

## Clofarabine Has Apoptotic Effect on T47D Breast Cancer Cell Line via P53R2 Gene Expression

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### Abstract

**Purpose:** Clofarabine, a purine nucleoside analogue and inhibitor of Ribonucleotide Reductase (RR), is used for treatment of leukemia. Clofarabine-induced defect in DNA replication, induces p53 and subsequently P53R2 genes as subunit of RR. clofarabine deregulated P53R2 gene expression leading to the elevated levels of P53R2 which impose resistance to DNA damaging drugs. In this study the apoptotic and cytotoxic effects of clofarabine has been investigated on breast cancer cell line.

**Methods:** Cofarabine cytotoxicity on T47D cells has been studied by MTT assay. T47D cells were exposed to the different concentrations of clofarabine for 24, 48 and 72 hours intervals. Relative expression of P53R2 gene has been studied using real-time PCR. Moreover, after treating with clofarabine the apoptotic and necrotic cells were detected using Annexin V and propidium iodide (PI) reagents by flowcytometry technique.

**Results:** MTT assay results showed that the clofarabine IC50 on T47D cell line were 3 and 2.5 μM after 48 and 72 h exposure, respectively. Clofarabine did not show any significant cytotoxic effect after 24 h exposure. The analysis of qRT-PCR showed a significant increase in P53R2 gene expression in treated cells with both 2.5 and 3 μM doses and also, the results of flowcytometry revealed 26.91 and 74.46 percent apoptosis induction in 48 and 72h treatments respectively in comparison to the control groups.

**Conclusion:** Our results showed that apoptotic and cytotoxic effects of clofarabine on T47D cell line were in time and dose dependent manner; therefore it could be considered a new candidate in breast cancer therapy.

### Introduction

After surgery, the standard procedure for treatment of breast cancer is chemotherapy. Clofarabin, one of the compounds that are used in chemotherapy, is a purine nucleoside analogue that has a strong inhibitory effect on wide-spread leukemia and some of solid tumors such as ovary, prostate, kidney and breast.<sup>1-3</sup> In the unphosphorylated form, clofarabine passes through the cell membrane using several transporting system including diffusion, facilitated diffusion and active transportation, and it is phosphorylates by intracellular kinases to its mono, di and three phosphates forms.<sup>1</sup> From these forms, only clofarabine-3 phosphate is therapeutically active, and the remaining forms act as intracellular clofarabine reservoir.<sup>2-4</sup>

Clofarabine-3P plays its roles through different mechanisms including: a) it competes with dATP during DNA replication and repair. b) it binds to allosteric site of regulatory subunits of Ribonucleotide Reductase (RR) enzyme and inhibits dCTP, dATP formation. Reducing the dCTP level limits DNA synthesis and reactivates deoxyCytidinekinase (dCK) and subsequently increases formation of clofarabine-3P. Also, high clofarabin - 3P/dATP concentration ratio results in priority of clofarabine-3P to dATP in competition for DNA-polymerase. c) It induces apoptosis through inhibition of DNA repair.<sup>2-5</sup>

P53R2, a p53-induced peptide, is a homologue of R2 subunit, which can substitute with R2 subunit in RR structure. RR is a tetramer composed of two different

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homodimers including hRRM1 (R1) and hRRM2 (R2).<sup>6,7</sup> P53, R1 and R2 are expressed in a cell cycle dependent manner. R1 and R2 expressed completely during the S-phase.<sup>8,9</sup> R2 gene is regulated by cell cycle-associated transcription factors, such as NF-Y and E2F;<sup>10</sup> however, P53R2 is regulated by p53 in response to genotoxic stresses such as irradiation.<sup>11</sup> After DNA damage, p53 not only causes to cell-cycle arrest in the G1 and G2 phases, but also induces P53R2 gene expression. Subsequently, P53R2 accumulates in cell nuclear and increases RR activity.<sup>12,13</sup> However, P53R2 activation may not be fast enough to provide dNTPs immediately, which can be completed during a few hours after DNA damage. It is shown that ATM phosphorylates P53R2 in response to genotoxic stress. This modification is essential for maintaining P53R2 protein stability against degradation by ubiquitylation.<sup>14</sup>

Because of the long half-life, R1 level is constant higher than R2 level throughout cell cycle.<sup>6,15</sup> In early G1-phase, R2, but not P53R2, is degraded by cadherin 1/anaphase promoting complex (Cdh1/APC).<sup>12</sup> Thus, in G1phase, P53R2 acts with R1 instead of R2 to provide dNTPs for DNA.<sup>15-17</sup> So there are two independent pathways in providing dNTPs: first, R2 provides dNTPs in S-phase and second, P53R2 provides dNTPs for DNA repair in G1 or G2-phase.<sup>12</sup> The maximum level of P53R2 has been found at G1/S transition.<sup>6</sup> Furthermore, P53R2 up-regulates P21 and down-regulates cyclin D,<sup>15</sup> causing cell cycle arrest in G1 and providing both time and dNTP for the repair of damaged DNA.

