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Original Article

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Evaluation of Cytotoxicity, Cell Cycle, and Apoptosis Induction of Methyl Thiosemicarbazone Complex with Copper on K562 Cell Line

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Abstract

***Background & Aims*:** Chronic human myeloid leukemia (CML) is caused by mutations and changes in stem cells. This study aimed to investigate the toxicity, apoptosis, and cell cycle of thiosemicarbazone complex with copper on the human chronic myelogenous K562 leukemia cell line.

***Materials & Methods*:** After culturing the human K562 cell line, it was exposed to the combination of methyl thiosemicarbazone complex with copper in different concentrations and durations. Trypan blue dye exclusion test and MTT were used to determine cell viability and cell growth inhibition. The occurrence of apoptosis was examined by dual acridine orange/ethidium bromide (AO/EB) fluorescent staining and fluorescence microscopy, cell cycle analysis, and dual PI/AnnexinV staining using flow cytometry.

***Results*:** The data obtained from the present study showed morphological changes resulting from apoptosis and cell cycle arrest in Sub G1 in the presence of phosphatidylserine in the outer leaflet of the cell membrane due to treatment with thiosemicarbazone compound. It also decreased the biological growth of the K562 cell line in a concentration-/ and time-dependent manner.

***Conclusion*:** effective at low concentrations and short duration of action, this compound can be a suitable candidate for future pharmacological studies on treating CML.

***Keywords*:** Chronic human myeloid leukemia, Cytotoxic Effects, K562, Pharmacological Studies, Thiosemicarbazone

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Introduction

Cancer is one of the leading causes of death in developing countries. The incidence of cancer in different countries is estimated at 8 million per year and this number is increasing. According to the World Health Organization (WHO), Cancer mortality rate is 45%, which will reach 65% in 2030 (1). The pattern of

cancer varies in different populations and depends on factors such as occupation, nutrition, economic and social, racial, and geographical issues (2).

Chronic myeloid leukemia (CML) is a type of adult leukemia that is reported in 1-2 cases per 100,000 people, accounting for approximately 15% of all cancers. According to annual statistics in the United States, it is estimated that by 2030 it will reach 180,000 cases (3).

CML leukemia is a type of blood malignancy that originates in hematopoietic stem cells (HSCs). Myeloid cells are formed at different stages of puberty by the oncoprotein BCR-ABL1, which is an unregulated tyrosine kinase, and can be detected in peripheral blood and bone marrow (4). CML leukemia occurs in 90-95% of the patients with Philadelphia chromosome crossover t(9; 22) (q34; q11.2) (5).

Cytogenetic abnormalities and blast increase represent the most consistent indicators of progression to end-phase CML. End-phase CML comprises early progression with emerging high-risk additional chromosomal abnormalities (ACA) and late progression with failing hematopoiesis and blast cell proliferation (6).

CML leukemia affects the bone marrow cells that make up the bone marrow tissue and has a chronic course. The disease most often affects adults but occurs at any age (7). The average age for CML diagnosis in Western countries is approximately 57-56 years. Patients over the age of 70 constitute more than 20% of all CML leukemia patients, and in developing countries with a young population, the average age is less than 50 years (9, 8).

There are different types of cell death, which are generally divided into two categories: physiological death and necrosis. Types of physiological death include apoptosis, autophagy, and several other types of death, among which the molecular mechanism of apoptosis has been studied the most (10). Apoptosis is a highly evolved defense mechanism against cell death triggered by a wide range of extracellular or intracellular stimuli including growth, environmental, and intracellular stress signals. Apoptosis as a genetic control process, plays an important role in tissue development and homeostasis, and has been considered as a potent mechanism of tumor protection (11). Apoptosis is one of the main mechanisms against cell carcinoma and is the main protective mechanism against uncontrolled grow and development of cancer cells by using various mechanisms such as increasing anti-apoptotic proteins and decreasing pre-apoptosis proteins. For this reason, apoptosis has attracted much attention in anti-cancer research (12, 13).

