Physiology and Pharmacology 30 (2026) 1-9
Experimental Research Article

Complex of zinc (II) methylthiosemicarbazone induces cytotoxicity, antiproliferative, and apoptosis effects in human K562 leukemia

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ABSTRACT

Introduction: Methylthiosemicarbazones (MTSCs) are compounds with various biological properties. Some studies have shown that their metal complexes have potential cytotoxic effects on numerous cancers. In this study, the effects of zinc (II)-methylthiosemicarbazone complex (Zn-MTC) on the human chronic myelogenous leukemia K562 cell line were evaluated.

Methods: In this experimental study, K562 cells were treated with different doses of Zn-MTC for 24, 48, and 72 hours. We investigated whether this compound could induce cytotoxicity and apoptosis in K562 cells using the MTT assay, fluorescence microscopy, and flow cytometry.

Results: Our findings indicated that Zn-MTC inhibited the growth of K562 cells and induced apoptosis in a time- and dose-dependent manner. The IC50 of the complex was 100 µM after 72 hours of incubation. Morphological changes and sub-G1 cell cycle arrest confirmed the induction of apoptosis in K562 cells treated with the IC50 concentration of this compound.

Conclusion: Given its antiproliferative and apoptosis-inducing effects, Zn-MTC can be proposed for further pharmaceutical evaluation in the treatment of chronic myeloid leukemia (CML) in the future.

Keywords:
Methylthiosemicarbazone
Apoptosis
Cytotoxicity
Cell cycle
K562 cell line

Introduction

Chronic myeloid leukemia (CML) is a slowly progressive and uncommon type of neoplasm that generally has comorbidities, which make patients ineligible for clinical trials. CML is caused by a reciprocal translocation between chromosomes 9 and 22 with an exchange of parts of the long arms of both chromosomes, creating a recombinant chromosome 22 called the Philadelphia chromosome (Kolenova et al., 2016; Lakshmipriya et al., 2018; Siegel et al., 2023). In 2022, leukemia was the second most common blood malignancy worldwide, after non-Hodgkin lymphoma. Using data from the

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Received 1 February 2025; Revised from 5 August 2025; Accepted 7 September 2025
Citation: Namvar Aghdash S, Mahdavi M, Foroughi G. Complex of zinc (II) methylthiosemicarbazone induces cytotoxicity, antiproliferative, and apoptosis effects in human K562 leukemia. Physiology and Pharmacology 2026; 30: 1-9.

"Cancer Prevalence in Five Continents" database along with GLOBOCAN 2022 estimates of leukemia in 185 countries, this study estimated the incidence of different types of leukemia by country, world region, and Human Development Index.

(Chhikara and Parang 2023). Sex-specific age-standardized incidence rates (ASIRs) per 100,000 population for children (0–19 years) and adults (20+ years). In 2020, 57.85% of the 67,008 new cases of CML reported worldwide were in men. The global ASIR for CML was 3.4 per 100,000 (3.9 in males, 3 in females). In addition, 58.86% of the 25,080 CML-related deaths occurred in men.

CML is a myeloproliferative disorder of the hematopoietic stem cells that progresses from the chronic phase, identified by the Philadelphia chromosome as the only genetic abnormality, to blast crisis, which is generally associated with other chromosomal and molecular secondary changes. (Clarke and Holyoake 2017; Deininger et al., 2020; Kaleem et al., 2015).

Apoptosis, a Pyridine-2-carboxaldehyde programmed cell death, is a promising target for anticancer therapy. In cancer, the apoptotic pathway is usually blocked through various mechanisms, including increased expression of antiapoptotic proteins and decreased expression of pro-apoptotic proteins. These changes cause intrinsic resistance to the most common anticancer therapies. Dysfunction of apoptosis pathways has been observed in several types of cancer (Green 2022; Hanahan and Weinberg 2011). Identification of apoptosis pathways will shed light on the molecular elements involved, which in turn could provide insight into cancer treatment approaches using different agents (Taylor et al., 2008). Numerous studies have been conducted on CML treatment methods using antiproliferative and apoptosis-inducing agents (Cao et al., 2023; Li et al., 2017; Xu et al., 2020).

