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# Digoxin Inhibits Retinoblastoma through Suppressing a Non-canonical TGFβ Signaling Pathway

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors MH, RK and FG designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors MS, AA and AS managed the literature searches, analyses of the study performed the spectroscopy analysis and authors GRM, MSK and MM managed the experimental process. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims***:* Retinoblastoma is a childhood ocular tumor rapidly developing from the immature cells of the retina due to loss of functional retinoblastoma protein. Digoxin, a cardiac glycoside, has been reported to be effective in inducing apoptosis, cell cycle arrest, and cytotoxic effects on human cancers. In this regard, the present study aims to investigate whether digoxin could suppress retinoblastoma cancer through the regulation of transforming growth factor-β (TGF-β) signaling pathway.

**Methodology:** The effects of digoxin on Y-79 cells, retinoblastoma cancer cell line, were investigated using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoli-umbromide) and BrdU (bromodeoxyuridine) assays to measure cellular cytotoxicity effects and cell apoptosis, respectively. Also, a qPCR assay was employed to analyze the mRNA expression levels of TGFβ signaling pathway including C-*MYC*, *P21*, *P15*, *TGFβRI*, *TGFβRII*, and *SMAD2*, 3, and *4* genes.

**Results:** The results of the cell function assays revealed that digoxin inhibited the cell viability and proliferation of Y-79 cells. In addition, it was found that digoxin significantly suppressed *C-MYC* expression and enhanced the expression of *P21*, *P15*, *SMAD2* and *SMAD4* genes in a dose-and time-dependent manner. However, the obtained results could not detect any significant effect of digoxin on *TGFβRI, TGFβRII* and *SMAD3* genes.

**Conclusion:** Taken together, the findings of the present study suggest that digoxin could be a potential therapeutic agent in the treatment of retinoblastoma by regulating the cell cycle genes via a non-canonical TGF-β signaling pathway.


*Keywords: c-Myc; eye cancer; gene expression; P21; P15; real-time PCR.*

## 1. INTRODUCTION

Retinoblastoma (Rb) is a rare form of eye cancer that originates from the immature cells of a retina. This intraocular malignancy is the most common childhood eye cancer. There are two forms of this cancer; bilateral (15% inheritable) and unilateral (85% non-heritable). In spite of the fact that this disease may occur as part of a familial cancer syndrome, the majority of retinoblastoma cases arise with no preceding family history [1]. Retinoblastoma is the first human malignancy that has been confirmed to have a nature of genetic origin [1,2]. The loss of Rb protein functions, owing to genetic alterations, has been accepted as a genetic factor for human cancer, particularly retinal cell malformation. However, recent studies suggested that amplification of the MYCN oncogene might initiate retinoblastoma in the presence of non-mutated *Rb* gene [3-7].

To date, major treatment strategies for retinoblastoma involve enucleation, external beam radiation therapy (RT), radioactive plaques (I-125 brachytherapy), cryotherapy, laser photoablation and chemotherapy [8-10]. Although these therapies have improved the survival rate of the cancer (up to 98%), chemotherapeutic resistance remains a significant hurdle in the treatment of retinoblastoma [11,12].

Cardiac glycosides (CGs) including digoxin, ouabain, bufalin and digitoxin have been purified from foxglove and have a long history used in the treatment of congestive heart failure and arrhythmia [13,14]. The effect of digoxin is mediated by the inhibition of the plasma membrane Na+ K+-ATPase (sodium-potassium adenosine triphosphatase), ion pump and stimulation of $Ca_2$+ influx [15,16]. Also, it has been suggested that digoxin exerts anti-proliferative and apoptotic effects against malignant cells *in vitro* and animal models [17-22].

Retinoblastoma like other types of cancer may be stratified into various biological subgroups based on their global gene expression profiles [10]. These subgroups vary in their genetic changes and may respond differently to anticancer agents. Given that the anticancer effects and molecular mechanisms of digoxin in retinoblastoma cancer cells are unknown, the present study aimed at evaluating the effects of digoxin on cellular function and mRNA expression of TGFβ target genes in Y-79, a retinoblastoma cell line.

