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Cytotoxicity and Apoptosis Prevailed by Tricyclohexyltin(IV) Dithiocarbamate Compounds Against Human Lung Carcinoma Cell Line (A549)

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Abstract: In Malaysia, lung cancer is a highly common kind of cancer and the leading cause of cancer-related deaths. This study examined the cytotoxicity of two new tricyclohexyltin(IV) dithiocarbamate compounds compounds, which are tricyclohexyltin(IV) *N*-methyl-*N*-benzyldithiocarbamate (C1) and tricyclohexyltin(IV) *N*-ethyl-*N*-benzyldithiocarbamate (C2), on lung carcinoma cell lines (A549) of humans. The cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cell death mode was determined by Annexin V-FITC/PI assay, with morphological observations done at 24 h using an inverted microscope. C1 showed strong cytotoxic effects with an IC_{50} of 0.58 μM, while C2 had an IC_{50} of 1.66 μM. Besides, this study observed apoptosis-like changes in cell morphology such as cell shrinkage, membrane blebbing and necrosis. According to the method used to evaluate cell death, C1 caused the A549 cells to stop growing, with 62% of the cells remaining alive, while C2 caused apoptosis in 92.47% of the cells. These results suggest that tricyclohexyltin(IV) dithiocarbamate compounds with different *N*-bound alkyl substituents exhibit varying levels of cytotoxicity against A549 cells, where both compounds demonstrated higher cytotoxicity compared to cisplatin. Therefore, this research is vital for uncovering the potential of novel tricyclohexyltin(IV) dithiocarbamate in cytotoxicity and apoptotic pathways *in vitro*, which could lead to the creation of new therapeutic drugs.

Keywords: Organotin (IV), Dithiocarbamate, A549 Cells, Cytotoxic, Apoptosis

Introduction

Lung cancer is the leading cause of cancer-related deaths, with a poor prognosis and no significant advancements in treatment outcomes. This is mostly because it is frequently diagnosed at an advanced stage, which further exacerbates the global public health burden (Saab *et al*., 2020). Lung cancer arises from the cells of the respiratory epithelium and is divided into two primary kinds: Small Cell Lung Cancer (SCLC), which accounts for 15% of occurrences and Non-Small Cell Lung Cancer (NSCLC), accounting for 85% of cases. NSCLC further includes subtypes such as adenocarcinoma, squamous cell carcinoma and large cell carcinoma (Dela Cruz *et al*., 2011). Cisplatin and pemetrexed are the conventional treatment for advanced non-squamous NSCLC (Gao *et al*., 2019). Despite cisplatin exhibiting potent antitumor activity, its limitations, such as drug resistance (Chen and Chang, 2019) and ineffectiveness in treatment (Oun *et al*., 2018), are becoming more evident. This has drawn researchers' attention to initiate studies on non-platinum metal-based compounds as substitutes for cancer chemotherapy treatment (Khan *et al*., 2020).

Furthermore, there has been an increasing fascination with the possibility of organotin(IV) dithiocarbamate compounds as drugs that can combat cancer. Multiple investigations have shown that organotin(IV) compounds have significantly lower toxicity, superior elimination capabilities and less severe side effects when compared to platinum medicines (Banti *et al*., 2019). The toxicity of

organotin(IV) dithiocarbamate compounds is seen in multiple cancer cell types, such as those found in the melanoma, lungs, colon, ovaries kidneys, breasts and prostate (Khan *et al*., 2015). The efficacy in suppressing tumors depends on the dosage of the compound. Organotin compounds with lower dosages have been shown to activate gene pathways, leading to a mechanism of action against cancer cells. Consequently, this has sparked new research on organotin compounds (Wiecek *et al*., 2010).

Presently, the focus of the research has shifted towards triorganotin compounds with dithiocarbamate ligands instead of organotin(IV) compounds. According to Hadjikakou and Hadjiliadis (2009), compared to diorganotin(IV), triorganotin(IV) demonstrates potent cytotoxicity due to its ability to form free coordination positions and undergo an unbalanced reaction in solution, resulting in R_4Sn dan R_2Sn $(IV)^{2+}$ types. Additionally, the majority of studies have indicated that triorganotin(IV) compounds exhibit potent cytotoxic effects with an IC_{50} lower than 1 μM (Awang *et al*., 2015; Haezam *et al*., 2021; Syed Annuar *et al*., 2022).

