Research Article

Design and *In Vitro* Biological Evaluation of a Novel Organotin(IV) Complex with 1-(4-Carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione

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A novel triphenyltin(IV) compound with 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione was synthesized and characterized by IR, NMR spectroscopy, mass spectrometry, and elemental analysis. *In vitro* anticancer activity of ligand precursor and synthesized organotin(IV) compound was determined against tumor cell lines: human adenocarcinoma (HeLa), human myelogenous leukemia (K562), and human breast cancer (MDA-MB-453), using microculture tetrazolium test (MTT) assay. The results indicate that complex exhibited very high antiproliferative activity against all tested cell lines with IC_{50} values in the range of 0.22 to 0.53 μM. The highest activity organotin(IV) compound expressed against the HeLa cells ($IC_{50} = 0.22 \pm 0.04\,\mu$M). The ligand precursor did not show anticancer activity ($IC_{50} > 200\,\mu$M). Furthermore, fluorescence microscopy analysis of HeLa cells reveal that organotin(IV) complex induced apoptosis as a mode of cell death, which is consistent with the increase of cells in the sub-G1 phase.

1. Introduction

Metal complexes have been successfully applied in medicine for the treatment of human diseases such as cancer, rheumatoid arthritis, and gastric and duodenal ulcers and also are in widespread use as imaging agents as diagnostics tools [1]. One of the most expanding areas in medicinal bioinorganic chemistry is research on the synthesis of new metal-based compounds and their applications in medicine as drugs [2–4]. Nowadays, cancer is one of the most threatening mankind diseases. During last five decades, over 500,000 synthetic chemical compounds have been tested for their antitumor activity, but only about 25 of these are in worldwide use today [5]. The application of the first and most worldwide used metal-based anticancer drug, cisplatin, discovered by Rosenberg, is limited by severe side effects such as neuro-, nephro-, and ototoxicity [6–11], and therefore, the research of novel metal-based complexes with reduced toxicity and improved clinical efficacy is the subject of intensive studies into anticancer drug investigations [12–16].

Recent studies have shown very promising *in vitro* antitumor properties of organotin(IV) compounds, against a wide panel of tumor cell lines of human origin [17–21]. The organotin(IV) complexes with carboxylate [22–29], thiolato

[30–36], and dithiocarbamato [37] ligands have been extensively studied, and it was found that (carboxylato)triphenyltin(IV) and tetraorganotin(IV) compounds show the highest cytotoxic activity [22, 38]. However, the possible application of the synthesized organotin(IV) derivatives was very limited due to their poor water solubility [39]. Also, significant biological studies of organotin(IV) derivatives with benzoic acids have been reported [40–46].

Many studies demonstrated that cyclic imides such as succinimides (pyrrolidine-2,5-diones) have promising biological properties such as nephrotoxic [47], anticonvulsant [48], antimutagenic [49], and analgesic [50] activities. Additionally, it was found that they inhibit a selective monoglyceride lipase and psychiatric disorders such as anxiety and depression [51]. Therefore, attention herein was drawn to aryl-N-succinimide with a carboxylate substituent as ligand compound. There are numerous reports showing that there is no single or well-defined number of ways in which organotin(IV) compounds interact with the cell membrane or constituents within the cell [52–55]. Thus, the mechanism of action of organotin(IV) compounds in effecting cell death remains unclear, and further work is necessary to identify the real apoptotic or necrotic pathways that cells treated with these compounds follow to their death.

In this study, the synthesis and characterization of a triphenyltin(IV) compound containing 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione anion (CEMPD$^-$) is reported. The evaluation of cytotoxic activity against tumor cell lines human cervix adenocarcinoma (HeLa), human myelogenous leukemia (K562), and human breast cancer (MDA-MB-453) has been performed. Additionally, the mode of cell death and cell cycle distribution of HeLa cells induced by synthesized complex were studied.

2. Materials and Methods

2.1. Chemicals and Methods. The ligand 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione, a racemate, was synthesized as described previously [56]. Elemental analyses were performed on an elemental vario EL III microanalyzer. A Nicolet 6700 FT-IR spectrometer and ATR technique were used for recording midinfrared spectra (4000–400 cm^{-1}) for complex. NMR spectra were recorded on Bruker Avance III 500 spectrometer. Chemical shifts for ^{1}H, ^{13}C, and spectra were referenced to internal standard TMS. Mass spectra of the compound were recorded with an Orbitrap LTQ XL instrument (Thermo Scientific, Bremen, Germany) in CH$_3$CN [57]. Reagents and solvents were of commercial reagent grade quality and used without further purification.

2.2. Synthesis of Complex. A suspension of the 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione (50 mg; 0.191 mmol) in distilled water (10 mL) was treated with LiOH·H$_2$O (8.1 mg; 0.191 mmol), the mixture was stirred for 3 h at 40°C, and a clear solution was formed. Then, 5 mL of Ph$_3$SnCl (73.8 mg; 0.191 mmol) methanolic solution was added dropwise into the reaction mixture. Afterwards, the solution was stirred for 3 h and white precipitate was

formed. The precipitate was filtered off, washed with 5 mL of distilled water, and then dried *in vacuo* over silica gel.

