

PhTAD-Substituted Dihydropyrrole Compounds Regulate Apoptotic Cell Death in MCF-7 Cells

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Abstract

Breast cancer is the most common type of cancer amongst women. Apoptosis is known as a programmed cell death and this mechanism induces cancer cell death. Dihydropyrrole compounds contain a heterocyclic structure and these molecules have many biological effects including functioning as antioxidants and anticancer molecules.

In this regard, the aim of this research was to investigate how PhTAD-substituted dihydropyrrole compounds affect the expression of apoptotic cell death proteins in the MCF-7 cells. The levels of Bax, Bcl-2, and cleaved caspase-3 proteins in the MCF-7 cells were measured using the ELISA method.

The results revealed that CI, CII, CIII, CV, CVII, CVIII, CXI and CXII increased Bax, while CXIII and CXIV markedly decreased Bax. In addition, compounds CI, CII, CIII, CVII, CVIII, CXI and CXII upregulated Bcl2. Conversely, CIV, and CXIV downregulated Bcl2. Moreover, CIV and CXIV increased the Bax/Bcl2 ratio. However, CVIII and CXIII decreased Bax/Bcl2 ratio. In addition, CI, CIV, CIX and CXII treatment increased cleaved caspase-3 in MCF-7 cells compared to the negative control. These findings indicate that the PhTAD-substituted dihydropyrrole derivative molecules induced apoptotic proteins as a potential regulator of cancer cell death.

Key Words: Breast Cancer, PhTAD-dihydropyrrole, Apoptosis, Bcl2 family, Caspase 3

PhTAD-Süstitüe Dihidropirol Bileşikleri MCF-7 Hücrelerinde Apoptotik Hücre Ölümünü Düzenler

Öz

Meme kanseri, kadınlar arasında en sık görülen kanser türüdür. Apoptoz, programlanmış hücre ölümü olarak bilinir ve bu mekanizma kanser hücrelerinin ölümünü indükler. Dihidropirol bileşikleri heterosiklik bir yapı içerir ve bu moleküllerin antioksidan ve antikanser gibi birçok biyolojik etkisi vardır.

Bu noktada, Çalışmamızın amacı, PhTAD türevli dihidropirol bileşiklerinin MCF-7 hücrelerindeki apoptotik hücre ölüm proteinlerinin ifadesini nasıl etkilediğini araştırmaktır.

Bax, Bcl-2 ve kesilmiş kaspaz 3'ün protein miktarları, MCF-7 hücre hattında ELISA yöntemi ile ölçülmüştür. Sonuçlarımız, bileşik (B)I, BII, BIII, BV, BVII, BVIII, BXI ve BXII'nin Bax'ı artırdığını, BXIII ve BXIV'ün

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ise Bax'ı önemli ölçüde azalttığını ortaya koymuştur. Ek olarak, BI, BII, BIII, BVII, BVIII, BXI ve BXII'nin Bcl2'yi yukarı regüle ederken BIV, BXIII ve BXIV'ün Bcl2'yi aşağı regüle ettiği tespit edilmiştir. Ayrıca, BIV ve BXIV'ün Bax / Bcl2 oranını arttırdığı, BVIII ve BXIII'ün ise Bax / Bcl2 oranını düşürdüğü belirlenmiştir. Negatif kontrole kıyasla BI, BIV, BIX ve BXII uygulanan MCF-7 hücrelerinde kesilmiş kaspaz 3 ekspresyonunun arttığı tespit edilmiştir.

Bulgularımız, PhTAD ile türevlendirilmiş dihidropirol moleküllerinin, kanser hücresi ölümünün potansiyel bir düzenleyicisi olarak apoptotik proteinleri indüklediğine işaret etmektedir.

Anahtar Kelimeler: Meme Kanseri, PhTAD-dihidropirol, Apoptoz, Bcl2 ailesi, Kaspaz 3

1. Introduction

Cancer disease caused by tumors is one of the most significant health problems that human beings are exposed to due both to its high frequency and the risk of death (Ma and Yu, 2006). Breast cancer is the most prevalent type of cancer in women (Bray et al., 2018). One in eight women (or about 13 percent) has a risk of developing breast cancer in their lifetime (Howlader et al., 2017).