Metal compounds bearing sulfur ligands, such as dithiocarbamates, exhibit unique stereo-electronic features that facilitate the transport of molecules to intended cells and result in prolonged retention periods (Adeyemi and Onwudiwe, 2020). The chelating effects of dithiocarbamate ligands can further diminish the polarity of metal ions, thereby enhancing their lipophilicity and permeability and in turn, enhancing the biological activity of organotin(IV) dithiocarbamate compounds (Cvek and Dvorak, 2007; Javed *et al*., 2016; Kamaludin *et al*., 2013).

The precise method by which this compound operates is incompletely comprehended. Research has demonstrated that organotin(IV) dithiocarbamate compounds possess potent anti-cancer effects by triggering programmed cell death, or apoptosis (Haezam *et al*., 2021; Hamid *et al*., 2020; Farahana Kamaludin *et al*., 2019; Rasli *et al*., 2023; Syed Annuar *et al*., 2022). This process involves various biological pathways, including the intrinsic mitochondrial pathway that is activated by DNA damage. This system plays a crucial role in starting apoptosis, which is programmed cell death. Subsequently, changes occur in the mitochondrial membrane potential, along with the activation of caspase-9 and caspase-3 in cancer cells (Syed Annuar *et al*., 2022). Furthermore, studies suggest that these substances can disrupt critical cellular mechanisms required for programmed cell death by stopping cell division at various stages including G_0/G_1, S and S-G2/M (Rasli *et al*., 2023; Syed Annuar *et al*., 2022). Thus, this study sought to establish the effect of tricyclohexyltin(IV) dithiocarbamate compounds on cytotoxicity and apoptotic cell death in the A549 human lung carcinoma cell line.

Materials and Methods

Research Compounds

Tricyclohexyltin(IV) dithiocarbamate compounds, which are tricyclohexyltin(IV) *N*-methyl-*N*-benzyldithiocarbamate (C1) and tricyclohexyltin(IV) *N*-ethyl-*N*-benzyldithiocarbamate (C2) were synthesized at the chemical laboratory of the environmental health and industrial safety program, faculty of health sciences, UKM. The chemical structures of C1 and C2 are illustrated in Fig. 1.

Culture of A549 Cells

A549 cells, a type of human lung carcinoma cell line, were procured from the American Type Culture Collection (ATCC) and cultivated in T-75 flasks under conditions of 37°C temperature and 5% CO_2 in a humidified environment. These cells were nurtured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, sodium bicarbonate ($NaHCO_3$), 1% penicillin and streptomycin, along with 10% Fetal Bovine Serum (FBS) obtained from GIBCO, USA. Initially, the cells were observed daily to monitor cell proliferation. This helps ensure that the cells are actively growing and healthy before any experimental treatments are applied. To prevent nutrient depletion and the build-up of hazardous metabolites, the culture medium was refreshed every 48 h. Additionally, the cells were regularly subcultured every 3-4 days to maintain cell growth at a confluency of 70-90% and prevent over-confluence, which can lead to contact inhibition and alterations in cellular behavior.

Stock Preparation

All stock compounds were prepared at 20 mM. C1 was weighed at 0.0113 g before being fully dissolved in 600 µL Dimethyl Sulphoxide (DMSO) and 400 µL acetone, while C2 was weighed at 0.0113 g before being fully dissolved in 1000 µL Dimethyl Sulphoxide (DMSO). All stocks were freshly prepared before the experiment and diluted to a specific concentration.