Also, P53R2 plays an important role in mitochondrial veracity. Based on previous studies, mutations in P53R2 are associated with severe depletion of mitochondrial DNA in both human and mouse cell lines.<sup>18,19</sup> Moreover, P53R2 may plays a role in scavenging reactive oxygen species (ROS) and thereby protecting mitochondrial membrane from oxidative stress.<sup>12</sup> In addition, P53R2 down-regulates MEK-ERK signal pathway through direct interaction with ERK-Kinase2.<sup>20</sup> MEK-ERK signaling pathway regulates various cellular processes including cell cycle progression and cell survival, thus inhibition of this pathway is another anti-cancer property of P53R2.<sup>21,22</sup>

Since the P53R2 is critical molecule in dNTP formation and DNA repair, we hypothesizes that clofarabine might plays a role in apoptosis and cancer therapy by altering the expression levels of P53R2 gene. To evaluate this hypothesis, T47D breast cancer cell line was treated with several concentrations of clofarabine in different times. The MTT assay, flowcytometry, and qRT-PCR were used for evaluation of cytotoxicity, apoptosis and gene expression, respectively.

## Materials and Methods

Human epithelial breast cancer cell line (T47D) was purchased from Pasteur Institute (Iran), RPMI1640

powdered cell culture medium was purchased from Gibco (England), Fetal Bovine Serum (FBS), Penicillin/ streptomycin 100X, Trypsin-EDTA 10X and Trypan-blue were acquired from Biosera (England); MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma (USA), PI was from CALBIOCHEM (Germany), cDNA synthesis kit and Real Time-PCR master mix were provided from Thermo Scientific (USA), RNX-Plus was purchased from Sinaclon (Iran). Clofarabine was obtained from Sigma (USA).

## Cell culture

T47D cells were propagated in RPMI1640 media supplemented with 10% fetal bovine serum (FBS), 2mM of glutamine, penicillin (100 U/ml) and streptomycin (100 g/ml), at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Then, cells were seeded into plates for MTT assay and into flasks (25 cm<sup>2</sup>) for gene expression and apoptosis studies, respectively.

## Cytotoxicity assay

Before doing MTT assay, the count and viability of cultured cells were determined using a hemocytometer. Then, for MTT assay, the cells were seeded at 5×10<sup>3</sup> cells per well in 96-well culture plates and were settled at 37°C for 24 h, followed by treatment with different concentrations of clofarabine (1.5 - 8μM). The concentration of clofarabine that inhibited cell proliferation by 50% (IC<sub>50</sub>) was determined from MTT assay data after 24, 48 and 72h treatments. Each experiment was done in triplicate manner.

## P53R2 gene expression

The cells were seeded in 7 flasks with concentration of 1×10<sup>6</sup> cells/flask. Then 3 flasks were treated with IC<sub>50</sub> of 48h exposure time for 24, 48 and 72h exposure, and other three flasks were treated with IC<sub>50</sub> of 72h exposure time for 24, 48 and 72h exposure times; and last flask was served as control group. Total RNA was extracted from cultured cells using RNX Plus kit and 1μg of total RNA was used as substrate for reverse transcription using Thermo Scientific cDNA synthesis kit according to the manufacturer's protocols. Quantification of genes expression was performed with master mix from Thermo scientific company using thermo-cycler Corbet 2000 real-time PCR machine. PCR primers were 5'-AGG CTC GCT TCT ATG GC-3' (forward) and 5'-TCT GCT ATC CAT CGC AAG GC-3' (reverse) for P53R2. Gene expression levels in each cDNA sample were normalized to the internal β-actin gene expression level (Forward primer: 5'ACCGTGAAAAGATGACCCAG3' and Reverse primer: 5'CCATACCCAAGAAGGAAGGC3'). The experiments were done in triplicate manner for each sample.

### Apoptosis assay

T47D cells were seeded at concentration of  $1 \times 10^6$  cells/ flask. One of these flasks was used as control and other two flasks were used for 48 and 72h treatment with clofarabine. The 24 h time exposure was not included in the experiments, because after treatments the 24 h exposure did not show significant cytotoxic effects on T47D cell line. After treatments, apoptosis detection kit (CALBIOCHEM, Germany) was used for apoptosis evaluation according to the manufacturer's protocol.

