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Downregulation of HSP27 by isoindole-derived pyrrolidines suppressing multidrug resistance (MDR) and inducing apoptosis in MCF-7 and DLD-1 cell lines

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In most cancer treatments, major problem arises from the prevention of cell death (apoptosis suppression) with the development of drug resistance. Anticancer agents that ensure elimination of drug resistance and drug-resistant cells to apoptosis, are among the main targets. Here, we evaluated a series of synthesized N-phenyl maleimide substituents in tetracyclic compounds as anticancer drug candidate. We selected compounds may lead to death and eliminate drug resistance in breast and colon cells. In MCF-7 and DLD-1 cell lines; multidrug resistance genes (*ABCB1*, *ABCC3*, *ABCC10*, *ABCC11* and *ABCG2*), apoptosis mechanism genes (*BAX*, *BCL-2*, *p53*, *PARP* and *CASP3*), heat shock genes (*HSP27*, *HSP40*, *HSP60*, *HSP70* and *HSP90α*) and endoplasmic reticulum (ER) chaperone genes (*GRP78* and *GRP94*) mRNA levels were determined by qPCR method. Amounts of proteins of apoptosis and signalling pathways were measured by human apoptosis antibody array. The compounds have been shown to have downregulation on multidrug resistance genes other than ABCC3. It was found that all compounds in MCF-7 and DLD-1 cells showed significant increase in p53, BAX and CASP3 gene expressions. Also, the compounds have the potential to reduce gene expression of heat shock genes (HSPs). While the compounds have been determined to increase protein expression in BAD, BAX, BID, BIM, Caspase-3, Caspase-7, Caspase-8, Cytochrome-C, Fas, TNF, TRAIL, p27, p38 and p53; decrease protein expression in AKT, BCL-2, ERK1/2, HSP27, HSP60, IGFs, JNK, NF_KB, PARP, TAK1, Survivin in MCF-7 and DLD-1 cells. The compounds stand out with their inhibition of HSP27 in DLD-1 cells and their inhibition with HSP27 and NFκB in MCF-7 cells. Overall, it has been shown that these compounds increase intrinsic and extrinsic proapoptotic proteins, decrease antiapoptotic proteins, decrease HSPs and some growth factors, and they may serve as potential anticarcinogenic molecules.

Keywords: Breast cancer, Colon cancer, Heat shock proteins (HSPs), Isoindole

Breast cancer is a serious health problem that accounts for about 32% of cancers among women in developed countries with Western lifestyles worldwide[1,2]. Colon cancer is a disease of economically "advanced" populations, and it is one of the most common cancer types in both men and women in Europe[3,4]. Women rank 1st in breast cancer and 3rd in colon cancer in terms of estimated new cases, and in estimated deaths they rank 2nd and 4th, respectively. Men rank 3rd in colon cancer in terms of estimated new cases as well as deaths[2]. The most important problem encountered in cancer treatment methods is that the cells develop resistance to treatment or drugs[5,6]. Drug transport proteins; causes suppression of apoptosis, increased proliferation, changes in drug-target interaction, drug inactivation, and resistance to cytotoxic factors[6,7].

The ABC transport family is represented by 49 genes in the human genome. The gene structures, number and organization of domains are divided into seven subfamilies based on amino acid sequences[8]. These transporters prevent the drug from accumulating in the tumour tissue by removing

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Abbreviation: ATM: ataxia telangiectasia mutated; AKT: protein kinase B; BAD: Bcl-2-Associated Death; BAX: Bcl-2-associated X protein; BCL-2: B-cell lymphoma 2; CD40: Cluster of differentiation 40; cIAP-2: cellular inhibitor of apoptosis protein 2; CHK: Checkpoint kinase; cytoC: Cytochrome C; DLD-1: Dukes' Type C, colorectal adenocarcinoma; DR6: Death receptor 6; eIF2a: Eukaryotic Initiation Factor 2; ERK: extracellular signal-regulated kinase; GRP: glucose-regulated protein; HSP: heat shock protein; HTRA: high-temperature requirement; IGF: insulin-like growth factor; IGFBP: Insulin-like growth factor binding protein; JNK: c-Jun N-terminal kinase; MCF-7: human breast adenocarcinoma; MDR: multidrug resistance; MRP: multidrug resistance protein; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; PARP: Poli (ADP-riboz) polimeraz; sTNF-R: soluble TNF receptor; p53/TP53: Tumor suppressor protein 53; SMAC: Second mitochondria-derived activator of caspase;TAK1: transforming growth factor-b-activated kinase-1; TNF: Tumor necrosis factor; TRAILR: tumor necrosis factor-related apoptosis-inducing ligand; XIAP: X-linked inhibitor of apoptosis protein

chemotherapy drugs from the cells and there fail the applied chemotherapy[9].

Inactivation of programmed cell death or apoptosis is central to cancer development[10]. Apoptosis plays a critical and natural physiological role in eliminating abnormal cells in tissue homeostasis. The development of target drugs directly targeting cell death mechanisms in tumours is an important way of cancer treatment[11]. BCL-2 family proteins consist of both anti- and pro-apoptotic members. BCL-2, BCL-XL, and BCL-W antiapoptotic members inhibit activation of BAX/BAK, while BAD, BID, BIK, and BIM proapoptotic members promote BAX/BAK activation[12]. Caspase-3, -6 and -7 form effector groups and are activated by -8 or -9 of the initiator caspases "downstream" and cut the functions of target proteins (non-caspase) in the cells from their special places[13,14]. Extrinsic apoptotic pathways: the transmembrane death receptors mediated by external apoptotic signalling pathways or death receptor pathways[15,16]. Intrinsic apoptotic pathways: replicate intracellular signals controlled by mitochondria[17].

Research has shown that p53 can induce apoptosis in cancer cells by upregulating proapoptotic proteins like BAX and regulating the BAX/BCL-2 ratio[18]. p53, the commonly distributed cell division inhibitor and apoptosis inducer (TP53)[19], prevents genetic mutation and genomic instability[20] and, generally, upregulated or mutated in cancer cells, especially MDR cancer cells[21]. HSP27 can inhibit the accumulation of p21 and suppress cell death in response to p53 activation, indicating a regulatory role of HSP27 in the p53 pathway[22].

Rel/NF-kappaB (NF-κB) transcription factors are key regulators of programmed cell death (apoptosis)[23]. Released NF-κB dimers enter the nucleus, where they regulate the transcription of different genes that encode cytokines, growth factors, cell adhesion molecules, and proapoptotic and antiapoptotic proteins[24].

Insulin-like growth factors (IGFs) act mainly as growth hormones, regulate the growth of human cells and tissues, and affect their lifespan[25]. Along with other signalling pathways, IGFs also play a role in determining the balance between apoptosis and cell survival. IGFBPs direct IGFs through the IGF receptor to target tissues where they promote cell growth, proliferation, differentiation, and survival[26]. Antiapoptotic and pro-survival effects are of great importance for the development and progression of some types of cancer[25-27].