[Ph$_3$Sn(CEMPD)] yield: 75 mg (62%), white solid. Anal. calcd. for SnC$_{32}$H$_{29}$NO$_4$ × 2H$_2$O: C, 59.47; H, 5.15; N, 2.17. Found: C, 59.60; H, 5.19; N, 2.13. IR (ATR, cm^{-1}): 1716 (s) (ν C=O), 1642 (s) (ν_a COO$^-$), 1390 (s) (ν_s COO$^-$), 449 (s) (ν Sn–O). ^{1}H NMR (500 MHz, DMSO): $|\delta|$ ppm = 7.90 (d, 2H, –C$_6$H$_4$), 7.86 (m, 6H, o-H in SnPh$_3$, ^3J(^{1}H–Sn) = 34 Hz), 7.39 (m, 9H, m- and p-H in SnPh$_3$, ^5J(^{1}H–Sn) = 77 Hz), 7.26 (d, 2H, –C$_6$H$_4$), 2.78–2.62 (AB q, 2H, –C^{5}H$_2$–), 1.70–1.58 (m, 2H, –C^{2}H$_2$CH$_3$), 1.27 (s, 3H, –C^{3}H$_3$), 0.85 (t, 3H, –CH$_2$C^{1}H$_3$); ^{13}C NMR (125 MHz, DMSO): $|\delta|$ ppm = 181.92 (C6), 175.32 (C7), 169.35 (C12), 143.46 (C8), 137.58 (C2′ and C6′ in SnPh$_3$, ^2J(^{13}C–Sn) = 45 Hz), 130.13 (C11), 129.26 (C10), 128.69 (C1′ in SnPh$_3$, ^1J(^{13}C–Sn) = 67 Hz), 127,01 (C3′ and C5′ in SnPh$_3$), 44.12 (C4), 127.01 (C9), 40.39 (C5), 31.27 (C2), 23.32 (C3), 8.85 (C1). HR ESI–MS (CH$_3$CN), m/z: 634.10257 [M + Na]$^+$; 650.07690 [M + K]$^+$.

2.3. Preparation of Drug Solutions. DMSO (Sigma-Aldrich, St. Louis, MO, USA) was used for preparation of stock solutions for investigated compounds at the concentration of 10 mM. Afterwards, various working concentrations were prepared from the stock solutions diluting with the nutrient medium. The nutrient medium was RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L glutamine, 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) [57].

2.4. Cell Lines. Human cervix adenocarcinoma cell line (HeLa), human chronic myelogenous leukemia cells (K562), and human breast cancer cell line (MDA-MB-453) were grown in complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) as indicated in [57].

2.5. Determination of Cell Survival. Similarly to methods described in [57], HeLa (2500 cells/well) and MDA-MB-453 cells (3000 cells/well) were seeded into the wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after the cell adherence, different concentrations of investigated compounds were added to the wells, except for the controls, where only the complete medium was added. The final complex concentration range used in the experiments was 0.0625–1 μM (0.0625, 0.125, 0.25, 0.5, and 1 μM). The ligand concentration range used was 12.5–200 μM. The final concentration of DMSO was negligible for complex and never exceeded 1% for ligand, which is a nontoxic concentration for the cells [57]. For nonadherent K562 cells (5000 cells/well), the compounds were applied 2 h after cell seeding. The culture medium with corresponding concentrations of investigated compounds, but without cells, was used as blank. The cultures were incubated for 72 h, and the effects of the investigated compounds on cancer cell survival were determined using the microculture tetrazolium test (MTT), according to Mosmann [58] with modification by Ohno and Abe [59], 72 h after the addition of the

investigated compounds. Briefly, $20 \mu L$ of MTT solution (5 mg/mL of phosphate-buffered saline, PBS) was added to each well. Samples were incubated for additional 4 h at 37°C in a humidified atmosphere of 5% CO_2 (v/v). Afterward, 100 mL of 10 sodium dodecyl sulfate (SDS) was added in order to extract the insoluble formazan, which represents the product of the conversion of the MTT dye by viable cells. The number of viable cells in each well is proportional to the intensity of the absorbance (A) of light, which was measured in a microtiter plate reader at 570 nm, 24 h later [57]. To determine cell survival (%), the A of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (the A of control cells grown only in the nutrient medium) and multiplied by 100. The A of the blank was always subtracted from the A of the corresponding sample incubated with the target cells. IC_{50} is defined as the concentration of an agent inhibiting cell survival by 50% compared with the vehicle-treated control [57]. All experiments were performed in technical and biological triplicates.