## 2. MATERIALS AND METHODS

### 2.1 Cell Culture

The human retinoblastoma Y-79 cell line was obtained from National Cell Bank of Iran (NCBI)

affiliated to Pasteur Institute (Tehran, Iran). The cells were grown in suspension at a concentration of $10^5$-$10^6$ cells/ml in RPMI-1640 medium (Gibco; Germany) containing 10% fetal bovine serum (FBS) (Gibco; Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). Cell culture condition designed at 37℃ in a humidified atmosphere of 5% CO2-95% air.

### 2.2 Digoxin Treatment

The treatment of cells with digoxin (Sigma-Aldrich, Belgium) was conducted as previously described [23]. Briefly, digoxin was dissolved by dimethylsulfoxide (DMSO) with a concentration of 10 µM and stored at -20℃. The Y-79 cells in each experiment were treated using a different range of digoxin concentrations (as explain in the below sections). The concentration of DMSO was equal in all conditions with the exception of the untreated group.

### 2.3 MTT Assay

The cytotoxicity effect of digoxin was measured by the MTT assay as previously described [22,24]. Briefly, Y-79 cells were seeded in 96-well flat-bottom microtitration plates (SPL Life Sciences; South Korea) at a density of $4\times10^4$ cells/well (200 µl media/well). After reaching to ~85% confluence, the cells were treated with various concentrations of digoxin (0.0, 0.5, 1, 2 and 3 µM). In all *in vitro* experiments, control cells were incubated with dimethylsulfoxide (DMSO) alone (with a final concentration 0.2%).

### 2.4 BrdU Assay

The BrdU assay was performed using BrdU ELISA kit as previously described [24]. Briefly, the Y-79 cells were seeded in 96-well flat-bottom microtitration plates at a density of $4\times10^4$ cells/well (100 µl media/well). Then, the cells were treated with digoxin (0.0, 0.5, 1, 2 and 3 µM) and the cell apoptosis calculated as previously described [24].

### 2.5 RNA Isolation and cDNA Synthesis

To extract RNA from cells under study, the TriPure Isolation Reagent (Roche) was used. After treatment of Y-79 cells with digoxin followed time 24, 48 and 72 hours, RNA was extracted from cells and quantity spectrophotometric assay was done using the ND-1000 Nanodrop. The reverse transcription reaction was completed (Revert Aid First Strand cDNA Synthesis Kit, Takara BIO) as previously described [25,26]. Total RNA was transcribed to cDNA in a reverse transcription reaction. In brief, 1 µ total RNA with 2 µl dNTPs, 1 µl oligo dT, 1 µl RTase M- MULV (200 U / µl ), 1 µl RNase inhibitor (20 U / µl ) and 4 µl reaction buffer 5X. The synthesized cDNA was stored at -20℃ until use.

### 2.6 Real-Time PCR Test

Real- time PCR test was performed to analyze the expression level of genes in the treated and non-treated cells as described previously (25]. Briefly, Real-time PCR reaction in a volume of 20 µl including 10 µl of SYBR Premix Ex Taq II (Takara Bio), 4 µl of the product cDNA, 1µl of each primer (10 pmol) and 4 µl nuclease-free water was completed. Temperature conditions consisted of an initial activation step at 95℃ for 30 seconds, followed by 40 cycles of denaturation (10 seconds at 95℃), annealing (30 seconds at 60℃) and extension (45 seconds at 72℃). To evaluate the specificity of the amplified products, the melting curve of a temperature of 55 to 95 degrees was evaluated. The sequences of specific primers for studied genes are listed in Table 1. Quantitative gene expression data were normalized to the expression levels of control/housekeeping gene, GAPDH.

### 2.7 Statistical Analysis

Statistical analysis for multiple comparisons of differences between treatment groups was performed with SPSS software. Data analysis for gene expression was performed by $2^{-\Delta\Delta ct}$ method. The standard error of means was computed and analysis of variance (ANOVA, Tukey'spost tests) completed via GraphPad Prism 5.0 software. P values less than 0.05 were considered to indicate statistically significant differences between data sets.