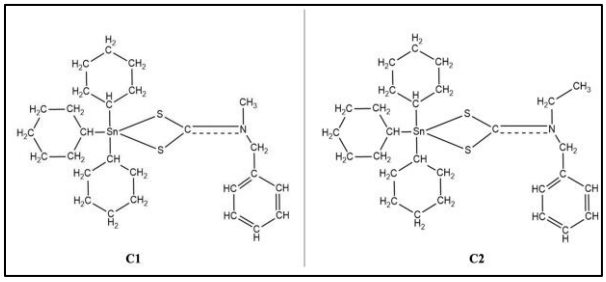

Fig. 1: Chemical structure of tricyclohexyltin(IV) *N*-methyl-*N*-benzyldithiocarbamate (C1) and tricyclohexyltin(IV) *N*-ethyl-*N*-benzyldithiocarbamate (C2)

Thiazolyl Blue Tetrazolium Bromide (MTT) Assay

The viability of A549 was determined using the MTT assay developed by Mosmann (1983). Prior to the assay, the cells were checked to ensure they covered 85-90% of the flask base. Later, the cells were treated with trypsin and then spun at a speed of 1200 rpm for 5 min. The resulting solid mass was then mixed with culture media in order to determine the number of living cells in a volume of 1 mL, using trypan blue staining. Following this, A549 cells were plated in a sterile 96-well microplate at a density of 5×10^4 cells per milliliter and incubated at 37°C with 5% CO_2 for 24 h. Following incubation, the cells were treated with different concentrations of both compounds, ranging from 0.0781-5.000 μM, for an additional 24 h using a serial dilution technique. Cisplatin was utilized as the positive control, whereas untreated cells were utilized as the negative control. After a 24-h incubation period, 20 μL of a 5 mg/mL MTT salt solution was added to each well. The cells were then incubated for another 4 h to allow for formazan crystal formation. After this incubation, approximately 180 μL of supernatant was extracted from each well and 180 μL of DMSO was used to liquefy the formazan crystals, followed by a 15 min incubation. The Optical Density (OD) of each well was then measured using an ELISA microplate reader at a wavelength of 570 nm.

The percentage of cell viability was calculated using the formula: Cell viability (%) = 100× (absorbance compound/absorbance control). This data was then graphed against compound concentration to show the connection between cell viability and compound effectiveness. The IC_{50} value indicates the concentration needed to reduce cell proliferation by 50% after 24 h. These tests were done in triplicate and the IC_{50} values (in micromolar units) represent the mean ± Standard Error of the Mean (SEM), serving as a quantitative measure of the compound's anticancer potential based on its impact on cell viability. It is a crucial metric in pharmacology and toxicology studies, especially in assessing the potency of anticancer compounds. In this context, lower IC_{50} values indicate greater cytotoxicity towards cancer cells, implying a higher potency of the compound in inhibiting cancer cell proliferation.

Morphological Observation

The cells were placed in a clean T-25 flask at a concentration of 5×10^4 cells mL^{-1} and given 24 h to attach. Then, the A549 cells were treated with the respective IC_{50} values of the compounds obtained from the MTT assay. The IC_{50} value for C1 was 0.58 μM, C2 was 1.66 μM and cisplatin was 32 μM. Following incubation for 24 h, the morphological changes were observed using a light-inverted microscope at 40 X magnification.

Apoptosis Detection Via Annexin V-FITC/Propidium Iodide

The annexin V-FITC and PI Dual Staining assay was used to evaluate the mode of cell death in A549 cells based on a modified procedure by Chan *et al*. (2006). The cells were placed in a clean T-25 flask at a concentration of 5×10^4 cells mL^{-1}. After a 24-h period of growth, the cells were subjected to both compounds at concentrations that inhibit 50% of their activity (IC_{50} values). The cells that underwent treatment were kept in a controlled environment for the next 24 h. Following the incubation period, the cells were collected and put into a centrifuge tube and subjected to spinning at a speed of 1200 rpm for a duration of 5 min. After removing the supernatant, 5000 μL of cold PBS was inserted into every tube and the cells were spun for 5 min. Following the removal of the liquid portion, the solid residue was washed with 1 mL of chilled PBS and subjected to centrifugation under the same conditions of speed and duration. The pellet was then resuspended in 100 μL of Annexin V binding buffer and stained with 2.5 μL of annexin V-FITC (BD biosciences) for 12 min at room temperature. Subsequently, 5 μL of propidium iodide was introduced to the cells, followed by an additional incubation period of 3 min. Subsequently, 300 μL of annexin V binding buffer was introduced to the sample and the resulting combination was relocated to a falcon tube. The final step involved analyzing the sample using a BD FACS Canto II flow cytometer. The samples were maintained in a dark environment throughout the staining procedure to minimize light interference.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA and results were expressed as mean ± Standard Error of the Mean (SEM). A p-value of less than 0.05 was considered statistically significant.