Gene families that play a critical role in the protection of cells against environmental factors are heat shock proteins (HSP) and glucose regulated proteins (GRP)[28]. Major HSPs with chaperone function; HSP90, HSP70, HSP60, HSP40 and small HSPs (HSP27)[29]. Expression of HSP plays a role in the regulation of apoptosis and multiple drug resistance[18].

Heterocyclic compounds have exhibited notable bioactivities, such as antitumor, antiviral, antimicrobial, and antitubercular properties[31,32]. Zhao *et al*.[33] reported that two new isoindole compounds showed antitumor activity against HepG-2 cells. Pesquet *et al*.[34] demonstrated that the synthesized isoindole ring compounds exhibited inhibitory activity on human farnesyltransferase at IC_{50} values of 1.5 nM. Min *et al*.[35] conducted PARP inhibition and cellular proliferation assays against MDA-MB-436 and CAPAN-1 cell lines with pyrrolidine-derived compounds. The compounds are reported to exhibit cytotoxic and anticancer activity.

In previous work, we examined the antioxidant and cytotoxic activity in breast cancer cells by synthesizing the compound isoindole pyrrolidine, a heterocyclic compound[36,37]. In this study, we tried to elucidate the mechanisms of genes and proteins that are thought to trigger death pathways (BAX, BCL-2, p53, PARP ve CASP3) through drug resistance (ABCB1, ABCC3, ABCC10, ABCC11 ve ABCG2), heat shock genes (HSP27, HSP40, HSP60, HSP70 ve HSP90α) and endoplasmic reticulum (ER) chaperone genes (GRP78 ve GRP94) of isoindole-derived pyrrolidine compounds in MCF-7 (breast cancer) and DLD-1 (colon cancer) cell lines. Amounts proteins of apoptosis and signalling pathways (intrinsic pathways, extrinsic pathways, pro- and anti-apoptotic proteins, TNFs, TRAILRs, HSPs, IGFs, IGFBPs, etc.).

Materials and Methods

Chemistry

The starting materials methyl 2-((bis(propylthio) methylene)amino) acetate (I) and methyl 2-((bis (methylthio)methylene)amino)acetate (III) were synthesized following Elemes group working methods (litx); methyl-1,3,5,7-tetraoxo-2,6-diphenyl-8-(propylthio) decahydro-4,8-epiminopyrrolo[3,4-f]isoindole-4(1H)-carboxylate and methyl-1,3,5,7-tetraoxo-2,6-di-

phenyl-8-(methylthio)decahydro-4,8-epiminopyrrolo [3,4-f]isoindole-4(1H)-carboxylate were taking previously work[36,37]. Compound I and III (starting), which are synthesized as glycine methyl ester derivatives, differ in their alkyl groups attached to the sulphur atom, as well as having a similar skeleton. Compound II and IV (ending) are bridged compounds and in these structures, the active N-H bond at the bridgehead is the region that will cause the greatest difference from the starting material. It caused the structure to acquire acidic, basic, inducing, and reducing properties (Fig. 1).

Cell culture

MCF-7 (ATCC® HTB22™) (breast adenocarcinoma) DLD-1 (colon cancer) (ATCC® CCL221™) human cell lines were used in this study. MCF-7 and DLD-1 cells were cultured in 10% fetal bovine serum (FBS) and RPMI-1640 (Roswell Park Memorial Institute) medium, 5% CO_2 incubator, 24 h and 37°C[38]. Compounds whose cytotoxic dose was determined as 50 μM were applied to the cells.

qPCR assay

In MCF-7 and DLD-1 cell lines; multidrug resistance proteins (ABCB1, ABCC3, ABCC10, ABCC11 and ABCG2), apoptosis mechanism proteins (BAX, BACL-2, p53, PARP and CASP3), heat shock proteins (HSP27, HSP40, HSP60, HSP70 and HSP90α) and endoplasmic reticulum (ER) chaperone proteins (GRP78 and GRP94) mRNA levels were determined by qPCR method.

Total RNA isolation

RNA isolation was studied in MCF-7 (breast cancer) and DLD-1 (colon cancer) cells by the method recommended in the "Thermo Fisher; RNA isolation kit (K0731)" using 2×10^6 cells. The concentration and purity of the isolated RNA were carried out with the aid of a nanodrop (Thermo Fisher Scientific/Multiskan) device. In the process of measuring RNA samples with nanodrop, firstly, RNA samples diluted at appropriate concentrations (ng/μL) were read at 260 and 280 nm.

The RNA concentration was calculated by the formula (μg/mL) = [OD (Optical density) 260 × dilution rate × 40 (μg/mL)]. A260/A280 ≅ 2.0 RNA samples were included in the study.

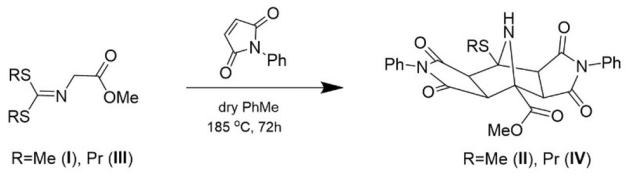


Fig. 1 — Chemical structures and names of compounds used in the study

cDNA synthesis

cDNA synthesis was performed according to the kit protocol using "Thermo Fisher; cDNA synthesis kit (K1622)" by Thermal Cycler Prime.

qPCR

Gene sequences were determined by NCBI database. In Table 1, the information about the primers was shared. The primers required for qPCR were designed using the NCBI primary blast program (https://www.ncbi.nlm.nih.gov/tools/primer-blast). "Thermo Fisher; Maxima SYBR Green/ROX qPCR Master Mix (2X) kit K0221" was used for qPCR analysis and analyzes were performed in accordance with the kit protocol. Threshold cycle (CT) was determined by PikoReal™ Real-Time PCR System (Thermo Scientific). Relative gene expressions of multidrug resistance, apoptosis, HSPs and GRPs genes were calculated as a fold change using $2^{-\Delta\Delta CT}$ methods.

Antibody array

Amounts proteins of apoptosis and signalling pathways (BAD, BAX, BID, BIM, Casp3, Casp7, Casp8, CD40, CD40L, cIAP-2, cytoC, DR6, Fas, FasL, HTRA, p38, p27, p53, SMAC, sTNF-R1, sTNF-R2, TNF-alpha, TNF-beta, TRAILR-1, TRAILR-2, TRAILR-3, TRAILR-4, ATM, CHK1, CHK2, eIF2a, SMAD BCL-2, BCL-W, HSP27, HSP60, HSP70, IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, IGF-1sR, Livin, p21, Survivin, XIAP, AKT, Erk1/2, IkBa, JNK, NFKB, PARP, TAK1) were measured by human apoptosis antibody array (Human Apoptosis Antibody Array C1 Kit and Human Apoptosis Signaling Antibody Array C1 Kit, RayBiotech, USA).

Protein isolation

MCF-7 (breast cancer) and DLD-1 (colon cancer) cells were incubated for 24 h at 37°C and seeded to flasks by calculating the number of 5×10^6 cells. Compounds whose cytotoxic dose was determined as 50 μM were applied to the cells. The proteins isolated by adding 500 μL of RIPA buffer (Thermo Fisher Scientific, 9806) to the cells were kept at –20°C.

