

Mitochondrial Apoptosis and G0/G1-Phase Blockade: Key Mechanisms Underlying Triphenyltin(IV) Dithiocarbamate-Mediated Cytotoxicity in Human Lymphoblastic Leukemia Cells

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**Mitochondrial Apoptosis and G₀/G₁-Phase Blockade: Key Mechanisms
Underlying Triphenyltin(IV) Dithiocarbamate-Mediated Cytotoxicity in
Human Lymphoblastic Leukemia Cells**

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Abstract

Recent findings highlight organotin(IV) compounds as effective anticancer agents, especially for leukemia, offering a potential advancement in cancer therapy by inhibiting cell proliferation with reduced toxicity and resistance issues. Five novel di- and triphenyltin(IV) dithiocarbamate compounds (**OC1-OC5**) were tested against Jurkat E6.1 human lymphoblastic leukemia cells. All compounds produced potent cytotoxic effects by inducing 55 % - 86% of apoptotic events at their

respective IC₅₀ values, 7.1 μM (**OC1**), 0.1 μM (**OC2**), 2.8 μM (**OC3**), 0.2 μM (**OC4**) and 0.35 μM (**OC5**). **OC2** [**Ph₃Sn**(*N,N*-diisopropylthiocarbamate)] was the most potent, with an IC₅₀ of 0.1 μM and a selectivity index (SI) of 5.5, leading to further investigation. Mechanistic studies revealed that **OC2** triggers apoptosis in Jurkat cells via the intrinsic pathway by disrupting mitochondrial membranes. This was initiated by DNA damage, leading to excessive reactive oxygen species (ROS) production and activation of caspase cascade (-9, -8, -3). Pretreatment with the antioxidant *N*-acetyl-L-cysteine (NAC) significantly reduced apoptosis, confirming oxidative stress involvement. **OC2** also caused G₀/G₁ cell cycle arrest and upregulated cleaved-PARP and p21. These findings reveal the anti-proliferative ability of **OC2** in a Jurkat T lymphoblastic leukemia cell model, which warrants further investigation for its antileukemic potential and therapeutic value.

Keywords: Anticancer; apoptosis; cell cycle; DNA damage; organotin(IV); leukemia

1.1 Introduction

Cancer is characterized by dysregulated cell proliferation. A thorough understanding of apoptosis, a regulated cell death process, is crucial for comprehending cancer progression [1,2]. Mutations in genes can lead to changes in the cellular phenotype, disrupting the normal apoptotic response [1,2]. This abnormal cellular activity creates a microenvironment that fosters genetic instability and DNA mutations, accelerating the progression of cancer. As a result, therapies that promote apoptosis, like chemotherapy, hold potential in battling this disease [2]. Apoptosis is preferred over necrosis as a means of cell death because the latter can trigger inflammation, which can harm nearby healthy cells [3].

Apoptosis is a precisely regulated and evolutionarily preserved process of cellular demise, wherein a cell undergoes self-destruction [4,5]. It plays a critical role in multicellular organisms by eliminating unnecessary or excessive cells during development and neutralizing cells with DNA damage, thus hindering the progression towards cancer [5,6]. Proper regulation of apoptosis is vital for preserving the normal equilibrium and integrity of cells. While apoptosis is crucial for maintaining a healthy body, malfunctions in its regulation can lead to a domino effect. Uncontrolled cell death can trigger chronic inflammation, contribute to the hardening of arteries (atherosclerosis), and even be a factor in the onset of cancers and respiratory diseases. Additionally, it's implicated in neurodegenerative conditions like Alzheimer's and Parkinson's [6].

Organotin(IV) compounds hold promise for various medical applications due to their ability to act as effective drugs when paired with specific molecules called ligands. Research suggests they have potential as anticancer agents [7,8], anti-inflammatory drugs [9], antifungals [10], and antibacterials [11]. This versatility stems from the synergistic effects observed when ligands amplify the biological effects of these compounds. These findings suggest that organotin(IV) compounds hold promise as potent and adaptable therapeutic agents across various medical domains. Among various organotin(IV) compounds, organotin(IV) dithiocarbamates stand out due to their wide range of uses. They are valuable in agriculture, biology, and even as catalysts. Additionally, they can be used to create nanoparticles made of tin sulfide. Additionally, these derivatives are valued for their capacity to maintain distinct stereochemistry within their complexes [12, 13, 14].

Dithiocarbamate is classified as a mono-anionic ligand that can create enduring complexes with a wide range of transition metals, as well as most main group elements, lanthanides, and actinides. Additionally, its cationic characteristics make dithiocarbamate an effective solvent in

both water and organic solvents [15]. The use of dithiocarbamate is widespread due to its potential as a chemotherapeutic agent, pesticide, and fungicide [16]. According to Awang et al. [7], dithiocarbamate ligands are prized in inorganic chemistry for their versatility in bonding with a wide range of metals. This chelating ability is attributed, in part, to the presence of two sulfur atoms within the ligand's structure.

In a recent investigation conducted by Annuar et al. [17], we documented the antitumor activities of three series of phenyltin(IV) dithiocarbamate compounds against the K562 human erythroleukemia cell line. Triphenyltin(IV) diisopropyl dithiocarbamate (**OC2**) exhibited the highest level of cytotoxicity among the compounds tested, while also showing moderate selectivity towards K562 cells. **OC2** induced intrinsic mitochondria-mediated apoptosis by activating the caspase cascade and blocking cell cycle progression at the S phase checkpoint. This promising outcome motivates us to explore the potential of phenyltin(IV) dithiocarbamate compounds as antileukemic drugs. Consequently, this study aims to investigate the cytotoxic effects of these compounds in another human leukemia cell line.

The Jurkat cell line originates from a patient diagnosed with T-cell acute lymphoblastic leukemia (ALL). Acute lymphoblastic leukemia (ALL) is a set of conditions that affect blood cells with lymphoblastic lineage. T-cell ALL (T-ALL) and B-cell ALL (B-ALL) are two subtypes of ALL that can be distinguished based on the lymphoblastic lineage that is impacted. The survival of T-ALL patients has increased because of substantial advancements in T-ALL treatment during the past few years. However, a significant part of pediatric T-ALL patients and an even higher percentage of adult patients still encounter therapeutic failure [18, 19]. Immortalized T cell lines, including Jurkat cells, T-cell clones, and hybridomas, are popular *in vitro* models for studying apoptosis [20]. Furthermore, in this study we present an investigation into the molecular

mechanism of cytotoxicity, specifically examining the role of the mitochondrial pathway involves in cell death and cell growth regulation caused by organotin(IV) in the Jurkat human leukemia cell model. These findings enhance the potential value of organotin as a promising antileukemic agent.

2.1 Material and methods

2.1.1 Compounds

Five novel compounds namely, di- and triphenyltin(IV) dithiocarbamate that represented as Ph_nSnL_2 (where $\text{Ph} = \text{C}_4\text{H}_9, \text{C}_6\text{H}_5$; $n = 2, 3$; $\text{L} = N,N$ -dithiocarbamate), Ph_2Sn (N,N -diisopropylidithiocarbamate) (**OC1**), Ph_3Sn (N,N -diisopropylidithiocarbamate) (**OC2**), Ph_2Sn (N,N -diallyldithiocarbamate) (**OC3**), Ph_3Sn (N,N -diallyldithiocarbamate) (**OC4**), and Ph_2Sn (N,N -diethyldithiocarbamate) (**OC5**), were synthesized and characterized as described previously [17].

Table 1 represents the chemical structures of **OC1 – OC5**, based on their corresponding ligands.

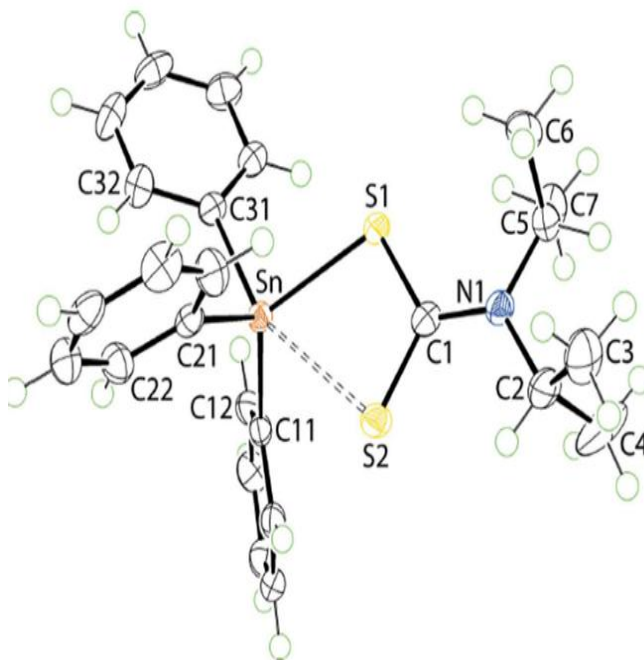
Table 1. Chemical Structures of OC1-OC5

Di- and Triphenyltin(IV)	Chemical Structure
Dithiocarbamate	
Compound 1 Diphenyltin(IV) diisopropyl dithiocarbamate MW: 625.26 g/mol	

Compound 2

Triphenyltin(IV) diisopropyl
dithiocarbamate [21]

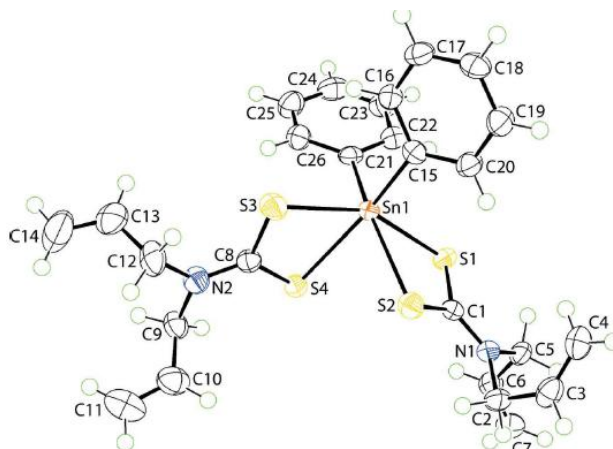
MW: 526.11 g/mol



Compound 3

Diphenyltin(IV) diallyl
dithiocarbamate [22]

