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Investigation of the apoptotic and cell cycle effects of sorafenib and doxorubicin on URG4/URGCP in leukemia cells

Sorafenib ve doxorubicin'in lösemi hücrelerinde URG4/URGCP üzerindeki apoptotik ve hücre döngüsü etkilerinin incelenmesi

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Abstract

Purpose: The aim of this study is to investigate the effects of anticancer drugs such as Sorafenib (SOR) and Doxorubicin (DOX) on URG4/URGCP mRNA levels in K562 and HL-60 leukemia cells, elucidating their effects on apoptosis and cell cycle. The effects of these drugs on apoptosis and the cell cycle in leukemia cells have been explored. This research aims to understand the cellular effects of drugs used in leukemia treatment and contribute valuable insights to the drug development processes in leukemia therapy.

Materials and methods: DOX and SOR were evaluated for their IC50 values in K562 and HL-60 cell lines using the CellTiter-Glo assay (Promega, USA), based on ATP measurement. Total RNA isolation was performed using Trizol reagent in both control and dose groups of each treated cell line. Following RNA isolation, cDNAs were synthesized using the "Transcriptor High Fidelity cDNA Synthesis Kit". Subsequently, changes in mRNA expression levels were examined using specific primers for URG4/URGCP, Casp-3, Casp-8, Casp-9, FADD, DR4, TRADD, CCDN1, CDK4, CDK6, PTEN, P53, and Rel-A genes.

Results: In the groups treated with Sorafenib, the IC50 dose for HL-60 cell line was calculated as 40 μM at the 24th hour, and for K562 cell line, it was calculated as 40 μM at the 48th hour. In the groups treated with Doxorubicin, the IC50 doses were calculated as 50 μM at the 48th hour for HL-60 cell line, and as 50 μM at the 72nd hour for K562 cell line. Significant increases were observed in the mRNA expression levels of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes in the groups treated with SOR, while a decrease was observed in the mRNA expression levels of URG4/URGCP, CCDN1, CDK4, and CDK6 genes. In the groups treated with DOX, significant increases were observed in the fold changes of Casp-3, Casp-8, P53, and PTEN genes. However, a significant decrease in mRNA expression levels was observed in URG4/URGCP, CCDN1, and CDK4 genes.

Conclusion: As a result, it has been demonstrated that both SOR and DOX may play a role in regulating the mRNA expressions of URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A, and FADD genes in HL-60 and K562 cells.

Keywords: Doxorubicin, sorafenib, cell cycle, apoptosis, URG4/URGCP.

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Öz

Amaç: Çalışmanın amacı, Sorafenib (SOR) ve Doxorubicin (DOX) gibi antikanser ilaçlarının K562 ve HL-60 lösemi hücrelerinde URG4/URGCP mRNA düzeyleri üzerindeki etkilerini inceleyerek, apoptoz ve hücre döngüsü üzerindeki etkilerini açıklığa kavuşturmaktır. Bu ilaçların lösemi hücrelerinde apoptoz ve hücre döngüsü üzerindeki etkileri araştırılmıştır. Bu araştırma, lösemi tedavisinde kullanılan ilaçların hücresel etkilerini anlamak ve lösemi tedavisinde ilaç geliştirme süreçlerine değerli bilgilerle katkıda bulunmayı amaçlamaktadır.

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Gereç ve yöntem: DOX ve SOR'in K562 ve HL-60 hücre hatlarında IC50 ATP ölçümüne dayanan CellTiter-Glo (Promega, ABD) tarafından değerlendirildi. Uygulama yapılan her iki hücre hattının kontrol ve doz gruplarında Trizol kimyasalı kullanılarak total RNA izolasyonu yapıldı. RNA izolasyonunun ardından "Transcriptor High Fidelity cDNA Sentez Kiti" ile cDNA'lar sentezlendi. Daha sonra URG4/URGCP, Casp-3, Casp-8, Casp-9, FADD, DR4, TRADD, CCDN1, CDK4, CDK6, PTEN, P53, Rel-A genlerine özgü primerler kullanılarak mRNA düzeyindeki ekspresyon değişiklikleri incelendi.
Bulgular: Çalışmada Sorafenib uygulanan gruplarda IC50 dozu HL-60 hücre hattı için 24'üncü saatte 40 µM, K562 hücre hattı için ise 48'inci saatte 40 µM olarak hesaplandı. Doksorubisin uygulanan gruplarda ise IC50 dozları, HL-60 hücre hattı için 48'inci saatte 50 µM, K562 hücre hattı için ise 72'inci saatte 50 µM olarak hesaplanmıştır. SOR uygulanan gruplarda Casp-8, Casp-9, TRADD, DR4, Rel A ve FADD genlerin mRNA ekspresyon düzeylerindeki kat değişimlerinde önemli derecede artış gözlemlenirken, URG4/URGCP, CCDN1, CDK4 ve CDK6 genlerinin mRNA ekspresyon düzeylerinde azalma olduğu gözlemlendi. DOX uygulanan gruplarda, Casp-3, Casp-8, P53 ve PTEN genlerinin kat değişimlerinde önemli derecede bir artış olduğu gözlemlenmiştir. Ancak, URG4/URGCP, CCDN1 ve CDK4 genleri üzerinde mRNA ekspresyon düzeylerinde önemli bir azalma meydana gelmiştir.
Sonuç: Sonuç olarak hem SOR hem de DOX' in HL-60 ve K562 hücrelerinde URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A ve FADD genlerin mRNA ekspresyonları düzenlenmesinde rol alabilecekleri gösterilmiştir.