Thiosemicarbazones have good performance in the field of medicine and are very important due to their pharmacological properties; they play an important role in the treatment of some bacterial, antifungal, antiviral infections, including HIV. They also have anti-cancer and anti-inflammatory properties. The biological functions of Thiosemicarbazones are well known, and many studies have been performed in this field (14).

According to some studies on the Thiosemicarbazones and the biological effects of metal derivatives of thiosemicarbazones, we decided to design a study to investigate the toxicity, apoptosis, and cell cycle of thiosemicarbazone complex with copper on the human chronic myelogenous K562 leukemia cell line.

Materials & Methods

RPMI 1640 culture media and Fetal Bovine Serum (FBS) were purchased from Gibco BRL UK, dimethyl sulfoxide from Merck, Germany, and K562 cells from Pasteur Institute, Iran. MTT powder, penicillin / streptomycin antibiotics, propidium iodide powder, acridine orange, agarose powder, and phosphate buffer were purchased from Sigma, USA, and Trypan Blue from suvchem, India.

Cell Growth:

For the growth of K562 cell line, RPMI1640 culture media enriched with L-glutamine, fetal bovine serum (FBS) 10%, along with penicillin and streptomycin antibiotics with concentrations of 100 U/mL and 100 g/mL, respectively, were used. The cells were incubated with 5% CO2 and 95% humidity at 37 ° C during culture.

Cell Proliferation and Cell Viability Tests:

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for proliferation and bioavailability. This colorimetric test is based on the breakdown of the yellow MTT tetrazolium salt into purple formazan crystals by metabolically active cells. This cell reduction is mediated by the involvement of the pyridine nucleotide cofactor and mitochondrial dehydrogenases NADH and NADPH. The formed formazan crystals are soluble in Dimethyl sulfoxide (DMSO), and the adsorption of the resulting dye solution is quantitatively investigated using ELISA. This test can be used to determine the toxicity of the substance to cells as well as to determine the half maximal inhibitory concentration (IC50) (15). In order to perform this test, 3×10^4 K562 cells were cultured in 96-well plates and then were treated with different concentrations of the desired composition for 24, 48, and 72 hours. Upon completion of different times after treatment, 20 µl of MTT solution was added to each well. The reaction of conversion of MTT to formazan was then performed over a period of 4 hours. Next, the supernatant was decanted and 200 µl of DMSO was used to lysing the existing cells and dissolve the formed formazan crystals. After dissolving the crystals, Sample absorbance of each well was measured at a wavelength of 570 nm using a microplate reader.

Cell Cycle Analysis:

Flow cytometry was used to evaluate the cell cycle and DNA content of the treated cells. For this purpose, the cells were cultured in containers of 24 wells and treated with different concentrations of the drug in different time periods. After collection and centrifugation at 800G, the cells were washed with cold PBS and fixed with 70% ethanol. Cells were then stored at -20 °C until analysis day. To evaluate the cell cycle stages, the control and treated samples were mixed with 20 mg of propidium iodide (PI) and 20 mg of RNase (dissolved in 1 ml of PBS) for 30 minutes and examined by flow cytometry (16). Partec PAS-III flow cytometer (Partec, Munster, Germany) was employed.

Study of Apoptosis Using Fluorescent Microscope:

The occurrence of apoptosis was studied performing a DNA fragmentation apoptosis assay. Initially, boric acid-treated cells were exposed to 10 mM Tris-HCl EDTA lysis buffer, SDS 0.1 W/V, at pH 7.4. After centrifugation, DNA was extracted using phenol-chloroform-isoamyl alcohol combination. Isolated DNA was precipitated with absolute ethanol and 5 M NaCl overnight. Finally, DNA precipitate was dissolved in TE buffer (10 mM Tris-HCl, and 1 mM EDTA) and loaded on 1.5% agarose gel at 10 volts.