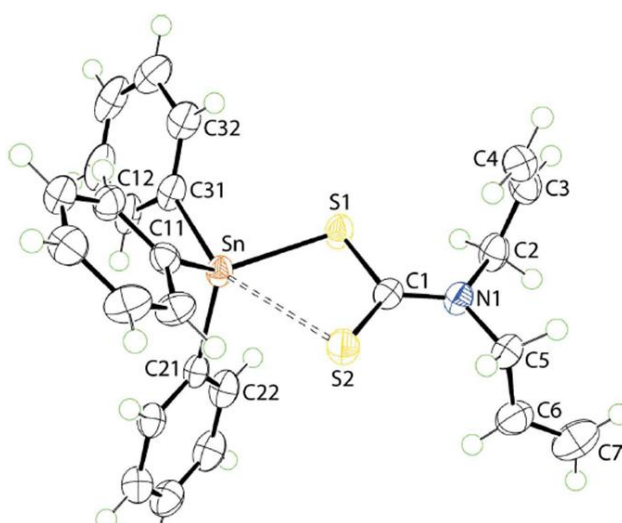
MW: 617.26 g/mol



Compound 4

Triphenyltin(IV) diallyl
dithiocarbamate [22]

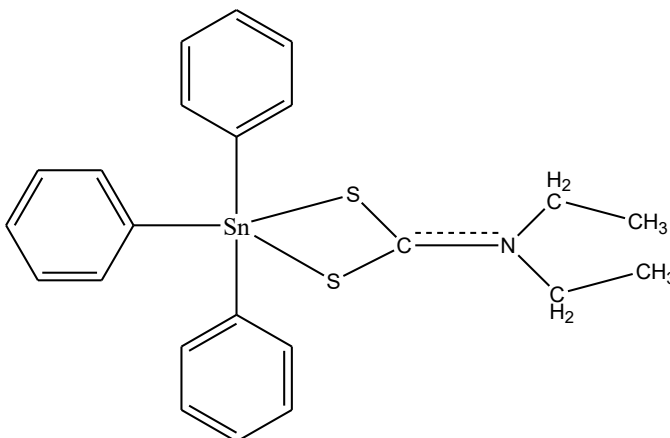
MW: 522.11 g/mol



Compound 5

Triphenyltin(IV)
diethyldithiocarbamate

MW: 498.09 g/mol



103

104 2.1.2 Cell Culture

105 Jurkat T lymphoblastic leukemia cells (clone E6-1) were obtained from the American Type Culture
106 Collection (ATCC, TIB-152) and maintained in RPMI-1640 medium supplemented with L-
107 glutamine, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin solution. The cells were
108 cultured at 37°C in a humidified incubator containing 5% CO₂.

2.1.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

The viability of Jurkat cells was determined using a MTT assay, following a modified protocol from Mosmann [23]. This colorimetric assay provides an indirect measure of cell metabolic activity. A serial dilution of the compounds was prepared in culture media, followed by plating in sterile 96-well microplates at a range of concentrations. **OC1-OC3** were tested at a maximum concentration of 10 mM, while **OC2**, **OC4** and **OC5** were examined at 5 mM. Jurkat cells (1×10^6 cells/mL) were then plated in the same wells and incubated at 37°C with 5% CO₂ for 24 hours. After incubation, an MTT assay was performed to measure cell viability. Briefly, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours. The medium was then removed and replaced with 180 µL of DMSO to solubilize formazan crystals, followed by a 15-minute incubation. The optical density (OD) was measured at 570 nm using an ELISA Microplate Reader. The IC₅₀, the concentration inhibiting 50% of cell growth, was determined for each compound based on the methodology by Annuar et al. [17].

2.1.4 Selectivity index (SI)

Effective anticancer drugs should preferentially target cancer cells [24]. To assess this preferential targeting, we compared the concentration required to inhibit growth by half in normal-human B lymphocyte cells (WIL2-NS, ATCC, CRL-815) to the concentration required for the same effect in Jurkat cells.

2.1.5 Annexin V-FITC/propidium iodide labelling

Annexin V-FITC/PI staining was used to assess the specific cell death pathway triggered by the compound. This assay employs established protocols with minor modifications [17]. Each

compound was applied to Jurkat cells at a concentration previously determined to inhibit their growth by 50% (IC₅₀). This incubation lasted for 24 hours. Following incubation, the cells were harvested by centrifugation (220 x g, 5 minutes, 4°C) and washed with chilled PBS to remove residual media. Following a subsequent centrifugation, the cell pellet was resuspended in 150 µL Annexin V Binding Buffer and stained with 2 µL Annexin V-FITC, a marker for phosphatidylserine exposure during early apoptosis, for 13 minutes at room temperature in the dark. Next, 10 µL Propidium Iodide (PI), a marker for necrotic and late apoptotic cells with compromised membranes, was then added and incubated for an additional 2 minutes. After incubation in the dark, the samples were diluted with 350 µL Annexin V Binding Buffer and analyzed using FACSCanto II flow cytometry (Becton Dickinson, USA).

2.1.6 Cell cycle checkpoint analysis

To evaluate the compound's effect on cell cycle distribution, propidium iodide (PI) staining with RNase treatment was performed, adapting a published protocol [25]. Jurkat cells were exposed to their IC₅₀ concentration for two and four hours. Following harvest, cells were fixed in 70% ethanol overnight at -20°C to permeabilize membranes and preserve DNA. After washing, cells were stained with PI and RNase to differentiate between DNA content in G₀/G₁, S, and G₂/M phases. Flow cytometry (FACSCanto II, Becton Dickinson, USA) then quantified the distribution of at least 10,000 cells across the cell cycle.

2.1.7 Alkaline Comet assay

DNA damage induced by the compound was assessed using a modified alkaline Comet assay based on the protocol by Singh et al. [26]. Cells were treated with the IC₅₀ concentration for various time points (0.5 to 4 hours). After incubation, cells were collected by centrifugation, washed, and

suspended in low-melting-point agarose. This mixture was spread onto pre-coated slides and allowed to solidify. Following overnight lysis, the slides underwent high pH (>13) buffer treatment for 20 minutes to unwind DNA. Electrophoresis at 25 V and 300 mA separated fragmented DNA. Subsequently, the slides were neutralized, stained with ethidium bromide, and visualized under a fluorescence microscope. The Comet assay, analyzed with CometScore software (Tritek Inc.), was employed to quantify DNA damage. This technique measures the tail moment parameter, where a longer tail signifies a greater extent of DNA breaks.

2.1.8 Flow cytometric analysis of mitochondrial function and oxidative stress in response to OC2

Intracellular ROS levels and mitochondrial membrane potential ($\Delta\psi_m$) were assessed using flow cytometry after treatment with the compound at its IC_{50} for varying time points (0.5 to 6 hours). We employed established methods for dihydroethidium (DHE) and tetramethylrhodamine ethyl ester (TMRE) staining, incorporating minor adjustments based on previous works by [17,27]. Briefly, cells were harvested, washed, and resuspended in 1000 μ L fresh media. Then 1 μ L of either 10 mM DHE or 50 μ M TMRE was then added for staining, followed by 15-minute incubation at 37°C in the dark. After washing, cells were analyzed using a FACSCanto II flow cytometer (Becton Dickinson, USA).

2.1.9 Cells pre-treated with *N*-acetyl-L-cysteine (NAC)

The involvement of intracellular oxidative stress was evaluated by pre-treating cells with the antioxidant NAC. Cells were exposed to either 20 μ M or 50 μ M NAC for 1 hour, followed by treatment with the compound at its IC_{50} concentration for 24 hours. Subsequent steps for cell harvesting and analysis mirrored the Annexin V-FITC/PI assay protocol.

2.1.10 Determination of caspases -3, -8 and -9 activities

To identify the specific cell death pathway induced by the compound, Jurkat cells were treated with the compound's IC₅₀ concentration for 2 and 4 hours. A CaspGLOW fluorescein active caspase staining kit (BioVision) was then employed to analyze caspase activation, a key indicator of apoptosis. Following incubation, cells were collected, washed, and resuspended in fresh media. Specific caspase activation (caspase-8, -9, or -3) was assessed by staining with 1 µL of the corresponding FITC-conjugated inhibitor (FITC-IETD-FMK, FITC-LEHD-FMK, or FITC-DEVD-FMK) for 1 hour at 37°C. Cells were then washed, resuspended in buffer, and analyzed using a BD FACSCanto II flow cytometer.

2.1.11 Western blot analysis

To investigate protein expression changes, Jurkat cells were treated with the compound at its IC₅₀ concentration for 30 minutes to 6 hours. Following treatment, cells were washed (ice-cold PBS) and lysed with RIPA buffer. After centrifugation (13,500 rpm, 30 minutes, 4°C), protein lysates were collected and stored at -80°C. Protein concentration was determined using a Bradford assay. Western blot analysis was performed: 25 µg of protein per lane were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS-T (1.5 hours), followed by overnight incubation (4°C) with primary antibodies. HRP-conjugated secondary antibody (anti-rabbit) was then added (1 hour incubation). β-actin served as a loading control. Protein bands were visualized using an ECL kit.

2.1.12 Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis employed one-way analysis of variance (ANOVA) using SPSS software (version 25.0). Differences between groups were considered statistically significant at $p < 0.05$.

3.1 Results

3.1.1 Differential cytotoxicity of di- and tri-phenyltin(IV) dithiocarbamates against Jurkat leukemia and WIL2-NS cells

Jurkat cells exhibited pronounced cytotoxicity after 24-hour treatment with the tested compounds (Figures 1 & 2). Table 2 summarizes the IC_{50} values, demonstrating potent cytotoxic activity against Jurkat cells. Etoposide (ETO), the positive control, had an IC_{50} of 0.87 μ M. Notably, Table 3 highlights the selectivity of these compounds. Among them, **OC2** displayed the highest selectivity towards Jurkat cells based on its IC_{50} value on the non-cancerous WIL2-NS cell line and the corresponding selectivity index (SI).