Results

Cytotoxic Effects of Tricyclohexyltin(IV) Dithiocarbamate Compounds Using MTT Assay

Cytotoxicity is quantified by half-maximum inhibitory concentration (IC_{50}) values, representing the concentration of the potential anticancer drug that induces the death of 50% of the cells. A lower IC_{50} value indicates higher toxicity of the compound to cancer cells (Florento *et al*., 2012). The cytotoxic effect of tricyclohexyltin(IV) *N*-methyl-*N*-benzyldithiocarbamate (C1) and tricyclohexyltin(IV) *N*-ethyl-*N*-benzyldithiocarbamate (C2) toward A549 cells after 24 h treatment were expressed in Figs. 3-4, respectively. Figure 2 shows the graph of cisplatin cytotoxicity on A549 cells using the MTT assay method. At concentrations 50, 25, 12.5, 6.25, 3.125 and 1.5625 μM, the average viability values (%) of A549 cells ± Standard

Error of the Mean (SEM) obtained were 38.41±1.66, 55.62±1.45, 71.92±3.96, 81.55±1.61, 87.5±1.96 and 97.82±4.10%, respectively.

Figure 3 shows the graph of the cytotoxic effect on A549 cells treated with C1 after 24 h of treatment. The mean of cell viability (%) ± SEM for C1 were 79.10±2.06, 69.75±4.84, 59.61±4.77, 48.07±2.90, 28.78±0.81, 22.54±0.82 and 21.13±0.41%. At concentrations of 0.08, 0.16, 0.31, 0.63, 1.25, 2.50 and 5.00 µM, respectively. Next, the graph in Fig. 4 shows the cytotoxic effect on A549 cells treated with C2 after 24 h of treatment duration. C2 cell viability (%) at concentrations 0.08, 0.16, 0.31, 0.63, 1.25, 2.50 and 5.00 µM were 98.56±1.89, 98.77±0.56, 96.02±0.70, 89.15±1.21, 65.26±4.00, 28.00±0.45 and 16.12±2.17%, respectively. Both C1 and C2 exhibited the ability to reduce the average viability of A549 cells at the highest concentrations (5 µM) from 100% viability to 21.13±0.41 and 16.12±2.17%. Table 1 shows the IC_{50} values of C1, C2 and cisplatin on A549 cells.

The statistical analysis revealed a noteworthy disparity ($p<0.05$) in the vitality of A549 cells treated with both compounds in comparison to the cells without undergoing treatment. C1 showed a significant difference at all concentrations tested while C2 indicated no notable variance ($p>0.05$) at the three lowest concentrations: 0.08, 0.16 and 0.31 µM.

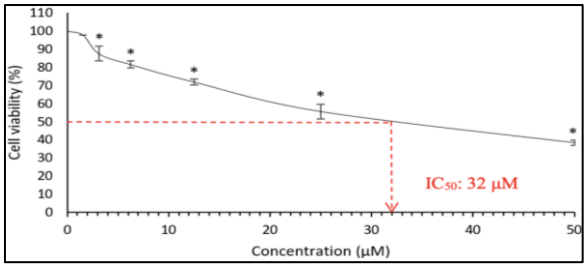

Fig. 2: The cytotoxicity of cisplatin after 24 h of treatment in A549 cells. Values are the cell viability (%) ± SEM of three repeated experiments; * The variance was statistically significant, where the p-value was less than 0.05 in comparison to the negative control