Protein quantification

Protein determination was made in accordance with the "Thermo Fisher Scientific BCA Protein Assay" protocol. Absorbance values of proteins at 562 nm in 96-well plates were measured.

Membrane associated protein array

It was performed in accordance with the protocol of the Human apoptosis antibody array (RayBiotech). Protein samples were diluted with Blocking Buffer to 300 µg/mL. All incubations and washings were performed under slight rotation/shaking (~0.5-1 rev/s). One mL of protein samples was pipetted onto the membrane and after incubation, serial washes were made using wash buffers; and one mL of biotinylated antibody cocktail (Human Apoptosis Antibody Array C1 Kit) and detection antibody cocktail (Human Apoptosis Signaling Antibody Array C1 Kit) were added onto the membrane and serial washes were made using wash buffers after incubation. 2 mL 1X HRP-streptavidin (Human Apoptosis Antibody Array C1 Kit) and 1X HRP-Anti-Rabbit IgG (Human Apoptosis Signaling Antibody Array C1 Kit) was added to the membrane and after incubation, serial washes were made using wash buffers. 250 µL Detection Buffer-C and 250 µL Detection Buffer-D were pipetted onto the membrane by mixing and membrane images were determined with a chemiluminescence device (Vilber Lourmat). Protein spots in membrane images were measured with the help of the Image J program. The intensity of every duplicated array spot was calculated with the ImageJ software program, and the averaged intensity was measured by RayBio® Analysis Software Tools subtracting the averaged background signal. The fold change was acquired by comparing treatment samples with the untreated control.

Results

In our previous study, we demonstrated high cytotoxic and antioxidant activity[3] of these compounds[6,37]. In this study, The N-phenyl maleimide substituent in tetracyclic compounds represented an interesting drug active agent and compound II was found to have an effect on the MCF-7 (breast cancer) and DLD-1 (colon cancer) cell lines on multidrug-resistant proteins (MRPs) and apoptosis mechanism.

Effects of isoindole derivated pyrrolidine compounds on multi-drug resistance genes in cancer cells

In the MCF-7 cell compared to the control group; the expression of ABCB1, ABCC10, ABCC11, and ABCG2 mRNA were decreased 0.5-fold by compound I; the expression of ABCB1, ABCC10, and ABCC11 mRNA were decreased 0.5-fold approximately by compound II; the expression of ABCB1, ABCC10, ABCC11 and ABCG2 mRNA were decreased 0.5-fold approximately by compound III; the expression of ABCB1, ABCC10 and ABCC11 mRNA were decreased 0.5 -fold approximately by compound IV (Fig. 2A).

In the DLD-1 cell compared to the control group; the expression of ABCB1 and ABCG2 mRNA was decreased 0.5-fold by compound I; the expression of ABCB1 and ABCG2 mRNA was decreased 0.5-fold approximately by compound II; the expression of ABCB1 and ABCG2 mRNA 0.5-fold were decreased by compound III; the expression of ABCB1, ABCC10, ABCC11 and ABCG2 mRNA were decreased 0.5-fold approximately by compound IV (Fig. 2B).

Effects of isoindole derivated pyrrolidine compounds on apoptotic HSPs and GRPPs genes in cancer cells

In the MCF-7 cell compared to the control group; p53 mRNA expression was 8-fold, BAX mRNA expression 2-fold, and CASP3 mRNA expression 1.5-fold were increased by compound I. But BCL-2 mRNA expression is 0.9-fold, PARP mRNA expression is 0.6-fold, HSP27 mRNA expression 0.3-fold, HSP40 mRNA expression 0.6-fold, HSP60 mRNA expression 0.4-fold, and HSP90 mRNA expression 0.7-fold were decreased. In addition, there

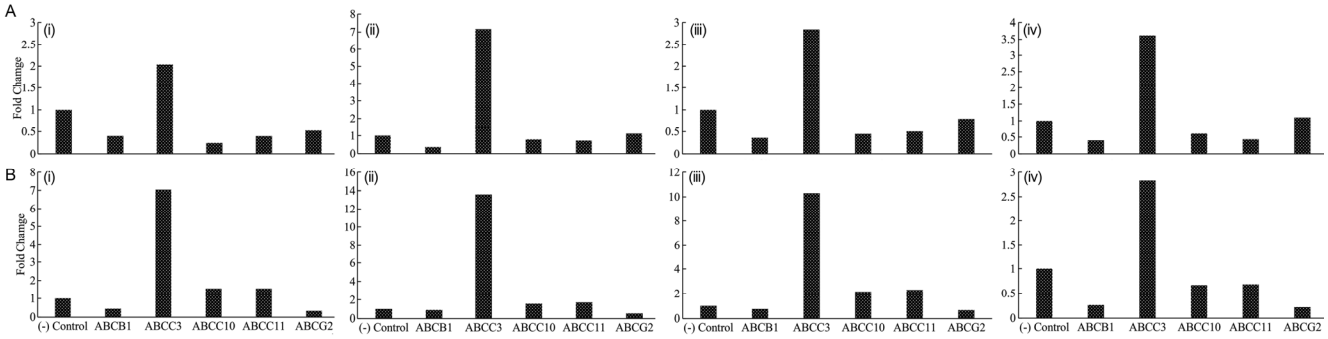


Fig. 2 — Effect of the isoindole derivated pyrrolidine compounds on mRNA expression of multidrug resistance genes in (A) MCF-7 and (B) DLD-1 cell lines. [Relative gene expressions were calculated as a fold change by using $2^{-\Delta\Delta CT}$ methods]

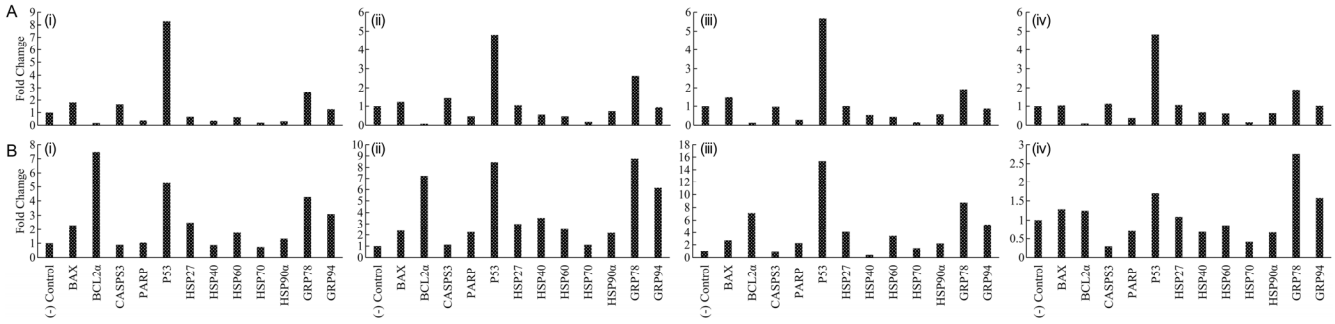


Fig. 3 — Effect of the isoindole derivated pyrrolidine compounds on mRNA expression of apoptosis, HSPs and GRPs genes in (A) MCF-7 and (B) DLD-1 cell lines. [Relative gene expressions were calculated as a fold change by using $2^{-\Delta\Delta CT}$ methods]

