RESEARCH ARTICLE

Oxidative Stress-Induced Apoptosis in Chronic Myelogenous Leukemia K562 Cells by an Active Compound from the Dithio-Carbamate Family

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Abstract

Previous studies suggested that dithio-carbamates are potent apoptosis and anti-apoptosis inducing agents in various cancer cells. Here, the anti-proliferative and apoptosis inducing effects of a new derivative (2-NDC) from the dithio-carbamate family was examined in human leukemia K562 cells. We use thiazolyl blue tetrazolium bromide (MTT) to measure viability and cell growth inhibition. The 2-NDC showed effects on viability in a dose and time-dependent manner, inhibiting proliferation at concentrations of 10-30 µM after 24-48 hours of treatment and increasing values after 72 hours at 40-120 µM. The cytotoxic effect of the compound was calculated with an IC_{50} of 30 μM after 24-hour. Apoptosis induction was confirmed by acridine orange-ethidium bromide (AO/EtBr) staining, DNA fragmentation assay, flow cytometric assessment and also caspase-3 activation assay. Furthermore, enzymes level such as superoxide dismutase (SOD) and catalase (CAT) involved in oxidative stress were evaluated. The results of this study demonstrated insignificant increase of intracellular ROS levels for 24 hours and reduction after 48-72 hours. In addition to reduction of intracellular thiol, caspase-3 like activity was also decreased in a time-dependent manner in cells treated with 2-NDC. Thus 2-NDC can be considered as a good candidate for further pharmaceutical evaluations.

Keywords: Apoptosis - dithio-carbamate - oxidative stress - caspase-3 - K562 cells

Asian Pac J Cancer Prev, **17 (9)**, 4267-4273

Introduction

Chronic myeloid leukemia (CML) is a type of clonal bone marrow stem cells disorder. This disease involving increased unregulated growth of myeloid cells in the bone marrow and the accumulation of these cells in the blood (Wong and Witte, 2004). Over 90% of Chronic myeloid leukemia is the result of a reciprocal recombination between chromosomes 9 and 22; t(9;22) (q34;q11) which generate shortened chromosome 22 (Philadelphia) and an elongated chromosome 9 (Ghasemian et al., 2015). In the majority of patients with CML breakpoints in ABL gene on chromosomes 9 and breakpoint in the BCR gene on chromosomes 22 result in the formation of a BCR-ABL gene (van Oostveen et al., 1999). The BCR-ABL gene produces a dysfunctional protein called "Bcr-Abl tyrosine kinase." The Bcr-Abl tyrosine kinase, which leads to the abnormal regulation of cell growth and proliferation, is the reason for the development of CML (Gesbert et al., 2000; Macdonald et al., 2004).

Intracellular redox imbalance is one of the apoptosis reasons. Reactive oxygen species (ROS) are produced through aerobic metabolism and are mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Das, 2002). Also exogenous antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, vitamin A prevent excessive production of free radicals by combining with them. In fact when the redox balance is disrupted, creating a oxidative stress condition (Diplock, 1994). In oxidative stress condition, accumulation of oxidative damage to critical biomolecules (including genome, protein, lipid and carbohydrate) results in several biological disorders, such as alterations in signal transduction and gene expression to mitogenesis, mutagenesis, transformation and cell death (Hunt et al., 1998; Matés and Sánchez-Jiménez, 2000). A full knowledge of the correlation between redox state and apoptotic proteins could underpin the development of oxidative stress associated disorders treatments.

Dithio-carbamate (DTC) molecules exhibit both pro-oxidant and antioxidant activities (Nobel et al., 1995; Orrenius et al., 1996; Liu et al., 1996; Burkitt et al., 1998; Cereser et al., 2001). These compounds are organosulfur

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compounds with a general structure (R1R2)N-(C=S)-SX, that R group can be replace by an alkyl, alkylene, aryl, or similar other group, and X group usually can be replace by a metal ion (Edwards et al., 1991; Kamrin, 1997). For the first time dithio-carbamate (DTC) was used as fungicides during World War II (Ware and Whitacre, 2004). The DTC anions can react with other molecules containing SH groups and form metal chelates. The multisite interactions of DTC influence the biological activities of different proteins and enzymes. Some compounds of DTCs have been clinical applications (Morrison et al., 2010). They induce pro-oxidant effects by the disulfide bridges and the metal chelating and exerting antioxidant effects by the SH group (Orrenius et al., 1996; Elskens and Penninckx, 1997).

