CYTOTOXICITY AND MODE OF CELL DEATH INDUCED BY TRIPHENYLtin (IV) COMPOUNDS IN VITRO

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ABSTRACT

A series of newly synthesized organotin (IV) with N-alkyl-N-phenylthiocarbamate ligands namely triphenyltin (IV) ethylphenyldithiocarbamate (compound 1) and triphenyltin (IV) butylphenyldithiocarbamate (compound 2) were assessed for their cytotoxic effect against HT-29 human colon adenocarcinoma cells and human CCD-18Co normal colon cells. The cytotoxicity of these organotins in both cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay upon 24 h treatment. Both compounds demonstrated potent cytotoxicity towards HT-29 cells with the IC\textsubscript{50} of 0.18 µM for compound 1 and 0.20 µM for compound 2. Interestingly, compound 1 exhibited lower cytotoxicity towards CCD-18Co with IC\textsubscript{50} of 1.55 µM whereas no IC\textsubscript{50} was detected for compound 2 up to 2 µM treatment. The mode of cell death was determined based on the externalization of phosphatidylserine using flow cytometry. Cells treated with compound 1 and compound 2 were mainly viable and the apoptotic cell death was around 10% which suggests that both compounds induced growth arrest. In conclusion, this study demonstrated that both compounds were selective towards human colorectal cells by giving a strong cytotoxicity to cancer cells and low toxicity towards normal cells. Both compounds were suggested to induce growth arrest in HT-29 cells.

Keywords: Cytotoxicity, Apoptosis, Organotin (IV), Dithiocarbamate, HT-29, CCD-18Co

1. INTRODUCTION

The world cancer statistic in 2008 has estimated 12.66 million people were diagnosed with cancer. Among all types of cancers, colorectal cancer being the third most commonly diagnosed malignancy. An approximately 1.24 million people were suffered with this type of cancer throughout the year, accounting for 10% of overall cancer cases Cancer Research UK, 2011. The mortality resulted from cancers were 7.56 million whereby the colorectal cancer was ranked as the fourth most common cause of cancer-related death in 2008 Cancer Research UK, 2011 (Sasaki et al., 2010).

The increase number of human population will proportionally increase the incidence of cancer although the rates of current cancer incidence remain consistent Cancer Research UK, 2011. Therefore, the effective ways to combat the disease, specifically colorectal cancer are crucially needed and many researchers are currently paying a great attention into this matter. The current treatment used for colorectal cancers are surgery, chemotherapy, radiation therapy and targeted therapies (Hagan et al., 2013). Taken together, surgery excision remains as a basis for cancer treatment especially at the early stages of colorectal cancer (Hagan et al., 2013). Apart from that, chemotherapy is also recognized as one of the effective method used to treat colorectal cancer patients (Hagan et al., 2013; Tong et al., 2011). Several chemotherapeutic drugs used in colorectal cancer treatment are 5-Fluorouracil (5-FU), bevacizumab, cetuximab and oxaliplatin (NCI, 2013; Hagan et al., 2013). Among these, 5-FU have been the main options.
for chemotherapy in patients with stage II, III and IV of colorectal cancer (ACS, 2013).

Unfortunately, the colorectal cancer cells have been identified to become resistant in response to 5-FU treatment. In addition, there were some cases reported the toxicity caused by this drug on normal cells. These have become a major problem to ensure a successful chemotherapy among colorectal cancer patients (Srimuangwong et al., 2012). Therefore, more effective anticancer agents are subjected to be developed in order to treat this type of cancer. Many studies are currently focusing on other alternatives to find the most effective way to enhance the efficacy of 5-FU, as well as to reduce its toxicity towards normal cells (Srimuangwong et al., 2012). Hence, some approaches have been introduced to overcome these problems including the combination of 5-FU with other drugs or new compounds (Srimuangwong et al., 2012). On the other hands, the newly synthesized compounds are also suggested as the potential chemotherapy drugs and being investigated worldwide.