was no significant decrease in GRP78 and GRP94 mRNA expression. p53 mRNA expression 5-fold, BAX mRNA expression 1.5-fold and CASP3 mRNA expression 1.5-fold were increased by compound II. But BCL-2 mRNA expression 1-fold, PARP mRNA expression 0.5-fold, HSP40 mRNA expression 0.5-fold, HSP60 mRNA expression 0.5-fold, and HSP90 mRNA expression 0.3-fold were decreased. In addition, there was no significant decrease in HSP27, GRP94, and GRP78 mRNA expression. p53 mRNA expression 5.5-fold and BAX mRNA expression 1.5-fold were increased by compound III. But BCL-2 mRNA expression 0.9-fold, PARP mRNA expression 0.7-fold, HSP40 mRNA expression 0.5-fold, HSP60 mRNA expression 0.6-fold, and HSP90 mRNA expression 0.4-fold were decreased. In addition, there was no significant increase in CASP3, HSP27, GRP78, and GRP94 mRNA expression. p53 mRNA expression 5-fold was increased by compound IV. There was no significant increase in BAX and CASP3 mRNAs expression. But BCL-2 mRNA expression 0.9-fold, PARP mRNA expression 0.6-fold, HSP40 mRNA expression 0.3-fold, and HSP60 mRNA expression 0.4-fold were decreased. In addition, there was no significant decrease in HSP27, GRP78, and GRP94 mRNAs expression (Fig. 3A).

In the DLD-1 cell compared to the control group; p53 mRNA expression 5-fold and BAX mRNA expression 2.5-fold were increased by compound I. In addition, there was no significant increase in CASP3 and decrease in BCL-2, PARP, GRP78, GRP94, and HSP mRNAs expression. p53 mRNA expression 8.5-fold and BAX mRNA expression 2.5-fold were increased by compound II. In addition, there was no significant increase in CASP3 and decrease in BCL-2, PARP, GRP78, GRP94, and HSP mRNAs expression. p53 mRNA expression 15.5-fold and BAX mRNA expression 3-fold were increased by compound III. But HSP40 mRNA expression 0.5-fold were decreased. In addition, there was no significant increase in CASP3 and decrease in BCL-2, PARP, GRP78, GRP94, and HSP40 mRNA expression. p53 mRNA expression 2-fold and BAX mRNA expression 1.5-fold were increased by compound IV. However, a significant decrease was observed in HSP mRNA expressions other than PARP and HSP27. In addition, there was no significant increase in CASP3 and decrease in BCL-2, GRP78, GRP94, and HSP27 mRNA expression (Fig. 3B).

Array analysis of isoindole-derivated pyrrolidine compoundson apoptosis and related signaling pathway proteins in MCF-7 cell

In the MCF-7 cell compared to the control group; BAX 5-fold, BID 4-fold, BIM 1.8-fold, Casp3 (Asp175) 2.8-fold, CASP8 4.2-fold, CD40 4.5-fold, CD40LG 3.5-fold, cIAP-2 2.5-fold, cytoC-1.6-fold, DR6 (TNFRSF21) 4-fold, FAS 3-fold, FASLG 3.7-fold, HTRA2 1.5-fold, P27 (T198) 3.3-fold, p53 1.8-fold, P53 (S15) 2.6-fold, SMAC 1.8-fold, sTNF-R2 7-fold, TNF-alpha 4-fold, TNF-beta 8.6-fold, TRAILR-1 4.1-fold, TRAILR-2 4.5-fold, TRAILR-3 5.3-fold, TRAILR-4 6-fold, IGFBP1 5.8-fold, IGFBP2 1.9-fold, IGFBP3 1.8-fold, IGFBP4 6.3-fold, IGFBP5 1.3-fold and IGFBP6 1.9-fold protein amounts increased by compound I. But no significant increase in the BAD (S112), p38 (T180/Y182), IkBa (S32), and sTNF-R1proteins was detected. ERK1/2 (T202) 0.5-fold, HSP27 (S82) 0.2-fold, NFKB (S536) 0.7-fold and TAK1 (S412) 0.5-fold protein amounts decreased by compound I. In addition, there was no significant decrease in the of BLC-2, BCL-w, ATM (S1981), Casp7 (Asp198), CHK1 (S280), CHK2 (T68), eIF2a (S52), SMAD2 (S245/250), HSP27, HSP27 (S82), HSP60, HSP70, IGF1, IGF2, IGF1sR, livin, survivin, p21, XIAP, AKT (S473), JNK (T193) and PARP (ASP214) proteins.

BAX 2.8-fold, BID 1.8-fold, CASP8 1.7-fold, CD40 4-fold, CD40LG 2.6-fold, cIAP-2 1.5-fold, DR6 (TNFRSF21) 2-fold, FAS 2.1-fold, FASL 2.7-fold, p27 2-fold, sTNF-R2 3.3-fold, TNF-alpha 2.3-fold, TNF-beta 4.3-fold, TRAILR-1 3.5-fold, TRAILR-2 3.3-fold, TRAILR -3 4.2-fold, TRAILR-4 4.2-fold, IGFBP4 2.7-fold, IGFBP6 1.3-fold, eIF2a (S52) 1.2-fold, P27 (T198) 3-fold and P53 (S15) 3-fold proteins amounts increased by compound II. But there was no significant increase in the amount of BAD, BIM, CASP3, cytoC, HTRA2, p53, SMAC, IGFBP1, IGFBP2, IGFBP3, IGFBP5, IkBa (S32), sTNF-R1 ATM (S1981), BAD (S112), CASP3 (ASP175), CASP7 (ASP198), CHK1 (S280), CHK2 (T68), P38 (T180/Y182) and SMAD2 (S245/250) proteins. HSP27 0.4-fold, survivin 0.5-fold, ERK1/2 (T202) 0.4-fold, HSP27 (S82) 0.3-fold, and NFKB (S536) 0.6-fold protein amounts decreased by compound II. But there was no significant decrease in the of BLC-2, BCL-w, HSP60, HSP70, IGF1, IGF2, IGF1sR, livin, p21, XIAP, AKT (S473), JNK (T193), PARP (ASP214) and TAK1 (S412) protein amount.