## 3. RESULTS AND DISCUSSION

### 3.1 Digoxin Induces the Apoptosis as well as Decreases Growth and Proliferation of Y-79 Cells

To determine the inhibitory effect of digoxin on cell proliferation in Y-79, the present study analyzed the cell growth by performing MTT assay with various conditions. The data generated from the present study clearly showed that digoxin treatment significantly reduces the viability of Y-79 cells in a dose- and time-dependent manner (Table 2). Fig. 1 shows the

cell response to different drug concentrations (0.0, 0.5, 1, 2 and 3 µM) for a time course of 24, 48 and 72 h. The results of cell function assays revealed a significant change in the cell viability in the treated versus untreated Y-79 cells ($P<0.05$) at 48h and 72h.

Moreover, to examine whether cardiac glycosides can promote apoptosis in Y-79 cells, the researchers analyzed apoptotic index using a BrdU experiment in the digoxin treated cells compared to controls (Fig. 2). The apoptotic findings revealed a significant change in the cell death in the treated versus control cells ($P<0.05$) at 48h and 72h.

The obtained results showed that the cytotoxicity and apoptosis effects of digoxin on retinoblastoma cell line are time-and dose-dependent (Table 2). Interestingly, the findings of the present study revealed that the cytotoxic effect of the digoxin is not considerable after 24 h incubation regardless of the concentration in the cell line. Besides, its apoptotic effect like its toxicity seems to be a time-and dose-dependent manner.

### 3.2 Digoxin Regulates mRNA Expression of TGFβ Signaling Related Genes, p21, p15 and c-Myc

To study the ability of the digoxin as an anticancer agent at the molecular level, we evaluated its effects on mRNA expression of TGFβ signaling-related genes including cyclin dependent kinase inhibitors (CDKI), *p21*, *p15* and *c-Myc* genes which are involved in cell cycle progression. As Fig. 3 shows, digoxin could up-regulate *p15* and *p21* ($P<0.05$) while down-regulating *c-Myc* ($P<0.05$) at the mRNA expression level in retinoblastoma cells.

It is now well established that members of the cardiac glycosides including digoxin, bufalin and ouabain are currently used in the treatment of congestive heart failure and arrhythmia. Also, digoxin has been suggested to possess anticancer activity in some human cancer cells by the inhibition of cell cycle process [27-33]. The cell function assays conducted in this study revealed that digoxin significantly reduced the cell viability and induced apoptosis in Y-79 cells. The digoxin had the highest time- and dose-dependent cytotoxicity and apoptosis on the cells. As the results indicated, the effect is more significant after 48 h incubation regardless of the concentration. The potent toxic activity of digoxin and more than 2600 drugs in retinoblastoma cells has already been reported in previous studies [23]. Accordingly, the ranges of the treatment conditions in this study were designed around the reported IC50.

To further examine the ability of the digoxin as an anticancer drug, here, we evaluated its effects on mRNA expression of the genes involved in cell cycle progression in retinoblastoma cells. The findings revealed the capability of digoxin in the up-regulation of *p21* and *p15*, tumor suppressor genes, and down-regulation of *c-Myc* mRNA expression in retinoblastoma cells. Interestingly, regulation of these genes was detected as part of TGFβ signaling pathway by digoxin treatment but not on the other related genes, *TGFβRI*, TGFβRII and SMAD3.

Research efforts in the last decade have postulated various mechanisms to explain the digoxin-mediated control of tumor cell proliferation. However, the exact mechanism of the anti-tumor activity of digoxin remains unclear. Previous studies suggested that cardiac glycosides bind to Na+, K +-ATPase pump, which in turn triggers a complex cellular signaling cascade that results in the activation of MAPKs (mitogen-activated protein kinases). Subsequently, the activation of MAPKs could lead to increased expression of the cell cycle inhibitor of *p21* [34].