Thiosemicarbazones (R1R2C2=N3-N3(H)-C1(=S) N1R3R4), which are classified as monothiosemicarbazones, bis-thiosemicarbazones and are Schiff base compounds, demonstrate a variety of biological activities. Several studies have been conducted on thiosemicarbazones and their potential in cancer treatment. The antitumor properties of Pyridine-2-carboxaldehyde thiosemicarbazone has been shown in L1210 leukemia (Brockman et al., 1956b). This compound was also tested on human leukemia cell lines KG1 and K562, and an apoptosis effect was observed in these cells. This effect was mainly due to the activity of the nickel complex, which was identified as the most active. The apoptotic effect of the copper methylthiosemicarbazone complex was also demonstrated (Hosseini-Yazdi et al., 2017). In this study, we investigated the antiproliferative and apoptosis-inducing effects of Zn^{2+} methylthiosemicarbazone complex (Zn-MTC) on the human chronic myeloid leukemia- derived K562 cell line.

Despite several studies on thiosemicarbazone metal complexes and their anticancer effects, the cytotoxic and apoptosis-inducing potential of Zn-MTCcomplex on CML cells has not been thoroughly investigated. This study aimed to fill this gap by evaluating the antiproliferative and pro-apoptotic effects of Zn-MTC on the human K562 cell line and provide new insights into its potential as a therapeutic agent for CML. Our findings helped expand the understanding of metal-based complexes in the treatment of leukemia and provide a foundation for future drug development (Nath et al., 2022; Ngoepe and Clayton 2021)

Material and Methods

Chemicals and Drugs

RPMI-1640 medium and penicillin/streptomycin were purchased from Gibco. Culture plates were purchased from SPL. Acridine orange/ethidium bromide (AO/EtBr) and proteinase K were purchased from Sigma . Annexin V FITC Apoptosis kit was purchased from Roche and the cell extraction buffer was purchased from Invitrogen. Propidium iodide (PI), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma-Aldrich. Finally, the K562 cell line was obtained from the Pasteur Institute of Iran.

Cell culture

K562 cell line was initially cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 µg/mL penicillin. The cells were then incubated at 37ºC in a humidified atmosphere with 5% CO_2.

Cytotoxic Activity

The cytotoxicity of the complex was examined using the MTT assay. K562 cell line (5×10^4 cells/mL) was cultured in 96-well plates and then treated with different

concentrations of Zn-MTC. The IC_{50} value was determined by adding dimethyl sulfoxide (DMSO) to each well, followed by incubation for 4 hours at 37ºC. After this time, MTT was reduced to formazan crystals. Next, DMSO was used to dissolve the formazan crystal. Then, a multiwell plate reader was used to measure the absorbance at 570 nm to assess cell viability (Sheth 2022).

DNA Fragmentation Assay

DNA fragmentation occurs in apoptotic cells and confirms the occurrence of apoptosis. K562 cells were exposed to 100 µM Zn-MTC, which corresponds to the IC_{50} value. Cells were centrifuged 72 hours later, then collected and washed with phosphate-buffered saline (PBS). In dying cells, DNA is degraded by endonucleases that fragment the chromatin into nucleosomal units. In this study, agarose gel electrophoresis was conventionally used to analyze fragmented DNA in cells (Yazdanparast et al., 2005).