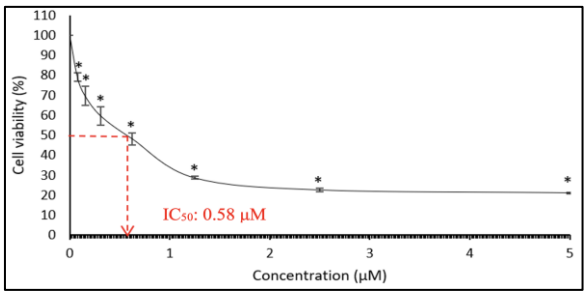

Fig. 3: The cytotoxicity of C1 after 24 h of treatment in A549 cells. Values are the cell viability (%) ± SEM of three repeated experiments; * The variance was statistically significant, where the p-value was less than 0.05 in comparison to the negative control

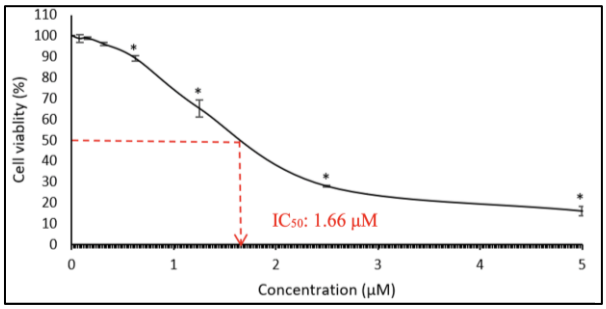

Fig. 4: The cytotoxicity of C2 after 24 h of treatment in A549 cells. Values are the cell viability (%) ± SEM of three repeated experiments; * The variance was statistically significant, where the p-value was less than 0.05 in comparison to the negative control

Table 1: The IC50 value for C1, C2 and cisplatin on A549 cells

Compounds	IC_{50} value (µM) ± SEM
C1	0.58±0.06
C2	1.66±0.08
Cisplatin	0.32±1.53

Morphological Changes of A549 Cells Induced by Tricyclohexyltin(IV) Dithiocarbamate Compounds

The alteration in the structure and form of A549 cells were examined using an inverted microscope after being exposed to the compounds at a concentration that inhibits 50% of cell growth (IC_{50} value). The IC_{50} values for C1, C2 and cisplatin were 0.58, 1.66 and 32 µM, respectively. Examples of morphological changes observed in A549 cells included cell shrinkage, cell swelling, apoptotic bodies and membrane blebbing, whereas A549 cells without treatment did not exhibit significant morphological changes. Figure 5a shows the morphology of untreated cells (negative control) while (Fig. 5b) shows the morphology of A549 cells after being treated with an IC_{50} value of C1. Next, (Fig. 5c) displays changes in A549 cells following C2 treatment whereas (Fig. 5d) displays changes in A549 cells following cisplatin treatment.

Cell Death Pathways Elicited by Tricyclohexyltin(IV) Dithiocarbamate Compounds in A549 Cells

The Annexin V-FITC/PI assay was conducted to determine the mode of A549 cell death induced by the IC_{50} concentration of each tricyclohexyltin(IV) dithiocarbamate compound over 24 h of treatment. Apoptosis and necrosis were two distinct kinds of cell death. Based on Fig. 6, both compounds induced apoptotic cell death by 35.60±4.06% for C1 and 92.47±1.67% for C2. In addition, there were notable variations in the proportions of viable cells and apoptosis cells between untreated cells and A549 cells treated with C1, C2 and cisplatin.

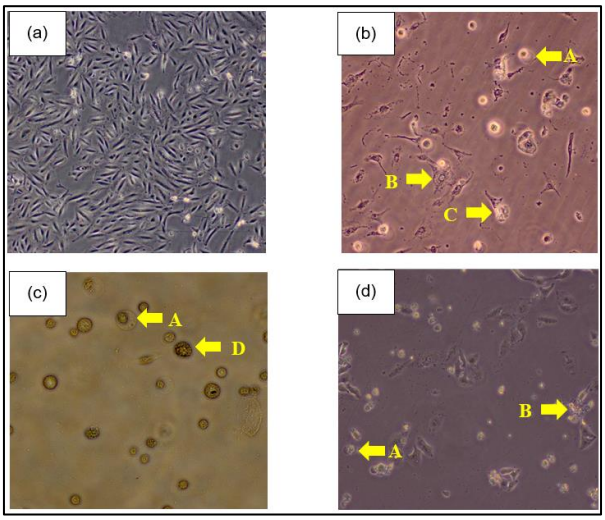

Fig. 5: Morphology of A549 cells; (a) without treatment; (b) with treatment of C1; (c) with treatment of C2; (d) with treatment of cisplatin after 24 h of treatment using IC_{50} value

