Dependent Kinase 4 (CDK4) pathways are vital regulators of tumor cell proliferation and survival. The present study was accordingly designed to use dual inhibition of such pathways to kill thyroid cancer cells.

Methods and materials: The effects of each inhibitor on human ATC and BCPAP cell lines were evaluated by MTT assay. The right concentrations of inhibitors were determined and synergistic effects of such inhibitors were evaluated by bax/bcl-2 mRNA ratio, Caspase-3, and Caspase-9 activity assay as well as Akt, mTOR, and CDK4. Our finding showed that both ATC and BCPAP cell proliferation is significantly inhibited by PD-332991 (PD) and NVP-BEZ235 (NVP) in a time and concentration-dependent manner (P<0.05), however, the BCPAP cells were more sensitive than ATC cells. Our data also revealed that NVP treatment significantly decreased the phosphorylation state of both AKT and mTOR. Nonetheless, PD treatment alone increased activation of both AKT and mTOR whereas combination treatment of NVP and PD1 significantly decreased AKT and mTOR phosphorylation (P<0.001). NVP and PD treatment either alone or in a combination also decreased Retinoblastoma phosphorylation. Combination of NVP and PD similarly decreased Cyclin D1 expression. In addition, our data of Caspase 8 and Caspase 3 as well as Bax/Bcl-2 ratio indicated that the combination of NVP and PD induced cell apoptosis significantly (P<0.05).

Conclusion: The present study suggests that inhibition of PI3K and CDK4 is the effective treatment for both resistant and sensitivethyroid cancer cell line.

Key words: Thyroid cancer, Carcinoma, Apoptosis, NVP-BEZ235, PD-332991
Introduction

Thyroid cancer arising from follicular and parafollicular cells is one of the most rapidly growing endocrine cancers all over the world (Nix et al., 2005; Zarkesh et al., 2018). It has been predicted that the incidence rate of thyroid cancer will rise by the year 2030 to make it the fourth leading cancer prevalence (Rahib et al., 2014). Histological study of this cancer indicated that it can be classified into four major types, including papillary carcinoma follicular carcinoma, medullary carcinoma and undifferentiated carcinoma (Hedinger et al., 1989). Despite numerous therapeutic advances, it seems to be an incurable cancer. To date, surgery followed by the administration of systemic chemotherapy agents remains an effective treatment for the advanced stage of thyroid cancer (Mazzaferri and Kloos, 2001). Unfortunately, not only is the initial response to chemotherapy is satisfactory, but the tumors become resistant to chemotherapeutic agents (Wartofsky and Van Nostrand, 2016). Nevertheless, advances in molecular biology have provided a possibility to develop a novel effective therapeutic strategy for thyroid cancer.

Phosphoinositide 3-kinase (PI3K) is a class of kinases that play a key role in the cell growth regulation, proliferation, and differentiation. Based on sequence homology and substrate specificity, the kinase family of enzyme is divided into three classes as follows: class I, which is activated downstream of cell surface receptors, Class IA consisting of the catalytic subunits p110α, p110β, and p110δ and class IB, which operates downstream of GPCRs (Brown and Toker, 2015). A large body of experiments have shown that the PI3K pathway is greatly activated in thyroid tumors by several different mechanisms including somatic activating mutation, amplification of genes encoding key components, and upstream receptor tyrosine kinase overexpression (Nozhat et al., 2018). The targeting PI3K pathway using pharmacological small molecule inhibitors has become a potential therapeutic strategy (Wu et al., 2015). NVP-BEZ235 (NVP) is a small molecule PI3K inhibitor which inhibits ATM and Rad3-related (ATR) as well. Emerging evidence has indicated that glioblastoma treatment is likely to be significantly improved through the combination of ionizing radiation (del Alcazar et al., 2014). Unlike other specific PI3K inhibitors, this is currently used in phase I/II clinical trials as an mTOR inhibitor in animal study (Peyton et al., 2011). Cyclin-dependent kinases (CDKs) are a family of protein kinases characterized by needing to a separate subunit called Cyclin, which plays a vital role in the cell cycle. CDKs are divided into three cell-cycle-related subclasses (Cdk1, Cdk4, and Cdk5) and five transcriptional subfamilies (Cdk7, Cdk8, Cdk9, Cdk11, and Cdk20). A growing number of studies in the present field provide evidence to support the hypothesis that deregulation of these proteins can lead to several malignancies. For instance, Pojo and associates have shown that CDK4 is up-regulated in ATC tumor cells. Further, they have indicated that CDK4 inhibitors such as Palbociclib (PD-0332991) act as an effective reagents to ATC treatment (Pojo et al., 2017).