Morphological Study of K562 cells

The K562 cells were exposed to 100 µM Zn-MTC (IC_{50} value) for 24-72 hours to induce apoptosis. After treatment, the cells were centrifuged and washed with cold PBS. Then the collected cells were stained with fluorescent dyes, 100 µg/mL acridine orange and 100 µg/mL ethidium bromide (AO/EtBr). Finally, 5 µL of the cell suspension was placed on a laboratory slide and observed under a fluorescence microscope (Mahdavi and Yazdanparast 2007)

Analysis of Cell Cycle Distribution

In summary, the K562 cells were cultured in 96-well plates for various periods (24-72 hours), after which each well containing 1×10^4 cells/well was treated with 100 µM of the chemical complex at the IC_{50} value. Cells were collected and washed twice with cold PBS and then fixed by exposure to cold 70% (V/V) ethanol. Fixed cells were stored at -20ºC for several weeks before analysis. Subsequently, control and treated cells were incubated for two hours in a dark room at 37ºC with 50 µg/mL propidium iodide (PI) and 20 µg/mL RNase A (Mahdavi M et al., 2016). Finally, the stained K562 cells were analyzed using flow cytometry (BD FACSCalibur ™, BD Biosciences, CA, and USA).

Annexin V/PI Double Staining Assay

K562 cells were cultured in 96-well plates and exposed to either control conditions or treatment with 100 µM Zn-MTC (the determined IC_{50} value) for varying durations of 24, 48, or 72 hours.Following the treatment periods, both control and treated cells were collected, washed twice with PBS, and then stained with Annexin-V-FITC and PI in the dark for 15 minutes at room temperature to assess apoptosis. Finally, the stained cells were analyzed by flow cytometry (Mahdavi et al., 2016).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9. Data are presented as mean ± SD. The unpaired Student's t-test was employed to compare the means of two independent groups. A significance level of $p < 0.05$ was considered statistically significant. Significance levels are indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

Cell viability

The MTT assay determines the number of viable cells by assessing the MTT that living cells convert into formazan. We treated K562 cells with various concentrations of Zn-MTC for different periods. As shown in Fig. 1, the Zn-MTC complex was cytotoxic to K562 cells and inhibited their proliferation. The IC_{50} value of the Zn-MTC compound was 100 µM after 72 hours.

Qualitative study of apoptosis

To evaluate the apoptotic-inducing effects of the complex, the K562 cell line was exposed to 100 µM (the IC_{50} value) of the complex and analyzed using a fluorescent microscope. As shown in Fig. 2A, untreated cells are uniformly green due to the plasma membranes of living cells containing only AO. Apoptotic cells showed bright green and orange colors that originated from the nuclei of treated cells and were likely created by condensed DNA fragments that occur during apoptosis. Fig. 2A shows that the number of viable cells decreased. In contrast, the number of viable apoptotic cells increased in a time-dependent manner, confirming that the chemical complex induced apoptosis in K562 cells. During apoptosis, DNA is fragmented, producing nucleosome fragments of approximately 180 base pairs. Activation of caspase-3 during the apoptosis process also activates CAD in the nucleus, causing DNA fragmentation (Elmore 2007). Electrophoresis can show whether DNA