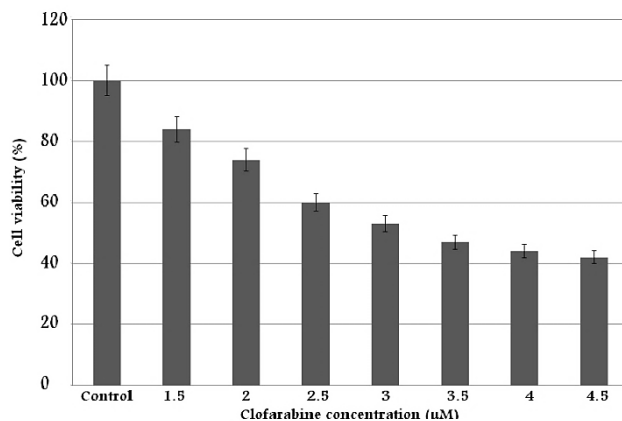
### Statistical analysis

The alteration of P53R2 gene expression was analyzed by  $\Delta\Delta Ct$  method and version 16 of SPSS (with  $p < 0.05$ ) was used for statistical analyses. The results of apoptosis experiment were analyzed by Flowjo software.

### Results

#### Clofarabine has cytotoxicity effect on T47D cell line

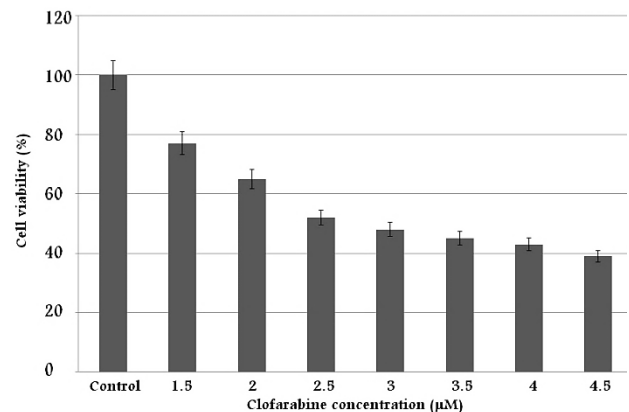
Clofarabine induces DNA damage and therefore it can impose cytotoxicity effect on T47D cells. After treatment of T47D cell line with different concentrations of clofarabine for 24, 48 and 72h, clofarabine  $IC_{50}$  was calculated as  $3 \mu M$  for 48h treatment and  $2.5 \mu M$  for 72h treatment. In this study, after 24h treatment, clofarabine did not show cytotoxic effect on T47D cell line (Figures 1 and 2). These results show that clofarabine at the longer treatment duration has a stronger cytotoxic effect on T47D cell line.



**Figure 1.** The results of MTT assay after 48h treatment with different concentrations of clofarabine.  $3 \mu M$  clofarabine is cytotoxic to half of cellular population ( $IC_{50}$  of 48h).

#### Clofarabine induces P53R2 gene expression

Clofarabine inhibits RR complex that results in gradual reduction of dNTPs pool. Low level of dNTPs inhibits or defects repair of damaged DNA which induces p53 and p53R2 proteins, subsequently. Upon binding of P53R2 to R1, they form an active RR complex that provides dNTPs for repair of DNA.



**Figure 2.** The results of MTT assay after 72h treatment with different concentrations of clofarabine.  $2.5 \mu M$  clofarabine induces cell death in half of cellular population ( $IC_{50}$  of 72h).

P53R2 expression after 24, 48 and 72h treatments with two clofarabine concentrations equal to acquired  $IC_{50}$ s ( $2.5$  and  $3 \mu M$ ) has shown in Table 1 and Figure 3. Based on the results, the higher concentration of clofarabine and longer treatment duration induce p53R2 gene expression more efficiently, but it seems that the effect of treatment duration is more significant than the effect of concentration (Figure 3). In other words, in longer treatment duration, the P53R2 gene expression was increased regularly.

**Table 1.** The effect of clofarabine on P53R2 gene expression in T47D cell line

samples	clofarabine concentration ( $\mu M$ )	P53R2 gene expression (fold change)
control	0	1
24h	$3 \mu M$	3.83
	$2.5 \mu M$	8.39
48h	$3 \mu M$	28.24
	$2.5 \mu M$	18.76
72h	$3 \mu M$	76.10
	$2.5 \mu M$	61.39

The level of P53R2 gene expression after treatment with different clofarabine concentrations ( $3 \mu M$  and  $2.5 \mu M$ ), shows that clofarabine induces P53R2 gene expression and it's level is directly related to treatment duration so that in treatment of 24h the gene expression is after the lowest level and after treatment of 72h is in the highest level.