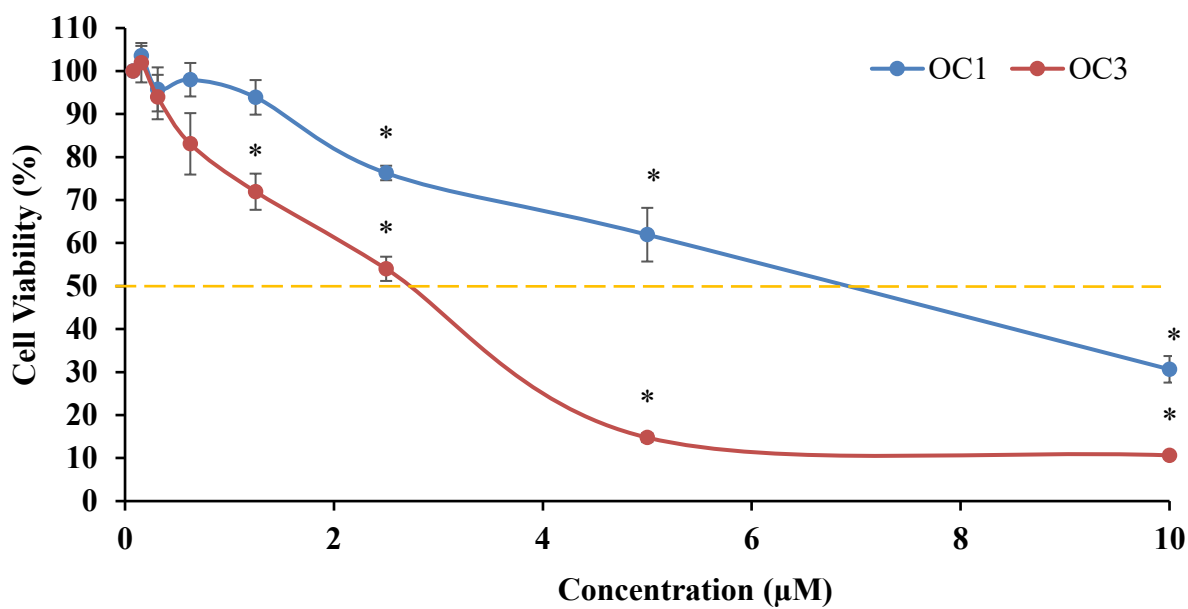


Fig. 1 Jurkat cells viability after 24-hour treatment with **OC1** and **OC3**. Data represent mean cell viability (%) $\pm$ SEM from three independent experiments.

* $p < 0.05$ against Neg

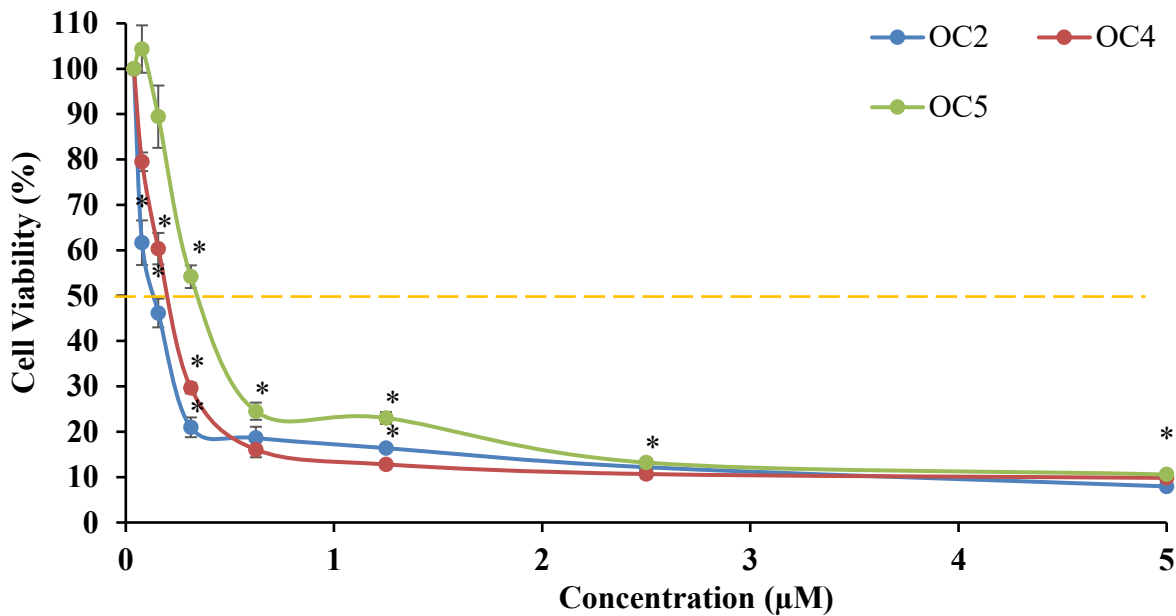


Fig. 2 Jurkat cells viability after 24-hour treatment with **OC2**, **OC4**, and **OC5**. Data represent mean cell viability (%) $\pm$ SEM from three independent experiments.

* $p < 0.05$ against Neg

Table 2. Half-maximal inhibitory concentration (IC_{50}) values of compounds for Jurkat cell proliferation

	ETO	OC1	OC2	OC3	OC4	OC5
IC_{50} (μ M) $\pm$						
	0.87 ± 0.06	7.1 ± 0.6	0.1 ± 0.01	2.8 ± 0.13	0.2 ± 0.01	0.35 ± 0.01
S.E.M						

Table 3. IC₅₀ values and selectivity index (SI) of compounds in WIL2-NS cells

	OC1	OC2	OC3	OC4	OC5
IC ₅₀ (μM) ± S.E.M	2.6 ± 0.3	0.55 ± 0.02	1.2 ± 0.14	0.2 ± 0.016	0.2 ± 0.03
<i>SI</i>	0.37	5.5	0.42	1	0.57

3.1.2 Apoptosis induction by di- and tri-phenyltin(IV) dithiocarbamates

To determine if the observed cytotoxicity involved apoptosis, Jurkat cells were treated with the compounds for 24 hours. They were then stained with FITC-annexin V and propidium iodide (PI) for flow cytometric analysis. The findings depicted in Figure 3 demonstrate that all compounds induced apoptosis, leading to a range of apoptotic cell percentages from 55% to 86%. The treatment groups exhibited the following percentages of apoptotic events: 68.1 ± 4.2 % at 7.1 μM **OC1**, 55.6 ± 5.0 % at 0.1 μM **OC2**, 86.7 ± 2.9 % at 2.8 μM **OC3**, 84.5 ± 5.3 at 0.2 μM **OC4** and 73.4 ± 4.8 % at 0.35 μM **OC5**. In comparison, the negative control displayed a significantly lower percentage of apoptotic events at only 15.0 ± 2.5 % ($p < 0.05$).

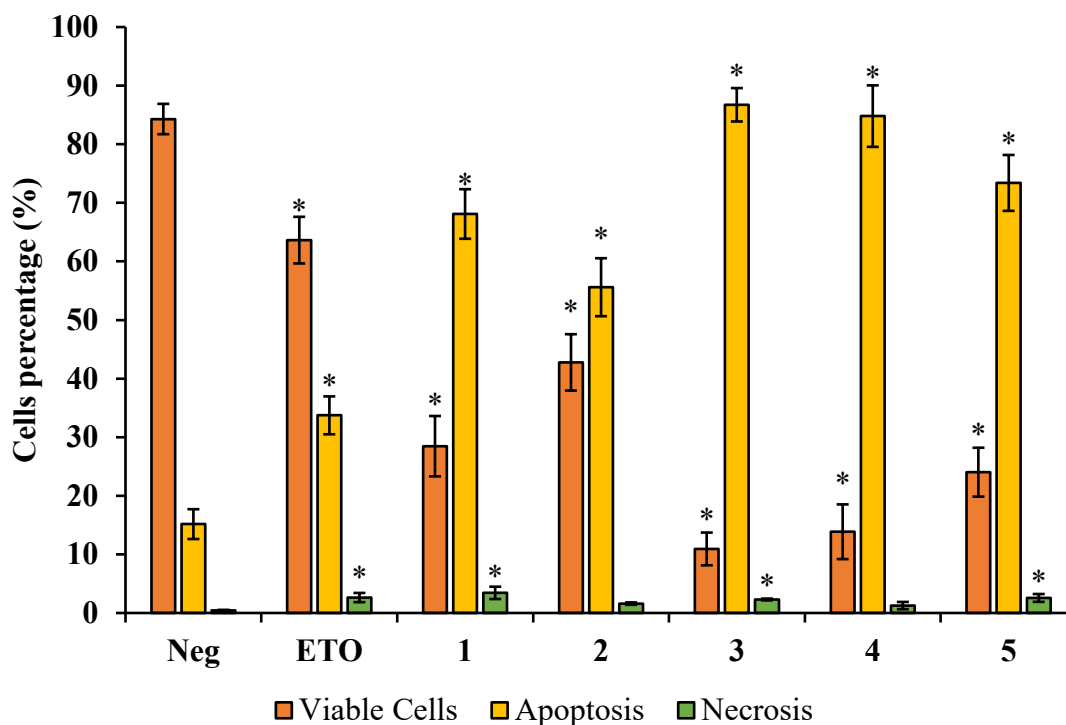


Fig. 3 Jurkat cell viability, apoptosis, and necrosis after 24-hour treatment with compounds at IC₅₀.

Data represent mean $\pm$ SEM from three independent replicates.

* $p < 0.05$ against Neg

3.1.3 OC2 treatment disrupts cell cycle progression

Triphenyltin(IV) diisopropyl dithiocarbamate (**OC2**) exhibited the most potent antiproliferative effects on Jurkat cells among the tested compounds, characterized by its potent cytotoxicity and ability to induce apoptosis. Notably, **OC2** triggered 55% apoptotic events at its lowest IC₅₀ concentration, highlighting its high efficacy. Furthermore, its selectivity for Jurkat cells was superior, as reflected by a selectivity index (SI) of 5.5. Consequently, **OC2** (IC₅₀ concentration) was chosen for further mechanistic investigation of phenyltin(IV) dithiocarbamates in this study.

As shown in Figure 4, **OC2** effectively halted the Jurkat cell cycle in the G₀/G₁ phase within 2-4 hours, with a significantly higher proportion of stained cells compared to the control.