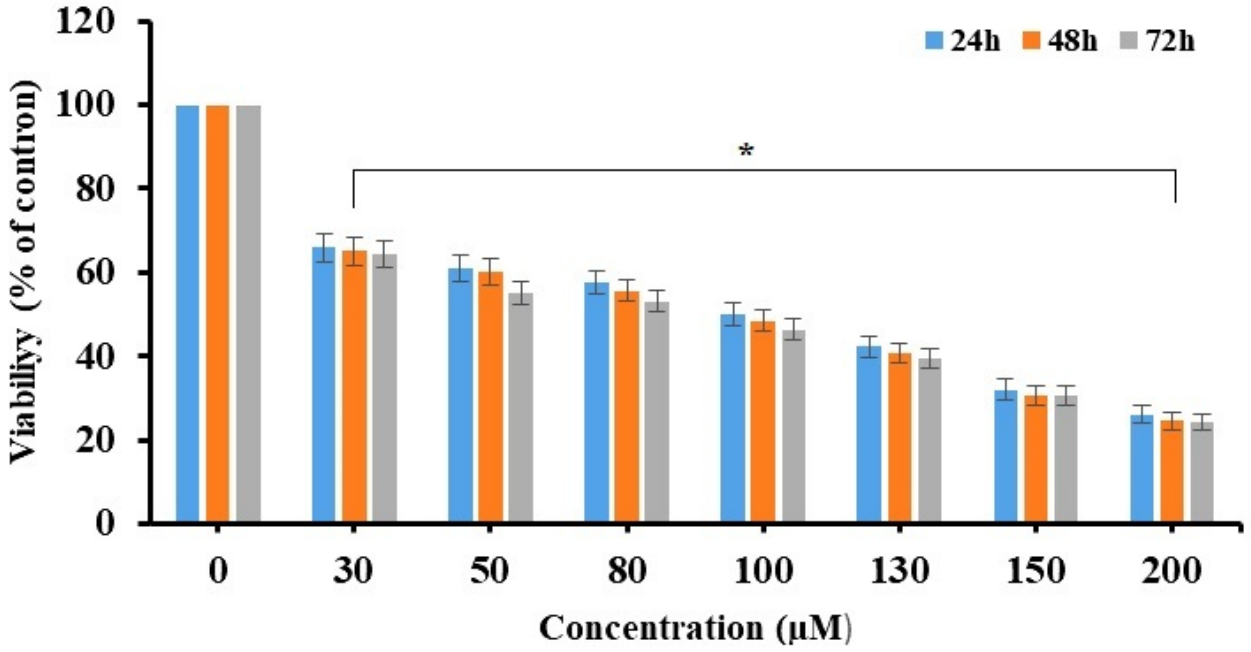

FIGURE 1. Effect of Zn-MTC on K562 cells. K562 cells were treated with different concentrations (30-200 µM) of the complex for 24, 48, or 72 hours, and cell viability was assessed using the MTT assay. Data are presented as mean ± SD (*P-value < 0.05).