Apoptosis controlled cell elimination that plays a critical role in embryonic development and adult tissue homeostasis (Zhang et al., 2005; Opferman, 2008; Fuchs and Steller, 2011; Ashkenazi, 2015; AnvariFar et al., 2017). Reducing or eliminating the activation of apoptosis or different cell deaths plays a fundamental role in cancer development (Brown and Attardi, 2005; Jan and Chaudhry, 2019). Apoptosis plays an important and inherent physiological role in eliminating diseased and unnecessary or abnormal cells. Apoptosis can be initiated by a variety of stimuli, including developmental signals, cellular stress, and cell cycle disruption. The development of drugs that target cell death mechanisms in tumors and direct targeting is an important means of cancer treatment (Karna and Yang, 2009). Two separate but interconnected signaling pathways control apoptosis by activating the nuclear intracellular mechanism of death proteases called caspases. The intrinsic apoptotic pathway interacts with caspases through members of the Bcl2 protein family and mitochondria in response to severe cellular damage or stress. The release of cytochrome c and apoptosis-activating factors from the mitochondrial membrane is regulated by proteins of the Bcl2 family. The balance between Bax (Bcl-2-associated X protein) and Bcl2 (B-cell lymphoma 2), which plays a role in the initiation of apoptosis in the intrinsic apoptosis pathway, is critical (Azimian et al., 2018). The extrinsic pathway of apoptotic cell death activates caspases through cell surface death receptors that act on death ligands, especially, through T lymphocytes (Debatin, 2004; Elmore, 2007; Wang and Youle, 2009; Fulda, 2013; Nikolettou et al., 2013; Siddiqui et al., 2015; Kondratskyi et al., 2015; Ashkenazi, 2015; Chen et al., 2018; Jan and Chaudhry, 2019). Apoptosis is initiated in two ways: the death receptor (extrinsic) and the mitochondria-dependent (intrinsic) route. The extrinsic pathway begins with the activation of caspase-8 by ligands that bind to death receptors on the cell surface. The intrinsic pathway causes the cytochrome C released from mitochondria to form apoptosome in the cytoplasm, resulting in the activation of caspase-9 and caspase-3, respectively. Caspase-3 cuts the effector caspases, leading to cell death (Wu et al., 2011; Kominami et al., 2012; Brentnall et al., 2013; Green and Llambi, 2015; Wei et al., 2016; Galluzzi et al., 2018; Elena-Real et al., 2018; Belkacemi et al., 2018). Cancer was first demonstrated to be linked to accelerated and irregular cell proliferation leading to an increase and accumulation of cell mass; it was later found that some cancers are caused by physiological lack of cell death (also known as cellular suicide or apoptosis), rather than an increased rate of cell proliferation (Burz et al., 2009). Currently, the disorder of two well-defined apoptotic signaling pathways, extrinsic/death receptors and the

intrinsic/mitochondrial pathway, are considered clinical problems that must be overcome in cancer treatment (Debatin, 2004; Fulda, 2013; Belkacemi, 2018).

Dihydropyrroles are a structure commonly found in naturally occurring alkaloids and biologically active substances (Wang et al., 2018). Dihydropyrrole compounds are known for their anticancer activities and suppress cell growth in cancer cells (Tarnavsky et al., 2003). Dihydropyrroles have the capacity to be defined as anticancer agents in cancer cells by causing mitotic arrest, which has an inhibitory effect (Garbaccio, et al., 2006). Dihydropyrrole compounds have inhibitory potential as new therapeutic agents in cancer (Sebastian, 2017).

In this regard, the aim of this research was to investigate how PhTAD-substituted dihydropyrrole compounds affect the expression of apoptotic cell death proteins in MCF7 (human breast adenocarcinoma) cells. The first breast cancer subtype, seen in 70% of breast cancer patients, is estrogen receptor (ER) and/or progesterone receptor (PR) positive but human epidermal growth factor receptor 2 (HER2) negative. At present many cell lines are used in breast cancer studies. The MCF7 cell line is generally preferred cell since it is positive for ER and PR receptors and negative for HER2 receptor. Because this receptor profile is most frequently observed in patients, MCF 7 cells were chosen for this study. Our findings indicate that the effects of PhTAD-substituted dihydropyrrole derivative molecules induce activity of apoptotic proteins as a potential regulator of cancer cell death. These compounds could be used as a potential molecule for anticancer research.