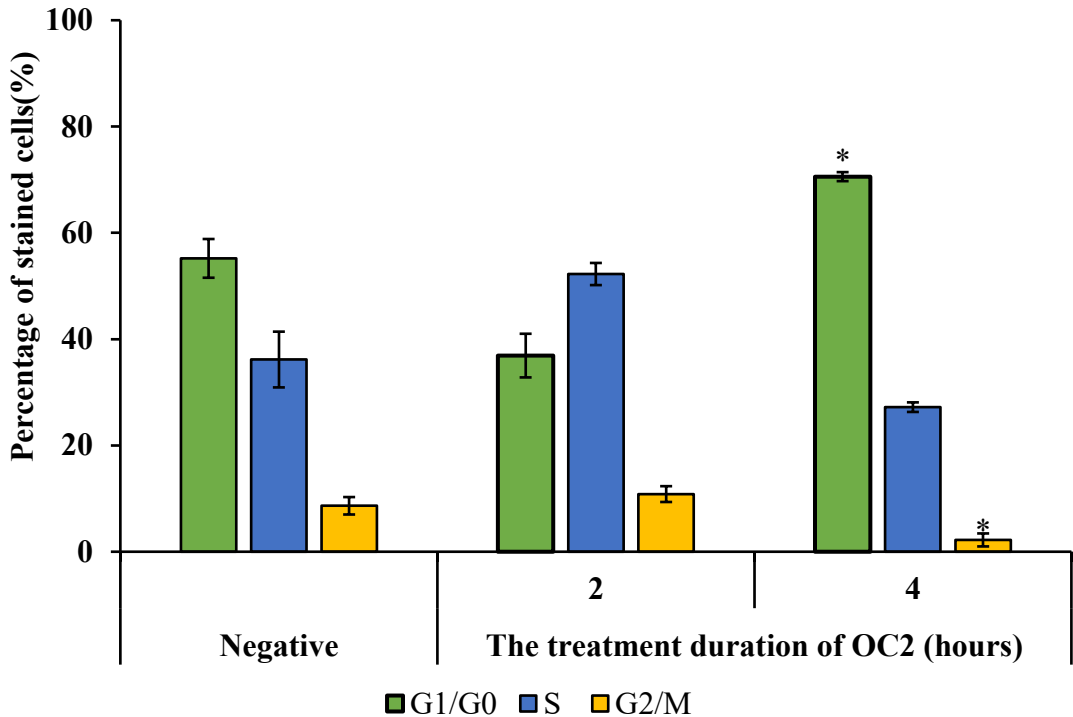


Fig. 4 Jurkat cell cycle distribution after **OC2** treatment for 2 and 4 hours. Data represent mean $\pm$ SEM from 3 independent replicates.

* $p < 0.05$ against Neg

3.1.4 **OC2** treatment induces early-stage DNA damage

The alkaline Comet assay revealed DNA damage in Jurkat cells treated with **OC2**. As shown in Figure 5A, damaged cells displayed comet-like tails due to DNA fragmentation following electrophoresis. **OC2** induced this DNA damage in a time-dependent manner, with visible effects as early as 30 minutes post-treatment. Furthermore, the length of the comet tails increased with longer **OC2** exposure (Figure 5B), suggesting a progressive accumulation of DNA breaks.

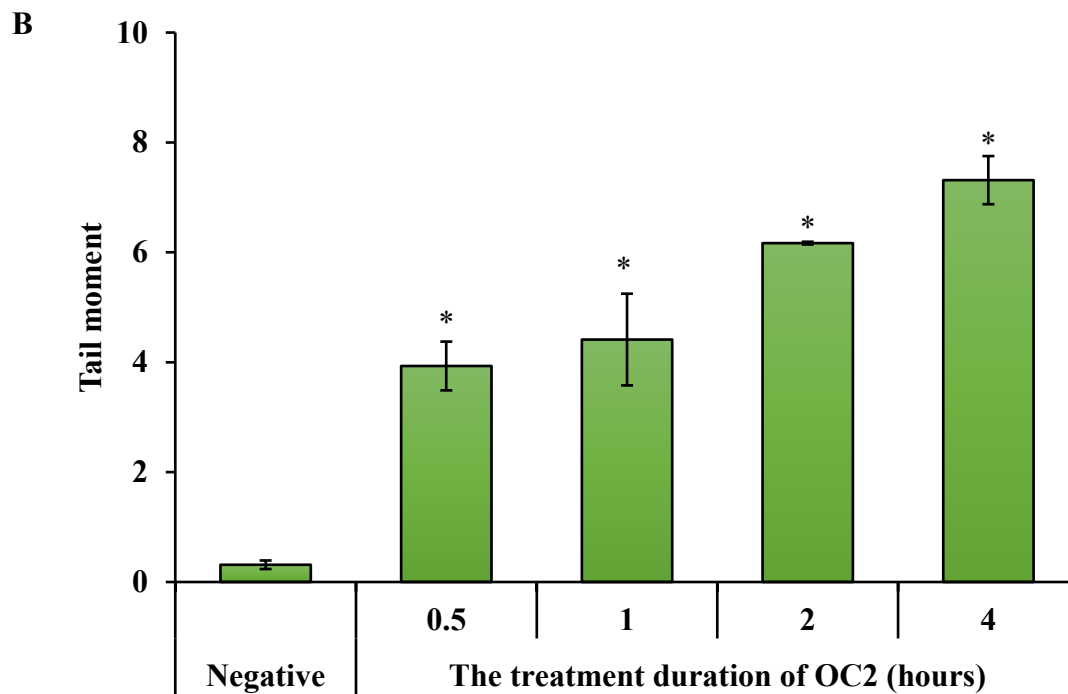
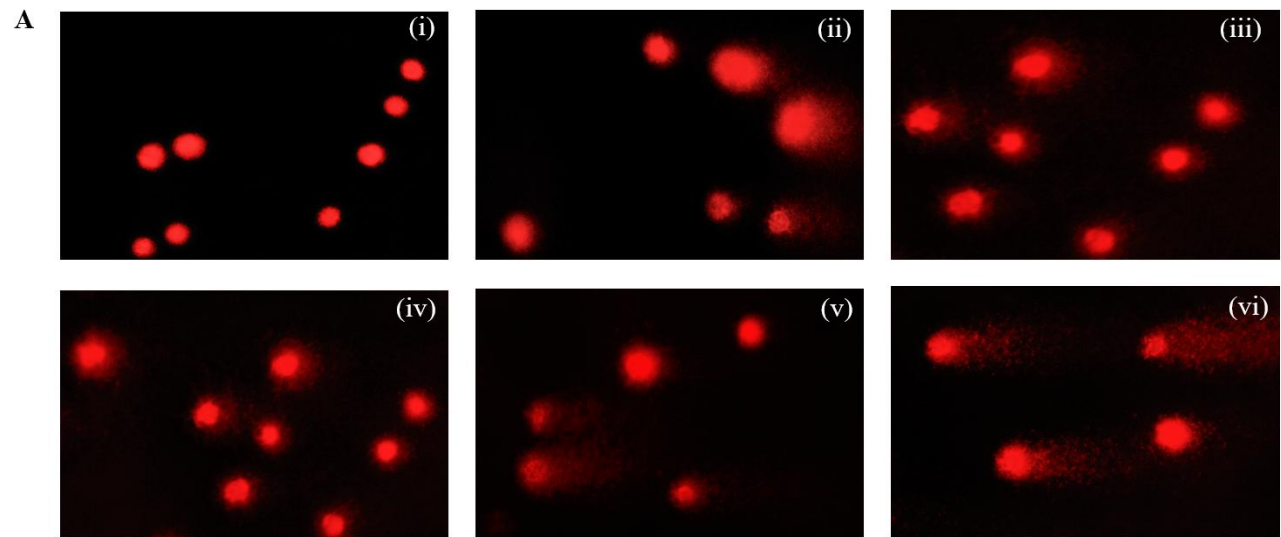


Fig. 5 Jurkat cell DNA damage after **OC2** treatment (0.5 to 4 hours). (A) Representative fluorescence microscopy images with EtBr staining: (i) control, (ii) etoposide (positive control), and (iii-vi) **OC2** treatment for increasing durations. (B) Quantification of DNA damage by tail moment (mean $\pm$ SEM, 3 replicates).

* $p < 0.05$ against Neg

3.1.5 Mechanism of OC2 mitochondrial membrane integrity

Figure 6 demonstrates the decrease in mitochondrial membrane potential ($\Delta\psi_m$) induced by **OC2** in Jurkat cells which treated with IC_{50} concentrations over time. The TMRE staining assay data revealed a slight increase in the average percentage of negative TMRE signal (indicating cells with reduced TMRE fluorescence) after a 1-hour treatment. This increase suggests a decline in $\Delta\psi_m$ as early as 1 hour of treatment, although it was not statistically significant ($p > 0.05$) compared to the negative control. Therefore, the treatment duration was extended to 2, 4, and 6 hours. After a 2-hour treatment, there was a significant decrease in $\Delta\psi_m$ ($p < 0.05$) in Jurkat cells, which amounted to $33.0 \pm 4.5\%$, compared to the negative control. Moreover, $\Delta\psi_m$ was found to significantly decrease ($p < 0.05$) compared to untreated cells at 4 and 6 hours of treatment, with percentages of $37.4 \pm 2.4\%$ and $43.2 \pm 5.5\%$.