The synthesized organotin derivatives have been identified as one of the promising candidates to combat cancers (Alama et al., 2009). They are getting more attentions due to their diverse structures and potent biological activities (Shahzadi et al., 2006). Interestingly, the organotin biological activities are greatly influenced by their various molecular structures and most of them are generally very toxic even at very low concentrations (Pellerito et al., 2006). Previous studies by (Gielen et al., 2000; Abdellah et al., 2009) using organotin derivatives had shown a significant cytotoxicity and anticancer properties against various types of human cancerous cells in in vitro studies including HT-29 colon adenocarcinoma cells (Girasolo et al., 2010). All novel organotin (IV) derivatives, with the exception of dimethyltin (IV), showed significant cytotoxicity in HT-29 and was suggested to induce cell death via apoptosis (Girasolo et al., 2010). To date, the synthesized di- and tri-n-butyltin (IV) carboxylates derivatives against HT-29 colon adenocarcinoma cell line showed a potent anticancer activity via the inhibition of thioredoxin reductase at the micromolar range (Oliveira et al., 2013). These examples demonstrated the organotin (IV) derivatives as promising and potent new anticancer drugs that promote a new dimension in anticancer drugs development.

In this study, the in vitro cytotoxicity and the mode of cell death of the newly synthesized organotin (IV) compounds namely triphenyltin (IV) ethylphenyldithiocarbamate (compound 1) and triphenyltin (IV) butylphenyldithiocarbamate (compound 2) were assessed. The chemical structures of both compounds are shown in Fig. 1 and 2. The cytotoxicity of both compounds were done in HT-29 colon adenocarcinoma cells and human CCD-18Co normal colon cells. The observation of cell morphological changes and the mode of cell death in both cells were carried out.

![Fig. 1. The chemical structure of compound 1 (Kamaludin et al., 2012)](image-url)
2. MATERIALS AND METHODS

2.1. Method for Compounds Synthesis

Both compounds were synthesized via in-situ method using the respective secondary amines, carbon disulphide and triphenyltin (IV) chloride salts. The formation of the compounds were confirmed via elemental analysis (C,H,N and S), infrared and nuclear magnetic resonance (\(^1\)H and \(^{13}\)C) spectroscopies and X-ray crystallography study (Kamaludin et al., 2011; 2012).

2.2. Cell Culture and Reagents

HT-29 human adenocarcinoma cells and human CCD-18Co normal colon cells were obtained from American Type Culture Collection (ATCC). The HT-29 cells were maintained in McCoy’s 5a Modified medium containing L-glutamine and 10% Foetal Bovine Serum (FBS) (GIBCO, USA). The CCD-18Co cells were maintained in Eagle’s Minimum Essential Medium (MEM) supplemented with sodium bicarbonate (NaHCO\(_3\)), 1% sodium pyruvate, 1% non-essential amino acid, 1% penicillin/streptomycin and 10% Foetal Bovine Serum (FBS) (GIBCO, USA). The cell lines were maintained at 37°C in 5% CO\(_2\) atmosphere according to the recommended protocols by ATCC.

2.3. MTT Cytotoxicity Assay

The viability of HT-29 and CCD-18Co cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were seeded in a sterile 96-well microplate at a density of 5 \(10^4\) cells mL\(^{-1}\) and incubated at 37°C in 5% CO\(_2\) for 24 h. Then, the medium in each well was removed and replaced with a fresh medium containing tested compounds at various concentrations ranging from 0 to 2 µM. After 24 h of treatment, 20 µL of 5 mg mL\(^{-1}\) MTT solution was added to each well prior to 4 h incubation.

An approximate of 180 µL media in each well was removed and replaced with 180 µL of DMSO to dissolve the crystal formazan. After 15 min of incubation, the plate was agitated using orbital shaker for 5 min to ensure the crystal formazan was completely dissolved. The Optical Density (OD) of each well was measured at 570 nm wave length using iMark Microplate Reader (BioRad, USA). The inhibitory concentration that killed 50% of cell population (IC\(_{50}\)) was calculated and used as a parameter to compare the relative cytotoxicity of each compound (Thati et al., 2007).