2. Materials and methods

2.1. Structure of compounds

PhTAD-substituted dihydropyrrole compounds (C) were synthesized according to our previous studies (Gul et al., 2017; Ayar et al., 2021). Thus, a 1 mmol concentration of dihydropyrrole solution in dichloromethane was added to the 1.1 mmol concentration of PhTAD at 25°C. After completion reaction (4-8 h), the crude products were purified by column chromatography. The structures of these compounds are as shown in Figure 1.

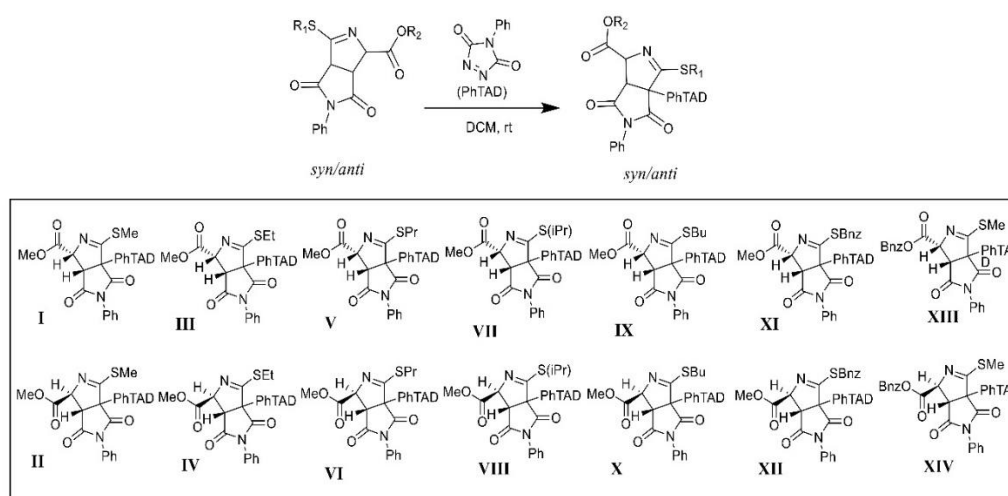


Figure 1. Structure of *syn* and *anti* PhTAD-substituted dihydropyrrole compounds.

2.2. Cell culture

In this study, the human breast adenocarcinoma cell line (MCF-7, ATCC® HTB22™, Manassas, VA, USA) was used for cell culture. MCF-7 cells were cultured in appropriate media containing 25 g/100 mL sodium bicarbonate (Sigma–Aldrich), 10 % fetal bovine serum (Sigma–Aldrich), RPMI-1640 (Sigma–Aldrich), penicillin/streptomycin (Sigma–Aldrich). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in the air. After, the cell populations had grown to 80% saturation, the cells were washed with phosphate-buffered saline (PBS) and detached from the flasks with 1X Trypsin-ethylenediaminetetraacetic acid (EDTA). Nearly 2×10⁶ cells were seeded per well of the 6-well culture plate in 3 mL of growth medium. Cells were incubated at 37 °C and 5% CO₂ and 90% humidity for 24 h until PhTAD-substituted dihydropyrrole compounds were applied.

2.3. Protein isolation and determination

PhTAD-substituted dihydropyrrole compounds were appropriately weighed, dissolved in Dimethyl sulfoxide (DMSO) and diluted in the culture medium. The final concentration of DMSO in the treated groups was below 0.1% and was not cytotoxic. According to the IC₅₀ results obtained in our previous studies appropriate doses were applied on MCF-7, as shown in Table 1 (Ayar et al., 2021).

Table 1. Applied IC₅₀ doses of PhTAD substituted dihydropyrrole compounds.