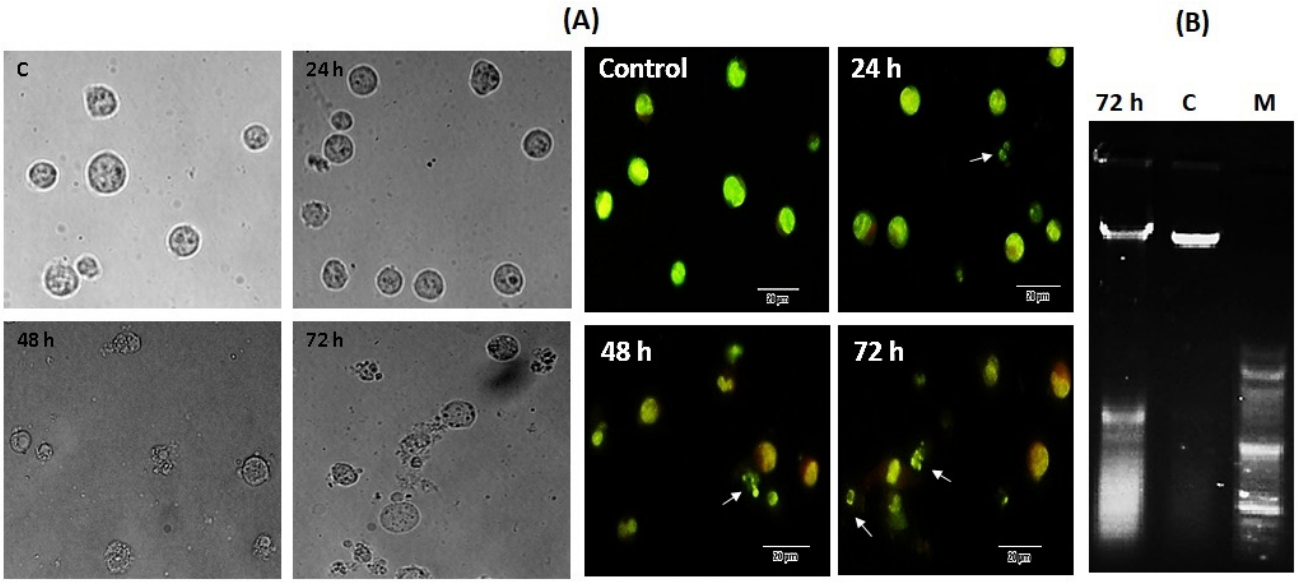

FIGURE 2. (A) Morphological study of the K562 cells treated with 100 µM of the Zn-MTC for 24, 48, and 72 hours, observed under a light microscope. 4-MTC induced morphological changes in K562 cells compared to control cells, including cell deformation and an increase in the number of cells with membrane shrinkage. Apoptotic cells are marked with white arrows. (B) Fluorescence microscopy image of K562 cells treated with 100 µM Zn-MTC for 72 hours, stained with acridine orange/ethidium bromide (AO/EB). This staining method distinguished live, apoptotic, and necrotic cells based on membrane integrity and nuclear morphology. DNA fragmentation in apoptotic cells was qualitatively detected by agarose gel electrophoresis (1: Ladder 5 bp, 2: Control, 3: Zn-MTC treatment for 72 hours).