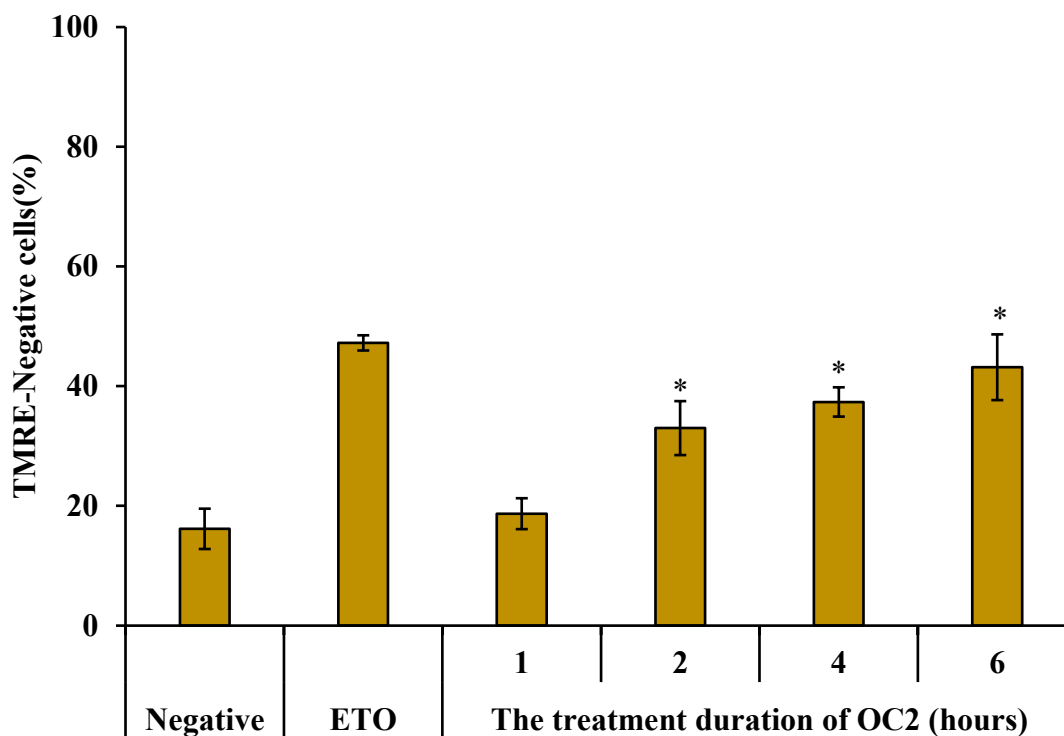


Fig. 6 The loss of $\Delta\Psi_m$ in **OC2**-treated Jurkat cells for 0.5 h – 6 h of treatment. The data are presented as mean ($\pm$ S.E.M) values obtained from three independent experimental replicates.

* $p < 0.05$ against Neg

3.1.6 Oxidative stress in OC2-mediated cytotoxicity

To explore the potential contribution of oxidative stress to **OC2**-induced apoptosis, we measured intracellular ROS levels. Jurkat cells treated with **OC2** displayed a time-dependent increase in ROS production compared to the negative control (Figure 7A). This suggests **OC2** promotes ROS generation, potentially linking it to the observed mitochondrial dysfunction ($\Delta\Psi_m$ loss). To further explore this connection, cells were pre-treated with the antioxidant NAC before **OC2** exposure. Pre-treating cells with NAC significantly decreased the number of apoptotic cells compared to the

control (Figure 7B). These findings suggest that ROS contribute to the apoptotic pathway triggered by OC2.

A

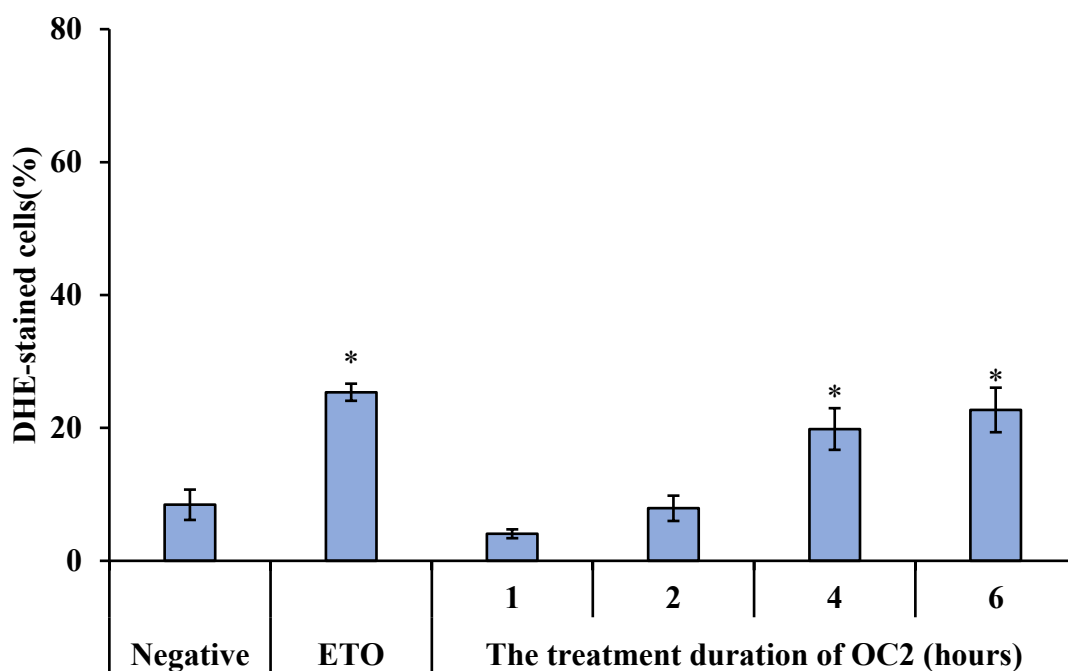


Fig. 7A The ROS production in Jurkat cells after being exposed with OC2 for 0.5h – 6h. The data are presented as mean ($\pm$ S.E.M) values obtained from three independent experimental replicates.

* $p < 0.05$ against Neg

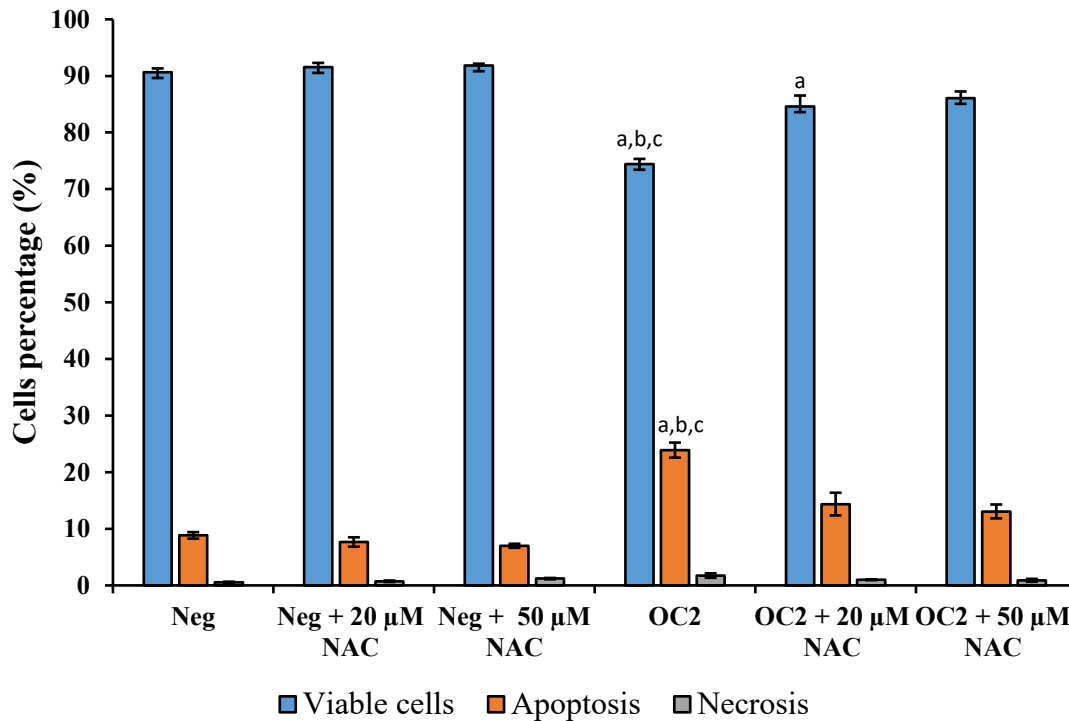


Fig. 7B Jurkat cell viability, apoptosis, and necrosis following pre-treatment with NAC (20 μ M and 50 μ M). Data represent mean $\pm$ SEM from 3 independent replicates

a. $p < 0.05$ against Neg

b. $p < 0.05$ against 20 μ M NAC + **OC2**

c. $p < 0.05$ against 50 μ M NAC + **OC2**

3.1.7 Caspase cascade activation during OC2-mediated apoptosis

The study assessed caspase activation, a hallmark of apoptosis, using fluorescence-tagged caspase-specific substrates. Jurkat cells treated with **OC2** exhibited time-dependent activation of caspase-9, a key executioner caspase, as shown in Figure 8. The percentage of cells with active caspase-9 steadily increased from 2 to 4 hours post-treatment. Notably, caspase-8 activation, associated with the extrinsic apoptotic pathway, remained unchanged compared to the control up to 4 hours.

However, a significant increase in caspase-3 positive cells, another executioner caspase, was observed after 4 hours of **OC2** treatment, suggesting its involvement in the later stages of apoptosis.

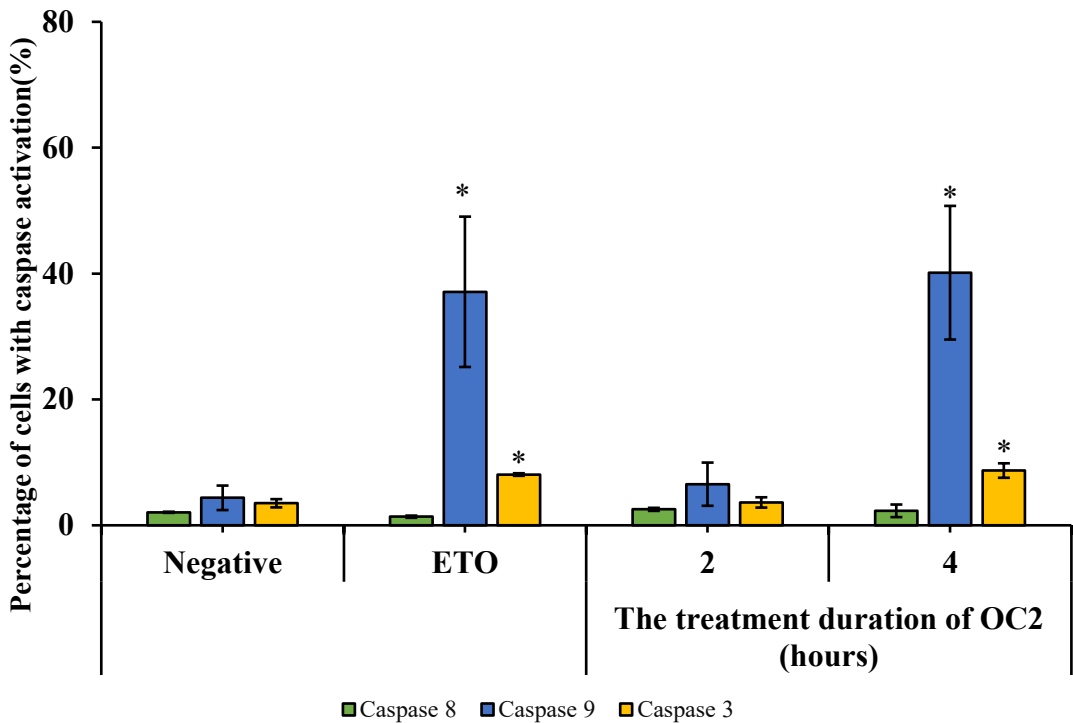


Fig. 8 Jurkat cell caspase activity after **OC2** treatment (2 and 4 hours). Caspase-8, -9, and -3 activation are shown. Data represent mean $\pm$ SEM from 3 independent replicates.