#### Clofarabine induces apoptosis in T47D cell line

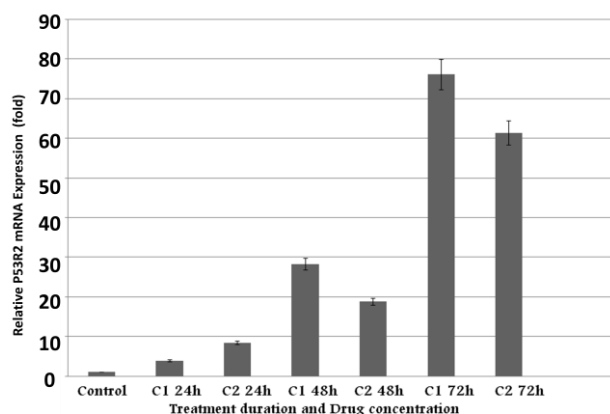
As mentioned earlier, clofarabine causes DNA damage by RR complex inhibition. And it can induce P53 gene expression that induces.

As shown in Table 2, after 48 and 72h treatments, clofarabine induced apoptosis in T47D cell line about 26.91% and 74.46%, respectively. These results show that treatment in longer duration is more effective for induction of apoptosis. The flowcytometry results for control, 48 and 72h treatments are shown in Figures 4-6.

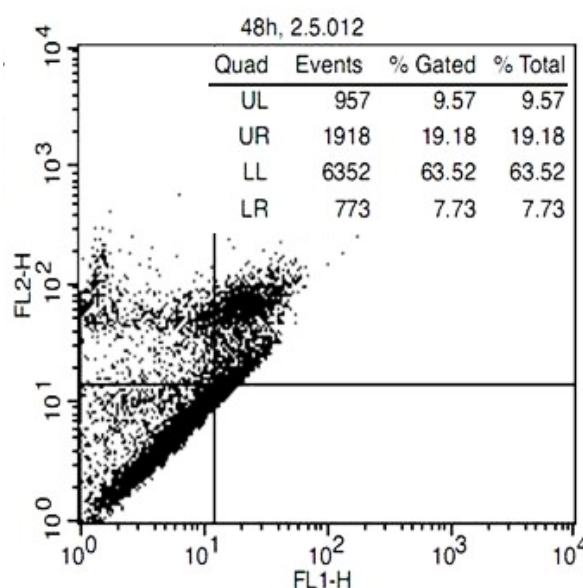
**Table 2.** The apoptotic effect of clofarabine on T47D cell line

Cell situation (%)	Live cells (LL)	Apoptotic cells (LR)	Late apoptotic and necrotic cells (UR)	Necrotic cells (UL)
Control	97.24	0.09	0.68	1.99
After 48h treatment	63.52	7.73	19.18	9.57
After 72h treatment	21.15	7.59	66.87*	4.45

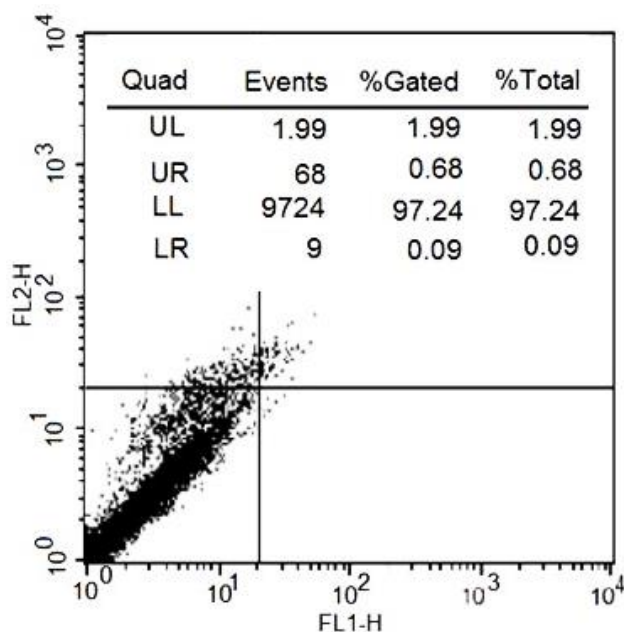
The results of flowcytometry (the percentage of live cell, early apoptotic, late apoptotic, and necrotic cells) after treatment of T47D cell line with 3µM clofarabine. The number of dead cells in the treated samples after 48 and 72h is remarkably more than the control sample. On the other hand, the number of apoptotic cells after 72h treatment is more than 48h treatment which suggests that clofarabine induces more apoptosis in longer duration treatment time. \*: apoptosis induction in the treated cells is significant in comparison to the control group.