Anahtar kelimeler: Doksorubisin, sorafenib, hücre döngüsü, apoptoz, URG4/URGCP.

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Introduction

Leukemia is a type of cancer caused by abnormal proliferation of blood cells. It is characterized by disrupting the body's normal blood functions by replacing abnormally proliferating blood cells as opposed to normal blood cells found in the bone marrow [1]. This type of cancer usually affects leukocytes, but in different cases it can also affect other blood cells. Leukemia is usually treated with stem cell transplants, radiotherapy and chemotherapy [2]. Chemotherapy is a drug treatment method widely used in cancer treatment [3]. The primary goal of this treatment method is to eliminate cancer cells, but it also causes serious damage to normal healthy cells during the treatment process. When the treatment is administered, the drugs enter the bloodstream and can either stop or hinder cell division during mitosis, causing serious damage to the cells [4, 5]. Chemotherapy employs a variety of anticancer medications, each with distinct mechanisms of action.

SOR is an anti-cancer drug belonging to the class of biaryl urea compounds [6]. While initially identified as a RAF kinase inhibitor, SOR has been found to have a broad spectrum of activity by affecting different cellular signaling pathways [7]. SOR can inhibit "*vascular endothelial growth factor receptors* (VEGFR) *1, 2,* and *3, platelet-derived growth factor receptor β* (PDGFRβ), *RET receptor tyrosine kinases*, *c-Kit protein* (c-Kit), and *FMS-like tyrosine kinase 3* (Flt-3)". Due to this wide spectrum of activity, it has become an effective drug used in cancer treatment [8]. The antiproliferative activity of SOR varies in different tumor types (lung cancer, hepatocellular carcinoma etc.) and is generally associated with oncogenic signaling pathways that regulate tumor cell proliferation. Additionally, SOR initiates the process of programmed cell death (apoptosis) in many tumor cell lines [9, 10].

DOX is an antitumor drug derived from the *bacterium Streptomyces peucetius* [11]. This drug is commonly utilized in chemotherapy for the treatment of breast, ovarian, bladder, acute lymphoblastic leukemia, and acute myeloblastic leukemia [12]. DOX hinders macromolecular biosynthesis by interacting with DNA through intercalation. It also prevents the enzyme topoisomerase II from making progress and loosens supercoils in DNA, which increases the accessibility of DNA for transcription [13]. By breaking DNA strands, it stabilizes the topoisomerase II complex, preventing the DNA double helix from resealing. This mechanism can halt the replication process in cells, ultimately leading to cell death [14].

Oncogenes are genes that control the normal division and growth of cells, but when these genes are mutated they can lead to cancer [15]. Typically, oncogenes encode proteins that play significant roles in foundational cellular processes such as cell proliferation, programmed cell death or cell cycle control [16]. Despite a considerable number of identified genes, there are still new genes that remain unidentified [16, 17]. One such gene recently studied and identified is Up-regulated Gene 4 (URG4) and Up-regulator of Cell Proliferation (URGCP), located on chromosome 7 [17, 18]. When overexpressed, URG4/URGCP proteins stimulate cell growth and initiate DNA synthesis in the cell cycle. [19]. This process occurs through cyclin D1 (CCND1) protein, which is associated with the cell cycle. CCND1 plays a crucial role in regulating the checkpoints of the cell cycle at both the G0/G1 and S phases. CCND1 controls cell proliferation and division by affecting CDK4/CDK6 in the cell cycle. CCND1 forms a complex with CDK4/6, promoting the cell's progression to the S phase [20]. Another protein that suppresses cyclin-dependent kinase activity is encoded by the P21 gene [21]. These genes are typically activated by another protein involved in the cycle, p53, when DNA damage or other stress factors are detected in the cell cycle. The p53 gene detects DNA damage in cells, leading to responses such as halting the cell cycle, initiating DNA repair, or triggering cell apoptosis [22].

Apoptosis is a physiological process known as programmed cell death [22]. When a cell receives an apoptosis stimulus, it moves away from the environment and disconnects from other cells, resulting in chromatin condensation and a pyknotic appearance [23]. Apoptosis, as a vital component, leads to the controlled death of damaged cells under the influence of immune system development, cell cycle regulation and various chemical entities. There are two families of proteins involved in apoptosis. These are proapoptotic (BAX, PUMA, BAK, NOXA, BID, etc.) and antiapoptotic (BCL-XL, BCL-2, etc.) proteins [24, 25]. Apoptosis occurs through two different pathways, internal and external pathways. In the internal pathway, BID proteins inactivate BCL-2 and BAX are activated [24]. By regulating the integrity of mitochondrial membranes, these proteins contribute to the release of pro-apoptotic cytochrome-c (Cyt-C). Cyt-C, upon release into the cytoplasm, collaborates with Apaf-1 to activate caspase-9 (Casp-9), thus initiating the apoptosis process by subsequently activating caspase-3 (Casp-3) [26]. In the extrinsic pathway, death receptors (DR4, DR5, TNFR, FADD, TRADD, etc.) transmit death signals (FasL, TNF-Alpha, etc.). Subsequently, the complex formed by these receptors activates caspase-8 (Casp-8). Casp-8 can directly activate Casp-3 and also activate BID proteins, triggering the intrinsic pathway as well [27, 28].