Compounds	Dose (μM)
I	25
II	25
III	25
IV	25
V	50
VI	12.5
VII	12.5
VIII	12.5
IX	12.5
X	12.5
XI	6.25
XII	12.5
XIII	25
XIV	25
Negative Control	-

These compounds were applied to the 2×10⁶ cells at the specified doses. After this the cells were incubated at 37 °C for 24 hours. Then the cells were removed with 1X trypsin-EDTA and centrifuged at 12500 rpm. The supernatant was discarded and 1000 μL of cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Cat. No. 89900) was added to the pellet and incubated on ice for 30 min. After incubation, centrifugation was performed for 10 minutes at 12500 rpm and the supernatant was added into a clean tube. Protein concentrations were measured with a bicinchoninic acid (BCA) protein assay kit

(Pierce BCA Protein Assay Kit, Cat. No. 23225) according to the manufacturer's instructions. The protein concentration in the samples was calculated according to the standard curve in $\mu\text{g/mL}$. Protein isolates were diluted to 1/10 with RIPA buffer.

2.4. Detection of apoptosis

The levels of Bax (MyBioSource, Catalog No: MBS2513810), Bcl2 (Invitrogen, Catalog No: BMS244-3) and cleaved caspase-3 (Invitrogen, Catalog No: KHO1091) in the MCF-7 cells were analyzed using an ELISA kit according to the manufacturer's instructions. Briefly, standards, and controls or diluted protein samples were added in a well plate and incubated at room temperature for 2 h on a rotating shaker. Then the upper liquid was discarded from the plate. Biotin-conjugate was added to wells and they were incubated at room temperature for 2 h on a rotating shaker. The upper liquid was then aspirated from the plate. Well plates were washed 5 times with wash buffers. Streptavidin-HRP was added to the well plates and they were incubated at room temperature for 1 h on a rotating shaker. The upper liquid was aspirated from the plate and the well plates were washed 5 times with wash buffers. TMB Substrate Solution was added, and the well plates were incubated at room temperature for 10 min on a rotating shaker (dark). Stop solution was added to each well. The absorbance of each microwell was measured at 450 nm using a spectrophotometer (Thermo Multiskan Go). All standards, controls and samples were independently measured in duplicate and the average levels were calculated. Afterwards, the amounts of Bax, Bcl2 and cleaved caspase-3 were normalized by proportioning to the total protein amount.

2.5. Statistics

Differences between the groups were evaluated statistically by t test using the GraphPad Prism 8.0 software. A p value <0.05 was accepted to be statistically significant. Data are presented as mean \pm SD in the figures.

3. Results

3.1. PhTAD substituted dihydropyrrole compounds alter Bax protein expression in MCF-7 cells

CI, CII, CIII, CV, CVII, CVIII, CXI, and CXII treatment significantly increased Bax protein expression in the MCF-7 cells approximately 0.5-fold compared to the negative control, as shown in Figure 2. CXIII and CXIV decreased Bax protein levels by 95% and 70% respectively.

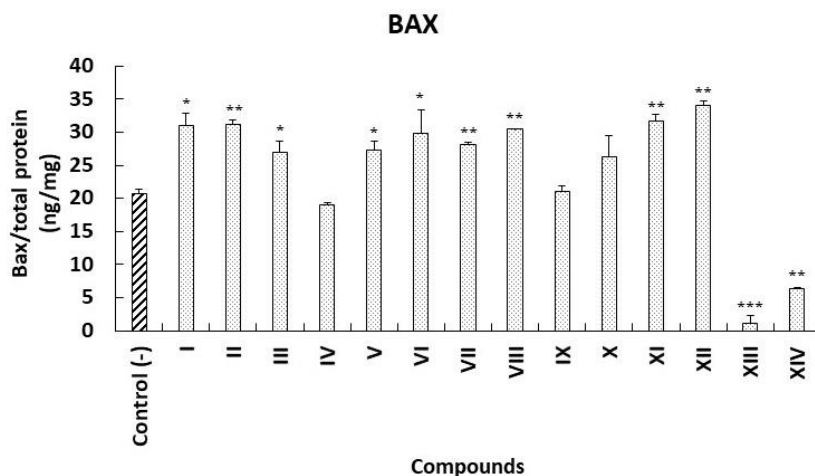


Figure 2. PhTAD-substituted dihydropyrrole compounds alter Bax protein in MCF-7 cells. Data are expressed as mean \pm S.D. t test. *** p <0.001, ** p <0.01 and * p <0.05 vs negative control. (n= 2).