Emerging data contributed to identifying a strategy to approach anti-proliferative ability against tumor cells. For instance, combined inhibition of PI3K and mammalian target of rapamycin complex (mTORC) may enhance the clinical activity to reduce tumor cell resistance (Elkabets et al., 2013). Moreover, co-treatment of PI3K and mTOR inhibitors have made their way into clinical trials (Markman et al., 2012).

Our present study was designed to identify additional strategies that may increase the efficacy of treatment against thyroid cancer cell lines proliferation. The current study also tried to investigate the molecular mechanism underlying synergistic activity of PI3K and CDK4.
inhibitors by the evaluation of downstream pathways to support the hypothesis for the treatment of thyroid cancer.

Experimental

Cells and materials

Human ATC and BCPAP as the resistant and sensitive thyroid cancer cells were provided from Pasteur Institute of Iran (Tehran, Iran). The Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin were provided from Gibco (Life technology, USA), while phosphate buffered saline (PBS), the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, penicillin-streptomycin, and ethidium bromide, NVP, and PD were purchased from Sigma Aldrich (MO, USA). Caspase activity assay kits and antibodies were also obtained from (Cell Signaling Technology, Inc., Danvers, MA, USA). The anti-Akt, pAKT, mTor, CDK4 and GAPDH respective secondary antibodies were purchased from Santa Crus (CA, USA). The chemiluminescent detection system was purchased from TaKaRa (Tokyo, Japan) and other chemicals used in this study were obtained from Merck (Darmstadt, Germany).

Cell culture

The BCPAP and ATC cells as a sensitive and resistant thyroid cancer cells were used for this study. Following previous research (Gheysarzadeh and Yazdanparast, 2012), BCPAP were cultured in RPMI 1640 (w L-glutamate) completed by fetal bovine serum (10%), penicillin/streptomycin (2%) and amphotericine B (1%), and in line with our previous study (Nozhat et al., 2018), the ATC cells were cultured in Dulbecco’s Modified Eagle Medium, completed by fetal bovine serum (10%), penicillin/streptomycin (2%), amphotericine B (1%).

Cell viability assay

The cells (5×10⁴ cells per well) were cultured into a 96-well plate and incubated in a CO₂ incubator at 37 °C for 24 h as described previously (Gheysarzadeh and Yazdanparast, 2012). The indicated concentrations of NVP and PD were added to each well and then after indicating the time, 10 μl MTT (5 mg/ml) was added to each sample and incubated again for 4 h at 37°C in dark. Finally, the production of Formazan by the viable cells was measured at 570 nm using a multiwell plate reader (BioTek, Winooski, VT, USA).

Caspase-3 and 9 activity assay

The treated and untreated cells (5×10⁵) were homogenized on ice followed by lysing with cell lysis buffer and incubated on ice for 1 h. The homogenates were then centrifuged at 14,000g for 15 min at 4°C. The pellets were discarded and obtained supernatant was then subjected to Caspase-3 and 9 activity evaluation according to the manufacturer’s instruction (Cell Signaling Technology, Inc., Danvers, MA, USA). Protein content was assessed by the BCA protein assay method (Hamzeloo-Moghadam et al., 2015). Results of at least three independent experiments were first normalized based on protein content using the BCA protein assay method and their values were expressed as fold change in compared with the untreated cells.
Analysis of gene expression by real-time quantitative PCR

