Carbenoxolone induces apoptosis and inhibits survivin and survivin-ΔEx3 genes expression in human leukemia K562 cells

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ABSTRACT

Background and the purpose of the study: Leukemia is a malignant disorder of the blood progenitor/stem cells which is characterized by abnormal proliferation of white blood cells. Although anti-cancer drugs induce apoptosis in cancerous cells, drug resistance is the significant problem mainly due to over-expression of inhibitors of apoptosis proteins (IAPs) such as survivin. In this content, it has been reported that an anti-inflammatory drug, Carbenoxolone (CBX), could induce apoptosis and growth inhibition in several types of cancerous cells. In the present study, effects of CBX on apoptosis and level of the expression of survivin gene and its ΔEx3 splicing variant have were evaluated in K562 cells.

Methods: K562 cells were cultured and treated with different concentrations of CBX (50-300 µM) at different time intervals (12-48 hrs). Trypan blue exclusion test was used to evaluate cell viability. Fluorescent microscopy (Acridine Orange/Ethidium Bromide double staining) and DNA fragmentation assay were used to study apoptosis. The expression level of survivin and its ΔEx3 splice variant were studied by RT- PCR.

Results and Major Conclusion: It was found that both growth inhibition and apoptosis occurred in K562 cells. In addition, down-regulation of survivin and survin-ΔEx3 were observed, after 2-4 hrs treatment with 150 µM of CBX. However, the expression level of survivin and its ΔEx3 splice variant increased in subsequent time (6-12 hrs) nearly to the level of control cells. From the results of this study, it may be concluded that CBX can be considered as a candidate for further studies in CML treatment, especially in the case of drug- resistant leukemia cells.

Keywords: Apoptosis, Carbenoxolone, Leukemia, Survivin.

INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant hematopoietic disorder caused by uncontrolled proliferation of blood progenitor/stem cells (1). Although, several chemotherapeutic drugs such as imatinib mesylate (Gleevec, STI571) are being used for CML treatment, drug resisting and relapse are usualy serious obstacles (2). It has been reported that drug-resistant CD34+ stem cells is the main problem in treatment of CML patients. Indeed, some drug-resisting proteins and inhibitor of apoptosis proteins (IAPs), such as survivin are highly expressed in CD34+ leukemia cells (3).

Survivin is a member of IAPs and its gene locates on chromosome 17. This gene consists of four main exons which are named E1 to E4 (4). Transcription of this gene and alternative splicing produce survivin and five other splice variants which are named 2B, 3B, 2α, 3α and ΔEx3 (4). Survivin has important role in regulation of two cellular processes; inhibition of apoptosis and promotion of cell proliferation (5).

Survivin-ΔEx3 is an anti-apoptotic splice variant of survivin which its over-expression have been reported in leukemia, lymphoma, medulloblastoma, renal and gastric carcinoma cell lines (6). There is some evidence for over-expression of survivin and survivin-ΔEx3 is associated with poor response to chemotherapy and reduced overall survival in leukemic patients (7).

Carbenoxolone (CBX) is a semi-synthetic drug which is derived from the natural triterpene compound, Glycyrrhetinic acid and has steroid-like structure (Fig. 1) (8, 9). CBX induce apoptosis by promoting oxidative stress process through mitochonderial permeability transition (MPT) and releasing cytochrome complex (cyt *c*) (10). Recently,

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some studies have represented documents that show CBX is able to induce growth inhibition as well as apoptosis in several cancerous cells, including breast, and lung cancers (11, 12).

Despite these findings, there is no organized study concerning anti-cancer effects of CBX in CML. Previously some effects of CBX on leukemic cells was reported briefly (13) and in the present article these effects were evaluated in detail. In order to examine potency of new drugs and treatment protocols, study was focused on human cancerous cell lines. K562, which is a universal experimental model of blast phase of CML and its unique characteristics such as P53 mutation and overexpression of drug-resisting proteins make it suitable model for CML stem cells (14, 15).This study was designed to investigate the inhibitory efficacy of CBX on expression of survivin and survivin-ΔEx3 in K562 cells.