In the present study, we demonstrated oxidative stress-induced apoptosis of 2-Nitro – 1- Phenylethylpiperidine -1- Carbodithioate (2-DTC) (Figure 1); the active compound from dithio-carbamate family in human leukemia K562 cells, by using cell cycle and DNA fragmentation assay different methods. We also evaluated anti-oxidant and pro-oxidant effects of 2-DTC through assay of intracellular ROS levels using flow cytometery and assessment of proteins and enzymes level that are involved in oxidative stress.

Materials and Methods

Materials

The cell culture medium (RPMI 1640), fetal bovine serum (FBS), ethidium bromide (EtBr) and Acridine orange (AO) were purchased from sigma chemical Company (Germany). Penicillin–streptomycin was purchased from Biowest France. The culture plates were obtained from SPL (Korea). MTT [3-(4, 5-dimethyltiazol-2-yl)-2, 5 diphenyltetrazolium bromide] and dimethyl sulfoxide (DMSO) were purchased from sigma aldrich (Germany). Propidium iodide (PI), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), cell extraction buffer and agarose were obtained from Invitrogen Company (USA). Caspase-3 activity assay kit was purchased from Roche (Germany). DTNB (5,5'-Dithiobis-(2-Nitrobenzoic Acid)), EDTA (Ethylenediaminetetraacetic acid), Tris base and Tris-Hcl, coomassie brilliant blue, pirogalol, phosphoric acid, citric acid, boric acid, H2O2, methanol, ethanol, isopropanol, chloroform were purchased from Merch company (Germany). DNA extraction kit (contain CL, DS and DE buffers) was provided from Research Center of Tabriz University of Medical Sciences. Caspase-3 activity assay kit was purchased from Roche (Germany). The K562 cells were obtained from Pasture Research Institute, Tehran, Iran.

Cell culture and treatments

K562 cells were cultured in RPMI 1640 medium added with FBS (10%, v/v) and 1% (v/v) penicillin-streptomycin (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) at 5% CO_2 and 37 °C. For treatment of the K562 cells, drugs were dissolved in a minimum quantity of DMSO (less than 0.1% (v/v)) followed by dilution with RPMI 1640 medium and then added at various concentrations and times.

Cell viability assay

MTT was used as an reagent for determining viability by its mitochondrial-dependent reduction to formazone (Denizot and Lang, 1986). For evaluation of the cytotoxicity effect of the 2-DTC, the K562 cells were seeded at a density of 5×10^4 cells/ml in a 96-well plate. The cells incubated in RPMI 1640 with 10% FBS and treated with different concentrations of the compound for 24-72 hours. 20μL of MTT (5 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed and the formazone crystals were dissolved using DMSO. The absorbance was read at 570 nm with a microplate reader (Bio-Tek, USA).

Apoptotic cells morphology and DNA fragmentation assay

Acridine orange/ethidium bromide (AO/EB) staining is used to distinction of apoptotic cells (Kasibhatla et al., 2006; Rahimi et al., 2015). For evaluation of apoptotic cells 5×10^4 cells/ml were treated with indicated concentration of 2-NDC for 24 h. After this time, the cell morphology was detected by an inverted microscope (Olympus, Germany). The cells were also stained with 1 µl of AO/EtBr solution (100 mg mL-1), then viewed using a fluorescence microscope (Olympus BX41, Germany).