* $p < 0.05$ against Neg

3.18 Analysis of protein expression p21 and cleaved-PARP

Western blot analysis revealed increased p21 and cleaved-PARP protein expression in **OC2**-treated Jurkat cells, as shown in Figure 9. While a slight increase in p21 was observed after 2 hours, it became statistically significant ($p < 0.05$) at later time points (4 and 6 hours) compared to the control. Cleaved-PARP expression also exhibited a similar pattern, showing a marked rise

($p < 0.05$) at 6 hours compared to the control. These findings suggest **OC2**-induced apoptosis through the mitochondrial pathway, involving caspase activation and p21 upregulation, potentially contributing to cell cycle disturbance in G_1/G_0 phase.

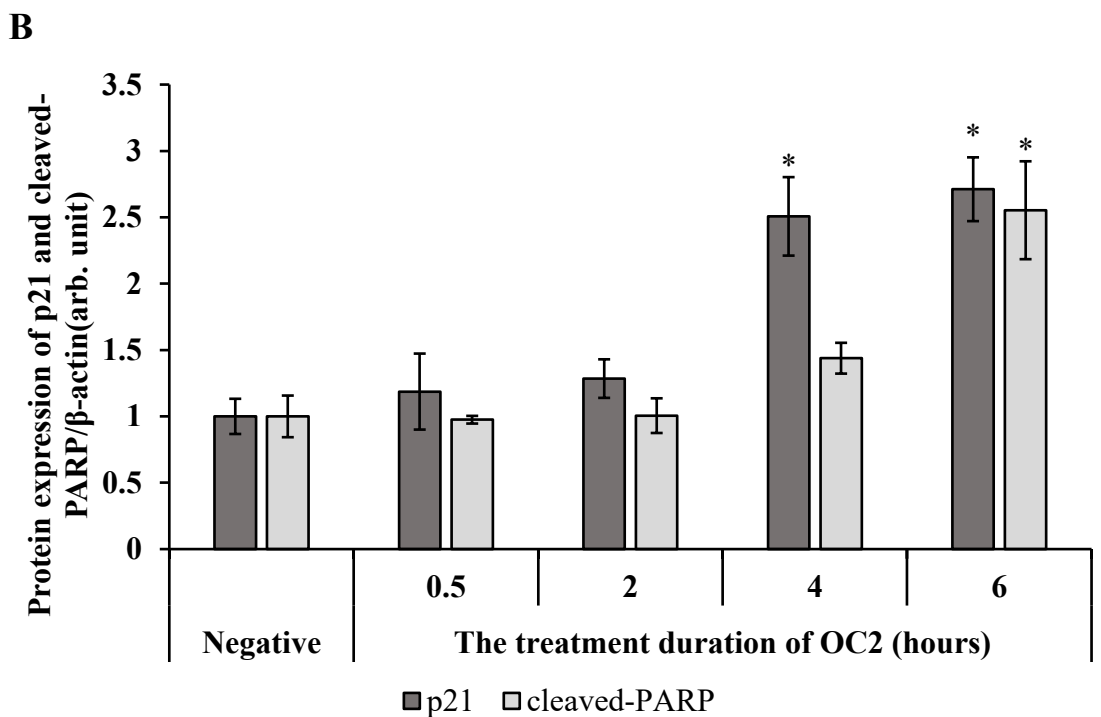
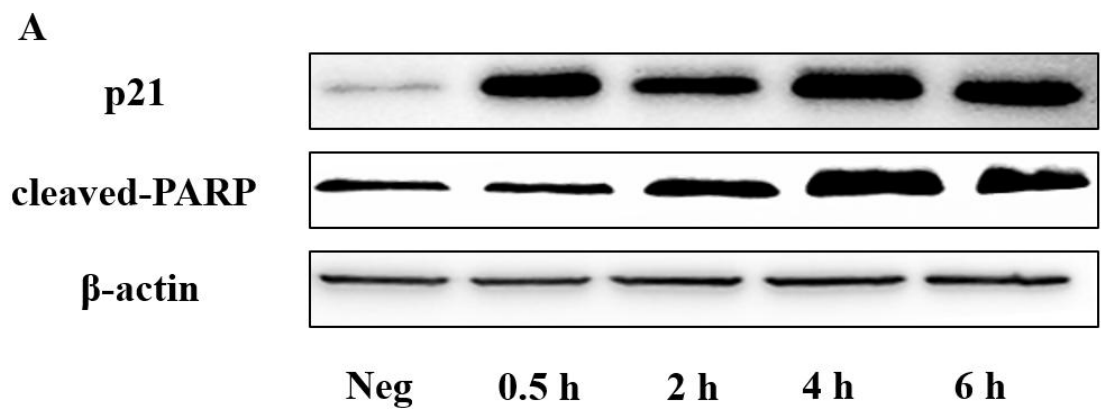


Fig. 9 The expression of p21 protein and cleaved-PARP in Jurkat cells treated with **OC2** at IC₅₀ dose. Figure A illustrates the protein bands of p21 and cleaved-PARP expression. Meanwhile, Figure B presents the bar graph of p21 protein and cleaved-PARP expression (arb. unit). The data are presented as mean ($\pm$ S.E.M) values obtained from three independent experimental replicates. Uncropped western blot images corresponding to Fig. 9A can be found in Supplementary Information File.

* $p < 0.05$ against Neg

4.0 Discussion

The success of cisplatin, a platinum-based chemotherapeutic drug, has highlighted the potential of metal compounds in revolutionizing cancer treatment. Its potent antitumor activity has proven effective against a wide range of cancers, significantly improving patient outcomes. However, cisplatin's effectiveness is hampered by significant limitations. These limitations include the drug's ability to cause DNA damage in healthy cells along with the concerning development of drug resistance in cancer cells over time [14, 27, 28]. This has driven the exploration of alternative metal-based drugs, particularly non-platinum compounds, with the goal of achieving improved efficacy and reduced side effects.

Beyond platinum, other metals like copper, gold [29], gallium, germanium, tin, ruthenium, iridium, and lanthanum [30] demonstrate promising activity in cancer treatment. However, to date, none of them have entered the clinical trial stage [14]. Gold and tin derivatives have gained significant interest among researchers due to their demonstrated high potential as anticancer drugs [31]. In a prior study, our group [32], demonstrated the cytotoxic activity of a novel phenyltin(IV) diallyldithiocarbamate compound in human colon adenocarcinoma cells (HT-29). This compound

displayed selectivity towards HT-29 cells, with a high selectivity index (SI) of 9.03, and induced apoptosis-mediated cell death. Awang et al. [33] also revealed that all three triphenyltin(IV) dithiocarbamate compounds studied were able to inhibit 50% of CCL-119 human leukemia cells' growth at low IC₅₀ concentrations (<1 µM). Moreover, all of these compounds induced apoptosis within 24 hours of treatment at the IC₅₀ concentration. While phenyltin(IV) compounds have demonstrated significant cytotoxic effects on various types of human tumor cells, their specific mechanisms of action have not been thoroughly studied yet.

Di- and triphenyltin(IV) dithiocarbamate compounds demonstrated a potent capacity to suppress the proliferation of Jurkat cells, effectively halting their growth at low micromolar concentrations ranging from 0.1 µM to 7.1 µM, as determined by MTT assays. This potent dose-dependent inhibition of Jurkat cell growth classifies these compounds as highly potent (IC₅₀ < 7.34 µM) according to established guidelines [8, 34]. Notably, the observed order of potency was **OC1** < **OC3** < **OC5** < **OC4** < **OC2**, suggesting that structural variations may influence their efficacy. This sequence clearly demonstrates that triphenyltin(IV) compounds have stronger cytotoxic effects compared to diphenyltin(IV), consistent with studies reported by Abd Aziz et al. [35], Hadi et al. [36], and Sirajuddin et al. [37], where groups treated with triorganotin(IV)-substituents demonstrated the most potent toxicity. Adeyemi et al. [38] also observed that organotin(IV) compounds with longer alkyl or aryl chains exhibit greater biological activity.

The presence of phenyl groups, known for their hydrophobic nature, likely enhances the lipophilicity of these compounds. This property can influence their cellular uptake and distribution within the organism [39]. This can be explained by the Tweedy chelation theory, which suggests that the metal ion's polarity decreases when it forms a complex, leading to increased lipophilicity of the complex. Moreover, this increased lipophilicity allows the complex to penetrate the cellular

membrane more easily [40]. Therefore, as the number of aryl substituents increases from two in **OC1** and **OC3** to three in **OC2**, **OC4** and **OC5**, the polarity decreases, resulting in increased lipophilicity of the complexes. Referring to previous studies, the degree of lipophilicity is associated with cytotoxic activity, where higher lipophilicity of a compound corresponds to stronger cytotoxic effects [41, 42, 43].