MATERIAL AND METHODS

The RPMI 1640 cell culture medium and Fetal Bovine Serum (FBS) were purchased from Biosera (Germany). Penicillin, streptomycin and RNX Plus were obtained from Cinnagen (Iran). K562 cell line was obtained from Pasteur Institute of Iran (Tehran). The culture plates were obtained from SPL life science (South Korea). Acridine orange (AO), ethidium bromide (EtBr) and trypan blue were obtained from Sigma (Germany). Ethylenediamine tetraacetic acid (EDTA), Tris, Sodium Dodecyl Sulfate (SDS), chloroform, isopropanol and ethanol were purchased from Merck (Germany). RNase A/T1, proteinase K, Oligo (dT) and RevertAidTM M-MuLV Reverse Transcriptase were obtained from Fermentase (Germany).

Cell Culture

The human erythroid leukemia K562 cells were cultured in RPMI 1640 medium supplemented with FBS (10% v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml) in 5% CO_2 humidified atmosphere at 37°C.

Cell growth and viability assay

CBX stock solution was prepared with RPMI 1640 medium. Cells were seeded into 96-well plates at density of 5×10^4 cells/well and incubated with different concentrations of CBX for various time intervals (12, 24 and 48 h). Cells were counted based on ability of the cells to exclude trypan blue using a hemocytometer.

Morphological study of the apoptotic cells

Apoptosis was determined morphologically after staining with AO/EtBr by fluorescence microscopy. Briefly, cells were washed in cold PBS and adjusted to a cell density of 1×10^6 cell/ml of PBS. AO/EtBr solution (1:1, v/v) was added to the cell suspension at a final concentration of 100µg/ml. The cellular morphology was evaluated using fluorescence microscope (Ziess-Germany) (16).

DNA fragmentation assay

DNA fragmentation in K562 cells was measured after extraction of DNA from a constant number of cells. After treatment, cells were collected and treated by 20 µl lysis buffer (100 mM EDTA, 20 mM Tris, pH=8, 0.8% SDS), followed by10 µl of RNase A/T1 and proteinase K, separately and then incubated at 50 °C overnight. The extracted DNA was separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml).

RNA extraction and RT-PCR

Total RNA of each sample was extracted from leukocytes by adding RNX Plus according to the manufacturer´s instructions and using chloroform as well as isopropanol.

For cDNA synthesis, oligo $(dT)_{18}$, primer (MWG, Germany) and RevertAidTM M-MuLV Reverse Transcriptase were used in a 20 ml reaction. PCRs were performed using cDNA with *Taq* polymerase (Cinnagen), $MgCl_2$, dNTPs, primer and buffer supplied by the company in 25µl reaction volumes. Survivin (NM-001168): Forward Primer 5'-TGGCAGCCCTTTCTCAAG-3'. Reverse Primer: 5'-GAGAGAGAGAAGCACCAC-3'.Forward primer Nested: 5'ACCACCGCTCTACATTC-3'. Reverse Primer Nested: 5'CTGGTGCCACTTTCAAGAC-3'. β2m (NM-00114048): β2m Forward primer 5'-CTACTCTCTCTTTCTGGCCTG-3'. β2m Revers primer:5'-GACAAGTCTGAATGCTCCAC-3'. Expression of β2m (candidate housekeeping gene) was monitored as internal control. PCR products were visualized under ultraviolet illumination using 1.5% agarose gels containing ethidium bromide. Results were expressed as Survivin/β2m ratios by uvitec software. Each result is average of three tests.

Statistical analyses

Data were statistically analyzed with Student's-t-test using Microsoft Excel 2003 and SPSS (version 14) software. Data with $P<0.05$ were considered significant.

RESULTS

Inhibition of growth and viability of K562 cells by CBX

The effects of different concentrations of CBX on growth and viability of K562 cells were studied for various time intervals. As shown in figure. 2, cell viability and growth were inhibited in K562 cells by CBX in a time- and dose-dependent manner. For example, the growth inhibitory effects of CBX in K562 cells were observed after 12 hrs exposure and continued to increase in a time-dependent manner.