**Table 1. The specific primers used for studied genes**

| Gene name | Forward & reverse primers sequences | Product size (bp) |
|---|---|---|
| P15 | FW: 5 '- AGGTGGACCTGGAGACTCTCAG -3'<br>RV: 5 '- TCCTCTTGGAGAAGATCAGCCG -3' | 205 |
| P21 | FW: 5 '- ACGGAGTCAACCGTTTCGGGAG -3'<br>RV: 5 '- GGTCGGGTGAGAGTGGCAGG -3' | 218 |
| c-Myc | FW: 5 ' - AAGGTCAGAGTCTGGATCAC-3'<br>RV: 5 ' -TAACTACCTTGGGGGCCTTT-3' | 140 |
| GAPDH | FW: 5’- CACCAGGGCTGCTTTTAAC-3’<br>RV: 5’-ATCTCGCTCCTGGAAGAT-3’ | 190 |

**Table 2. Data resulted from cell function assays**

| **MTT assay** | | **24h** | | | **48h** | | | **72h** | |
|---|---|---|---|---|---|---|---|---|---|
| **Concentrations of digoxin(µM)** | **OD value (mean±SEM)** | **P value** | **Inhibition rate(IR)%** | **OD value (mean±SEM)** | **P value** | **Inhibition rate(IR)%** | **OD value (mean± SEM)** | **P value** | **Inhibition rate(IR)%** |
| Untreated | 0.053±0.004 | | | 0.059±0.002 | | | 0.074±0.002 | | |
| Control | 0.051±0.003 | 0.958 | 3.77 | 0.056±0.002 | 0.854 | 5.08 | 0.068±0.006 | 0.788 | 8.10 |
| 0.5 | 0.050±0.002 | 0.832 | 4.86 | 0.56±0.001 | 0.643 | 6.34 | 0.066±0.004 | 0.601 | 10.01 |
| 1 | 0.047±0.001 | 0.590 | 11.32 | 0.52±0.001 | 0.194 | 11.86 | 0.057±0.003 | 0.037 | 22.97 |
| 2 | 0.044±0.002 | 0.326 | 16.98 | 0.048±0.001 | 0.020 | 18.64 | 0.052±0.004 | 0.016 | 29.72 |
| 3 | 0.043±0.001 | 0.247 | 18.86 | 0.045±0.001 | 0.005 | 23.72 | 0.050±0.002 | 0.005 | 32.43 |
| **BrdU assay** | | **24h** | | | **48h** | | | **72h** | |
| **Concentrations of digoxin (µM)** | **OD value (mean±SEM)** | **P value** | **Inhibition rate(IR)%** | **OD value (mean±SEM)** | **P value** | **Inhibition rate(IR)%** | **OD value (mean±SEM)** | **P value** | **Inhibition rate(IR)%** |
| Untreated | 0.092±0.003 | | | 0.114±0.001 | | | 0.124±0.003 | | |
| Control | 0.088±0.003 | 0.937 | 4.34 | 0.108±0.003 | 0.850 | 5.26 | 0.117±0.002 | 0.732 | 5.64 |
| 0.5 | 0.087±0.002 | 0.78 | 5.01 | 0.106±0.002 | 0.76 | 6.43 | 0.116±0.002 | 0.0654 | 9.43 |
| 1 | 0.084±0.002 | 0.417 | 8.69 | 0.098±0.004 | 0.136 | 14.03 | 0.097±0.005 | 0.027 | 21.77 |
| 2 | 0.081±0.001 | 0.259 | 11.95 | 0.091±0.003 | 0.031 | 20.17 | 0.092±0.005 | 0.012 | 25.80 |
| 3 | 0.080±0.003 | 0.137 | 13.04 | 0.084±0.004 | 0.013 | 26.31 | 0.088±0.002 | 0.007 | 29.03 |