DNA fragmentation assay is based on fragmentation morphological features of apoptotic cells. DNA fragmentation was measured by extracting genomic DNA from a constant number of cells (Basnakian and James, 1994). The K562 cells were treated with a single dose (at IC50 value) of the 2-NDC for 24-72 hours. The cells were washed with PBS and the cell pellet was incubated with 500 μl of lysis buffer (CL buffer) for 10 min at 50 ºC. After added 1 ml chloroform/ isoamyl alcohol (24:1) and centrifugation at 12,000 ×g for 10 min, three-phase was formed. The upper phase was contains DNA. It was isolated, then incubated with 500 μl of DS buffer on ice for 30 min followed by centrifugation at 12,000 ×g, 4 ºC for 10 min. The dry sample pellets were resuspended in DE buffer and loaded into the 2% agarose gel containing ethidium bromide, and then electrophoresed for 30 min.

Cell cycle analysis

This approach is based on propidium iodide (PI) DNA staining. This method distributes the cells in four major phases of the cycle (sub-G1, G1, S and G2/M) based on the amount of DNA in the cell. Increasing sub-G1 cell population of treated cells predicate to enhancement of apoptotic cells (Nicoletti et al., 1991). The K562 treated cells were fixed in 70% ethanol at -20 °C and washed twice with PBS. The cells were then stained with 50 µg/ml propidium iodide for 10 min in the darkroom at 37 ºC. The stained cells were analyzed by flow cytometry. The results represent the percentage of cells in each phase.

Assay of intracellular ROS level

2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for measuring intracellular ROS level. This dye can permeate into cells and hydrolyzed by intracellular esterase to

DCFH which is trapped within cells. In the presence of hydrogen peroxide, peroxidases can oxidize DCFH2 to 2'-7'-dichlorofluorescein (DCF). The amount of peroxide produced can be measured by an increase in DCF fluorescence at 530 nm when the sample is excited at 485 nm (Curtin et al., 2002; Ko et al., 2004). In the present study, cells were treated with 30 μM of the 2-NDC for 24-72 hours. The cells were incubated with 50 μl DCFH-DA (10 μM) for 30 min. Then, after twice washing with PBS amount of fluorescence was detected using flow cytometry.

Antioxidant enzymes assay

Superoxide dismutase (SOD) activity was measured according to the method described by Beutler (Jing and Zhao, 1995). In this assay pyrogallol uses as a reagent. In alkaline environment, pyrogallol is autoxided and produces superoxide anions. SOD converts the highly reactive radical superoxide into the less reactive peroxide. The inhibition of pyrogallol oxidation by SOD was measured at 420 nm, and the amount of enzyme producing 50% inhibition was determined as one unit of enzyme activity (IU/mg protein). Briefly, 1 ml Tris-EDTA buffer 5 mM (pH=8.0), 50 μl of sample and 50 μl pyrogallol 0.2 mM added to cuvette. Kinetic absorbance was read in 420 nm.

Catalase (CAT) was measured by Aebi method, that monitoring the decomposition of hydrogen peroxide (Aebi, 1984). 1 ml Tris-EDTA buffer 5 mM (pH=7.0) and 25 μl hydrogen peroxide (10 mM) added to cuvette. Absorbance decrease based on the decomposition of H2O2 was measured at a wavelength of 240 nm for 30 second.

Total SH groups assay

Total -SH groups were measured using DTNB (5, 5´-dithiobis- 2- nitro benzoic acid) as Ellman reagent (Sedlak and Lindsay, 1968). This reagent reacts with the SH groups and produces a yellow color. Its absorbance was measured with spectrophotometrically at 412 nm. To perform this assay, 1 ml Tris-EDTA buffer (pH 8.6) was added to 50 μl of sample and absorbance of sample was read at 412 nm (A1). Then, 25 μl DTNB reagents (10 mM) was added to the cuvette mixture and after 15 min (stored in laboratory temperature), absorbance was read again (A2). The absorbance of DTNB reagent was read as a blank (B).