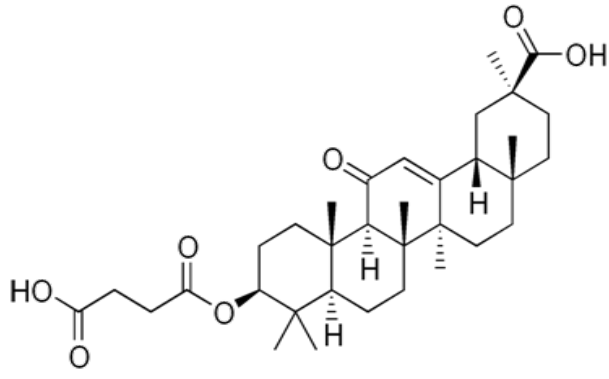

Figure1. The structure of carbenoxolone (CBX)

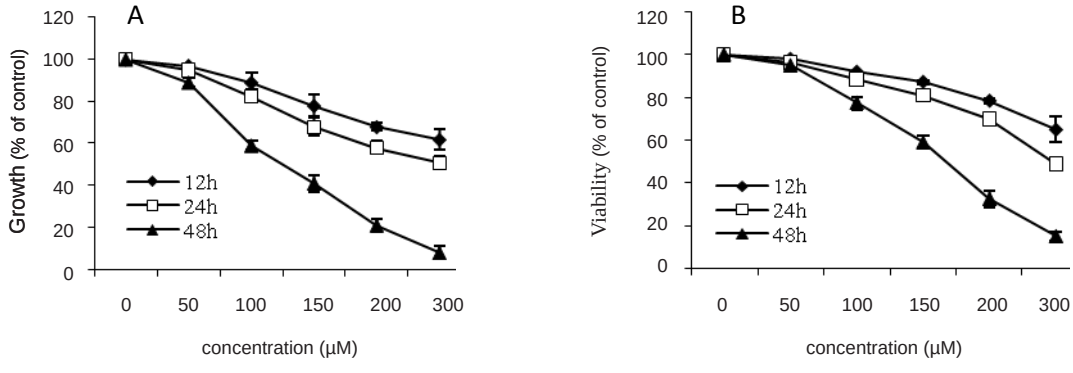

Figure 2. Effects of CBX on growth inhibition (A) and viability (B) of erythroleukemia K562 cells. Cells were exposed to the indicated concentration (50-300µM) for 12 h (♦), 24 h (□) and 48 h (▲). The number of viable cells was determined by Trypan blue exclusion test. Cell viability and growth inhibition in each treatment was expressed as a percentage of the control. Each value represents the mean ±SEM of three independent experiments.

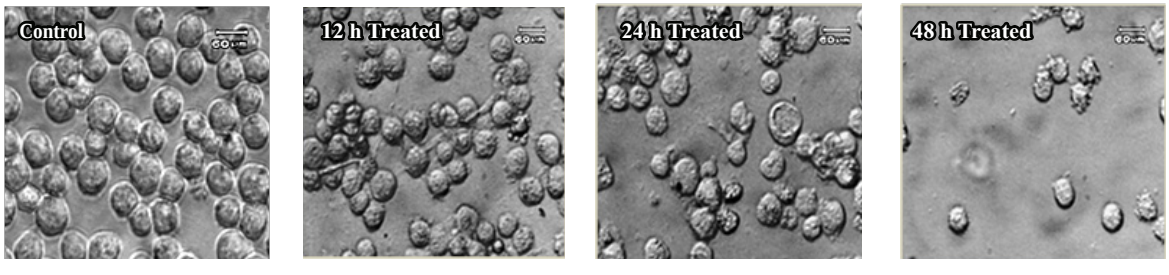

Figure 3. Morphological changes of K562 cells treated with CBX. K562 cells treated with 150 µM of CBX. Photomicrographs of the cells were taken by a light microscope at a magnification of 40×. The dead cells are shown by black arrows.

Indeed, the growth of the cells was inhibited by 38%, 46% and 92% with 300 µM CBX at 12, 24 and 48 hrs, respectively (Fig. 2A). The viability of cells after 48 hrs of treatment with concentrations of 50, 100, 150, 200 and 300 µM CBX, decreased by almost 5%, 22%, 41%, 67% and 85%, respectively (Fig. 2B). IC_{50} (the concentration inhibiting 50% of in vitro cell growth) was about 150 µM after 48 hrs of treatment.