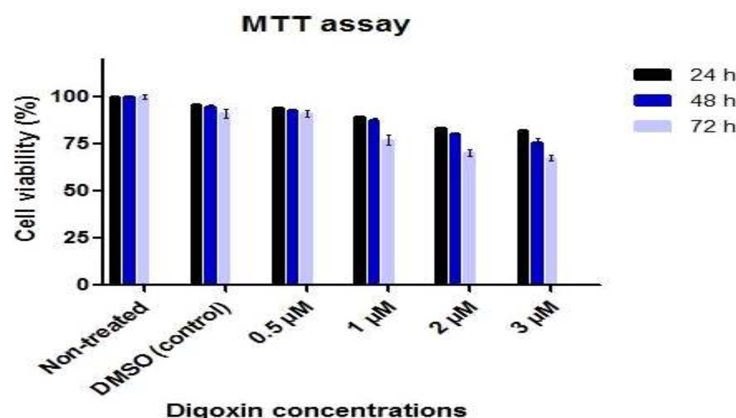


**Fig. 1. Effect of digoxin on the cell viability of the Y-79 cells**

*Y-79 cells were treated by 0.0, 0.5, 1, 2 and 3 µM for different time courses. The cell toxicity was examined by MTT assay as described in methods. All data are reported as the percentage change in comparison with the controls. Analysis of one-way ANOVA was used to compare the cell viability of Y-79 cells in different concentrations of digoxin compared to controls. Results are expressed as the mean±SEM from three independent experiments. P value < 0.05 was regarded as statistically significant*

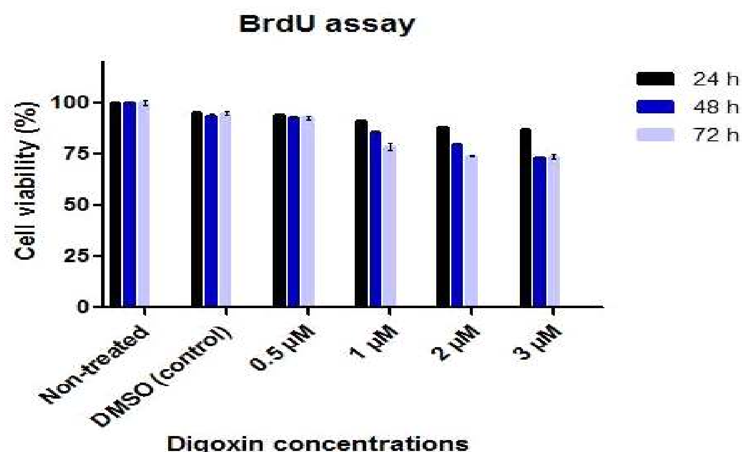


**Fig. 2. Impact of digoxin on retinoblastoma cell proliferation**

*The Y-79 cells were treated by 0.0, 0.5, 1, 2 and 3 µM for different time. Cell apoptosis was examined by BrdU assay as described in methods. Analysis of one-way ANOVA was used to compare the cell apoptosis of Y-79 cells in different concentrations of digoxin to control. Results are expressed as the mean±SEM from three independent experiments. P value < 0.05 was regarded as statistically significant*

Various studies demonstrated that *p21* could be regulated by multiple approaches and factors such as TGFβ, p53-dependent and p53-idependent manners [35-37]. There are typically several transcription-binding sites in the regulatory region of *p21*such as p53, STAT3, Sp1, Stat1 and SREBP [38-40]. Also, other molecular mechanisms and signaling pathways such as activations of PI3K/Akt MAPKs and ERK1/2 pathways could regulate the *p21* gene expression [34,41-43]. Kumar and colleagues demonstrated an up-regulation of *p21* via PI3K/Akt pathway in digoxin-treated human glioma cell lines (LN-18 and LN-229 cells [41]. Kometiani reported that digitoxin concentration close to or at the plasma level regulates *p21* gene expression in breast cancer cell line [34]. The most recent study showed the effects of digoxin on lung cancer cells via inhibiting multiple *src*-related signaling pathways [22]. The findings of the present study, in agreement with previous studies, revealed the up-regulation of *p21* in the retinoblastoma treated with digoxin compared to controls.