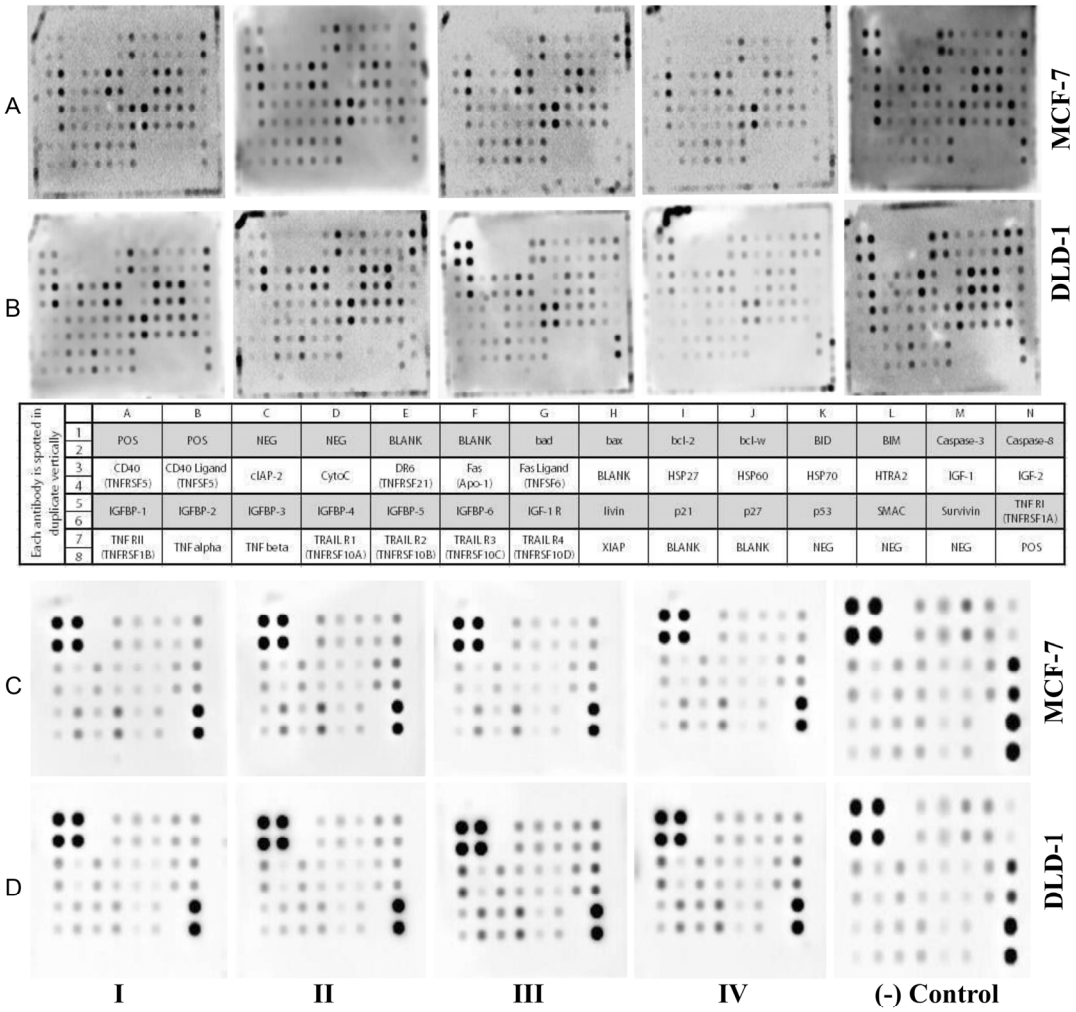
BAD 2.6-fold, BAX 14-fold, BID 14-fold, BIM 3-fold, CASP3 2.8-fold, CASP8 9.3-fold, CD40 11-fold, CD40LG 7.8-fold, cIAP-2 5.2-fold, cytoC 2.6-fold, DR6 (TNFRSF21) 2.9-fold, FAS 6.7-fold, FASLG 8.7-fold, HTRA2 2.2-fold, p27 5.5-fold, p53 2.5-fold, SMAC 4.7-fold, sTNF -R2 6.6-fold, TNF-alpha 6.9-fold, TNF-beta 17.9-fold, TRAILR-1 10-fold, TRAILR-2 11-fold, TRAILR-3 18.5-fold, TRAILR-4 11.5-fold IGFBP1 3.7-fold, IGFBP2 1.9-fold, IGFBP3 2.8-fold, IGFBP4 8.8-fold, IGFBP5 2.6-fold, IGFBP6 4.4-fold, P27 (T198) 2.3-fold and P53 (S15) 2 proteins amounts increased by compound III. But there was no significant increase in of the sTNF-R1, ATM (S1981), BAD (S112), CASP3 (ASP175), CASP7 (ASP198), CHK1 (S280), CHK2 (T68), eIF2a (S52), P38 (T180/Y182) and SMAD2 (S245/250) proteins. AKT (S473) 0.2-fold, ERK1/2 (T202) 0.7-fold, HSP27 (S82) 0.3-fold, JNK (T193) 0.3-fold, NFKB (S536) 0.7-fold, PARP (ASP214) 0.3-fold and TAK1 (S412) 0.4-fold protein amounts decreased by compound III. But there was no significant decrease in of the BLC-2, BCL-w, HSP27, HSP60, HSP70, IGF1, IGF2, IGF1sR, IkBa (S32), livin, survivin, p21 and XIAP protein.

BAD 1.3-fold, BAX10,3-fold, BID 4.2-fold, BIM 2-fold, CASP3 2-fold, CASP8 6-fold, CD40 4.5-fold, CD40LG 5.8-fold, cIAP-2 2.2-fold, cytoC 2-fold, DR6 (TNFRSF21) 3.7-fold, FAS 4.2-fold, FASLG 4.8-fold, HTRA2 1.7-fold, p27 2.1-fold, p53 1.8-fold, SMAC 2-fold, sTNF-R2 4.2-fold, TNF-alpha 1.8-fold, TNF-beta 13-fold, TRAILR-1 9.8-fold, TRAILR-2 8.5-fold, TRAILR-3 12.8-fold, TRAILR-4 10-fold, IGFBP1 3.3-fold, IGFBP2 1.2-fold, IGFBP3 1.4-fold, IGFBP4 2.4-fold, IGFBP5 1.2-fold, IGFBP6 1.9-fold, P27 (T198) 2.2-fold and P53 (S15) 12-fold proteins amounts increased by compound IV. But there was no significant increase in of the sTNF-R1, ATM (S1981), BAD (S112), CASP3 (ASP175), CASP7 (ASP198), CHK1 (S280), CHK2 (T68), eIF2a (S52), P38 (T180/Y182) and SMAD2 (S245/250) proteins. IGF1 0.4-fold, AKT (S473) 0.3-fold, ERK1/2 (T202) 0.6-fold, HSP27 0.9-fold, HSP27 (S82) 0.4-fold, JNK (T193) 0.3-fold, NFKB (S536) 0.7-fold, PARP (ASP214) 0.2-fold and TAK1 (S412) 0.3-fold protein amounts decreased by compound IV. But there was no significant decrease in of the BLC-2, BCL-w, HSP60, HSP70, IGF2, IGF1sR, IkBa (S32), livin, survivin, p21 and XIAP proteins (Figs. 4 A & C and 5 A & B).