Induction of morphological changes in K562 cells by CBX

As it is shown in figure 3, CBX induced morphological changes in K562 cells in a time-dependent manner. Indeed, after short exposure time (12 hrs), some of cells died. By increasing the

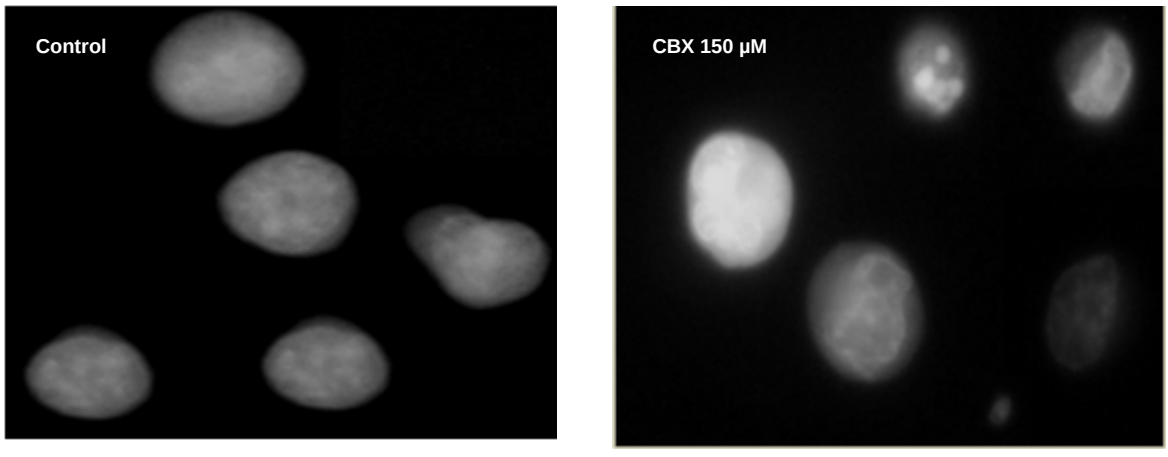

Figure 4. Fluorescence microscopic analyses of K562 cells. The cells were incubated with 150 µM of CBX for 24 h, along with membrane permeable acridine orange and ethidium bromide which is impermeable to the normal membrane but stain the nuclei of late apoptotic cells (orange color). Magnification 100×.

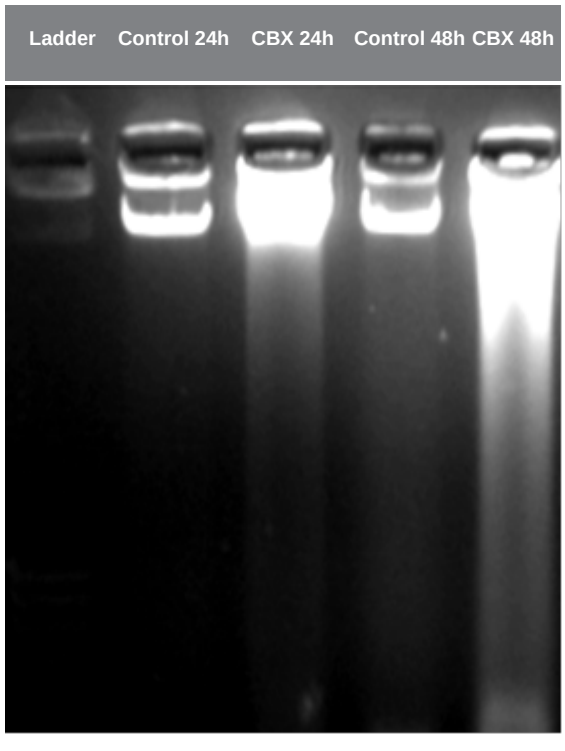

Figure 5. The effect of CBX on DNA fragmentation. The K562 cells (5×10^5 cell/well) were seeded in 24 wells plates and treated with the 150 µM of CBX for multiple and different time intervals. DNA was extracted as explained in materials and methods.

exposure time (24-48 hrs) most of the cells showed morphological features of cell death.