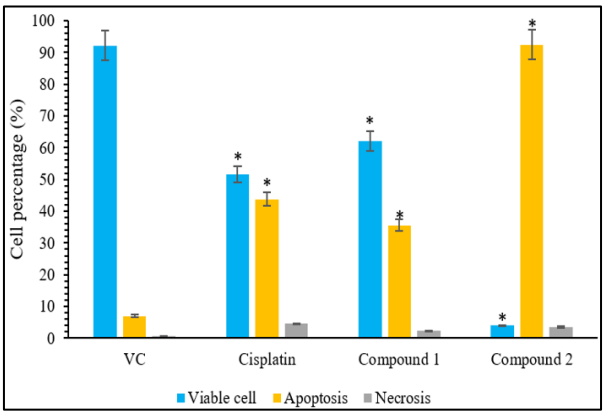

Fig. 6: The percentage of viable, apoptotic and necrotic cells in A549 cells upon being treated with C1, C2 and cisplatin at IC_{50} concentration for 24 h. Data represents the mean ± SEM of at least three independent experiments; * The variance was statistically significant, where the p-value less than 0.05 in comparison to the negative control

Discussion

How *et al*. (2008); Kamaludin *et al*. (2013) classified compounds as potent if their IC_{50} values were below 5 µg cm-^{3} or 8.70 µM. The MTT assay results indicated that C1 showed greater toxicity than C2 on A549 cells, possessing IC_{50} values of 0.58 and 1.66 µM, respectively, after 24 h treatment. This could be attributed to the significance of structure-toxicity relationships in inducing cytotoxic effects. The toxicity of organotin is dependent on the quantity of alkyl or aryl groups connected to the Sn atom. Specifically, tri-alkyl/aryl exhibits high toxicity, followed by di-alkyl/aryl and then monorganotin(IV) compounds (Abd Aziz *et al*., 2023). Furthermore, the lipophilic nature of triorganotin(IV) compounds, as revealed by Ferreira *et al*. (2014; 2013), may also contribute to their enhanced cytotoxicity. The large surface area of organotin(IV) compounds enables them to efficiently cross the plasma membrane, thereby increasing their ability to interact with cellular components and exert cytotoxic effects. In addition, dithiocarbamate ligands are also involved in influencing the toxicity of organotin(IV) compounds, which contribute to lipophilic properties and the stabilization of metal compounds (Galanski *et al*., 2005).

In this study, both tricyclohexyltin(IV) compounds possess different dithiocarbamate ligands. C1 features an *N*-methyl-*N*-benzyldithiocarbamate ligand while C2 incorporates an *N*-ethyl-*N*-benzyldithiocarbamate ligand. The cytotoxic effect increases as the alkyl length decreases (Abd Aziz *et al*., 2023; Koch *et al*., 2009). Therefore, the finding of this study is congruent with previous research (Awang *et al*., 2015), demonstrating that C1 exhibits higher toxicity than C2 due to its shorter alkyl length. Furthermore, cisplatin was utilized as the positive control towards A549 cells in this study. Despite their presumed similarity in molecular structure, both compounds C1 and C2 showed higher cytotoxicity on lung cancer cells compared to cisplatin (Hamid *et al*., 2020). Cisplatin, a ground-breaking metal chemotherapeutic medication, exerts its harmful effects by liberating a chloride ligand, binding to DNA to create intra-strand DNA adducts and impeding DNA synthesis and cellular production (Brown *et al*., 2019). In contrast to cisplatin, the organotin complex binds to DNA through intercalation (Devi and Pachwania, 2018) rather than cross-linking as seen in cisplatin (Adeyemi and Onwudiwe, 2018).