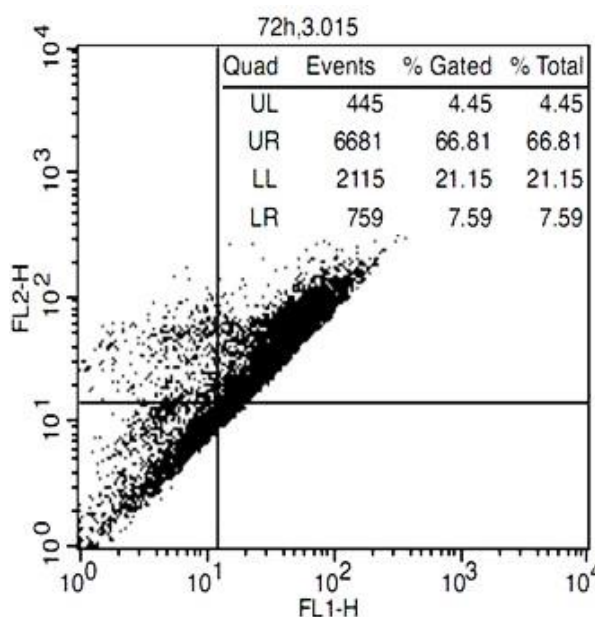
**Figure 3.** The clofarabine effect on P53R2 gene expression. As this figure shows, in higher clofarabine concentrations and treatment durations, the expression of P53R2 increases. P53R2 expression is more dependent on treatment duration than clofarabine concentration (C1=3µM and C2=2.5µM). \*: increasing in P53R2 gene expression is significant in comparison to the control group.



**Figure 5.** The flowcytometry result after 48h treatment with 3µM of clofarabine. After treatment there is a significant cell death.



**Figure 4.** The flowcytometry result for the control sample. It shows there is an acceptable amount of alive cells in the control sample (%97.24), on the other hand the number of necrotic and apoptotic cell are not remarkable.



**Figure 6.** the flowcytometry result for 72h treatment with 3µM of clofarabine. The number of dead cells has increased significantly in comparison with control and treatment of 48h samples.



## Discussion

Clofarabine is a therapeutic agent in treatment of leukemias, also, has a good inhibitory effect up on several solid tumors such as ovary and colon cancers.<sup>23,24</sup> The results of the present study show that clofarabine inhibits T47D cell growth. It means that clofarabine might have anti-cancer effects on breast cancer. It has been shown that clofarabine had anti-cancer effect by induction of p53 and P21 in MDA-MB-231 breast cancer cell line.<sup>25</sup> Considering the results of the present study, in T47D breast cancer cell line, clofarabine induces DNA damage and P53R2 gene expression. Therefore, clofarabine could play cytotoxic effect by induction of DNA damage and gene expression on T47D cells line.

Based on the results of flowcytometry, clofarabine can induce apoptosis in T47D cell line through induction of DNA damage and subsequent activation of internal apoptosis pathway. Deregulated expression of P53R2 has been shown in various cancer types.<sup>20</sup> P53R2 repairs DNA and acts as scavenger of ROS, maintaining cell survival of both cancerous and non-cancerous cells. Also, other studies showed opposite effects and in some cancers elevated P53R2 level can cause cancer progression through exerting resistance to DNA damaging therapies such as clofarabine and radiotherapy. Therefore, it is possible that clofarabine-induced P53R2 gene expression, attenuates cytotoxic effect of clofarabine on T47D cell and other solid cancer, and interestingly negligible cytotoxic effect of clofarabine on T47D cells during 24 h exposure confirms anti-apoptotic behavior of P53R2 protein in breast cancer.

## Conclusion

Clofarabine possess cytotoxic effect on T47D cell line and induces its apoptosis. On the other hand, it acts as an inducer for P53R2 gene expression. Some studies have reported that P53R2 can improve cancer cell response to therapy. Regarding to anti-growth effect of clofarabine on T47D cell line as in vitro model of breast cancer, further studies are required to study anti-cancer efficacy of clofarabine on breast cancer animal models, before using it in the clinical trial studies.

## Acknowledgments

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## Ethical Issues

Not applicable.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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