Induction of apoptosis in K562 cells by CBX

Because the CBX exerted profound effects on growth and viability of K562 cells, induction of apoptosis in CBX-treated cells was also investigated. Analysis of the drug-treated K562 cells by AO/EtBr staining showed that CBX could induce apoptosis in the cells (Fig. 4). Despite the resistance of K562 cells to most anti-cancer agents, apoptosis was clearly observed in these cells after treatment with CBX (150 µM). Early apoptotic cells were green and contained bright green dots in their nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells uptake EtBr and therefore stain orange (Fig. 4). In addition to AO/EtBr staining, DNA fragmentation assay also confirmed apoptosis. Although, oligonucleosomes were not detected in the treated K562 cells, DNA degradation was documented in these cells (Fig. 5) (16). Cell death was observed after 6 hrs of treatment (data not shown).]

Inhibition of survivin and survivin-ΔEx3 expression in K562 cells by CBX

The expression level of survivin and its splice variant, survivin-ΔEx3, were studied using semiquantative RT-PCR (Fig. 6). The RT-PCR analysis revealed that CBX (150 µM) inhibited gene expression level of survivin and survivin-ΔEx3 after 2-4 6 hrs. However, the expression level of survivin and survivin-ΔEx3 increased (to the level of control cells) after prolonged treatment (6-12 6 hrs).

DISCUSSION

Although, various drugs have been proposed for treatment of CML, adverse effects and drug-resistance are pivotal obstacles for complete remission. Therefore, many efforts are in progress to find new drugs with capability of inducing apoptosis (17). According to the results, CBX at 50-300 µM concentrations inhibited K562 cells growth and viability in a dose- and time-dependent manner with IC_{50} values of 150 µM after 48 hrs of exposure. The growth inhibitory effects of CBX have been reported in other cancer cells (18). Apoptosis induction in K562 cells by CBX have been shown by using DNA fragmentation assay and fluorescence microscopy. Internucleosomal DNA