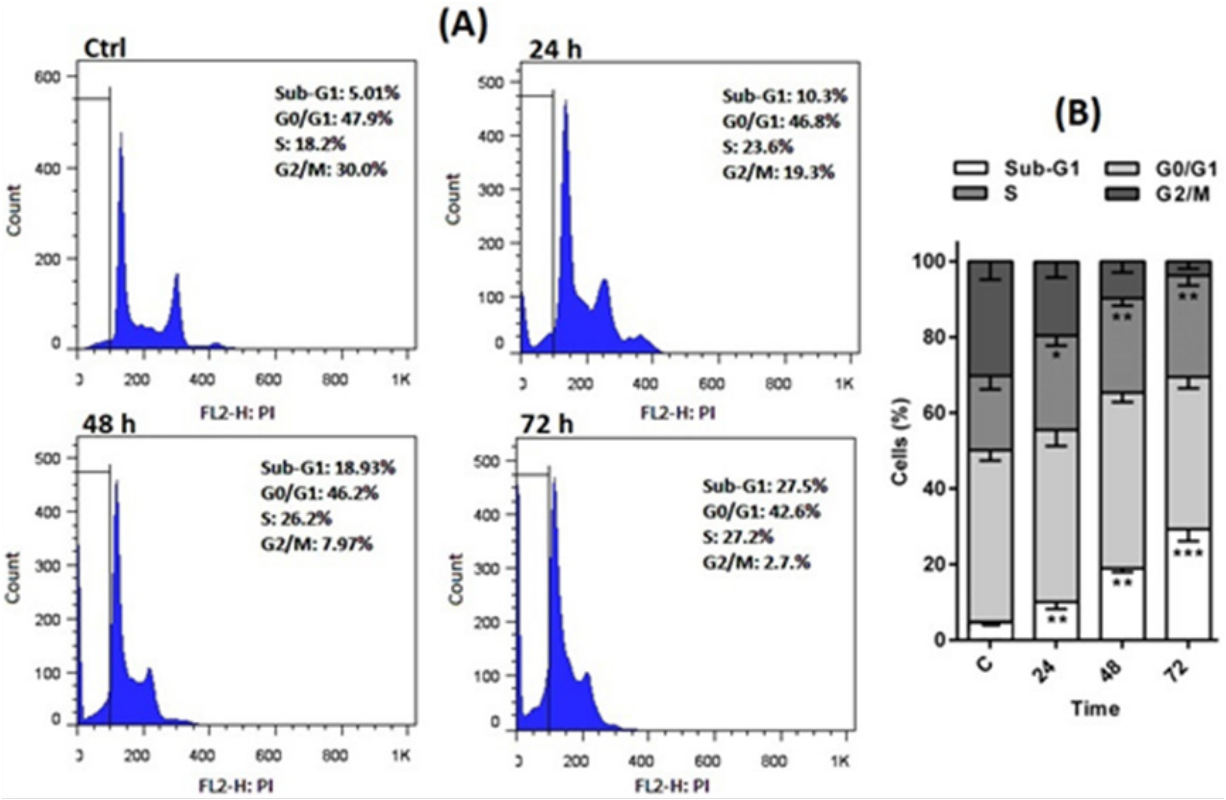

FIGURE 3. Cell cycle distribution in K562 cells after treatment with Zn-MTC. (A) The cells were analyzed using flow cytometry, and the percentage of cells in each phase of the cell cycle was determined. (B) The graph shows the quantitative statistical analysis of the results after exposure to the complex. The percentage of K562 cells in the sub-G_1 phase in the untreated and treated groups (24, 48, and 72 hours) was 5.01%, 3.62%, 18.93%, and 22.72%, respectively. The statistical significance is indicated as follows: * P-value < 0.05, ** P-value < 0.01 and *** P-value < 0.001.

fragmentation occurred in treated cells. To assess DNA fragmentation, DNA was extracted and loaded into an electrophoresis gel. This confirmed that apoptosis was induced in K562 cells exposed to Zn-MTC (Fig. 2B).

Analysis of the cell cycle

To further our investigation, the cell cycle of the K562 cell line was inspected using flow cytometry. We found that Zn-MTC induced apoptosis in K562 cells in a time-dependent manner (Fig. 3). Untreated cells were approximately 5.01%, 47.9%, 18.2%, and 30% in sub-G1, G0/G1, S, and G2/M phases, respectively. Treatment of the K562 cell line for 24 hours resulted in cell cycle distribution of 10.3%, 46.8%, 23.6%, and 19.3% in sub-G1, G0/G1, S, and G2/M phases, respectively (Fig. 3). This indicated that S phase arrest occurred after treating cells with Zn-MTC. The sub-G1 rate, a measure used to confirm the occurrence of apoptosis, also increased from 5.01% in untreated cells to 18.93% and 27.5% in cells treated for 48 hours and 72 hours, respectively (Fig. 3).

Quantitative analysis of apoptosis

The Annexin V/PI assay detects phosphatidylserine, a kind of lipid located on the surface of cell membranes. This test confirmed apoptosis in Zn-MTC-treated K562 cells. Cells in the lower left quadrant (LL) (Annexin V− /PI−) represent viable cells, the lower right quadrant (LR) (Annexin V+ /PI-) represent early apoptotic cells, the upper left quadrant (UL) (Annexin V- /PI+) represent necrotic cells, and the upper right quadrant (UR) (Annexin V+ /PI+) represent late apoptotic cells.

The rates of early apoptosis (Annexin V+/PI-) and late apoptosis (Annexin V+/PI+) in cells treated with Zn-MTC were 4.67%, 17.3%, and 26.1 and 0.051%, 2.67%, and 4.47% after 24, 48 and 72 h, respectively (Fig. 4).