Array analysis of isoindole-derivated pyrrolidine compounds on apoptosis and related signaling pathway proteins in DLD-1 cell

BAX 2.5-fold, CASP8 2.9-fold, CD40 3.3-fold, CD40LG 2.3-fold, cIAP-2 4-fold, cytoC 3.2-fold, DR6 (TNFRSF21) 2.5-fold, FAS 1.5-fold, FASLG 3.4-fold, HTRA2 1.8-fold, p27 2-fold, p53 1.5-fold, SMAC 2.4-fold, sTNF-R1 1.5-fold, sTNF-R2 4.8-fold, TNF-alpha 7.2-fold, TNF-beta 2.2-fold, TRAILR-1 3.5-fold, TRAILR-2 1.8-fold, TRAILR-3 5.8-fold, TRAILR-4 1.3-fold, IGFBP3 1.3-fold, IGFBP4 5-fold and IGFBP6 2.3-fold proteins amounts increased by compound I. But there was no significant increase in of the BAD, BID, BIM, CASP3, IGFBP1, IGFBP2, IGFBP5, ATM (S1981), BAD (S112), CASP3 (ASP175), CASP7 (ASP198), CHK1 (S280), CHK2 (T68), eIF2a (S52), P27 (T198), P38 (T180/Y182), P53 (S15) and SMAD2 (S245/250) proteins. AKT (S473) 0.8-fold, ERK1/2 (T202) 0.5-fold, HSP27 0.4-fold, HSP27 (S82) 0.5-fold, JNK (T193) 0,5-fold, NFKB (S536) 0.8-fold, PARP (ASP214) 0.5-fold and TAK1 (S412) 0.8-fold protein amounts decreased by compound I. But there was no significant increase in of the BLC-2, BCL-w, HSP60, HSP70, IGF1, IGF2, IGF1sR, IkBa (S32), livin, survivin, p21 and XIAP proteins.

BAX 1.5-fold, CASP8 1.6-fold, CD40 1.4-fold, CD40LG 1.2-fold, cIAP-2 2.3-fold, cytoC 1.5-fold, DR6 (TNFRSF21) 1.2-fold, FASLG 1, 6-fold, p27 1.3-fold, SMAC 1.3-fold, sTNF-R2 1.6-fold, TNF-



Each antibody is spotted in duplicate vertically

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	BLANK	BLANK	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase-3	Caspase-8
2														
3	CD40 (TNFRSF5)	CD40 Ligand (TNFSF5)	cIAP-2	CytoC	DR6 (TNFRSF21)	Fas (Apo-1)	Fas Ligand (TNFSF6)	BLANK	HSP27	HSP60	HSP70	HTRA2	IGF-1	IGF-2
4														
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1 R	livin	p21	p27	p53	SMAC	Survivin	TNFRI (TNFRSF1A)
6														
7	TNFRII (TNFRSF1B)	TNFalpha	TNFbeta	TRAILR1 (TNFRSF10A)	TRAILR2 (TNFRSF10B)	TRAILR3 (TNFRSF10C)	TRAILR4 (TNFRSF10D)	XIAP	BLANK	BLANK	NEG	NEG	NEG	POS
8														

Each antibody is spotted in duplicate vertically

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	Akt (P-Ser473)	ATM (P-Ser1981)	BAD (P-Ser112)	Caspase-3 (Cleaved Asp175)	Caspase-7 (Cleaved Asp198)
2								
3	CHK1 (P-Ser296)	CHK2 (P-Thr68)	eIF-2a (P-Ser51)	ERK1 (P-T202/Y204) ERK2 (P-Y185/Y187)	HSP27 (P-Ser82)	IKBa (P-Ser32)	JNK (P-Thr183/Tyr185)	NFKBP65 (P-Ser536)
4								
5	PARP1 (Cleaved Asp214/Gly215)	p27 (P-Thr198)	P38 (P-Thr180/Tyr182)	P53 (P-Ser15)	SMAD2 (P-Ser245/250/255)	TAK1 (P-Ser412)	NEG	POS
6								

Fig. 4 — (A & B) Human apoptosis antibody array membrane images; and (C & D) Human apoptosis signaling antibody array membrane images in (A & C) MCF-7 and (B & D) DLD-1 cells. [Duplicate array spots were measured with the ImageJ and the averaged intensity was calculated by RayBio® Analysis Software Tools]

alpha 2.7-fold, TNF-beta 3.3-fold, TRAILR-3 1.3-fold, IGFBP4 2,7-fold, IGFBP6 1.6-fold, IkBa (S32) 1.7-fold, CASP7 (ASP198) 3.5-fold, CHK1 (S280) 2.6 -fold proteins amounts increased by compound II. But there was no significant increase in of the IGFBP3, IGFBP5, ATM (S1981), BAD (S112), CASP3 (ASP175), CHK2 (T68), eIF2a (S52), P27 (T198), P38 (T180/Y182), P53 (S15), SMAD2 (S245/250), BID, BIM, CASP3, FAS, HTRA2, p53, sTNF-R1, TRAILR-1, TRAILR-2, TRAILR-4, IGFBP1 and IGFBP2 proteins. HSP27 0.5-fold, HSP60 0.3-fold, HSP70 0.2-fold, survivin 0.3-fold, IGF1 0.4-fold, IGF2 0.2-fold, AKT (S473) 0.5-fold, NFKB (S536) 0.3-fold and TAK1 (S412) 0.1-fold protein amounts decreased by compound II. But there was no significant increase in the BLC-2, BCL-w, IGF1sR, p21, livin, XIAP ERK1/2 (T202), HSP27 (S82), IkBa (S32) 1.4-fold JNK (T193) and PARP

(ASP214) proteins. CD40 1.8-fold, CD40LG 1.2-fold, cIAP-2 5.2-fold, cytoC 1.2-fold, DR6 (TNFRSF21) 1.5-fold, FASLG 1.2-fold, sTNF-R2 2-fold, TNF-alpha 1.8-fold, TNF-beta 1.4-fold, ATM (S1981) 0.1-fold, CASP7 (ASP198) 5.7-fold, eIF2a (S52) 1.7-fold, P38 (T180/Y182) 1.5-fold, p53 (S15) 2.5-fold and SMAD2 (S245/250) 0.1-fold proteins amounts increased by compound III. But there was no significant increase in of the BAD, BAX, BID, BIM, CASP3, CASP8, FAS, HTRA2, p27, p53, SMAC, sTNF-R1, TRAILR-1, TRAILR-2, TRAILR-3, TRAILR-4, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, BAD (S112), CASP3 (ASP175), CHK1 (S280), CHK2 (T68) and P27 (T198) proteins. BLC-2 0.5-fold, BCL-w 0.2-fold, HSP27 0.7-fold, HSP60 0.5-fold, HSP70 0.6-fold, IGF1 0.6-fold, IGF2 0.8-fold, IGF1sR 0.5-fold, survivin 0.8-fold, and NFKB (S536) 0.1-fold protein amounts decreased by compound III. But there was no significant increase in livin, p21 and XIAP, AKT (S473), ERK1/2 (T202), HSP27 (S82), JNK (T193), PARP (ASP214) and TAK1 (S412) proteins.