The cells were cultured in 6-well plates in a density of $5 \times 10^5$ and after 24 h they were treated with indicated concentration of NVP and/or PD in the complete growth medium. Total RNA was then extracted from cells using TRIzol according to the manufacturer's instruction. Total RNA was then reverse transcribed to cDNA using TaKaRa transcription kit, followed by Quantitative real-time RT-PCR analysis as described previously (Ahmed, 2005). In brief, the reaction mixture (10 μl) containing 2 μl of cDNA template, 1.5 μl each of forward and reverse primers and SYBR Green RT-PCR master mix amplified based on SYBR Green method. Each cycle of amplification was as follows: denaturation at 95°C for 10 min and 35 cycles at 95°C for 30 s, and 60°C for 20 s. Primers used in the present study were as follows: \textit{bcl-2} forward, 5' CGA CTT CGC CGA GAT GTC CAG CCA G3'; \textit{bcl-2} reverse 5' ACT TGT GGC CCA GAT AGG CAC CCA G3'; \textit{bax} forward, 5' AGG GTT TCA TCC AGG ATC GAG CAG3'; \textit{bax} reverse, 5' ATC TTC TTC CAG ATG GTG AGC GAG3'; \textit{GAPDH} forward, CAG CCT CAA GAT CAT CAG C; \textit{GAPDH} reverse, GGC AGT GAT GGC ATG GAC T (Maleki et al., 2019). The melting curve was generated at the end of each examination. The gene expression was relatively quantified by $2^{-\Delta\Delta CT}$ method and finally values were reported as mean ± SD (Gheysarzadeh et al., 2018).

Western blotting

The cells were lysed using buffer containing 20mM TrisHCl (Ph7.5), 0.5% NonidetP-40, 0.5mM PMSF, 100mM b-glycerol 3-phosphate, and 0.5% protease inhibitor cocktail followed by centrifugation at 14000 g for 10 minutes. The resulting supernatant was collected, aliquoted and stored in -70 °C for western blotting analysis as indicated previously(Gheysarzadeh et al., 2019; Gheysarzadeh et al., 2019). An equal concentration of each sample was boiled for 10 min and loaded into a 10% SDS-PAGE gel followed by transferring into a PVDF membrane. Non-specific sites of the membrane were blocked with 3% bovine serum albumin in Tris-buffered saline for 2 h at room temperature and incubated with indicated primary antibodies. The samples were exposed to the secondary antibodies for 2 h and then the bound secondary antibodies were evaluated with the ECL western blotting detection system. The membrane was exposed to the X-ray film and all blots were normalized with GAPDH as a loading control using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analyses were performed using version 19 of SPSS software (Chicago, IL, USA). All examinations were set up in triplicates and the results were presented as the mean ± standard deviation (SD). The Caspase activity assay and cell viability values obtained per each treatment were converted to the percentage of control cells. The effect of each treatment sample was compared with treated and untreated cells by student t-test and One-Way ANOVA. The $P < 0.05$ was considered as statistically significant.

Results and discussion

Synergistic inhibition of cell growth by a combination of NVP and PD

In order to determine the effect of NVP and PD on cell viability, the exponentially growing Thyroid cancer cells were exposed to different concentrations of NVP and PD. Our results showed that the ATC cell proliferation is inhibited by 0–38% after 24 h exposure to 0.025–10
μM of PD, 0-52% for 48 h and 1-60% for 72 h and 2-69% for 96h. Our data also indicated that the ATC cell proliferation is inhibited by 0-28% after 24 h exposure to 1-400 μM of NVP, 0-51% for 48 h and 1-56% for 72 h and 3-68% for 96 h. The similar pattern of cell viability was seen for BCPAP cells. However, the BCPAP cells were more sensitive than ATC cells. For instance, 24 h incubation of BCAP cells to 5-2000 nm of PD leads to cell viability inhibition to 1-57% (Figure 1). In order to further analyze in a combination of these two compounds, the IC20 were used as indicated by a previous study (Ashton, 2015).