The aim of this research was to investigate how DOX and SOR impact apoptosis and the cell cycle in K562 and HL-60 leukemia cells through their effects on URG4/URGCP mRNA levels.

Material and method

Chemicals used

Sorafenib (Nexavar, USA) was dissolved in DMSO solution. Doxorubicin (Koçak Farma, TURKEY) was dissolved in distilled water. 100 mM stock solutions were prepared in advance and cell culture treatments were performed.

Cell Culture

In this study, experiments were performed on leukemia cancer HL-60 and K562 cell lines obtained from our stocks. These cell lines were propagated in appropriate culture medium. RPMI 1640 medium, 10% fetal bovine serum, 2 mM L-glutamine, and 1% Penicillin-Streptomycin were used as culture medium. These cells had incubated at 37°C in an oven containing 95% humidity and 5% CO_2.

Cell viability

HL-60 and K562 cell lines were counted using Trypan Blue stain and planted in 96-well plates at $1x10^5$ cells per well. The cytotoxic effect of DOX and SOR on K562 and HL-60 leukemia cell lines was evaluated by CellTiter-Glo (Promega, USA), a luminometric-based method based on ATP measurement [29]. To determine the IC50 dose, both drugs were applied to the cells in the range of 1 µM-50 µM in a manner dependent on both dosage and time.

Determination of transcription level (mRNA) expression of target genes

The effects of SOR and DOX on the mRNA level of cell cycle and apoptosis -related genes were analyzed using quantitative real-time PCR method. Firstly, total RNA isolation was performed from control and dose groups of both cell lines of K562 and HL-60 leukemia cancer using Trizol chemical and cDNAs were synthesized with "*Transcriptor High Fidelity cDNA Synthesis Kit"* after RNA isolation. Then, expression changes at mRNA level were examined by using primers specific for Casp-3, Casp-8, CCDN1, CDK-4, P53, PTEN and URG-4/URGCP genes for DOX and CCDN1, CDK-4, CDK-6, Casp-8, Casp-9, FADD, TRADD, DR-4, Rel-A and URG-4/URGCP genes for SOR in both cell lines. These expression changes were determined using Step one plus real time PCR device (Table 1).

Table 1. The preferred real-time PCR primer sequences in this study

Gene Name	Gene Base Sequence
Casp-3	Forward 5′TGTTTGTGTGCTTCTGAGCC3′
	Reverse 5′CACGCCATGTCATCATCAAC3′
β-Actin	Forward 5′CTGGAACGGTGAAGGTGACA 3′
	Reverse 5′AAGGAACTTCCTTGAACAATGCA3′
Casp-8	Forward 5′AGAGTCTGTGCCCAAATCAAC3′
	Reverse 5′GCTGCTTCTCTCTTTGCTGAA3′
Casp-9	Forward 5′CTGTCTACGGCACAGATGGAT3′
	Reverse 5′GGGACTCGTCTTCAGGGGAA3′
FADD	Forward 5′CTCCTGCGCGAGCTGCTCGC3′
	Reverse 5′GCCTTCTCCAATCTTTCCCCAC3′
DR4	Forward 5′TCCAGCAAATGGTGCTGAC3′
	Reverse 5′GAGTCAAAGGGCACGATGTT3′
TRADD	Forward 5'GCTGTTTGAGTTGCATCCTAGC3'
	Reverse 5'CCGCACTTCAGATTTCGCA3'
CCDN1	Forward 5'AGCTCCTGTGCTGCGAAGTGGAAAC3'
	Reverse 5'AGTGTTCAATGAAATCGTGCGGGGT3'
CDK4	Forward 5'ATGTTGTCCGGCTGATGGA3'
	Reverse 5'CACCAGCGTTACCTTGATCTCCC3'
CDK6	Forward 5'AGACCCAAGAAGCAGTGTGG3'
	Reverse 5'AAGGAGCAAGAGCATTCAGC3'
URG4/URGCP	Forward 5′CGGGAGATGGGACAGTTTTA3′
	Reverse 5′CATGGTGTTGAGGAGTGTGG3′
PTEN	Forward 5'CCCAGACATGACAGCCATC3'
	Reverse 5'TCTGCAGGAAATCCCATAGC3'
P53	Forward 5′ATCTACAAGCAGTCACAGCACA3′
	Reverse 5′GTGGTACAGTCAGAGCCAACC3′
Rel-A	Forward 5'AGCAGCGTGGGGACTACGAC3'
	Reverse 5'AGGCTGGGGTCTGCGTAGGG3'

Statistical analysis

Data analysis was conducted utilized the ΔΔCT method., and these assessments were executed utilizing the online "RT² Profiler™ PCR Array Data Analysis" software. Additionally, Volcano Plot analyses were also carried out using this program.