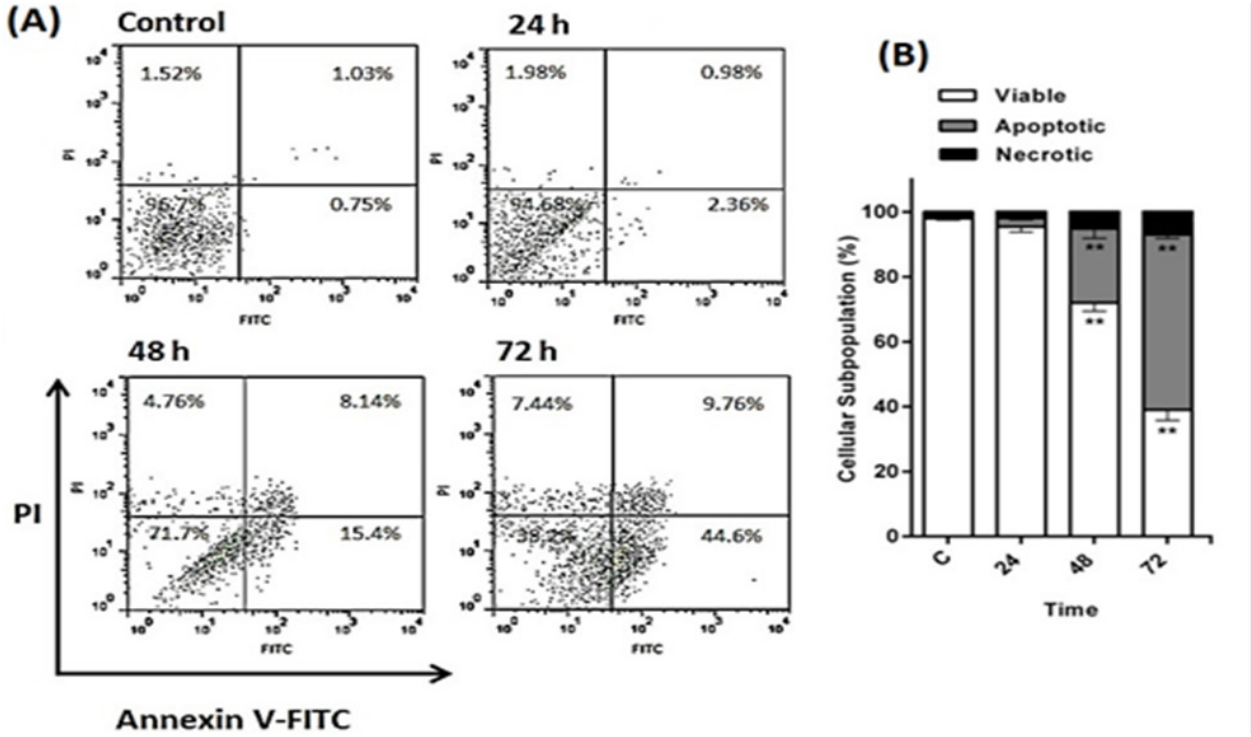

FIGURE 4. Zn-MTC induces apoptosis in K562 cells. (A) Quantitative analysis of apoptosis in K562 cells treated with Zn-MTC using Annexin V/PI double-staining assay. (B) The plot shows the quantified results of Annexin V/PI staining. Flow cytometric analysis showed that Zn-MTC induced apoptosis in K562 cells in a time-dependent manner. The statistical significance is indicated as follows: ** P-value < 0.01.

As shown in Figure 4, when K562 cells were treated with the Zn-MTC complex, the number of early and late apoptotic cells increased, while the number of viable cells (Annexin V-/PI-) decreased. In conclusion, these observations indicate that Zn-MTC can induce apoptosis in K562 cells.

Discussion

Thiosemicarbazones are a class of N, S-donor ligands. Many activities of this family, such as their antitumor, antibacterial, antiviral, and antiprotozoal effects, have been investigated (Kaur and Gupta 2018; Singh et al., 2021). This study evaluated the antitumor activity of Zn-MTC on the K562 cell line. Previous studies have reported the cytotoxicity of some compounds in this family against leukemia. For example, the anticancer effects of Meta- and para-nitrobenzaldehyde thiosemicarbazones have been tested on Adenocarcinoma 755 (Bonaccorso et al., 2019)

The Pyridine-2-carboxaldehyde thiosemicarbazone compound also exhibits antitumor activity against several experimental leukemia cell lines (Brockman et al., 1956a)

Recent studies have emphasized the potential of metal-thiosemicarbazone complexes in cancer therapy, highlighting their cytotoxic and apoptosis-inducing effects in various leukemia cell lines. For example, a recent study reported that copper-thiosemicarbazone complexes showed significant antiproliferative activity against K562 cells by inducing S-phase arrest and apoptosis, which is consistent with our findings on the mechanism of action of Zn-MTC (Parsa et al., 2020). Similarly, anather study showed that novel nickel-thiosemicarbazone derivatives induced apoptosis through mitochondrial pathways in human leukemia cells, supporting the induction of apoptosis observed in our study (Savir et al., 2021). These recent reports align well with our results, suggesting that metal coordination enhances the biological activity of thiosemicarbazones against CML(Parsa et al., 2020; Savir et al., 2021).