BIM 1.1-fold, CD40 2.1-fold, CD40LG 1.3-fold, cIAP-2 3.5-fold, cytoC 1.5-fold, DR6 (TNFRSF21) 2-fold, FASLG 1.6-fold, sTNF-R2 2.6-fold, TNF-alpha 2.2-fold, TNF-beta 1.7-fold, TRAILR-1 1.5-fold, TRAILR-3 1.4-fold, CASP7 (ASP198) 3.9-fold, CHK1 (S280) 1.2-fold, P38 (T180/Y182) 1.5-fold, and P53 (S15) 2-fold proteins amounts increased by compound IV. But there was no significant increase in of the BAD (S112), BAX, BID, CASP3, CASP8, FAS, HTRA2, p27, p53, SMAC, sTNF-R1, TRAILR-2, TRAILR-4, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, IkBa (S32), ATM (S1981), CASP3 (ASP175), CHK2 (T68), eIF2a (S52), P27 (T198) and SMAD2 (S245/250) proteins. BLC-2 0.2-fold, HSP60 0.3-fold, HSP70 0.5-fold, IGF1 0.6-fold, IGF2 0.8-fold, IGF1sR 0.5-fold, survivin 0.8-fold, ERK1/2 (T202) 0.3-fold, HSP27 (S82) 0.3-fold, JNK (T193) 0.3-fold, and NFKB (S536) 0.7-fold protein amounts decreased by compound IV. But there was no significant increase in the BCL-w, HSP27, p21, livin, XIAP AKT (S473), PARP (ASP214) and TAK1 (S412) proteins (Figs. 4 B & D and 5 C & D).

Discussion

Heterocyclic compounds, constituting an important class of organic chemistry, are compounds with known anticarcinogenic and antiproliferative properties that can be easily derivatized with other drug-active compounds. According to literature reviews, although there are many studies on various disciplines of heterocyclic compounds, biological activity studies of isoindole and pyrrolidine derivatives of this compound are rare.

The development of a drug resistance mechanism in cancer is related to the inability of the drug to reach sufficient concentration in the target area as a result of overexpression and activities of drug carriers and apoptosis resistance[39]. It has been reported that the mechanism of multidrug resistance in cancer cells is associated with the suppression of pathways leading to apoptosis or other forms of cell death[40].

This study; it was aimed to investigate the effects of isoindole and pyrrolidine-derived compounds, which are thought to have proapoptotic and anti-carcinogenic effects on MCF-7 and DLD-1 cancer cells, on multi-drug resistance (MDR), and apoptotic signalling pathways. The ABCG2 expression level of irinotecan which has a heterocyclic structure is an important drug for patients with colorectal carcinoma. It has been stated that irinotecan-based therapy may be a useful biomarker for chemotherapy resistance[41]. It is evaluated the P-gp interaction profiles of hetero-cyclic tetrahydroisoquinoline-derived compounds using HT29 and A549 cell lines. Immunoblot analysis method was performed against two ABC transporters, multidrug resistance-related protein-1 (MDR-1) and breast cancer resistance protein (BCRP). It has been shown to be very active and selective against P-gp[8]. It has been reported that the dacomitinib compound, which is thought to be a chemotherapy agent, significantly reverses drug resistance in the ABCB1 and ABCG2 genes at a dose of 1.0 mM. According to the research of Fan *et al*.[42] developed small molecular tyrosine kinase inhibitors (TKIs) for the treatment of various human cancers that overexpress the epidermal growth factor receptor (EGFR). The results showed that dacomitinib at 1.0 mM significantly reversed ABCB1 and ABCG2-mediated but ABCC1-mediated drug resistance. The adverse effect on ABCB1 overexpressing cells was found to be stronger than on ABCG2 overexpressing cells. Also, dacomitinib in reverse concentration did not affect the protein expression level and localization of ABCB1 and ABCG2[42]. The effect of the voruciclib compound on ABCB1 and ABCG2 gene expressions and on apoptosis of cells was determined by western blotting analysis. The compounds have been shown to drive

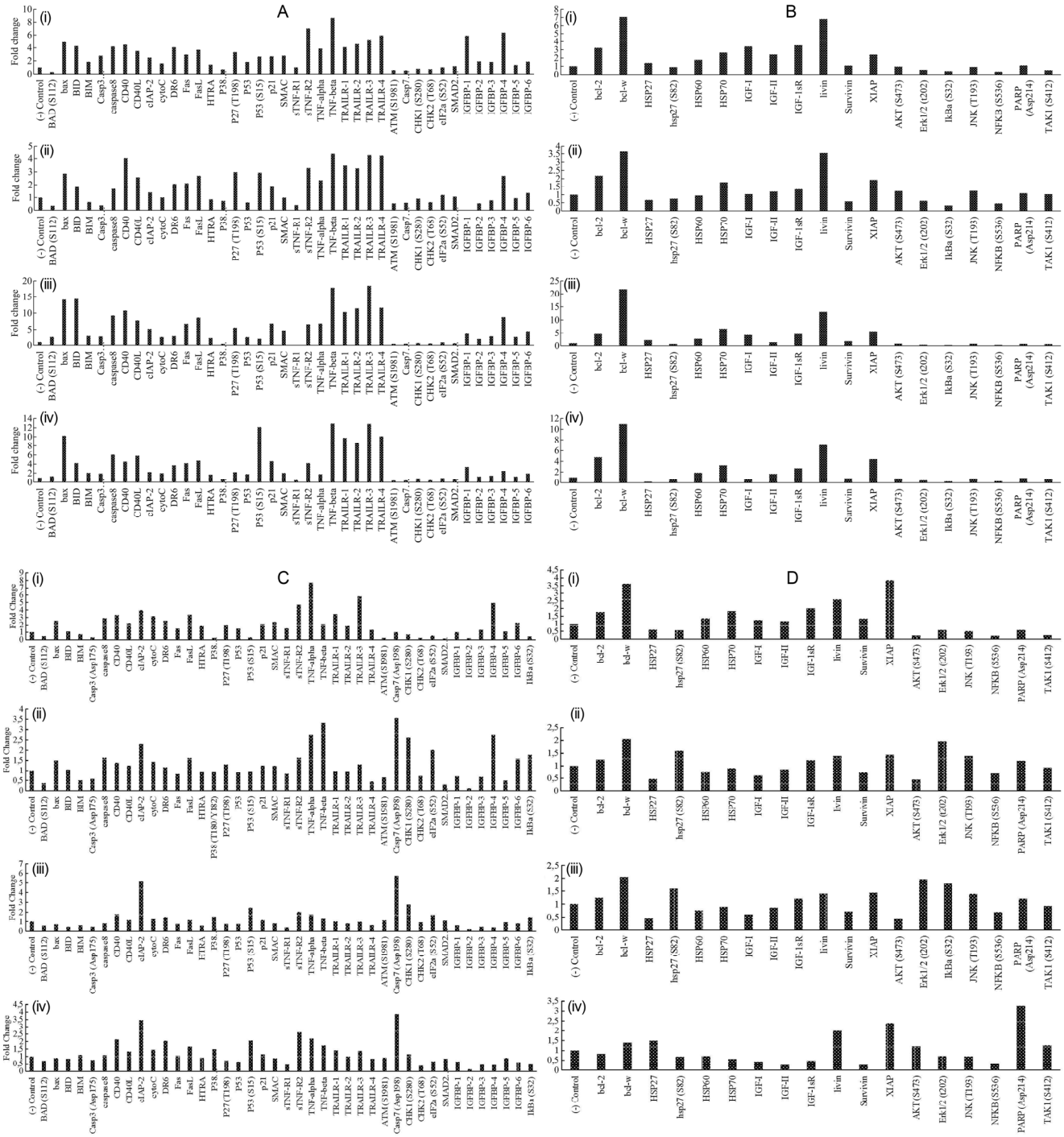


Fig. 5 — Effect of four compounds on (A & C) pro-apoptosis; and (B & D) anti-apoptosis mechanism in (A & B) MCF-7 and (C & D) DLD-1 cell lines. [The fold change was obtained by comparing compounds treatment samples with the untreated control]