Results

Cell viability

In the research, cell viability assays were performed employing the CellTiter-Glo kit with SOR on K562and HL-60 cell lines. Evaluations were performed at 24, 48, and 72 hours following treatment application. The effects of SOR were observed to vary dose-dependently and time-dependently in both cell lines. The IC50 dose of SOR in the treated groups was calculated as 40 µM at 24 hours for the HL-60 cell line and at 48 hours for the K562 cell line (Figure 1, Figure 2).

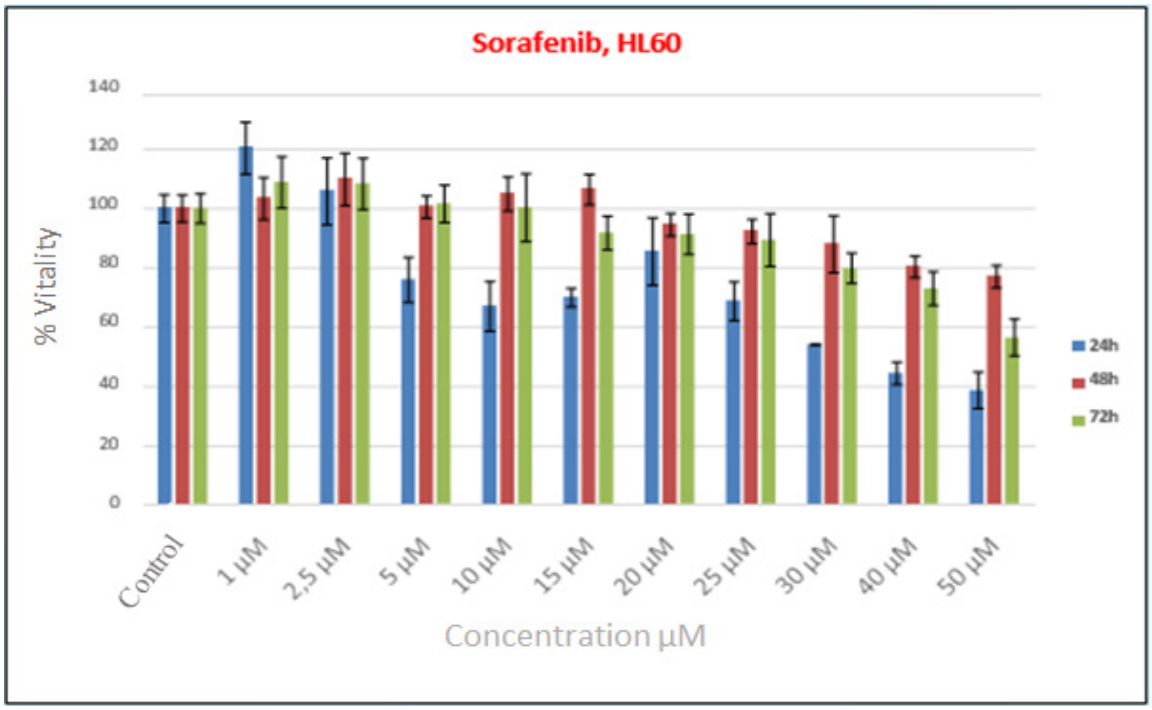


Figure 1. Cell viability percentage of Sorafenib on HL-60 leukemia cell line using the CellTiter-Glo kit

