

Conclusions

Taken together, our preclinical data highlights CDK4/6 as attractive therapeutic targets in ATC and suggests that Abemaciclib, as a CDK4/6 blocking agent, is a promising strategy against ATC. There is an urgent need to confirm the findings of this research in animal models and clinical samples in order to use the results to help patients affected by this disease.

Author contribution Elaheh S. Abutorabi: Data curation, Methodology, Writing- Original Draft; Arash Poursheikhani: Writing- Original Draft; Bahareh Kashani: Data analysis, Writing- Review & Editing; Sahar Shamsaieghakani: Methodology; Vahid Haghpanah: Investigation; Davood Bashash: Writing- Review & Editing; Seied A. Mousavi: Funding acquisition; Majid Momeny: Conceptualization, Data curation, Supervision; Seyed H. Gaffari: Project administration, Funding acquisition, Supervision and Responsibility for the final content. All authors have reviewed and approved the final manuscript.

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Availability of data and materials The authors declare that the datasets on which the conclusions of this manuscript rely are deposited in publicly available repositories.

Statements & declarations

Declaration of competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval and consent to participate Not applicable.

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