3.2. Effects of PhTAD substituted dihydropyrrole compounds on Bcl2 protein expression in MCF-7 cells

Bcl2 protein expression in the MCF-7 cells was upregulated by CI (1-fold), CII (1-fold), CIII (0.6-fold), CVII (1-fold), CVIII (2-fold), CXI (0.7-fold) and CXII (1-fold) treatment compared to the negative control. However, Bcl2 protein expression was downregulated by CIV (40 %) and CXIV (90 %), as shown in Figure 3.

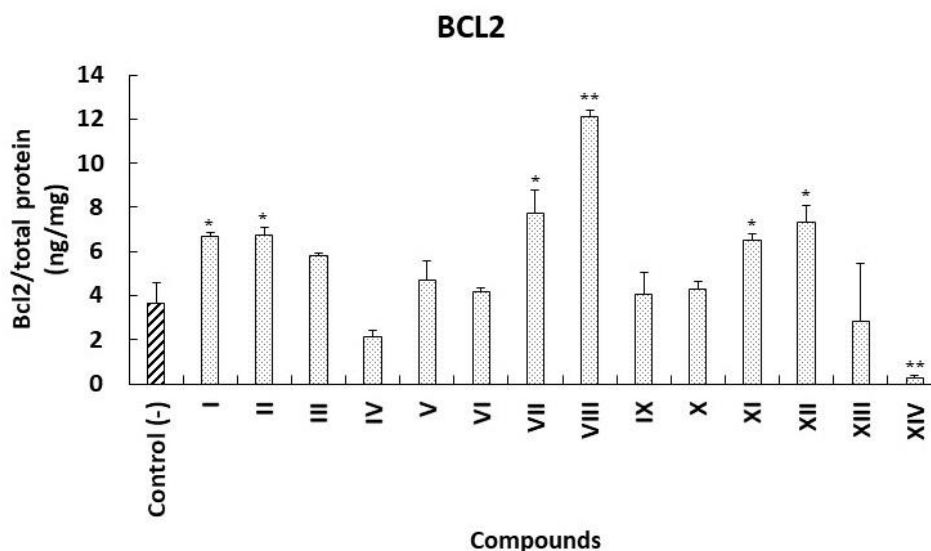


Figure 3. PhTAD-substituted dihydropyrrole compounds modulate Bcl2 protein in MCF-7 cells. Data are expressed as mean \pm S.D. t test. ** p <0.01 and * p <0.05 vs negative control. (n= 2).

3.3. Effects of PhTAD substituted dihydropyrrole compounds on Bax/Bcl2 ratio in MCF-7 cells

The Bax/Bcl2 ratio was increased by CIV (~0.5-fold) and CXIV (~3-fold) treatment compared to the negative control in the MCF-7 cells. The Bax/Bcl2 ratio was decreased by CVIII (~ 55 %) and CXIII (~ 90 %) treatment compared to the negative control. Also, this ratio did not change with the application of other compounds (Figure 4).