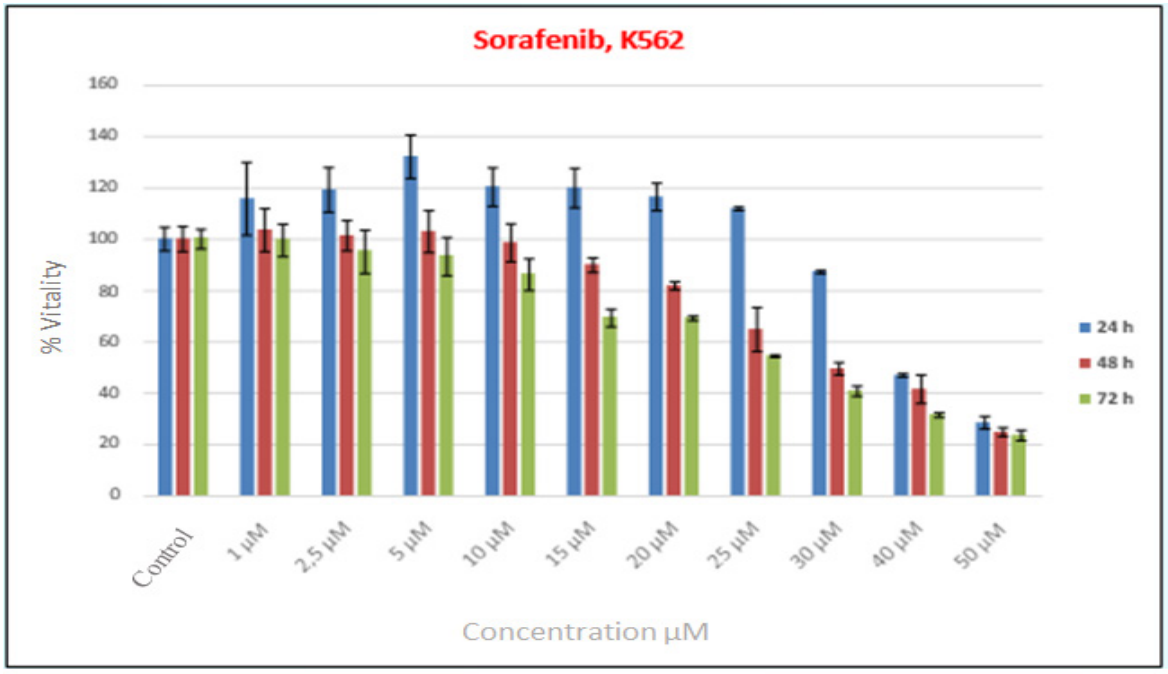


Figure 2. Cell viability percentage of Sorafenib on K562 leukemia cell line using the CellTiter-Glo kit

At the same time, cell viability tests were evaluated using CellTiter-Glo kit in HL-60 and K562 cell lines in DOX-treated groups. Cell measurements were recorded 24, 48, and 72 hours after treatment application. The effects of DOX showed dose- and time-dependent changes in both cell lines. IC50 doses in DOX treated groups were determined as 50 µM at 48 h for HL-60 cell line and 50 µM at 72 h for K562 cell line (Figure 3 and 4).

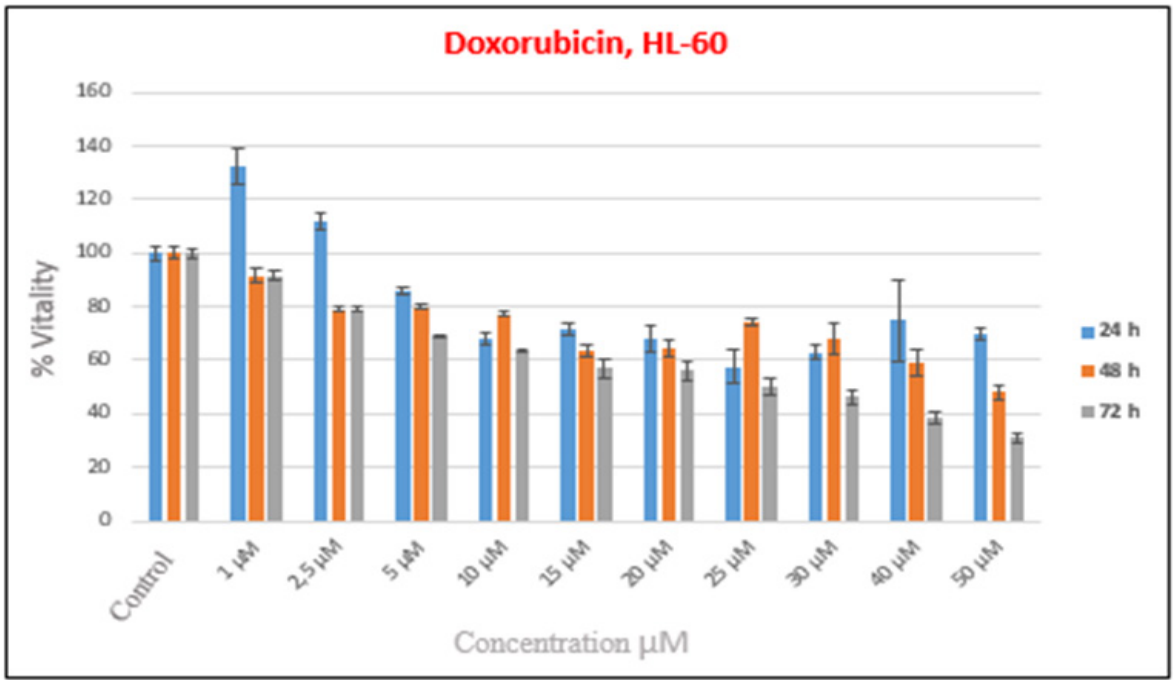


Figure 3. Cell viability percentage of Doxorubicin on HL-60 cell line using the CellTiter-Glo kit