Results

K562 Cells Growth and Viability Evaluation:

MTT assay was used to evaluate the effect of methyl thiosemicarbazone (4-MTC-Cu^{2+}) on K562 cell line. The MTT assay is generally used to measure cytotoxicity and cell proliferation. For this purpose, cell suspension was cultured in 96-well plates and treated with different concentrations of the drug (0-200 µM) for 24, 48, and 72 hours, and the absorbance of each well was read at 570 nm using ELISA reader. Survival percentage was calculated for each concentration at the respective time, relative to the control. According to the results presented in (Figure 1), this compound has a toxic effect on K562 cell line and reduces its viability. In addition, according to the presented results, the studied compound has a concentration-dependent and time-dependent toxic effect on the cells. So that, the IC50s after 24, 48, 72 hours were 130, 110, and 100 µM, respectively. The data are statistically significant compared to the control group. Subsequent tests were performed to determine cell death at a concentration of 100 µM. Figure 1 shows the data as mean ± standard deviation (SD) of three independent replications.

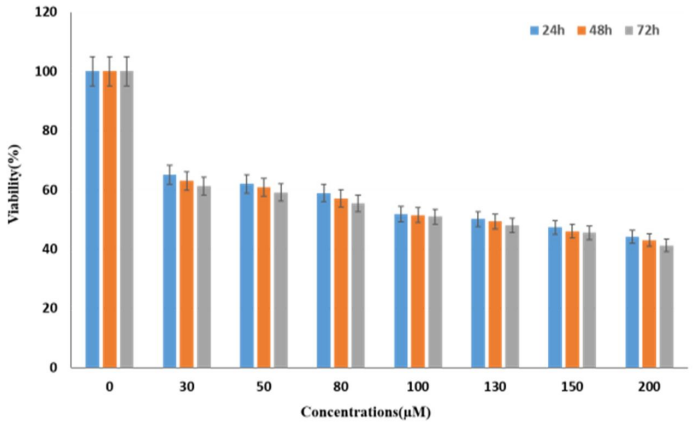

Fig. 1. Evaluation of the effect of 4-MTC-Cu2+composition on the K562 cells growth and viability. The values presented in the diagram are the means of three independent replications ± standard deviation.

Occurrence of Apoptosis Induced by Thiosemicarbazone:

Using a light microscope, the results showed apparent changes in K562 cells treated with IC50 (100 µM) over the time. Subsequent treating the cells with thiosemicarbazone for 72 hours, resulted considerable changes in their morphology. The treated cells are folded or wrinkled compared to the control cells and the dead cells are detectable (Figure 2).

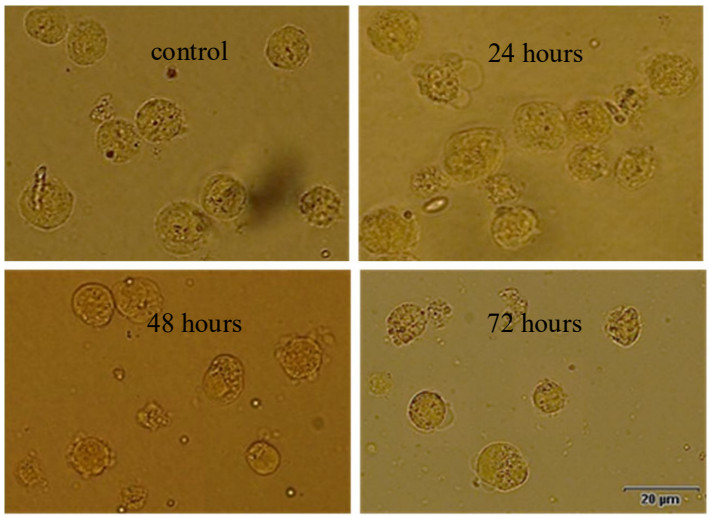

Fig. 2. Morphological changes of K562 cells treated with 100 µM of 4-MTC-Cu2+ in 24 to 72 hours.

Occurrence of Induced Apoptosis by the Studied Compound Using Fluorescence Microscopy:

Acridine Orange/Ethidium Bromide (AO/EB) Staining was used to detect the occurrence of cell death and to diagnose cell death as apoptotic. As shown in figure 2, cells with a uniform nucleus and green color are living cells; among the cells treated by thiosemicarbazone compound 100 µM after 24, 48, and 72, cells with green shrunken pyknotic and fragmented nuclei indicated early apoptosis. Cells that are in the secondary apoptotic stage have a fragmented nucleus and are yellow (reddish-orange). The uniform orange nucleus indicates cellular necrosis (Figure 3).