Total thiol concentration (mM) = (A2 - A1 - B) × 1.07/0.05 × 13.6

Caspase-3 activity assay

Caspase-3 activity was measured by the caspase-3 colorimetric assay kit, according to the manufacturer's instructions. Caspase-3 colorimetric substrate (DEVD-pNA) is cleaved upon caspase activation, releasing the pNA moiety, which can be measured at 405 nm, using a plate reader (Bio-TEK, USA). After 24-48 hours of treatment, the cells were collected by centrifugation at 250 ×g, the supernatant was removed and discarded, and the cell pellet was lysed in Tris-buffered salin (TBS) containing tween 20, at 4°C for 10 min. After centrifugation at 15,000 ×g for 1 min, the supernatant was harvested and the concentration of the proteins was determined by Lowry's method (Lowry et al., 1951). The supernatant was then incubated with DEVD-pNA at 37°C for 1 h. The cleavage of the peptide was spectrophotometrically quantified at 405 nm. Background readings from the cell lysates and buffers were deducted from the readings of both the treated and untreated samples before calculating the fold-increase/or decrease in caspase activities.

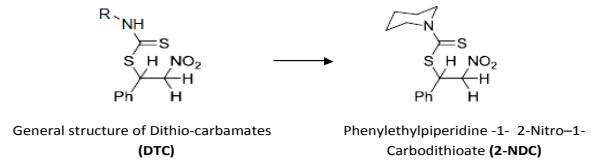
Statistical evaluation

Data are expressed as mean ± SD of three independent experiments and statistically analyzed using student's t-test. Differences were considered significant at $p < 0.05$.

Results

Growth inhibition and viability

The K562 cells were treated with different concentrations of the 2-NDC (10–120 μM) for 24-72 h. The cell viability was determined using MTT assay. The 2-NDC induced cell death in a dose and time-dependent manner (Figure 2). IC50 values, following 24 and 48 h exposure were found to be 30 ± 3.0 and 20 ± 2.5 μM respectively. The result showed that significant increase in cell growth and viability at high-dose (> 30 μM) and time (after 72 h treatment). Viability of the treated cells were decreased in 10-30 μM dose of the 2-NDC in comparison with untreated cells and increased in 40-120 μM dose compared to the treated cells by indicated doses (IC50 values) after 24 and 48 h. Viability of the treated cells was promoted after 72 h treatment compared with 24 and 48 h. In the presence of 30 μM of the 2-NDC for 24, 48 and 72 h, there were only 51.29±3.35, 46.7±4.03



General structure of Dithio-carbamates **(DTC)**

Phenylethylpiperidine -1- 2-Nitro–1- Carbodithioate **(2-NDC)**

Figure 1. General Structure of Dithio-carbamates and 2-Nitro–1- Phenylethylpiperidine -1- Carbodithioate (2-NDC)

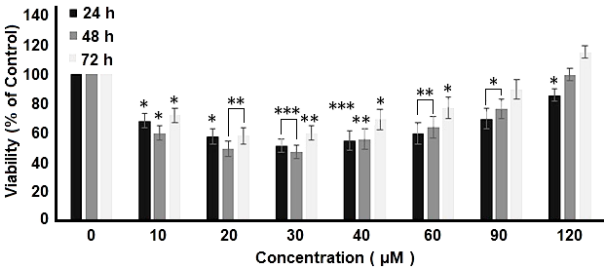


Figure 2. Viability of the K562 Cells Upon Exposure to the 2-NDC. The K562 cells were treated with various concentrations of 2-NDC (10-120 μM) for 24-72 h. The cell viability was determined by MTT reduction assay. The survival of untreated cells was normalized to 100%. Results are means ± S.D of three independent experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ vs

Table 1. Changes in the Activities of Catalase (CAT), Superoxide Dismutase (SOD) and Concentration Total Thiol (SH level) in the K562 Cells Treated with 2-NDC.

Groups	Control	24 h	48 h	72 h
CAT activity (U/mg protein)	27±5.37	29.40±6.71	19.14±4.66	14.28±3.81
SOD activity (U/mg protein)	356.01±42.81	451.49±32.65	268.22±39.72	235.51±37.21*
Total thiol (μM/mg protein)	54.49±5.37	59.22±6.71	52.73±4.66	39.79±3.81*

Results are means ± S.D of three independent experiments.* Significantly different from control ($p < 0.05$).