**Figure 1.** Growth inhibitory effects of NVP-BEZ235 of PD-332991 on human ATC cell line

The cells were cultured in 96-well plates and treated with the indicated concentrations of NVP-BEZ235 of PD-332991. Following 12, 24 and 36 h of incubation, cell viability was determined using an MTT assay. The data presented as the means ± SD of three independent experiments. Statistical significance: *P<0.05, **P<0.01, ***P<0.001, t-test.

**Effects of NVP and PD on signaling pathways**

To gain a molecular understanding on NVP and PD synergistic effects, we examined the effects of these two inhibitors and their combination on the expression and/or phosphorylation status (after 24h) of a number of relevant elements of the proliferation signaling pathway. PI3K/AKT pathway, as one of the most important signaling pathway, is generally accepted to mediate tumor cell growth and cell survival. The Akt and mTOR are also the key downstream elements of this pathway (Pópulo et al., 2012). According to Figure 2, NVP treatment, regardless of PD, catastrophically led to a decrease in the extent of phosphorylated both AKT and mTOR whereas no considerable alteration was observed in the expression of total AKT and mTOR (after 24h). Whereas PD treatment alone increased activation of both AKT and mTOR, combination treatment of NVP and PD significantly decreased AKT and mTOR phosphorylation (P<0.001). NVP and PD treatment either alone or in a combination also decreased Retinoblastoma phosphorylation (P<0.05). NVP in a combination of PD also decreased Cyclin D1 expression (P<0.05).
Cells were treated with indicated concentration of NVP-BEZ235 or PD-332991 either alone or in combination with each other for 24 h (A). Western blotting bound were quantified based on un-phosphorylated protein (for Akt, mTOR, and RB) and GAPDH for other proteins. The data reported as the means ± SD of three independent experiments.

### Effects of NVP and PD on apoptosis

To further investigate the combination effect of NVP and PD on apoptosis induction, the Bax/Bcl-2 ratio and Caspase activity assay were performed for ATC cells in response to NVP and PD either alone or in combination. The results indicated that NVP and PD incubation of cells for 24 h significantly increased the Caspase-8 and Caspase-3 activity (P<0.05). However, the combination of such compounds catastrophically increased both Caspase-8 and Caspase-3 activity (P<0.001), and this increase was significantly more than each compound alone (P<0.05). Similarly, BCAP cells were exposed to NVP and PD in a lower concentration. Likewise, the results showed that NVP and PD exposure of cells for 24 h increased the Caspase-8 and Caspase-3 activity in a significant manner (P<0.05) while the combination of such inhibitors increased both Caspase-8 and Caspase-3 activity (P<0.001), and that the Caspase activities for the combination of these compounds were significantly higher than each compound alone (P<0.05).
In addition, the Bax/Bcl-2 ratio also further confirmed the combination effects of NVP and PD on apoptosis induction. As illustrated in Figure 3B, NVP and PD incubation of ATC cells for 24 h significantly increased the Bax and reduced Bcl-2 mRNA (P<0.05). The combination of such compounds synergistically increased Bax and decreased Bcl-2 (P<0.001). However, the rise of Bax/Bcl-2 ratio for a combination of NVP and PD was significantly more than each compound alone (P<0.05). BCPAP cells exhibited a similar pattern.

Further, our finding showed that the Bax/Bcl-2 ratio increased significantly in the presence of NVP and PD for 24 h (P<0.05). The combination of such agents increased Bax/Bcl-2 ratio synergistically (P<0.001). However, the Bax/Bcl-2 ratio for the combination of these compounds was significantly higher than that of each compound alone (P<0.05).

![Figure 3](image_url)

**Figure 3**: Effect of NVP-BEZ235 and PD-332991 alone and in combination with together, on apoptosis of ATC cells.

The cells were treated with indicated concentration of NVP-BEZ235 of PD-332991 either alone or in combination with each other for 24 h and then the Caspase activity (A) and Bax/Bcl-2 ratio were also evaluated (B). The presented values were reported as the means ± SD of three independent experiments. Statistical significance: *P<0.05, **P<0.01, ***P<0.001, t-test.