Several other researchers have also stated that the relationship between the structure of a compound and its activity depends on the ligand and the substituent groups -R attached to the tin atom [44, 45]. The importance of ligands in modulating the cytotoxic properties of a complex is crucial. The lipophilicity and stability of metal complexes are highly dependent on the properties of the ligand system [46]. Therefore, the design of metal-based drugs and the selection of suitable ligand systems have significant implications in specificity, toxicity control, and bioavailability [47]. To achieve this goal, numerous ligand systems have been developed, among which dithiocarbamate has emerged as a favored option for diverse medical applications. These include its use as carbonic anhydrase (CA) inhibitors and its significance as a compound in cellular metabolism [48]. Johnson and colleagues [49] also stated that dithiocarbamate compounds are non-organic compounds that can bind to metal ions. Moreover, they function as ligands in *in vitro* models used as antioxidants, inhibitors, or inducers of apoptosis, as well as enzyme inhibitors. Furthermore, the property of dithiocarbamate compounds, which exhibit greater solubility in organic solvents (lipophilic) compared to water, facilitates the transportation of organotin(IV) compounds across cell membranes. The coordination of dithiocarbamate has also been reported to have potential in chemoprotection [50], treatment of bacterial and fungal infections, as well as addressing HIV-AIDS and cancer [51].

Our data from the Annexin V-FITC/PI assay demonstrate that **OC1-OC5** induce apoptosis. A previous study conducted by Ray et al. [52] also found that tri-*n*-butyltin(IV) benzoate compound induced apoptosis-mediated cell death in K562 cells. Our results are also corroborated by a previous study by Kamaludin et al. [53], which showed that organotin(IV) compounds induced a substantial amount of apoptotic cells in several human leukemia cell lines, including Jurkat E6.1, K562 and HL-60. Costa et al. [54] further strengthened the evidence for organotin(IV) compounds as apoptosis inducers in malignant melanoma. Their study showed that both diorganotin(IV) and triorganotin(IV) compounds triggered apoptosis in A375 human melanoma cells. Additionally, Awang et al. [55] also reported that triphenyltin(IV) dithiocarbamate compound triggers apoptosis in human colon adenocarcinoma cells, HT-29. Organotin(IV) derivatives have demonstrated efficacy as potential anticancer agents, effectively triggering apoptosis in diverse cancer cell lines [25, 56].

OC2 has shown the most potent cytotoxic effects on both K562 [17] and Jurkat leukemia cell lines, inhibiting 50% of cell growth at very low doses of 0.55 μ M (K562 cells) [17] and 0.1 μ M (Jurkat cells). Furthermore, at IC₅₀ concentration, **OC2** was able to induce apoptotic cells within K562 [17] and Jurkat cells, accounting for $57.1 \pm 4.5\%$ and $55.6 \pm 4.95\%$, respectively. Therefore, **OC2** was selected for a more detailed investigation of its mechanism of action in influencing cytotoxicity on Jurkat cells. This selection was not only based on the MTT and Annexin V-FITC/PI assays data, but also on the **OC2**'s ability to exhibit good selectivity towards both leukemia cell lines, with a selectivity index (*SI*) value of 1 for K562 cells [17] and 5.5 for Jurkat cells. **OC2** stands out as a promising candidate for development as an antileukemic drug, demonstrating potent antiproliferative activity against leukemia cells.

The cell cycle is regulated by a dual mechanism involving protein phosphorylation and degradation. Enzymes called cyclin-dependent kinases (CDKs) phosphorylate specific proteins, triggering events like DNA replication or cell division. Phosphatases then remove these phosphates, allowing the proteins to return to their inactive state. Additionally, the cell employs a cleanup crew called the ubiquitin-proteasome system, which targets and breaks down proteins no longer needed, ensuring a smooth cell cycle progression as described by Bai et al. [57]. These regulatory mechanisms prevent cells with DNA damage in the G₁ phase from progressing into the S phase, allowing time for DNA repair before entering the M phase. Consequently, chromosomes can be properly replicated before cell division occurs [57]. Apoptosis induction is a common feature of cytotoxic drugs and DNA-damaging agents. These agents achieve this by halting the cell cycle at different stages, such as G₀/G₁, S, or G₂/M phases [24].

Cell cycle analysis revealed that **OC2** treatment for 4 hours induced cell cycle arrest in Jurkat cells at the G₁/G₀ phase. This finding is consistent with a recent study by Attanzio et al. [58], which reported that the studied organotin(IV) compound disrupted the cell cycle at the G₁/G₀ phase. The G₁/G₀ phase arrest suggests that cancer cells undergo DNA damage by inhibiting DNA replication and transcription, ultimately leading to apoptosis [58]. Additionally, several other organotin(IV) derivatives have been shown to interfere with the development of various human cancer cell cycles at the G₀/G₁ and G₂/M phases [59-62]. The disruptions in different phases of the cell cycle emphasize the importance of both the organotin(IV) compound and its ligand in determining its biological activity.

In this study, **OC2** was able to induce DNA damage as early as the 30th minute after treatment in Jurkat cells. DNA damage plays a crucial role in determining cell fate. Mild damage, as reported by Jiang et al. [63] and Surova and Zhivotovsky [64], can disrupt cell cycle progression,

potentially allowing for repair mechanisms. However, extensive DNA damage can overwhelm these mechanisms, triggering apoptosis signaling pathways to eliminate the compromised cell. In some cases, severe damage can even lead to necrosis, a form of uncontrolled cell death. In line with previous findings [63], **OC2** treatment induced time-dependent DNA damage in Jurkat cells, as evidenced by comet tail formation in treated cells. DNA damage can occur due to the action of ROS, random errors during replication or recombination and exposure to environmental or therapeutic genotoxic substances [65]. Single-celled (unicellular) organisms react to DNA damage by triggering cell cycle checkpoints and repair processes, whereas multicellular organisms have additional potential to eliminate damaged cells by inducing cell death [65]. The DNA damage inflicted by genotoxic anticancer drugs, including methylating agents, ionizing radiation, and cisplatin, activates apoptosis [66]. These lesions disrupt essential cellular functions like transcription and DNA replication. Studies involving cells with deficiencies in DNA repair mechanisms due to mutations or the blockade of essential repair proteins, as well as studies involving cytotoxic modified nucleotide precursors incorporated into DNA, have demonstrated the ability of DNA lesions to trigger apoptosis [66].

OC2 treatment rapidly impaired mitochondrial function in Jurkat cells, with $\Delta\psi_m$ significantly dropping ($p<0.05$) within 2 hours. This early event was followed by a rise in intracellular ROS production after 4 hours, suggesting a potential link between mitochondrial dysfunction and ROS generation in the **OC2**-induced apoptotic pathway. Based on TMRE and DHE staining data, **OC2** showed slight differences compared to most previous studies in signaling the apoptotic pathway, where **OC2** was found to affect mitochondria-mediated apoptosis followed by ROS generation both in K562 [17] and Jurkat human leukemia cell lines. Studies conducted by Attanzio et al. [58], Wang et al. [62], Ge et al. [67], Girasolo et al. [68], Yusof et al. [69], and

Zhang et al. [70] have reported that organotin(IV) derivative compounds used induced apoptosis through ROS-mediated dysfunction of mitochondria, similar to the mechanism of action of cisplatin [71]. This study, alongside Annuar et al. [17], provides the first glimpses into the action mechanism of organotin(IV) compounds in human leukemia models. We demonstrate that these compounds trigger apoptosis by regulating $\Delta\Psi_m$, likely initiated by DNA damage.

Interestingly, the **OC2**-induced apoptotic pathway observed in both K562 [17] and Jurkat cells correlates with the findings of Roslie et al. [72]. The stilbene compound used by Roslie et al. [72] to treat K562 leukemia cells targeted mitochondria directly, resulting in a decrease in $\Delta\Psi_m$ followed by ROS expression, leading to cytochrome-c release and ultimately triggering the apoptotic pathway. This indicates that apoptotic regulators can control different signaling pathways of apoptosis. According to Circu and Aw, [73], it has been proven the significant involvement of mitochondria within living cells in the generation of free radicals. In the energy conversion process within the Krebs cycle, a fraction of electrons is spontaneously released to oxygen, leading to the formation of superoxide free radicals [74]. It is known that ROS are a natural byproduct of regular mitochondrial respiration, as highlighted by Chance et al. [75]. Approximately 1-3% of electrons within the mitochondrial electron transport chain are inadvertently leaked during the reduction of oxygen to water, resulting in the production of superoxide radicals [76].

Pretreatment with NAC, an antioxidant that scavenges ROS, notably attenuated in Jurkat E6.1 cells ($p<0.05$). This suggests ROS plays a role in **OC2**-induced apoptosis. Similar findings were reported by Li et al. [77] where NAC reduced ROS and apoptosis in gastric cancer cells treated with di-*n*-butyl-di-(4-chlorobenzohydroxamato)tin(IV). Consistent with our findings, Annuar et al. [17] reported a notable decrease in glutathione (GSH) levels ($p<0.05$) in K562 cells

treated with the IC₅₀ dose of **OC2** for 6 hours. This depletion of GSH was accompanied by a substantial elevation in ROS levels ($p < 0.05$) during the same treatment period. These observations suggest a potential link between GSH depletion, ROS production, and **OC2**-induced apoptosis. GSH is a recognized direct antioxidant and serves as a substrate for various antioxidant enzymes. The delayed rise in ROS production compared to the early decrease in $\Delta\psi_m$ suggests that ROS generation might be a downstream consequence of mitochondrial dysfunction in **OC2**-mediated apoptosis. NAC exerts its antioxidant activity through at least three mechanisms: (i) directly scavenging specific reactive oxygen species (ROS), (ii) indirectly boosting glutathione (GSH) synthesis by providing cysteine, a crucial precursor, and (iii) breaking disulfide bonds in proteins, thereby regenerating the pool of essential antioxidant thiol groups [78].

OC2 treatment for 4 hours stimulated the enzymatic activity of caspase-9 and -3 ($p < 0.05$) in Jurkat cells, indicative of the initiation of intrinsic mitochondrial apoptosis. Conversely, caspase-8 activation remained unchanged. Consistent with DNA damage, TMRE loss, ROS production, and this caspase profile, **OC2** likely triggers apoptosis through an intrinsic, DNA damage-initiated mitochondrial pathway.