2.4. Morphological Observation

Cells were seeded into a sterile 96-well microplate at a density of 5\(\times\)10\(^4\) cells mL\(^{-1}\) and allowed to attach within 24 h at 37°C in 5% CO\(_2\). Then, the cells were treated with a series of concentrations (0-2 µM) for 24 h. Finally, the cells were observed under light inverted microscope at 2000 magnification.
2.5. Mode of Cell Death

Annexin V-FITC/PI test was employed to distinguish between apoptotic and necrotic cells according to the method as previously described by Chan et al. (2012) with slight modifications. The HT-29 cells were seeded into a sterile 6-well microplate at a density of $5 \times 10^4$ cells mL$^{-1}$. After 24 h of incubation, the cells were treated with both compounds at IC$_{50}$ concentrations and further incubated for 24 h. Then, the media was collected into a centrifuge tube. The chilled PBS was used to wash the attached cells and it was also collected into the tube. The trypsin was added for 1 min to detach the cells. The trypsinization was stopped using the medium to prevent cell damages. The cells suspension were collected into the tube. The cells were centrifuged at 220 g for 5 min. The supernatant was removed. The pellet was washed twice with chilled PBS. Then, the supernatant was discarded and the cells were resuspended in 100 µL of Annexin V Binding Buffer. The cells were stained with 2.5 µL of Annexin V-FITC (BD Pharmingen) for 15 min at room temperature. Subsequently, 5 µL of Propidium Iodide (PI) (50 µg mL$^{-1}$) was added into the cells and further incubated for 2 min. The whole staining process was carried out in dark condition. Further, 400 µL of Annexin V binding buffer was added into the samples and transferred into a Falcon tube. The cells were analysed quantitatively via BD facsanto II flow cytometry (Becton Dickinson, USA).

2.6. Selectivity Index (SI)

The degree of selectivity of the compounds can be expressed by its SI value as suggested by Badisa et al. (2009).

$$SI = \frac{IC_{50, normal}}{IC_{50, cancer}}$$

2.7. Statistical Analysis

The data were expressed as the mean±Standard Error of Mean (S.E.M). Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 17.0 by employing one-way ANOVA. The data were considered statistically significant when p<0.05.

3. RESULTS

3.1. Cytotoxicity of Triphenyltin (IV) N-alkyl-N-Phenyldithiocarbamate Compounds in HT-29 and CCD-18Co Cells

Both compounds showed high cytotoxicity towards HT-29 cells in concentration dependent manner (Fig. 3). The IC$_{50}$ of compound 1 was 0.18 µM whereas the IC$_{50}$ value for compound 2 was 0.20 µM. This showed that the HT-29 cell line had higher sensitivity towards compound 1 as compared to compound 2. The statistical analysis for both compounds showed significant differences between the percentage of viability of treated and untreated cells (p<0.05) at all concentrations except 0.031 µM and 0.063 µM for compound 1 and compound 2, respectively.

In contrast to HT-29, only compound 1 gave high cytotoxicity towards CCD-18Co cells with IC$_{50}$ value of 1.55 µM upon 24 h of treatment (Fig. 4). There was no IC$_{50}$ value given by compound 2 up to 2 µM treatment. However, the statistical analysis for compound 1 and 2 showed that the percentage of cell viability for treated cells from 0.25 µM-2.00 µM and 0.5 µM-2.00 µM were significantly difference (p<0.05) compared to the percentage of cell viability exhibited by untreated cells, respectively.

3.2. Selectivity Index (SI)

Both compounds showed good selectivity against HT-29 and CCD-18Co cell lines with the SI value of more than 2 (Table 1). High SI value (>2) of a compound gives a selective toxicity towards cancer cells. While the compound with SI value <2 is considered to give general toxicity in which it also can cause cytotoxicity in normal cells (Badisa et al., 2009).