**Discussion**

Despite a decade of unremitting studies and clinical trials, such as surgical removal of tumor and chemotherapy, thyroid cancer treatment has not significantly improved. Such unfavorable outcomes are due in part to the invasive nature of tumor cell and their resistance to treatment (Tagliaferri et al., 2018). In addition, most of the alterations involved in thyroid...
cancer are due to large inactivation or down-regulation of tumor suppressor genes, and up-regulation or activation of oncogenic signaling cascades. These signaling alterations make the development of effective therapies very difficult (Brown et al., 2011). Anaplastic thyroid cancer (ATC) cells which have been thought to develop from papillary thyroid cancer is an extremely aggressive type of thyroid cancer with high mortality rate, exhibiting resistance to any chemotherapy or radiotherapy. Another papillary thyroid cancer cell in this study was BCPAP which was more sensitive to inhibitors (Lopes-Ventura et al., 2018).

The most frequent altered gene in thyroid cancers is PI3K signaling cascade (Vasko et al., 2004). This pathway seems to be activated by insulin and IGF-1 to induce cell growth (Ansari et al., 2017; Jafari et al., 2018). Still another signaling cascade such as epidermal growth factor is also involved in thyroid cancer (Maleki et al., 2018). The upstream element of this cascade is a complex of proteins present in cytoplasm catalyzing the phosphorylation of D3 position of phosphatidylinositol. Akt is a protein located in a downstream of PI3K. Phosphorylated Akt (p-Akt) is the activated form of Akt, which is able to enter cellular nuclei to phosphorylate a series of substrates and performs its biological functions (Hou et al., 2007). PI3K/Akt pathway is generally believed to act as a very important signaling pathway of cancer progression, which is closely related to cell proliferation, metabolism, and tumor growth. It also appears to activate other signaling pathways including Wnt-β-catenin, HIF1α, FOXO3, and NF-κB pathways. The activated form of PI3K is also capable of phosphorylating a series of downstream target proteins such as Bad, Caspase 9, p21, and mTOR which generally promote cell survival (Vivanco and Sawyers, 2002).

The most frequent altered signaling cascades in thyroid cancer are PI3K/Akt/mTOR and CDK4 which are responsible for the proliferation and cell cycle regulation, respectively. Other proliferative signaling pathways are Cyclin D1 and CDK4 which have been known to increase in aggressive thyroid cancer (Lee et al., 2018). It has therefore been suggested that dual inhibition of these pathways extremely inhibit cell growth.

Our results indicated that the CDK4/6 inhibitor reduced cell viability of both ATC and BCPAP cells of thyroid cancers (Figure 1). The similar pattern of growth inhibition was also shown for PI3K/Akt/mTOR inhibition conducting researcher to use in combination together. The effect of such inhibitors on signaling elements also indicated that PI3K inhibition led to a significant reduction on Akt and mTOR phosphorylation (Figure 2). As previously presented, CDK-4 inhibitor exerted its cytostatic effect by promoting the G0-G1 phase cell cycle arrest. Here we additionally presented that CDK-4 inhibition led to a slight reduction of Rb phosphorylation.

On the other hand, apoptosis is often accompanied by either PI3K or CDK4 inhibition. Our data also indicated that, as presented in Figure 3, the inhibition of such pathways is associated with apoptosis induction. A molecular mechanism by which Akt, mTOR, and RB can exert a proapoptotic function in a wide variety of cancer has been recently proposed. For instance, Rb has been shown to exert tumor suppression primarily through direct binding and suppression of the E2F transcription factor. In addition, PI3K inhibition resulted in inactivation of Akt, leading to a reduction of cell survival and promoting apoptosis. PI3K inhibition also eventually inactivates mTOR which is complicated to cell growth inhibition.

In conclusion, regardless of several limitations this study faced such as knocking down of genes of interest and apoptosis determination methods, it revealed that either inhibition of PI3K/Akt/mTOR or CDK4 reduces cell proliferation by inducing apoptosis. However, the combinations of these inhibitors reduce cell proliferation more effectively and induce apoptosis.

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