cells into apoptosis by reducing gene expression. Voruciclib exhibited a drug-induced apoptotic effect in ABCB1 or ABCG2 overexpressing cells. Its potential use with conventional anti-cancer drugs for cancer chemotherapy has been reported to be supported[43].

In our study, it has been shown that all compounds significantly reduce MDR mRNA expressions (except ABCC3 gene expression) in MCF-7 and DLD-1 cells in Figure 4, and these compounds may have an important role in suppressing chemotherapy resistance. When the compounds were examined,

compound II showed the most effective result in MCF-7 and DLD-1 cells. There are a total of three heterocycle structures in compound II. Heterocyclic structures are molecules that show a wide range of biological activity properties because they have an energy level that causes selective binding. The N-H pyrrolidine ring in the synthesized molecule is also effective due to its H-binding property, which increases this selectivity (Fig. 2).

It has supported the antitumor activity of isoindole-derived compounds p53 and MDM2 protein inhibition with biological data[44]. It has been stated that the spirooxindolepyrrolidine compounds may function as one of the good therapeutic agents used in the treatment of malignant tumours, particularly for the caspase-3 mediated apoptotic pathway[45]. PARP inhibition and cellular proliferation assays of pyrrolidine-derived have been performed and the compounds have been reported to exhibit potential anticancer activities against PARP-1 and PARP-2[35].

In our study, it was determined that all compounds in MCF-7 and DLD-1 cells showed a significant increase in p53, BAX, and CASP3 gene expressions leading the cell to death in Fig. 5. It has also been determined that the compounds have the potential to reduce the gene expression of heat shock proteins (HSP27, HSP40, HSP60, HSP70, HSP90α). The effects of the compounds on reducing the expression of the endoplasmic reticulum stress genes (GRP78 and GRP94) were not detected. When the gene expressions of the compounds were examined, compound II and compound IV gave effective results, and the compounds were found to have similar characteristics. Compounds II and IV are bridged compounds, and in these structures, the active N-H bond at the bridgehead is the region that causes the greatest difference. This region caused the structures to acquire acidic, basic, oxidizing, and reducing properties. In addition, it is thought that compound II has an effective structure due to the long alkyl chain, and compound IV gives an effective result in the structure due to the presence of the S-methyl group in the bridge pier (Fig. 3).

The apoptotic mechanism with carbazole alkaloid, which was isolated from *Murraya koenigii* Spreng on A549 lung cancer cells, was performed using a protein array antibody array (Human Apoptosis Antibody Array Kit, RayBiotech, USA) to investigate cell death. A significant effect of the compound on both internal and external pathways was determined. Besides p53, cell proliferation suppressor proteins, regulation of p27 and p21, and the important role of insulin/IGF-1 were identified. In addition, caspase-3 and -8 were found to be significantly activated[46]. In the human colorectal cancer cell line HCT 116, the proteomic approach of the Guggulsterone (GS) compound was determined by protein array antibody array (Human Apoptosis Signaling Array C1 Kit, RayBiotech, USA). Proteins related to cell proliferation/ migration, tumour formation, cell growth, metabolism, and DNA replication were found to be downregulated and found be upregulated in a functional role in exocytosis/tumour suppression[47].

In our study, the expression of pro-apoptotic proteins BAD, BAX, BID, BIM, caspase-3, caspase-7, caspase-8, cytochrome-C, Fas, TNF, TRAIL, p27, p38 and p53 was increased, and the expression of antiapoptotic proteins BCL-2, BCL-W, IGF, survivin, XIAP, AKT, ERK, JNK, NFKB, PARP and p21 was reduced in MCF7 and DLD-1 cells.

In MCF-7 and DLD-1 cells, all compounds are predicted to induce the cell to apoptosis by inducing Casp3, Casp7 and Casp8 signals through the death domains by increasing expression of FasL, TNF-α, TNF-β, TRAIL death ligands and Fas, TNF-R, TRAILR receptors. In MCF-7 and DLD-1 cells, all compounds are thought to lead to cell death by inhibiting *ABC* genes involved in multi-drug resistance, inducing p53 and p27 signal sequences and caspase pathways, and through survivin expression. It is predicted that compounds II, III, and IV in DLD-1 cells affect pro-apoptotic activity in the cell by decreasing the expression of HSPs, which are heat shock proteins, by inducing p38 and caspase pathways. By increasing the expression of compounds, I, III and IV in IGFBP growth factors in MCF-7 and DLD-1 cells, BAD, BAX, BID, BIM induce pro-apoptotic pathways. Thus, the compounds are thought to inhibit AKT, JNK, ERK1/2, and NF-kB signals in cells through the expression of BCL-2, cIAP-1, and survival (Figs. 4 and 5).

Conclusion

Remarkable results have been shown regarding gene expressions that play an important role in multidrug resistance and apoptotic and cell death-related signalling pathways of isoindole-derived pyrrolidine compounds in MCF-7 and DLD-1 cells.

All compounds significantly reduce MDR mRNA expressions (except ABCC3 gene expression), and these compounds may play an important role in suppressing chemotherapy resistance. When the compounds were examined, compound IV showed the most effective result in MCF-7 and DLD-1 cells. As a result of apoptotic and related signal pathways data; it was found that all compounds in MCF-7 and DLD-1 cells showed significant increases in p53, BAX, and CASP3 gene expression, dragging the cell to death. It has also been determined that the compounds have the potential to reduce gene expression of heat shock proteins (HSP27, HSP40, HSP60, HSP70, HSP90α). Thus, pro-apoptotic activity is activated in the cell by decreasing the expression of HSPs and inducing caspase pathways. When the gene expressions of the compounds were examined, compound II and compound IV gave effective results, and the compounds had a similar nature. The compounds significantly induced cell death in MCF-7 and DLD-1 cancer cells through the downregulation activity of HSP27. According to the molecular mechanism data regulating *in vitro* cell death in breast and colon cancer cells, it can be predicted that isoindole-derived pyrrolidine compounds can be used as HSP27 inhibition agents. Overall, it has been shown that these compounds increase intrinsic and extrinsic pro-apoptotic proteins, decrease antiapoptotic proteins, decrease HSPs and some growth factors, and may serve as potential anticarcinogenic molecules.

Conflict of Interest

Authors declare no competing interests.

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