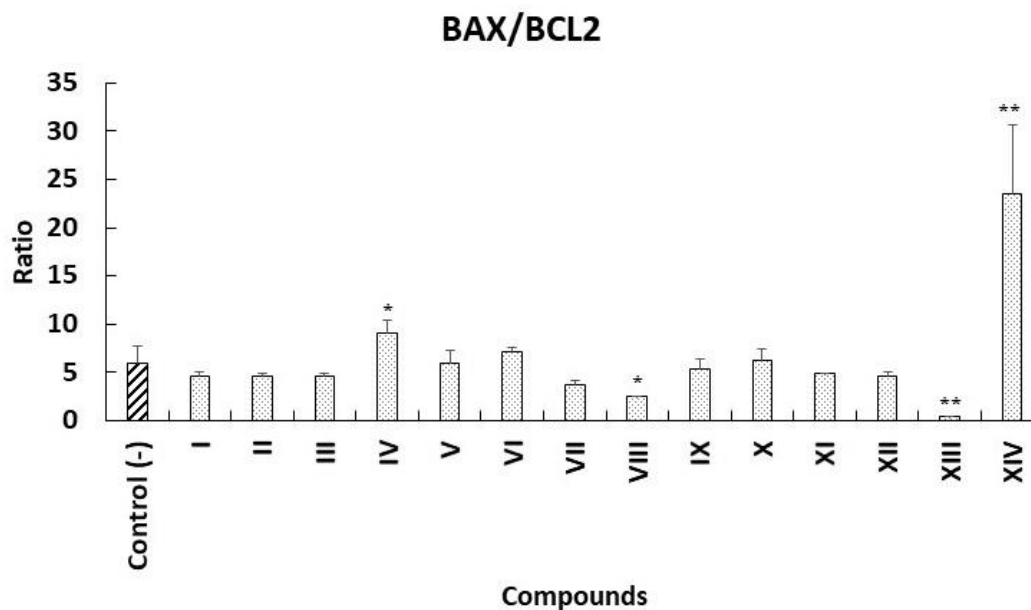


Figure 4. PhTAD-substituted dihydropyrrole compounds alter Bax/Bcl2 ratio in MCF-7 cells. Data are expressed as mean \pm S.D. t test. ** $p < 0.01$ and * $p < 0.05$ vs negative control. (n= 2).

3.4. PhTAD substituted dihydropyrrole compounds activate caspase-3 dependent manner apoptosis

Cleaved caspase-3 protein levels were increased by CI (~0.5-fold), CIV (~0.3-fold), CIX (2-fold) and CXII (0.5-fold) treatment compared to the negative control in the MCF-7 cells (Figure 5). However, cleaved caspase-3 protein levels were decreased by CVIII (20 %), CX (20 %), and CXI (15 %) treatment compared to the negative control.

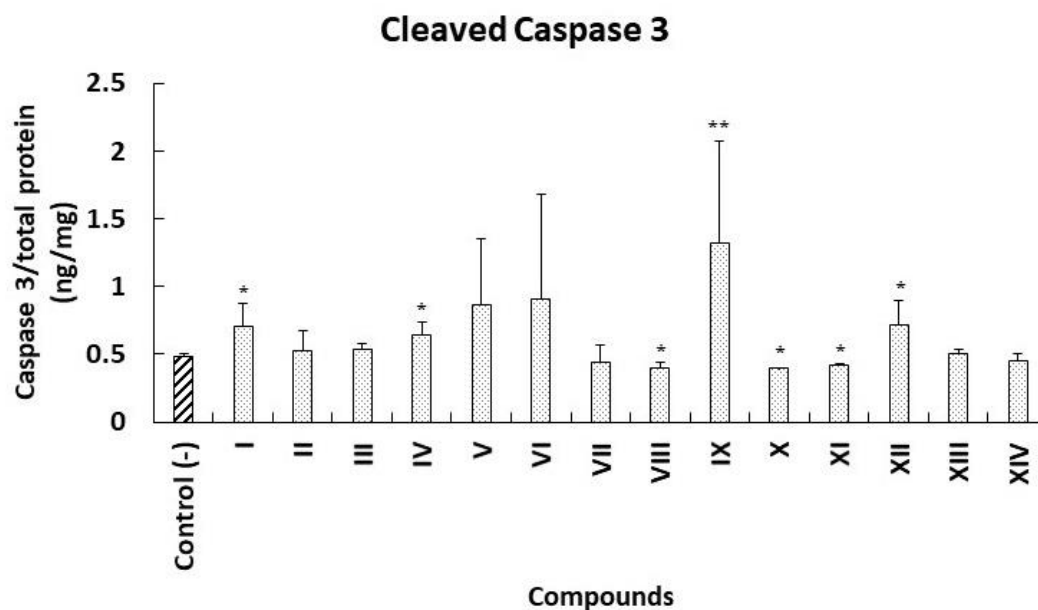


Figure 5. PhTAD-substituted dihydropyrrole compounds activate caspase-3 dependent manner apoptosis. Data are expressed as mean \pm S.D. t test. ** $p < 0.01$ and * $p < 0.05$ vs negative control. (n= 2).