Our results showed that Zn-MTC inhibits the growth and viability of K562 cells (Fig. 1). The IC_{50} value of this complex after 72 hours was 100 µg/ml (Hosseini-Yazdi et al., 2017). In morphological studies per-

formed with inverted microscopy and fluorescence, significant morphological changes such as chromatin condensation and nuclear fragmentation were observed in treated cells compared to untreated cells (Fig 2). This finding provides morphological evidence of qualitative induction of apoptosis by Zn-MTC in the K562 cell line. DNA ladder assay confirmed our morphological findings. DNA fragmentation was analyzed using agarose gel electrophoresis, confirming that apoptosis was induced in Zn-MTC-treated cells (Fig. 2B). Our findings are consistent with the results of another study showing that Fe^{2+}-thiosemicarbazone has significant potential chemotherapeutic activity in cancer cells (Shakya and Yadav 2020).

One hallmark of cell death is DNA damage. When DNA is destroyed or damaged by anticancer compounds, the cell's regulatory mechanisms prevent it from progressing to the next phase of the cell cycle for DNA repair (Moon et al., 2023). Before treatment with Zn-MTC, most of the cells were in G0/G1, S, and G2/M phases (Fig. 3). After 24 to 72 hours of treatment, the number of cells in G0/G1 and G2/M phases decreased, while the number of cells in Sub-G1 phase increased (Fig. 3). This indicates DNA damage and indicates that the cell was unable to enter these phases. In contrast, the number of cells in the Sub-G1 phase increased significantly (Mahdavi et al., 2016). In addition, the cell population in S-phase increased after 24 hours of treatment. It can be concluded that this compound caused an S-phase arrest and induced apoptosis (increased in sub-G1 population) in the K562 cell line.

During early apoptosis, phosphatidylserine (PS) is translocated from the inner surface of the cell membrane to the outer surface (Vermes et al., 1995). One way to detect phosphatidylserine is to use the annexin protein, which is attached to a fluorescent dye such as FITC (Fluorescent isothiocyanate). A qualitative study of apoptosis using flow cytometry showed that before treatment, most of the cells were in the LL region (living cells). After treatment with Zn-MTC, the cell population in the LL region decreased and shifted to the LR and UR regions, which correspond to early and late stages of apoptosis, respectively. These findings are consistent with previous studies on the anticancer activity and apoptosis induction of some thiosemicarbazones (Garbuz et al., 2025).

One way to detect phosphatidylserine is by using annexin protein, which is conjugated to a fluorescent dye such as FITC (Fluorescent isothiocyanate). The qualitative study of apoptosis by flow cytometry indicated that before treatment, most of the cells were in the LL region (viable cells). After treatment with Zn-MTC, the cell population in the LL region decreased and shifted to the LR and UR regions, corresponding to early and late stages of apoptosis, respectively. These findings are consistent with previous studies on the anticancer and apoptotic induction activities of some thiosemicarbazones (TSCs) (Kalındemirtaş et al., 2021; Kaur and Gupta 2018).

Despite the promising findings, this study has several limitations and challenges that should be acknowledged. First, all experiments were performed in vitro using the K562 cell line, which may not fully reflect the complexity of in vivo systems or other CML subtypes. Second, the molecular mechanisms and signaling pathways underlying the observed cytotoxic and apoptotic effects of Zn-MTC were not investigated in detail. Future studies including in vivo models and detailed molecular investigations, are necessary to better understand the therapeutic potential and safety of Zn-MTC.

Conclusion

During these studies, it was found that the water-soluble Zn-MTC complex has a cytotoxic effect on the K562 cell line and induces apoptosis. This compound causes morphological changes, cell cycle arrest in the S phase, and translocation of phosphatidylserine to the outer cell membrane. The evidence presented here suggests that Zn-MTC induces apoptosis in K562 cells. This compound could be considered for further drug research for the treatment of CML.

Conflict of Interest

None to Declare

Acknowledgment

None

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