An in-depth observation of cell morphology was conducted in A549 cells to further elucidate the cellular changes upon treatment with C1, C2 and cisplatin. After being exposed to these compounds at their respective IC_{50} values for 24 h, significant variations in cell shape were detected in the cells that underwent treatment. Apoptotic cell death was distinguished by cell shrinkage, the creation of apoptotic bodies and membrane blebbing (Park *et al*., 2021). These findings can be found on A549 cells treated with C1 Fig. 5b-c and cisplatin Fig. 5d. The morphological changes observed in A549 cells were comparable to those seen in the prior study (Hamid *et al*., 2020; Farahana Kamaludin *et al*., 2017). Besides that, (Fig. 5c) shows the characteristics of cell swelling, which is a major feature of necrosis (Miller and Zachary, 2017).

In order to ascertain the specific kind of cell death occurring in A549 cells as a result of tricyclohexyltin(IV) dithiocarbamate compounds, staining was performed using the Annexin V-FITC/PI technique. Anticancer drugs targeting apoptosis signaling pathways have been

identified as a crucial mechanism in cancer treatment (An *et al*., 2019). The Annexin V-FITC/PI result in this study revealed that C1 and C2 could induce apoptotic cell death at approximately 35-92% within 24 h of exposure at their respective IC_{50} concentrations. The results indicate that the proportion of cells undergoing apoptosis, as determined by the Annexin V-FITC assay in combination with Propidium Iodide (PI), does not correspond to the IC_{50} values attained from the MTT experiment. The use of different types of tests with different principles may contribute to this factor (Chan *et al*., 2006). MTT test measures cell proliferation by assessing the reduction of tetrazolium salts through the succinate dehydrogenase enzyme in the mitochondria, forming formazan crystals. In contrast, the Annexin V-FITC/PI test determines cell death by assessing the permeability of the plasma membrane (Mosmann, 1983; Van Engeland *et al*., 1998).

The study found that C1 does not predominantly kill cells, but instead inhibits the proliferation of A549 cells in a particular stage of the cell cycle, leading to cytotoxic effects (Awang *et al*., 2014). The G_0 phase of the cell cycle is a period during which cells do not experience growth and they have the ability to temporarily quit the cell cycle (Wu *et al*., 2021). Over 60% of A549 cells were found viable after being treated with the IC_{50} concentration of C1 and this potentially indicated that the cells were resting. Given that the percentage of A549 cell death for C1 was below 50% after being treated using the IC_{50} concentration, C1 might exert its effects by inhibiting cell proliferation rather than inducing cell death.

Conclusion

In conclusion, two new tricyclohexyltin(IV) dithiocarbamate compounds were successfully assessed for their potential cytotoxic effects and cell death mode using MTT and Annexin V-FITC/PI assays. Based on MTT results, the compounds demonstrated strong cytotoxicity in A549 cells, C1 was more potent (IC_{50} = 0.58 μM), compared to C2 (IC_{50} =1.66 μM). In comparison to cisplatin, both compounds displayed notable cytotoxic effects on A549 cells. Visual examination using an inverted microscope verified the alterations in the shape and structure of A549 cells following a 24-h treatment. A549 cells treated with C1 displayed signs of apoptosis, while those treated with C2 showed both apoptotic and necrotic patterns. Nonetheless, the IC_{50} values trend from the MTT assay did not align with the effects of either compound on the cells. Through Annexin V-FITC/PI result, this study suggested that C1 was linked to the suppression of cell proliferation in A549 cells, as evidenced by a lower percentage of apoptotic cell death (<40%). C2 caused more than 90% of cell death through apoptosis of A549 cells. Hence, it is necessary to conduct additional research on both compounds, which involves analyzing the cell cycle status, testing their cytotoxicity against normal cell lines and ascertaining their selective cytotoxicity. This will yield significant information regarding the potential of both compounds for cancer treatment.

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Author's Contributions

Normah Awang: Developed the conceptual framework; contributed to the manuscript editing and revision.

Nurul Amalina Abd Aziz: Conducted experimental work; performed data analysis; assisted in manuscript editing and revision.

Nur Fatini Nordin: Authored the original manuscript; carried out experimental procedures; analyzed data.

Nurul Farahana Kamaludin: Established the conceptual framework.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that the author has read and approved the manuscript and no ethical issues involved.

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