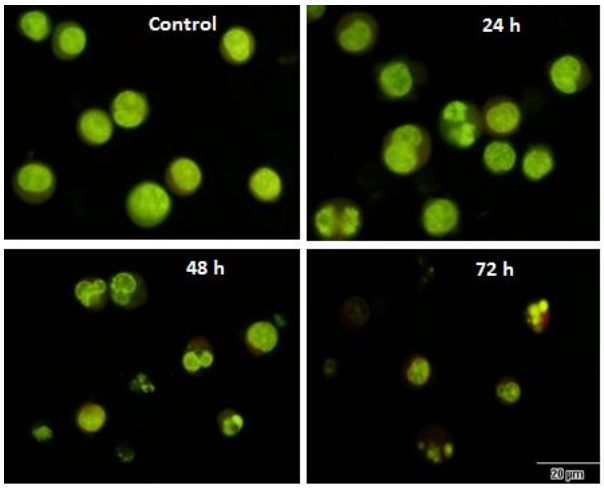

Fig. 3. Fluorescence microscopy image of K562 cells treated with 100 µM of methyl thiosemicarbazone compound. (4-MTC-Cu2+) for 24 to 72 hours.

The Effect of Thiosemicarbazone Compound on Different Stages of the Cell Cycle:

In order to investigate the effect of the desired compound on the cessation of the cell cycle at different stages, K562 cells were evaluated after treatment by PI dye using flow cytometry. In control mode most of the population was in phase G1 / G0. After 72 hours, a higher percentage of the population was observed below the peak G1 (SubG1). Accumulation of cells in this area indicated the induction of apoptosis in cells. Flow cytometry data analysis showed the presence of about 30% of the K562 cells in the Sub-G1 peak, compared to the controls after 72 hours (Figure 4).

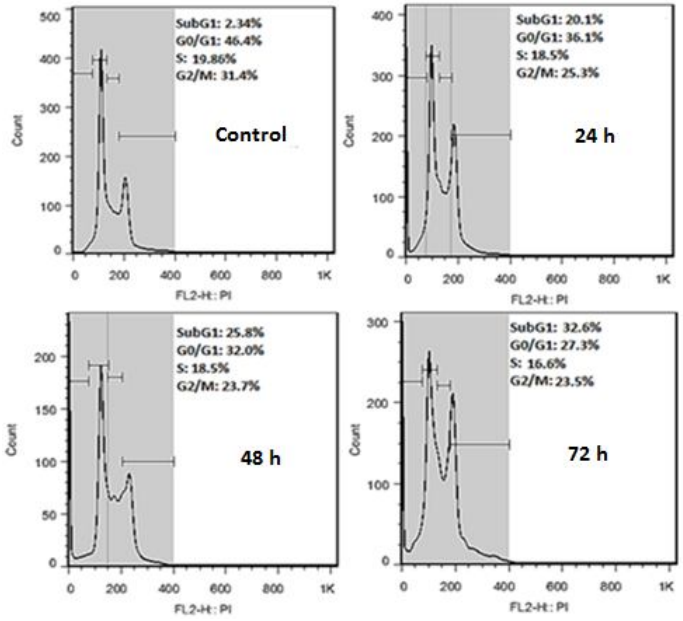

Fig. 4. Curve obtained from cell cycle analysis of K562 cells treated with 100 µM 4-MTC-Cu2+ in 24 to 72 hours.