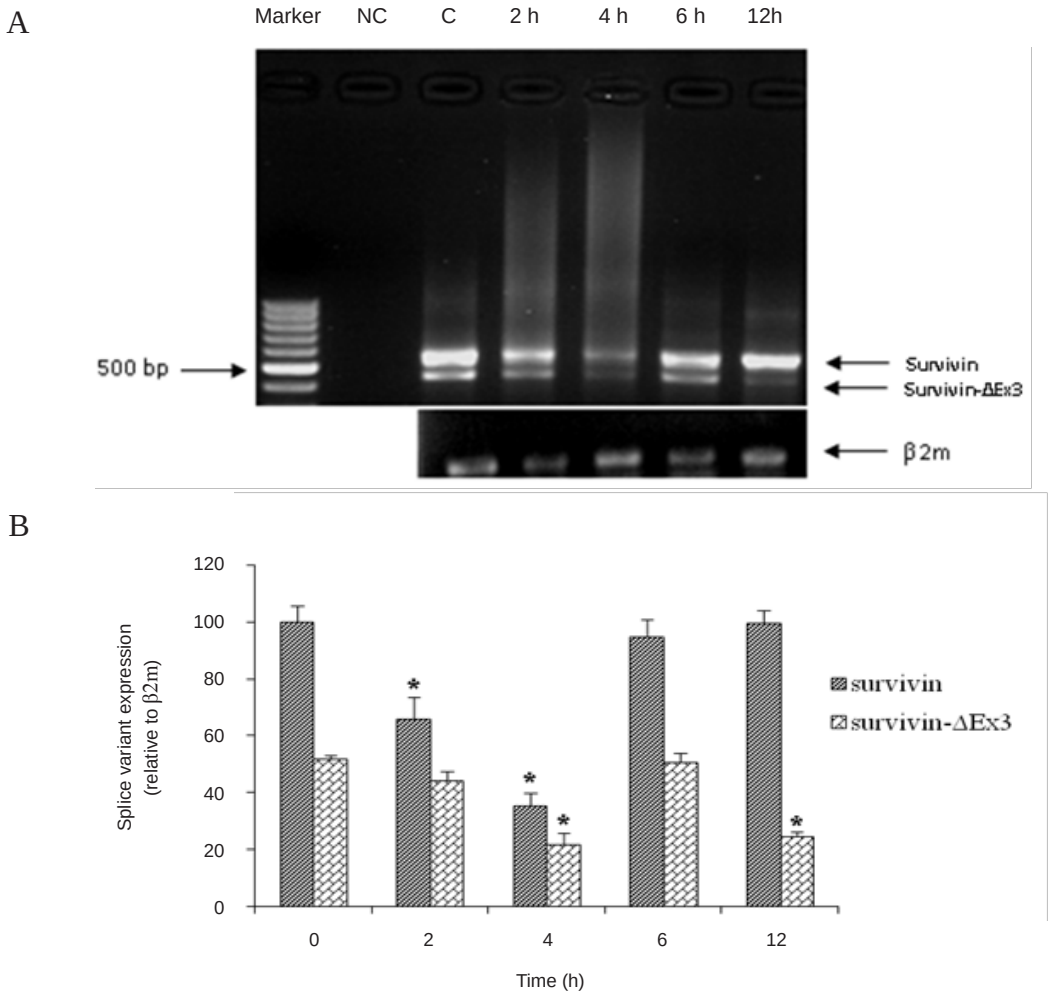

Figure 6. CBX effects on expression level of survivin and survivin-ΔEx3 genes. K562 cells were incubated with CBX (150 µM) for various times of treatment (212- h), the expression levels of survivin and survivin-ΔEx3 mRNA were detected by using RT-PCR (A) and were studied by Uvitec software (B). Expression level of β2m was used as internal control. The values were shown by ∗ are significant respect to the corresponding control, statistically. Each value represents the mean ±SEM.

fragments were not clearly observed in this type of cells mostly due to deactivation of caspase activated DNA (CAD) enzyme (19). Apoptotic effects of CBX have been reported in a few in vivo and in vitro models. For instance, apoptosis was induced using CBX at 20-300µM (similar to concentrations of this experiment) in rat hepatocytes and also in basal zone of rat placental cells (20, 21).

Drug-resisting is a major obstacle of curative treatment, and then many efforts are in progress to find new drugs that other than induction of apoptosis have the ability to overcome drug resistance. Survivin is one of the cancer-specific genes which have been reported that is up-regulated in almost all human tumors and leads to drug-resisting (21). Survivin-ΔEx3 as a novel anti-apoptotic splice variant of survivin was identified recently and it is highly expressed in some cancers such as leukemia (6). In the present study, effects of CBX on expression of survivin and survivin-ΔEx3 were investigated. It is noteworthy that CBX down-regulate these genes in K562cells in a special pattern. Indeed, it is shown that CBX (150 µM) down-regulate survivin and survivin-ΔEx3 in K562 cells after short incubation times (2-4 hrs), while the expression level of survivin and survivin-ΔEx3 increased after longer treatment times (6-12 hrs). Since the growth inhibitory and apoptotic effects of CBX was initiated 6 hrs after treatment, it may be concluded that K562 cells underwent apoptosis through down-regulation of survivin and survivin-ΔEx3 expression. However, compensatory mechanisms may increase the expression level of this gene up to the level of control cells due to prolonged treatment and further studies are needed to prove these mechanisms (22). Similarly one study has shown that daunorubicin and mitoxantrone induced down regulation of survivin in HL60 cells after 24 hrs incubation, while the

expression level of survivin increased after longer times (72 hrs incubation time) (23).

CBX behaves as a typical inducer of MPT and release of cyt *c*, so it can be considered as a potential pro-apoptotic agent (10, 24). Furthermore, it may induce apoptosis via interaction with glucocorticoid and mineralocorticoid receptors due to its structural similarity (25). Additionally, various experimental studies have confirmed inhibitory effects of CBX on gap junction-mediated intercellular communication (GJIC) which has some significant function in attached cells (26). From the results of this study it may be suggested that the mechanism of action of CBX in K562 cells (as suspended cells) might be genomics based, through down-regulation of survivin and survivin-ΔEx3 gene expression. In this regard, as mentioned about its growth inhibitory, apoptotic and down-regulatory effects on Survivin and Survivin-ΔEx3 expression, CBX can be used as a potential candidate for further studies in CML treatment.

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