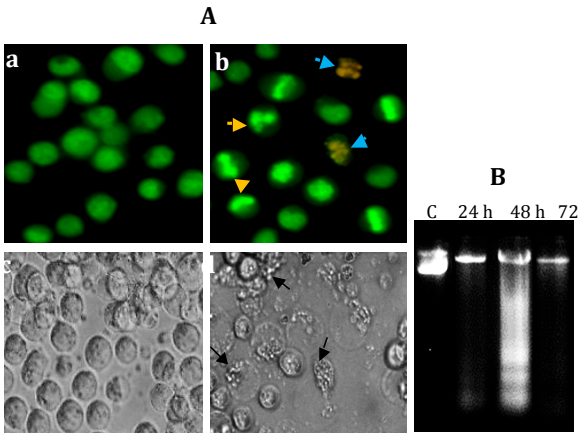


Figure 3. Morphological Changes of K562 cells Treated with 2-NDC (at IC_{50} value). A. Fluorescence microscopy of the control and treated K562 cells stained with AO and Et/Br (a and b) and light microscopy image of the control and treated K562 cells by (c and d) after 48 h. The viable cells were observed with white arrow; early apoptosis cells with orange arrow and late apoptosis whit blue arrow. B. DNA fragmentation pattern of the treated cells with the compound on the gel electrophoresis for 24-72 h

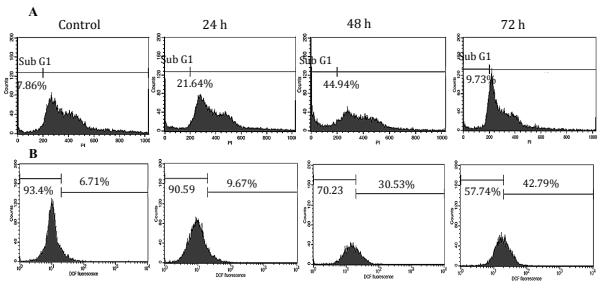


Figure 4. Flow Cytometric Analysis of Cell Cycle and ROS. (A) Flow cytometric analysis of the untreated and treated cells with indicated concentration (IC50) of the 2-NDC. Sub-G1 cell population indicated by M1 region. (B) Effects of the 2-NDC on intracellular ROS production. The K562 cells were treated with 30 μM 2-NDC for 24-72 h. Then, cells were incubated with DCHF-DA and detected by flow-cytometry

and 62.91±4.71% viable cells as compared to the control cells, respectively.

Confirmation of apoptosis by morphological changes

The morphological changes of the treated K562 cells with 2-NDC were studied by optical and fluorescence

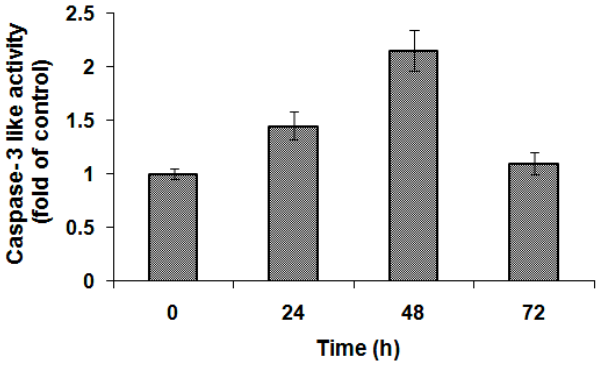


Figure 5. Evaluation of Caspase-3 Like Activity in 2-NDC Treated K562 Cells at Different Times (24-72 h). The K562 cells were treated with 2-NDC for 24-72 hours. Processing of the caspase-3 activation was inhibited after 72 h treatment. Results are means ± S.D of three independent experiments. ($p < 0.05$)

microscopy after 48 h (Figure 3). The control cells were round and crystal form while some of the cells exposed to drug were wrinkles and condensed (Figure 3A (c and d)). After staining the cells with AO and Et/Br, the control and viable cells were observed normal green color (white arrow); early apoptosis cells had condensed and fragmented bright green chromatin (orange arrow) and late apoptosis cells had condensed and fragmented orange chromatin (blue arrow), under the fluorescence microscope (Figure 3A (a and b)).