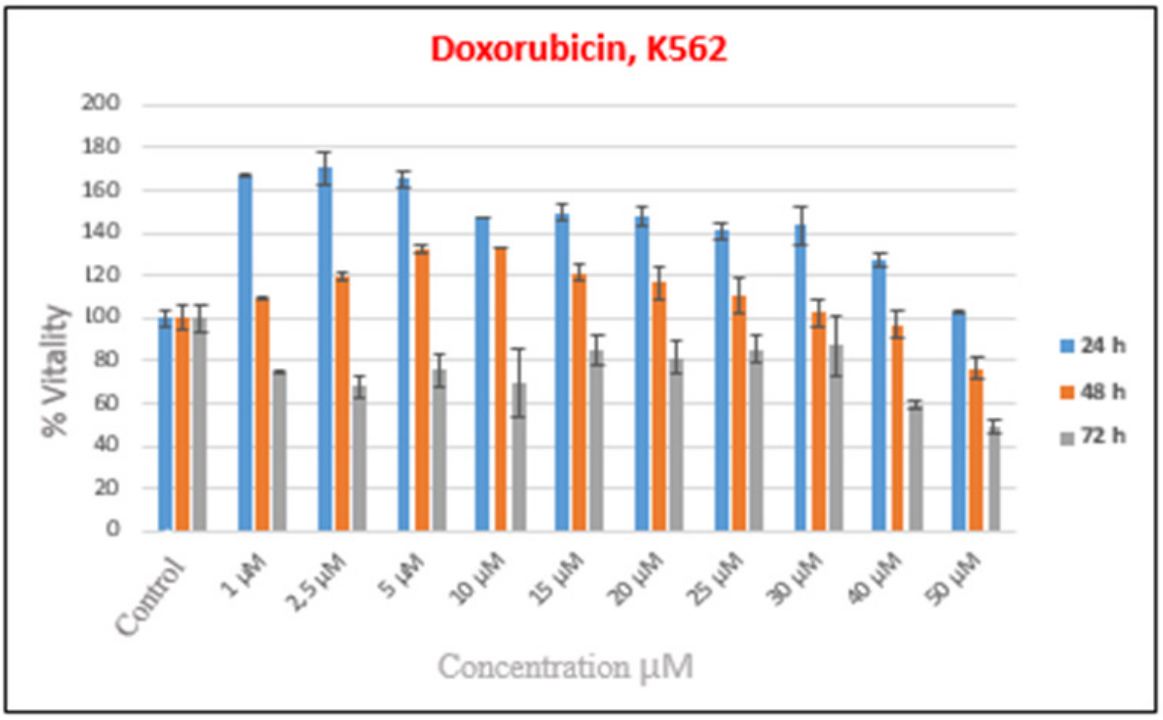
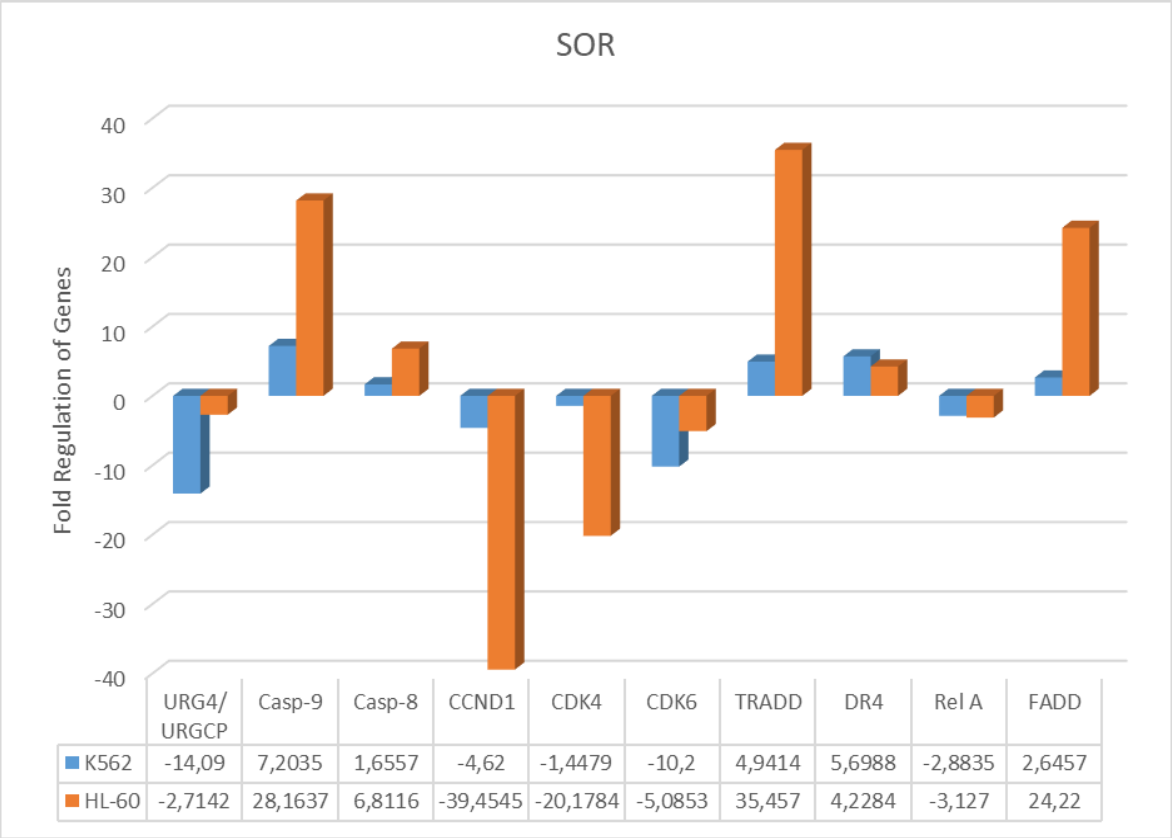