Organotin(IV) compounds, especially those containing tin(IV), have shown promise as potential anticancer agents due to their ability to trigger apoptosis in cancer cells through the activation of specific enzymes called caspases. For example, tributyltin (TBT) induces apoptosis by activating caspases-3 and -7 [79]. Additionally, studies have demonstrated that organotin(IV) compounds with specific structures, such as 4-nitro-*N*-phthaloylglutamine, can activate caspases-3 and -9 in a dose-dependent manner, suggesting the involvement of the mitochondrial pathway in their ability to induce apoptosis in liver cancer cells (HepG2 cells) [80]. Furthermore, these compounds have been shown to interact with DNA, potentially contributing to their apoptotic

effects. Similarly, trimethyltin(IV) compound (TMT) reported by Jenkins and Barone [81] is capable of inducing apoptosis through caspase-3 and caspase-9, along with p38 protein activity in PC12 cells. A study by Anasamy et al. [82] explored a tribenzyltin(IV) carboxylate derivative and its effects on various breast cancer cell lines (MCF-7, MDA-MB231, and 4T1). The compound significantly induced caspase-3 and -7 activation, hallmarks of the apoptotic (cell death) pathway. Interestingly, it also caused a rise in caspase-9 activity, indicative of mitochondrial pathway involvement, and a slight increase in caspase-8 activity at certain points, suggesting potential crosstalk between apoptotic pathways. This suggests the compound may trigger apoptosis in these cancer cells through two different pathways: the intrinsic pathway (internal cellular signals) and the extrinsic pathway (external signals).

PARP-1 acts as a target for caspases during apoptosis. Caspase-3 and caspase-7 specifically cleave PARP-1, leaving behind a signature fragment indicative of apoptosis. While most caspases can modify PARP-1 *in vitro*, caspases 3 and 7 appear to be the primary executioners in cellular contexts [83]. We evaluated the influence of **OC2** on cleaved-PARP protein levels over time. While no significant rise was observed from 0.5 to 4 hours, a noteworthy increase in cleaved-PARP was evident at the 6-hour mark. This time-dependent effect aligns with the findings of Stridh et al. [79], where tributyltin(IV) exposure also induced a gradual increase in cleaved-PARP protein. Moreover, the induction of cleaved-PARP expression by **OC2** closely correlates with the level of caspase-3 activity, which showed an increase throughout the treatment period. The data obtained from this study demonstrate that the apoptotic checkpoint occurs downstream through important nuclear effector molecules.

Playing a critical role alongside p53, the tumor suppressor protein p21 acts as a conductor of cell division. It accomplishes this by putting the brakes on cyclin-dependent kinases (CDKs),

essential proteins that drive cell cycle progression [84]. Therefore, the expression levels of p21 protein were determined in this study. Treatment with **OC2** for just 4 hours significantly boosted p21 protein levels in Jurkat cells. This rise in p21 coincided with cell cycle arrest at the G₁/G₀ phase, observed during the same timeframe. These findings suggest that **OC2** triggers p21 activation, leading to cell cycle inhibition. This aligns with previous studies by Li et al. [77] and Fanil et al. [85], where similar p21 upregulation was seen in human breast and gastric cancer cells upon treatment with *N*-(3,5-dichloro-2-oxidobenzylidene)-4-chlorobenzohydrazidatoaquatin(IV) chloride and di-*n*-butyl-di-(4-chlorobenzohydroxamato)tin, respectively.

In silico prediction using ADMETlab [17] suggests favorable pharmacokinetic properties for **OC2**. Both the triphenyltin(IV) and diisopropylthiocarbamate moieties contribute to its ability to permeate cell membranes, potentially due to the hydrophobic nature of organotin(IV) compounds [86]. This characteristic allows them to interact with various intracellular targets. The predicted CaCo2 permeability test results indicate that **OC2** [17] might possess good intestinal absorption. CaCo2 cells, a model for intestinal drug absorption, express transporter and efflux proteins alongside Phase II conjugation enzymes, reflecting potential absorption and metabolic pathways [87].

Moreover, **OC2** is unlikely to be a substrate or inhibitor of P-gp, potentially allowing for blood-brain barrier penetration [17, 88]. Additionally, **OC2** is predicted not to inhibit any major CYP450 enzymes, potentially minimizing drug interactions [17]. Furthermore, in silico models suggest that **OC2** may lack mutagenic and carcinogenic potential, and might not cause skin sensitization or liver injury [17]. However, *in vitro* and *in vivo* studies are necessary to confirm these predictions.

543 Our investigation revealed potent cytotoxic activity for all five synthesized di- and
544 triphenyltin(IV) dithiocarbamates, with **OC2** exhibiting particular selectivity towards Jurkat
545 leukemia cells. **OC2** triggered apoptosis via the mitochondrial pathway and halting cell division
546 at the G₁/G₀ checkpoints in Jurkat cells. Interestingly, **OC2**'s mechanism appears to differ from
547 some reported pathways, where DNA damage initiates the process. In our study, **OC2** caused a
548 decrease in $\Delta\psi_m$ followed by ROS production, potentially linking mitochondrial dysfunction to
549 ROS generation, as observed by Sinha et al. [89]. This imbalance in the Bax/Bcl-2 ratio disrupts
550 mitochondrial permeability, leading to cytochrome c release and apoptosome formation. Activated
551 caspase cascade (caspase-9, caspase-3, and PARP cleavage) ultimately leads to apoptosis. The
552 proposed mechanism of action for **OC2** at its IC₅₀ concentration is illustrated in Figure 10.
553 Notably, **OC2** also triggered p21 upregulation, potentially contributing to cell cycle arrest.

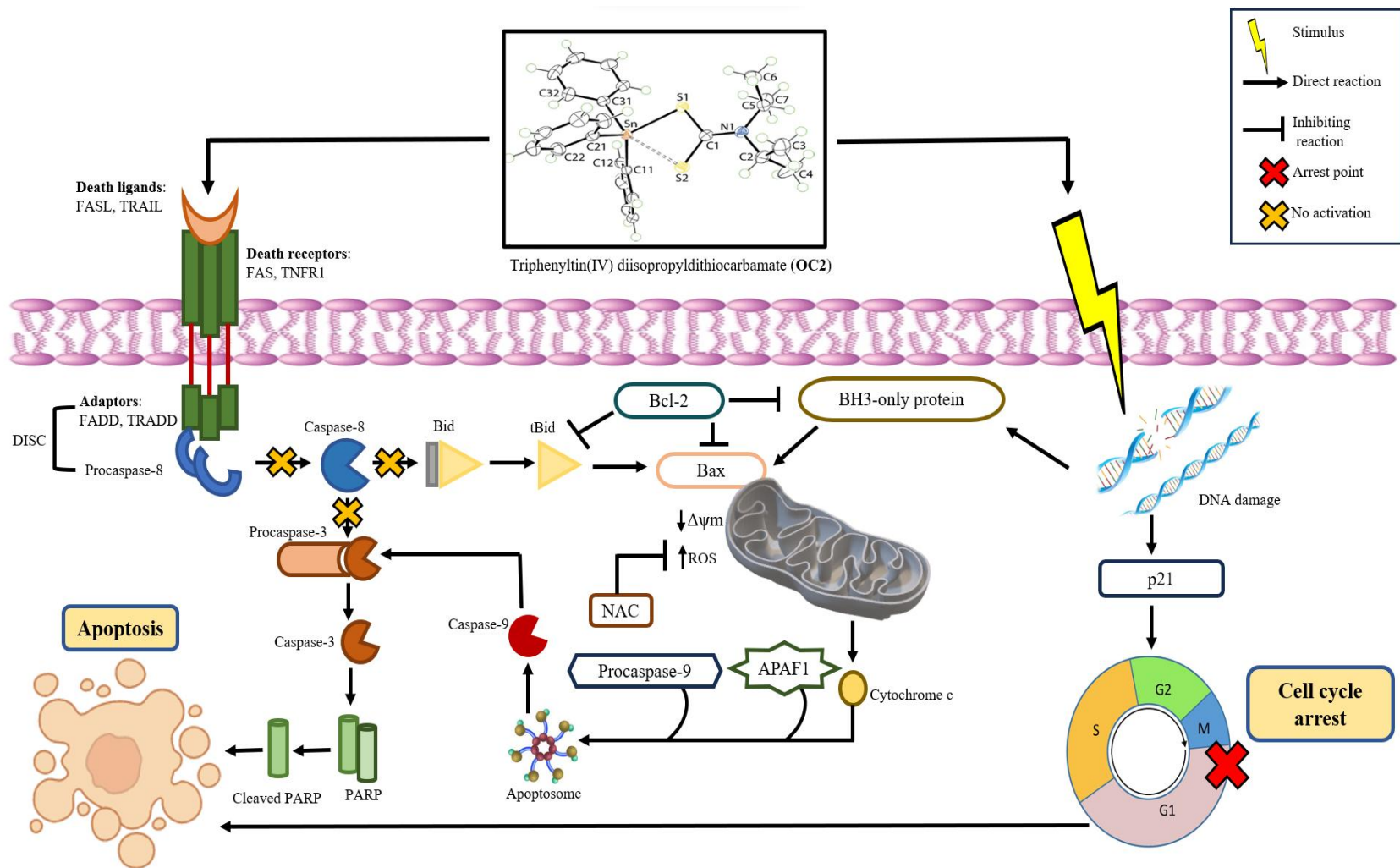


Fig. 10 Model of OC2-induced apoptosis in Jurkat cells, involving mitochondrial dysfunction and cell cycle regulation.

5.0 Conclusion

The five newly synthesized derivatives of phenyltin(IV) dithiocarbamate tested in this study effectively exhibited cytotoxicity against Jurkat E6.1 leukemia cell line at low micromolar concentrations, primarily through apoptosis. Notably, **OC2** displayed selectivity towards Jurkat cells compared to normal lymphocytes. **OC2**'s mechanism appears to involve initial DNA damage, leading to a cascade of events. It disrupts mitochondrial function ($\Delta\Psi_m$), triggers ROS production, and activates the Bax/Bcl-2 pathway. This ultimately results in cytochrome c release, apoptosome formation, and caspase activation, culminating in cell death. Interestingly, **OC2** also modulates p21 protein, causing cell cycle arrest within hours. These findings suggest organotin(IV) compounds hold promise as novel anticancer agents. Further exploration involving structural modifications and in-depth molecular characterization is warranted to develop effective and selective cancer therapies.

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CRediT author statement

SNSA carried out the experiment. NFK is the principal investigator of the project. SNSA wrote the manuscript with support and supervision from NFK, NA and KMC. All authors provided critical feedback and helped shape the context of the paper.

Declarations of interest

The author(s) declare no competing interests.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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