3.3. Morphological Changes of HT-29 Cells Induced by Triphenyltin (IV) N-alkyl-N-Phenyldithiocarbamate

The observation of cells morphology changes were assessed using the different concentration of compounds upon 24 h treatment (Fig. 5). The result of observation showed that the cells were treated using the highest concentration up to 2µM, the cell become shrink and formation of apoptotic bodies whereas no significant difference with the untreated cells when the cells were exposed to the lowest concentration 0.031µM.

3.3.1. Mode of Cell Death on HT-29 Cells Upon Treated with Triphenyltin (IV) N-alkyl-N-Phenyldithiocarbamate

The percentage of the externalization of phosphatidylserine in HT-29 cells, treated with IC$_{50}$ concentration of both compounds showed that the percentage of cells died via apoptosis were less than 10%, whereby the total cell death were less than 12% (Fig. 6). The cells were mainly viable and there was no significant difference among the percentage of live cells in treated and untreated cells.
Table 1. Selectivity Index (SI) for compound 1 and compound 2

|              | Compound 1     | Compound 2     |
|--------------|----------------|----------------|
| IC_{50}, CCD-18Co | 1.55 µM        | >2.00 µM       |
| IC_{50}, HT-29    | 0.18 µM        | 0.20 µM        |
| SI             | 8.61           | >10            |

Fig. 3. The cytotoxicity of compound 1 and 2 against HT-29 cells upon 24 h of treatment using MTT assay. Data represent the mean (±SEM) of at least three independent experiments.

Fig. 4. The cytotoxicity of compound 1 and 2 against CCD-18Co cells upon 24 h of treatment using MTT assay. Data represent the mean (±SEM) of at least three independent experiments.
Fig. 5. Morphological observations of HT-29 cells upon 24 h of treatment of compound 1 (b-h) and compound 2 (i-o) using 7 different concentrations at 20X magnification. (a) Represents the untreated cells. Both compounds induced morphological changes in HT-29 cells. The morphological changes that can be observed included cell shrinkage, cells became rounded and cells lysis.
Fig. 6. The percentage of viable, apoptotic and necrotic cells in HT-29 cells upon treated with compound 1 and 2 at IC\textsubscript{50} concentration for 24 h. Data represent the mean (± SEM) of at least three independent experiments.

4. DISCUSSION

The organotin derivatives are greatly being synthesized and their anticancer properties are extensively being studied. Organotin compounds have shown a marked cytotoxicity in various types of cell lines of human and animal origins. Several organotin compounds synthesized by our group also showed significant cytotoxicity towards HepG2 hepatocarcinoma, Jurkat T lymphoblastic, chronic myelogenous leukemia (K562) and thymoma murine (WEHI 7.2) cell lines (Kamaludin \textit{et al.}, 2013). Previous finding showed that organotin (IV) dithiocarbamate compounds displayed high toxicity towards Jurkat T lymphoblastic and K562 cells with IC\textsubscript{50} values of less than 9.2 µM (Kamaludin \textit{et al.}, 2013). Therefore, they were classified as very toxic compounds (How \textit{et al.}, 2008).

Similarly, the triphenyltin (IV) N-alkyl-N-phenylthiocarbamate compounds could also be classified as very toxic due to their efficacy to inhibit the growth of HT-29 cell's population as low as 0.18 µM. However, in CCD-18Co cells, high cytotoxicity was only given by compound 1 with IC\textsubscript{50} value of 1.55 µM. Compound 2 was expected to give median inhibitory concentration above 2.00 µM. Both compounds gave cytotoxicity in HT-29 and CCD-18Co cells whereby the higher cytotoxicity was reported by compound 1. This study suggested that the molecular structure of a compound plays a significant role in determining its cytotoxicity. The shorter length of alkyl substitution group in a compound increases its cytotoxicity (Biplob \textit{et al.}, 2008; Ray \textit{et al.}, 2000). This probably explained our finding that showed higher cytotoxicity of compound with ethyl group compared to butyl group in dithiocarbamate ligand, respectively.