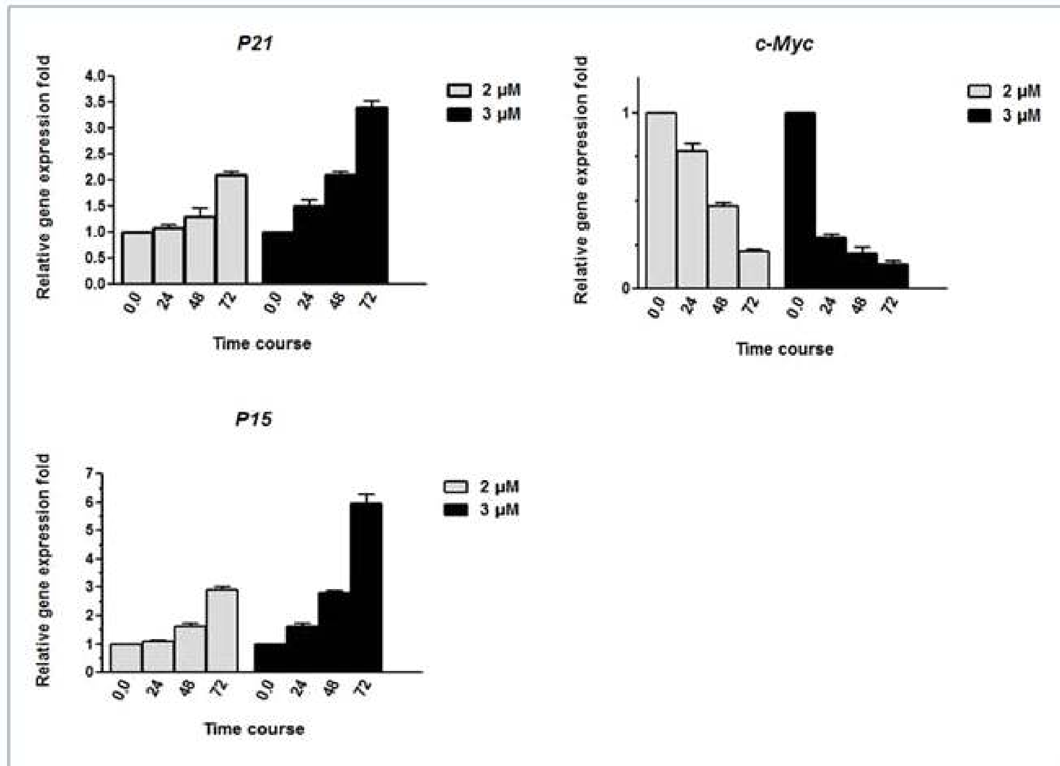


**Fig 3. Impact of digoxin on *P21*, *P15* and *c-Myc* gene expression in retinoblastoma**
*Digoxin treatment significantly regulated the expression of studied genes*

Additionally, we analyzed the expression of other TGFβ signaling related genes including *p15*, *c-Myc*, *TGFβRI*, *TGFβRII* and *SMAD2, 3, 4* genes with different digoxin concentrations (0.0, 0.5, 1, 2 and 3 µM) at three different incubation time (0.0, 24h, 48h and 72h). Interestingly, in addition to *p21*, there were significant up-regulation and down-regulation of *p15* and *c-Myc* genes, respectively. However, we could not observe any significant changes in the mRNA expression of other TGFβ related genes including *TGFβRI*, *TGFβRII* and *SMAD3*. In consistent with the results of the present study, different studies have indicated that the downstream of TGFβ signaling genes could be regulated by SMAD-independent manner [44-46].

The results obtained from this study raise a critical question: How could digoxin regulate above genes without the contribution of other TGFβ signaling compartments. With respect to the findings, there are several possibilities including: (1) drug-mediated targeted disruption of multiple protein activities through functional inhibition of the vital genes, (2) association of the TGFβRI, TGFβRII and SMAD3 mRNA expression with different miRNAs and/or long non-coding RNAs (lncRNAs), (3) the modulation of these genes under different histone modifications such as acetylation, methylation, ubiquitinilation, or (4) epigenetic (e.g., methylations) and genetic factors such as gene-gene and gene-environment interactions. Further studies are clearly required to determine whether digoxin acts as an anticancer in retinoblastoma via inhibiting Myc proto-oncogene and activating *p21* and *p15*, cell cycle inhibitors.

## 4. CONCLUSION

In conclusion, it was shown that digoxin was able to reduce cell proliferation and induce apoptosis in a dose and time-dependent manner in retinoblastoma. Moreover, as far as the authors are concerned, the results of the present study, for the first time, indicated that this chemotherapeutic agent could act via a non-canonical TGFβ signaling pathway in retinoblastoma.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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