To confirmation of apoptosis, cells were cultured in the presence of indicated concentration (IC50 values) of the 2-NDC. Observation of the DNA fragmentation on the gel electrophoresis further confirmed apoptosis in the treated K562 cells with 2-NDC (Figure 3B).

Analysis of DNA content

The result of DNA content analysis of the treated K562 cells with the 2-NDC revealed sub-G1 cell cycle compared to untreated cells. We performed flow cytometric analysis of DNA content to identify effects of the 2-NDC on the cell cycle. Following 24 and 48 cell treatment with 30 μM of the 2-NDC there was a 13.78%, 37.08% increase in accumulation of the treated cells in sub-G1phase of the cell cycle, respectively compared to untreated cells. Following 72 h exposure, this was associated with a decrease in sub-G1 proportion of the cells (Figure 4A). These results confirmed the previous data (viability and gel electrophoresis).

2-NDC induced oxidative stress in K562 cells

To examine ROS generation by treated K562 myeloid cells with the 2-NDC, we measured intracellular peroxide levels using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) staining. The K562 cells were treated with 30 μM of 2-NDC for 24-72 h. Then, cells incubated with DCFH-DA and the level of cellular ROS was determined by flow-cytometry. As shown in Figure 4B, 2-NDC (30 μM) enhanced ROS production 23.82% and 36.03% after 48 and 72 h treatment, respectively, in compared to control cells.

The effect of 2-NDC on redox state of K562 cell

In order to explore the effect of 2-NDC on K562 cell, antioxidant defense system capabilities and total thiol level were evaluated according to appropriate methods reported in materials and methods (Table 1). There was an elevation in the activity of CAT, SOD and total thiol (SH) level after 24 h and significantly decreased after 48-72 h of treatment, compared with corresponding control groups.

2-NDC inhibited caspase-3 activation

Caspase3 acts as an important indicator in apoptosis. This protein exist as inactive proenzyme which is processed and cleaved into 17 kDa and 12 kDa subunits and then actived by caspases8, -9 and 10. Association of two p17 and two p12 subunit lead to produce the active heterotetrameric enzyme (active caspase-3). To investigate caspase-3 activity in K562 cells treated with the 2-NDC (30 μM), we used caspase-3 enzyme assay. The results were evidenced to this fact that 2-NDC prevents of caspase-3 activity by over time. Exposure of K562 cells to 2-NDC for 72 h led to reduction of caspase-3 activity as compared to the treated cells after 24 and 48 h (Figure 5). Inhibitory effect of this compound on caspase-3 proenzyme processing was resulted in inhibition of apoptosis process. These findings confirm our previous data that this compound time dependently induces (24-48 h) and inhibits (72 h) apoptosis.

Discussion

Chronic myeloid leukemia is a malignant blood cancer which is often associated with a chromosomal disorder. With respect to the factors of drug resistance in blood cancer cells, extensive efforts have been used to find a specific chemical drug to inhibit proliferation of the malignant cells (Galluzzi et al., 2010). Dithio-carbamates are a family of effective compounds that have cell growth inhibition and apoptosis induction effects (Della Ragione et al., 2000; Cvek and Dvorak, 2007; Zahran et al., 2014; Lin et al., 2015). According to the dithio-carbamate structure, it is able to react with various molecules and protein. Hence, they perform their role by different pathway. Pyrrolidine dithiocarbamate (PDTC) and Disulfiram (DSF) as the dithio-carbamate family members, are known as the inhibitors of nuclear factor-κB (NF-κB) signaling pathway that cause of apoptosis induction (Cvek and Dvorak, 2007). PDTC increase accumulation of cell cycle regulatory proteins (p21 and p53) as well as induce apoptosis through down-regulating ubiquitin-proteasome system (Lin et al., 2015). Thalidomide dithiocarbamate is also a pro-apoptotic and anti-cancer compound that induce apoptosis through inhibition of histone deacetylases and activation of caspases in Hep-G2 and HCT-116 cells (Zahran et al., 2014). In this research, the ability of oxidative stress to induce apoptosis in treated K562 cells by 2-NDC from dithio-carbamate family was reported. This compound has cytotoxicity effect in low concentrations (10-30 μM) and proliferative inducing effect in high dose (> 30 μM). The effect of growth inhibition and induction of apoptosis by dithio-carbamate family members in K562 and other cell lines have been confirmed (ZHANG et al., 2009; Matsuno et al., 2012). It has been reported that induction and inhibition of proliferation in the cells treated with dithio-carbamate family is depend on dose and time (Denizot and Lang, 1986; Juan et al., 2005; Torres and Horwitz, 1998). For example, PDTC increase cell death by inhibition of ERK and NFκB pathway in indicated concentrations (100 μM). Also this compound causes enhancement of proliferation and viability in the higher dose of 100-150 μM through increase expression of phospho-ERK and NFκB activity (Chung et al., 2000).