Due to the different cytotoxicity exerted by both compounds, the Selectivity Index (SI) was measured to determine their selectivity property (Machana \textit{et al.}, 2011; Badisa \textit{et al.}, 2009). Both compounds had good selectivity against cancer cells with the SI values higher than 2. However, the SI value of compound 2 was greater than compound 1. This indicated that compound 2 probably more suitable to be further studied as a potent anticancer drug.

To further evaluated the cell changes upon treatment, the cell morphological observation was conducted in HT-29 cells. The morphological changes of the cells were detected to be varied in concentration-dependent manner. Slight morphological changes were found at 0.03125 µM and 0.0625 µM. Whilst, at 0.125 µM concentration, the cells began to appear in visibly different form as
compared to control. The cells started losing their shape with more intercell spaces were observed. Gradually, with the increment of treatment concentrations, the cells changed to round shaped cells and getting smaller in size or shrunk. At 2.00 μM concentration, dead cells floated and dirty background were observed suggesting the cells lysed. Cell shrinkage resembles the apoptotic characteristic (Pellerito et al., 2006), thus we suggested that the cells died via apoptosis.

To further confirm the mode of cell death, the Annexin V-FITC/PI test was conducted to determine the percentage of the cells death, either through apoptosis or necrosis. Apoptotic cell death is accompanied by the translocation of Phosphatidylserine (PS) from inner to the outer part of the plasma membrane thereby exposing PS to the external cellular activity (Alabsi et al., 2012). The binding ability of Annexin V to plasma membrane containing PS in the presence of calcium ion (Ca²⁺) indicates that the cells died via apoptosis (Engeland et al., 1998).

In this study, both compounds gave only small percentage of cells death which were less than 12% of overall cell death after treated with IC₅₀ values of both compounds within 24 h. These results displayed inconsistent trend with the IC₅₀ values obtained from the MTT assay. More than 80% of cells were detected as viable in Annexin V-FITC/PI assay as compared to the 50% cells viability as expected from MTT assay results. Hence, these data demonstrated that the different endpoints being assessed by two different methods would greatly influence the experimental data (Chan et al., 2006). The measurement of cell viability using MTT assay is based on reduction of tetrazolium salt by the mitochondrial succinate dehydrogenase enzyme to form crystal formazan, while the measurement of cell death in Annexin V-FITC/PI method is dependent on the integrity of plasma membrane (Mosmann, 1983; Engeland et al., 1998).

Based on the mode of cell death assessment, this study suggested that the cytotoxic effects exerted by these compounds were not by directly killing the cells, but most probably due to the growth inhibitory effects of HT-29 cells at the cell cycle level. Within this cycle, there is a phase known as G₀ phase whereby no proliferation occurs in living cells and the cells will leave the cycle temporarily (Pratt, 1994). This could be the possible reason to the observation made in this study whereby the HT-29 cells were still alive upon treated with IC₅₀ concentration, which could be due to the cells were in resting phase. Hence, the percentages of dead cells were less than 50% in HT-29 cells treated with IC₅₀ concentration of both compounds, suggesting that both compounds might act through the inhibition of cell proliferation instead of killing the cells.

5. CONCLUSION

The triphenyltin (IV) N-alkyl-N-phenyldithiocarbamate compounds demonstrated a strong cytotoxicity in HT-29 cells and showed good selectivity between HT-29 and CCD-18Co cells. In comparison between these compounds, the triphenyltin (IV) butylphenyldithiocarbamate (compound 2) was found to be more selective on cancer cells than triphenyltin (IV) ethylphenyldithiocarbamate (compound 1). However, the efficacy of both compounds to kill the HT-29 cells were not as expected whereby more than 80% of cells were remain viable upon treated using the IC₅₀ concentration. Hence, this study suggested that both compounds induced growth arrest in HT-29 cells due to the lower percentage of apoptotic cell death.

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