4. Discussion

Dihydropyrrole compounds are known for their anticancer activities and suppress cell growth in cancer cells. Olszewska et al. demonstrated that fluorinated pyrrole derivatives suppressed proliferation of MCF-7 and lung adenocarcinoma (A549) cells, halted the cell cycle, and shut off cancer cell proliferation. Moreover, these compounds induce caspase-3 activation and promote apoptosis of A549 cells. They have also been observed to markedly restrict the A549 and MCF-7 cancer cells migration levels (Olszewska et al., 2020). Moreover, Ji et al. found that pyrrole analogues showed a significant proliferation inhibitory effect in 10 different cancer cell lines. Furthermore, pyrrolidine stimulates cell cycle arrest and apoptosis mechanisms in both colorectal carcinoma (HCT116) and leukemia cells (HL60) (Ji et al., 2020).

Pyrrole compounds exhibited cytotoxicity in lung adenocarcinoma (PC-9), esophageal carcinoma (Eca-109), and gastric cancer (SGC-7901) cells. The compounds increased the Bax/ Bcl2 ratio and induced apoptosis via caspase-3 activation (Zhu et al., 2017). Pyrrole-derived compounds reduce cell viability and have a promising effect. In addition, the compounds induced apoptosis as a possible mechanism for antiproliferative effect (Badolato et al., 2017). Furthermore, Bavadi et al. indicated that the pyrrole compounds showed inhibitory activity and induced apoptosis against leukemia (HL-60), MCF-7, and human acute T lymphoblastic leukemia (MOLT-4) cell lines (Bavadi et al., 2017).

Moreover, compounds with pyrrole structure displayed strong cytotoxic activities in cancer cells in vitro rhabdomyosarcoma (RD), leiomyosarcoma (SK-LMS-1), gastrointestinal osteosarcoma (U-2 OS), Ewing's sarcoma (A-673) and stromal tumor (GIST-T1). These compounds led to both restriction of tubulin polymerization and the induction of a strong G2/M cell cycle arrest (Boichuk et al., 2016). Pyrrole conjugates are cytotoxic to different

types of cancer cells by promoting DNA damage in endothelial cells, arresting the cell cycle in the G2/M phase, activating apoptotic cell death mechanisms, and inducing vascular endothelial growth factor receptor 2 (VEGFR-2) (Chang et al., 2019).

Some studies have shown that pyrrole derivatives have been synthesized as inhibitors of various protein kinases including EGFR and VEGFR. These compounds induce apoptosis in colorectal cancer cells. Thus, these compounds could be used as inhibitors of EGFR and VEGFR, which may promote the realization of their pro-apoptotic and anti-cancer activities (Kuznietsova et al., 2020). In addition, another study found that pyrrole derivatives JG-03-14 showed reported anti-proliferative activity in MCF-7/caspase-3 cells and the MCF-7/ADR (multidrug-resistant) cell line. It has been demonstrated that this compound stimulates autophagic cell death and is active in cancer cells expressing multi-drug resistance proteins (Arthur et al., 2007). Furthermore, Proto et al. showed that the activity of pyrrole compounds potentiated the inhibitory effect of Nutlin 3, induced apoptosis and significantly impaired melanoma cell lines. It also reduced the p53-MDM2 interaction and induced p53-HSP90 complex formation (Proto et al., 2020).

In addition, pyrrole compounds have very good inhibitory activity against platelet-derived growth factor (PDGFR α), fibroblast growth factor receptor (FGFR-1), LYN, c-KIT, and VEGFR-1/2/3 kinases. Qin et al. demonstrated that the compounds markedly decreased phosphorylated AKT and ERK, strongly inhibited human liver cancer (HepG2), human colorectal adenocarcinoma (HT-29), and human stomach adenocarcinoma (MKN74) cells and encouraged apoptosis (Qin et al., 2020). Synthesized pyrrole-derived compounds were similarly found to be cytotoxic to colon carcinoma cells (HCT116) (Tikhomirov et al., 2020). Zhang et al. indicate that pyrrole-imidazole polyamides prevented proliferation of A549 and SGC-7901 cancer cells; they also suppressed the migration of cancer cells (Zhang et al., 2020).