Attention to the anti-oxidant and pro-oxidant effect of some dithio-carbamate derivative (Nobel et al., 1995; Moellering et al., 1999; Johnson et al., 1999; Xie et al., 2014) and the obtained result of viability assay that show increase and decrease apoptosis in the treated K562 cells by 2-NDC; lead us to conclude that 2-NDC has biphasic effect included pro-oxidant property in low dose and time, and anti-oxidant effect in high concentration and time. PDTC induces an increase in cellular GSH levels through increased activity of γ-glutamylcysteine synthetase at low concentrations (0–25 μM) on endothelial cells, whereas, GSH oxidation and apoptotic cell death occur at higher concentrations (Moellering et al., 1999). A dithio-carbamate derivative, 2-acetylamino-3-[4-(2-acetylamino-2carboxyethylsulfanyl thiocarbonylamino) phenylthiocarbamoylsulfanyl] propionic acid (2-AAPA) disturbed the balance of redox state by inhibited glutathione reductase and thioredoxin reductase activities and decreased the GSH/GSSG ratio in H9c2 rat cardiomyocytes (Xie et al., 2014).

The results of proteins and enzymes level measuring, as well as intracellular ROS levels analysis by flow cytometery demonstrated that 2-NDC was more potent at creating a state of oxidative stress induced apoptotic cell death. These results (Figure 4B, Table 1) did not confirm the data obtained from induction of apoptosis (Figure 3 and Figure 4A). Noble et al., demonstrated that PDTC and DSF are able to black activator(s) of caspase-3 (CD95, FAS/APO-1) and inhibit processing of the caspase-3 proenzyme while reducing intracellular thiol, in T-cells pre-treated with etoposide (as an inducer of apoptosis) (Nobel et al., 1997). The caspases are family of cysteine proteases that have cysteine residues in the caspase active site. Dithiocarbamate disulfide can through the formation of a mixed disulfide with thiol group of caspases inhibit activity of caspase.

The result of investigated caspase-3 cleavage in K562 cells treated with the 2-NDC showed a decrease in caspase-3 activity after 72 h of treatment. It suggests

that 2-NDC perhaps by reducing intracellular thiol leads to create a disulfide dithio-carbamate (DC). DC disulfide reacts with the free thiol group on a caspase-3 activator(s) and prevent from procaspase-3 cleavage. Thus, inhibition of caspase-3 activity led to decrease in apoptosis and followed by an increase in cell viability (Figure 2 and Figure 4A).

In conclusion, our study demonstrated that the new compound of dithio-carbamate family (2-NDC) exhibits an anti-proliferative effect on the human chronic myeloid leukemia K562 cells. According to our data, it shows that 2-NDC inhibits cell viability of the K562 cells associated with oxidative stress-induced apoptosis in a time and dose dependent manner. Our findings suggest that this compound can be considered as a great candidate for pharmaceutical evaluations.

Acknowledgements

The authors appreciate support of this investigation by the research council of University of Tabriz, Tabriz, Iran. We would also like to thank Dr. Saeed Balalaei for providing the 2-NDC.

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