Evaluation of Apoptosis in K562 Cells Treated with Thiosemicarbazone Using AnnexinV / PI Staining:

Flow cytometry performed after incubation of the examined cells in annexinV as well as propidium iodide, showed time-dependent induction of apoptosis with the mechanism of phosphatidylserine phospholipid surface increase and finally, membrane permeability. Based on what can be seen in Figure 4-5, the control or untreated cells were located in the lower left part (LL). This means that they have minimal annexin binding to the surface phosphatidylserine and minimal PI permeability into the cell. Cells treated with 100 µM thiosemicarbazone shift to the lower right (LR) region. The reason is that in the early stages of apoptosis, the surface Phosphatidylserine concentration increases but the membrane structure is not yet fully permeable. (Impermeability to PI); this area is considered the focus of cells that are in the early stages of apoptosis. Finally, the percentage of cells that have been treated for 72 hours with the test compound is concentrated in the upper right portion of the graph (UR). This area is associated with cells in the late stages of apoptosis or necrosis. These cells have the highest permeability to propidium iodide. The upper left region (UL) is also related to necrotic cells that have become permeable to PI due to injury (Figure 5).

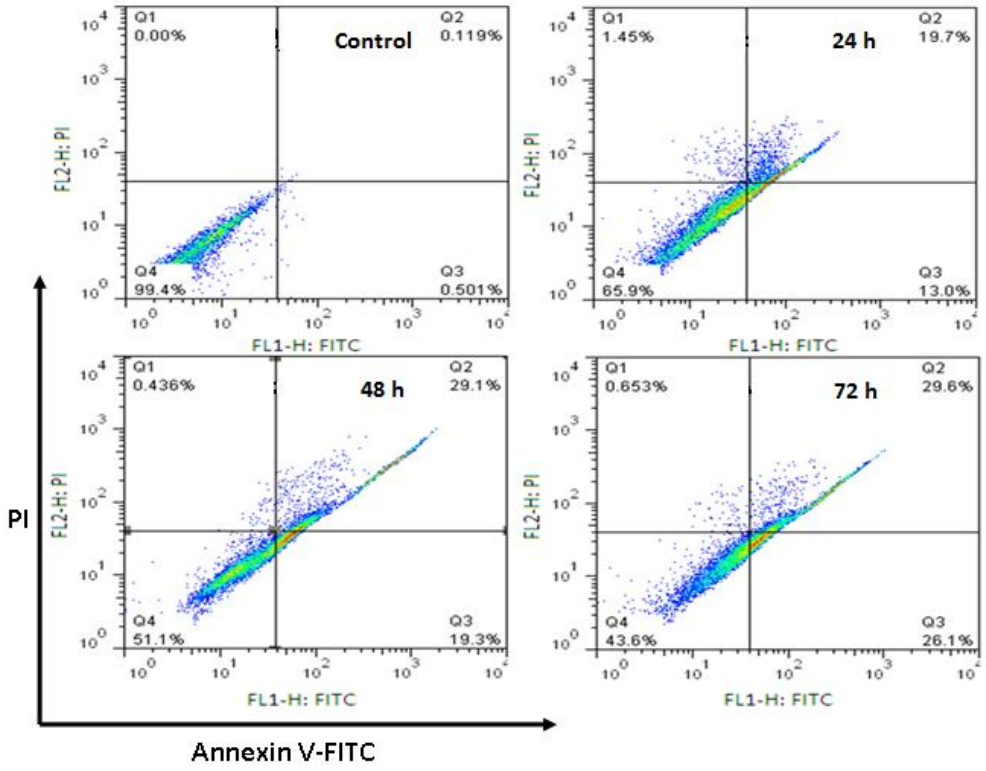

Fig. 5. Dispersion rate of K562 cells (control and treated with 100 µM of 4-MTC-Cu2+) after staining with AnnexinV / PI for 24 to 72 hours.

Discussion

Thiosemicarbazones, as a group of Schiff bases, form an interesting group of NS/NSO chelating bioligands. These compounds have a long history and a wide range of biological properties. Easy to prepare, excellent complexity, the presence of soft and hard atoms in the structure, variety of coordination methods, and having beneficial medicinal properties such as anti-

cancer and anti-bacterial effects have added to the attractiveness of these compounds (17).

Nowadays, various methods such as chemotherapy are used to treat leukemia. Inevitably, drug resistance, high cost and side effects, as well as the non-selectivity of the drugs, lead to the loss of a large percentage of healthy cells along with cancer cells, and in most cases cause recurrence of the disease (18).