Figure 4. Cell viability percentage of Doxorubicin on K562 cell line using the CellTiter-Glo kit

Real-time PCR analysis

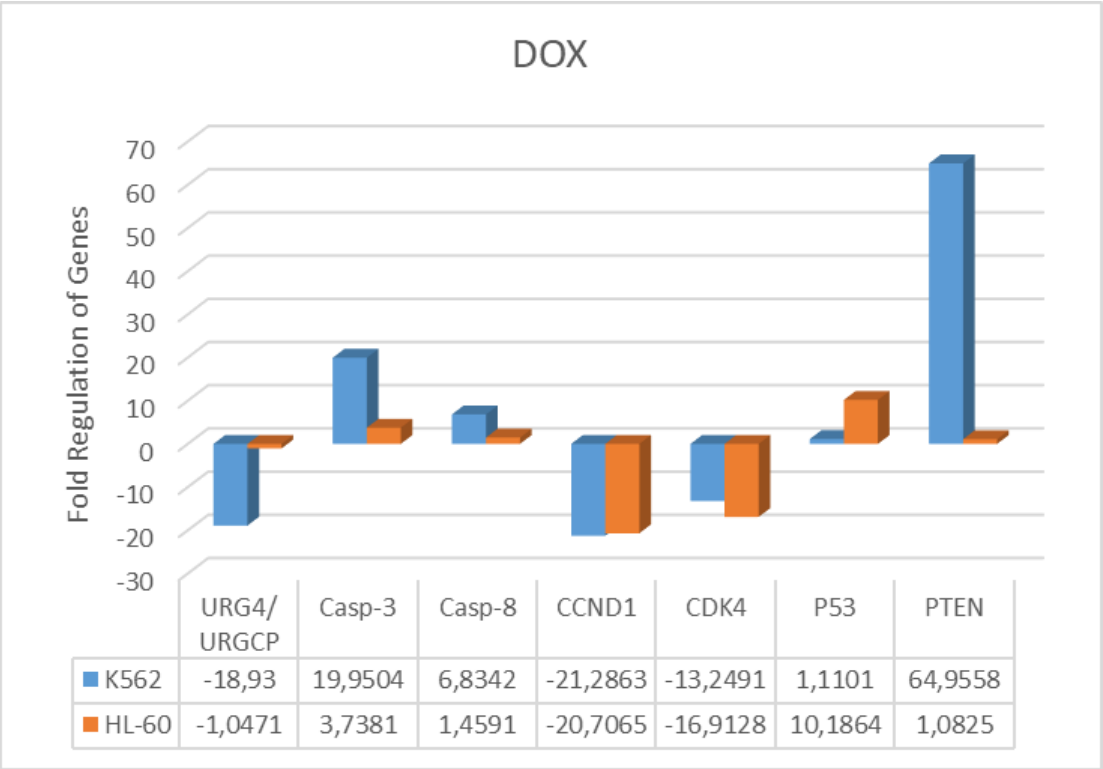
The obtained cDNAs for the SOR-treated groups were synthesized from total RNAs collected from the control and SOR-treated groups. In this research, the Real-Time PCR method was used to study the expression levels of specific mRNAs associated with cell cycle and apoptosis -related genes. Following SOR application to HL-60 and K562 cell lines, it was noted that the expression levels of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes increased, while the expression levels of URG4/URGCP, CCDN1, CDK4, and CDK6 genes decreased (Figure 5).



	URG4/ URGCP	Casp-9	Casp-8	CCND1	CDK4	CDK6	TRADD	DR4	Rel A	FADD
K562	-14,09	7,2035	1,6557	-4,62	-1,4479	-10,2	4,9414	5,6988	-2,8835	2,6457
HL-60	-2,7142	28,1637	6,8116	-39,4545	-20,1784	-5,0853	35,457	4,2284	-3,127	24,22

Figure 5. Fold regulation in mRNA expression levels of URG4/URGCP, Casp-9, Casp-8, CCND1, CDK4, CDK6, TRADD, DR4, Rel A, and FADD genes in K562 and HL-60 cell lines treated with Sorafenib

For the groups treated with DOX, cDNAs were synthesized from total RNAs collected from the control and DOX-treated groups. The Real-Time PCR method was used to analyze the expression levels of specific mRNAs associated with apoptosis and cell cycle-related genes. Within this research, the expression levels of genes related to the cell cycle and apoptosis, URG4/URGCP, Casp-3, Casp-8, CCDN1, CDK4, P53, and PTEN, were assessed in HL-60 and K562 cell lines treated with DOX. It was noted that the mRNA expression levels of Casp-3, Casp-8, P53, and PTEN genes exhibited an increase, whereas there was a notable reduce in the mRNA expression levels of URG4/URGCP, CCDN1, and CDK4 genes (Figure 6).