In this context, the aim of the current research was to investigate how PhTAD-substituted dihydropyrrole compounds affect the expression of apoptotic cell death proteins in the MCF-7 cells. Breast cancer, which is a heterogeneous disease, is categorized into three main subtypes in accordance with the presence or absence of the molecular receptor profile. The first subtype, which is observed in 70% of patients, is ER and/or PR receptors positive but HER2 receptor negative. At present 92 different cell lines are used in breast cancer studies. However, the MCF7 cell line is the most commonly used since it is positive for ER and PR, while negative for HER2 (Dai et al., 2017). Since this receptor profile is most frequently observed in patients, MCF 7 cells were chosen for this study. In our previous studies, two different cytotoxic activity tests were used to evaluate 14 different compounds on MCF-7 and MCF-12A cells. In the MTT and RTCA assays, the compounds were determined to have very high cytotoxic activity. The IC₅₀ value for the MTT assay of the compounds was between 5 and 54 μ M. The IC₅₀ value for the RTCA assay of the compounds was between 1.5 and 44 μ M. In the MTT assay, CII, CV, CVI, CIX, CX, and CXIV showed particularly high cytotoxic activity. In addition, in the RTCA assay, CI, CII, CIII, CIV, CXIII and CXIV showed high cytotoxic activity. We applied the appropriate doses on MCF-7 according to the IC₅₀ results obtained in our previous studies, as shown as Table 1. After that, the levels of Bax, Bcl2 and cleaved caspase-3 protein were determined. The results revealed that CI, CII, CIII, CV, CVII, CVIII, CXI and CXII increased Bax protein expression in MCF7 cells, while CXIII and CXIV markedly decreased Bax protein expression (Figure 2). In addition, CI, CII, CIII, CVII, CVIII, and CXII upregulated Bcl2 protein expression; conversely, CIV and CXIV downregulated Bcl2 protein levels (Figure 3). The Bax/Bcl2 ratio can be used as an indicator of cell death/or life, thus, we calculated this ratio. CIV, and CXIV increased the Bax/Bcl2

ratio; however, CVIII, and CXIII decreased the Bax/Bcl2 ratio (Figure 4). This ratio did not change with the application of other compounds. Caspase-3 is an executioner caspase. Caspase-3 activation induces the cleavage of many target substrates, such as lamins, tubulin, and PARP, which are crucial for the occurrence of apoptosis. We therefore measured the cleaved form of caspase-3. CI, CIV, CIX, and CXII treatment increased cleaved caspase-3 protein in the MCF-7 cells compared to negative control. In contrast, CVIII, CX, and CXI treatment decreased cleaved caspase-3 protein in the MCF-7 cells compared to the negative control (Figure 5). The structure of our compounds is very similar to the reference drugs prodigiosin and obatoclax, which are used as BCL2 inhibitors and cause cancer cell death (Mohammed, 2019; Schwartz-Roberts et al., 2013). Moreover, CIV upregulated both the Bax/Bcl2 ratio and caspase-3 protein levels. It is possible that these molecules induce an intrinsic apoptotic pathway in MCF 7 cells. Interestingly, CI, CII, CIII, CV, CIX, and CXII decreased the Bax/Bcl2 ratio, while increasing caspase-3 levels. These alterations may be modulated by either activation of the extrinsic apoptotic pathway or inhibition of Bcl2 protein.

In conclusion, our findings indicate that the effects of PhTAD-substituted dihydropyrrole derivative molecules induced the expression of apoptotic proteins as a potential regulator of cancer cell death. In particular, the CI, CIV CIX, and CXII compounds could be used as a potential molecule for anticancer research. Further research is needed to elucidate the mechanisms of action of these molecules on cancer.

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Conflict of Interest

All authors declare no conflict of interest.

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Authors' contributions

BY, SM, MA, AA, MG, and TY designed research, analyzed data, and wrote the article, MG synthesized compounds. SM, MA, and TY performed cell culture and protein isolation. BY, and AA conducted the protein determination and ELISA experiments.

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