The present study was conducted to investigate the anti-cancer effects of a new synthetic compound derived from the thiosemicarbazone family on K562 chronic myeloid leukemia cell line. Since K562 has the characteristics of cancer stem cells, it is a good model for studying the blast phase and drug-resistant CML. For this reason, any drug that can have an inhibitory effect on this cell line can affect cancer stem cells.

A 2019 study by Xia et al. examining two cell lines of SGC-7901 and BGC-823 from Schiff base copper coordinated compound (SBCCC) of gastric cancer found that the compound causes cancer cells to die by inhibiting NF-κB and ROS production. According to their data of the present study, the anti-cancer effects and induction of cell death of thiosemicarbazone-copper compounds in K562 cell line were reported (19).

The results of MTT test in the present study showed that the mentioned compound inhibits the growth of K562 cell line. In this test, it was found that the studied compound is a completely toxic compound for the studied cell line as a representative of CML, so much so that after 24 hours, 50% of the cells stopped growing and lost their viability. The results of the research of Hosseini-Yazdi et al. in 2016 by examining the phenyl thiosemicarbazone compounds on the K562 cell line showed that these compounds inhibit the growth and induce apoptosis, which is in consistent with the results of our study (20).

In 1980, Koeffler and Golde discovered and purified the K562 cell line from the embryonic fluid of a patient with chronic myeloid leukemia in the blast crisis phase. The K562 cell line is the result of glygophurin-rich undifferentiated blast cells. These cells are round and non-adherent cells that are Wright-Giemsa stained as cells with a diameter of 20 μm and have a basophilic cytoplasm without granules with two or more nuclei. K562 cells grow as single cells in culture suspension and have an average division and doubling time of about 12 hours. K562 cell membrane glycoproteins are very similar to erythrocytes, and in particular, these cells synthesize glygophorin A which is found exclusively in human erythrocytes. This cell line is differentiated into erythroid, monocyte-macrophage, and megakaryocyte blood types in the presence of various factors. Therefore, K562 cell line is a suitable model for CML in vitro studies and differentiating distinction of the factors (21).

In a 2019 study by Perondi et al., examining acute leukemia cell lines (ALL) K562 and Jurkat with a new thiosemicarbazone (LAP17) with cytotoxic and cell death effects, the results showed that the strong cytotoxic effect of LAP17 led to acute leukemia; it induces apoptosis and activates caspase-3 and DNA fragmentation. It also stopped the cell at G2/M phase, suggesting that LAP17 could be a promising candidate against leukemia. In their study, the cytotoxic effects of thiosemicarbazone in the K562 cell line caused a cessation of the cell cycle in Sub G1 and also reduced the viability and growth of the cell line (22).

The results of the study of Parsa et al. in 2019 on the anti-cancer activity of copper complex in water with thiosemicarbazone evaluated against two leukemia cancer cell lines K562 and KG1a, showed that copper complex with thiosemicarbazone (Cu-Tsc) inhibits cell growth and stops cell cycle in the G2/M and G0/G1 phases in two cell lines and in addition causes apoptosis and caspase-3 activation (23).

Conclusion

In this study, it was found that thiosemicarbazone causes a decrease in the biological growth of K562 cell line in a concentration- and time-dependent manner. According to the obtained data, these compounds induce monocyte-macrophage differentiation in K562 cells in the early times of the treatment. Thiosemicarbazone compounds also induce apoptotic cell death over longer periods of time. Therefore, it can be concluded that the derivatives first induce the cells to differentiate towards

the monocyte-macrophage and then lead to cell death. Altogether, as these derivatives induce both differentiation and apoptosis, they could be suggested as potent anti-cancer agents in the treatment of CML. Effective at low concentrations and short duration of action, this compound can be a suitable candidate for future pharmacological studies for the treatment of CML.

Acknowledgments

None declared.

Conflict of interest

None declared.

Data availability

The raw data supporting the conclusions of this article are available from the authors upon reasonable request.

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