	URG4/ URGCP	Casp-3	Casp-8	CCND1	CDK4	P53	PTEN
K562	-18,93	19,9504	6,8342	-21,2863	-13,2491	1,1101	64,9558
HL-60	-1,0471	3,7381	1,4591	-20,7065	-16,9128	10,1864	1,0825

Figure 6. Fold regulation in mRNA expression levels of URG4/URGCP, Casp-3, Casp-8, CCND1, CDK4, P53, and PTEN genes in HL-60 and K562 cell lines were treated with DOX

Discussion

Leukemia is a type of blood cancer characterized by the abnormal and uncontrolled proliferation of white blood cells [30]. This disease typically originates in the bone marrow, where immature leukocytes can replace normal blood cells, leading to adverse outcomes [31]. Furthermore, it is a widely prevalent form of cancer globally [32]. Cancer treatments commonly involve radiotherapy, immunotherapy, chemotherapy, or, in the case of leukemia cancer, additional bone marrow transplants (BMT) are administered [33]. However, these treatment methods not only affect rapidly proliferating cancer cells but also impact normally dividing healthy cells, causing damage. Particularly, many drugs used in chemotherapy can halt or hinder cell division in the cell cycle [34]. SOR is known as a multiple kinase inhibitor. This drug generally inhibits VEGFR, PDGFRβ, c-Kit and Flt-3 [35]. It is an active drug used in cancer treatment because it has many different properties. SOR's efficacy may vary in different cancer types. It is usually related to tumor-inducing signaling pathways that control the proliferation of cancer cells and also initiates the apoptosis process [36, 37]. DOX is an anti-cancer drug isolated from the *bacterium Streptomyces peucetius*. This drug is frequently used in chemotherapy in different cancer types [38]. It is known that DOX prevents the DNA double helix from closing during replication, leading to replication arrest and cell death. In short, DOX is utilized as a treatment in chemotherapy, but it has serious side effects such as DNA damage or cell death [39, 40].

The main aim of this study is to research the impacts of DOX and SOR on the mRNA level of K562 and HL-60 leukemia cell lines subsequent to determining suitable dosages. Specifically, we aim to examine their effects on certain genes associated withal the cell cycle and apoptosis. In the study, IC50 doses of cell viability for HL-60 and K562 cell lines treated with SOR and DOX were determined using the CellTiter-Glo kit. Measurements was conducted at 24, 48, and 72 hours post-drug application in both experimental groups. Changes in response to dose and time were observed in both cell lines upon drug application. The IC50 values for SOR-treated groups were found to be 40 μM at 24 hours for HL-60 and 40 μM

at 48 hours for K562 cell lines. In contrast, for DOX-treated groups, the IC50 values were 50 μM at 48 hours for HL-60 and 50 μM at 72 hours for K562 cell lines. After determining the IC50 values for the drug groups, the mRNA expression levels of genes associated withal the cell cycle and apoptosis were analyzed using the Real-Time PCR method. Specifically, in SOR-treated groups, genes affecting both the extrinsic and intrinsic pathways of apoptosis were investigated. A significant increase in the fold regulation in the mRNA expression of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes was observed in SOR-treated groups. Many studies have reported that SOR induces apoptosis and causes cell death [41, 42]. In this study, results supporting the literature were achieved. Simultaneously, it was observed that mRNA expression levels of genes URG4/URGCP, CCDN1, CDK4, and CDK6, known to be associated withal the cell cycle, decreased in SOR-treated groups. According to the results obtained, it can be inferred that SOR induces disruption in the cell cycle, leading to cell cycle arrest. Numerous findings have been documented regarding the impact of SOR on the cell cycle [43, 44]. A notable increase in the fold regulation was noticed in the mRNA expression levels of apoptosis-associated genes, including Casp-3, Casp-8, P53, and PTEN, in the DOX-treated groups. There are various studies in the literature demonstrating that DOX induces apoptosis [45, 46]. In this study, similar results to the literature were obtained regarding the effects of DOX on apoptosis. However, a significant reduce in mRNA expression levels of DOX on URG4/URGCP, CCDN1 and CDK4 genes was found. It supported the results obtained in studies managed in the literature [47, 48]. However, in a source different from the results we found in the literature, Zuryn et al. [49] examined the effects of DOX on HL-60 cell line and found an increase in CCDN1 expression level. They stated that the increase in CCDN1 expression may also be related with the reduce in the some of apoptotic cells correlated with increased hTERT and telomerase activity and the increase in S phase cells.

This study evaluated the effects of SOR and DOX on the cell cycle and apoptotic mechanisms in both cell lines (HL-60, K562). The results obtained demonstrate that both SOR and DOX effective in regulating the mRNA expressions of URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A, and FADD genes in both HL-60 and K562 cells. In this regard, this study carries originality and will provide an important foundation for future research, making a significant present to the literature.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

Y.D. have constructed the main idea and hypothesis of the study. Y.D., M.S., L.E. and N.D. conducted experiments. Y.D., C.B.A., G.B., and L.S.T. analyzed data. Y.D. and S.S. wrote the manuscript. In addition, all authors discussed the entire study and approved the final version.