2.6. Morphological Analysis (AO/EB Double Staining).
The mode of cell death induced by investigating compound was assessed with acridine orange (AO) and ethidium bromide (EB) double staining assay, according to the standard procedures and examined under a fluorescence microscope [60]. HeLa cells were seeded overnight on coverslips (100,000 cells/coverslip) in 2 mL of complete medium. The following day, cells were treated with complex in concentrations corresponding to $2 \times IC_{50}$ and $4 \times IC_{50}$ for the 72 h treatment. After 24 h, coverslips with target cells were stained with the acridine orange/ethidium bromide mixture ($3 \mu g/mL$ AO and $10 \mu g/mL$ EB in PBS) and visualized and photographed under a fluorescence microscope (fluorescence microscope-PALM MicroBeam systems, Carl Zeiss, Oberkochen, Germany) [57].

2.7. Cell Cycle Analysis.
HeLa cells were seeded in six-well plates (3×10^5 cells/well) and after 24 h of incubation treated with [Ph$_3$Sn(CEMPD)] ($2 \times IC_{50}$ concentration) at 37°C for the additional 24 or 48 h. The assay was performed as described in [57]. In short, after the incubation period, the cells were collected by trypsinization, fixed in ice-cold 70% ethanol for 1 h on ice, and incubated at −20°C for one week. Then, the cells were washed in PBS, and pellets obtained by centrifugation were treated with RNAse ($100 \mu g/mL$) at 37°C for 30 min and incubated with propidium iodide (PI) ($40 \mu g/mL$) for at least 30 min [57]. DNA content and cell-cycle distribution were analyzed using Becton Dickinson FACSCalibur flow cytometer. Flow cytometry analysis was performed using CellQuest (Becton Dickinson, San Jose, CA, USA) software with a minimum of 20,000 cells per sample [61].

3. Results and Discussion

3.1. Synthesis and Characterization.
In the reaction of Ph$_3$SnCl and equimolar amount of 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione, previously deprotonated with LiOH, the desired compound [Ph$_3$Sn(CEMPD)] was obtained as white product in moderate yield. The prepared compound was soluble in dichloromethane, chloroform, dimethyl sulfoxide, and acetonitrile. The synthesized compound was characterized by elemental analysis, IR, NMR spectroscopy, and mass spectrometry.

IR spectra of the synthesized compound showed strong ν(C=O) stretching band at 1716 cm^{-1} which was similar to the ligand precursor [56], indicating that coordination of carbonyl oxygen atoms to the tin(IV) center did not occur. Additionally, strong bands were found at 1642 and 1390 cm^{-1} which correspond to the asymmetric and symmetric vibrations, respectively, of the COO moiety (ligand precursor: 1682 and 1390 cm^{-1}). Moreover, the difference between the asymmetric and symmetric vibrations of more than 200 cm^{-1} indicates monodentate coordination of the carboxylate ligand to tin(IV) center [62]. The medium absorptions corresponding to the Sn–O stretching mode of vibration appear at 449 cm^{-1}.

In the ^{1}H NMR spectra, a set of two signals arising from the o-H and m-/p-H atoms of SnPh$_3$ moiety, at ca. 7.86 and 7.39 ppm, respectively, were observed. Furthermore, aliphatic protons of methyl and ethyl groups from the succinimide ring are on their anticipated positions [56]. Expectedly, methyl protons resonated as one strong singlet at 1.27 ppm, while those from the ethyl group were observed as multiplet (1.70–1.58 ppm) and triplet (0.88 ppm). Additionally, methylene protons in the succinimide ring (–CH$_2$–) were found as characteristic AB quartet in the range 2.78–2.62 ppm. Coupling with tin nucleus can be observed as satellites nearby resonances of o-H atoms from the Ph$_3$Sn moiety. ^{13}C NMR spectra of the synthesized compound showed expected signals at the appropriate positions. Numeration of carbon atoms is shown in Scheme 1. All carbonyl carbons are resonating at values higher than 160 ppm. Aromatic carbon atoms can easily be assigned due to visible coupling with tin through one ($ipso$-C) and two (o-C) bonds. Furthermore, HR ESI-MS was recorded in positive ion mode, and the $[M + Na]^+$ and $[M + Na]^+$ mass peak were detectable. Complete characterization of the synthesized compound can be seen in supplementary section (Figures S1–S7).

3.2. In Vitro Cytotoxicity.
The cytotoxic potential of ligand precursor 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione (CEMPDH) and its new synthesized organotin(IV) compound, [Ph$_3$Sn(CEMPD)], was studied in a panel of malignant cell lines. In Figure 1 the survival of HeLa, K562, and MDA-MB-453 in the presence of different concentrations of complex are presented. The IC_{50} values for CEMPDH, [Ph$_3$Sn(CEMPD)] and cisplatin, for comparison, against investigated cell lines are summarized in Table 1 (MTT test, 72 h incubation). Cisplatin was used as a positive control. The results indicate that the ligand precursor CEMPDH did not show cytotoxic activity against all tested cell lines ($IC_{50} > 200 \mu M$). On the other hand, [Ph$_3$Sn(CEMPD)] exhibits high activity against all three malignant cell lines, the
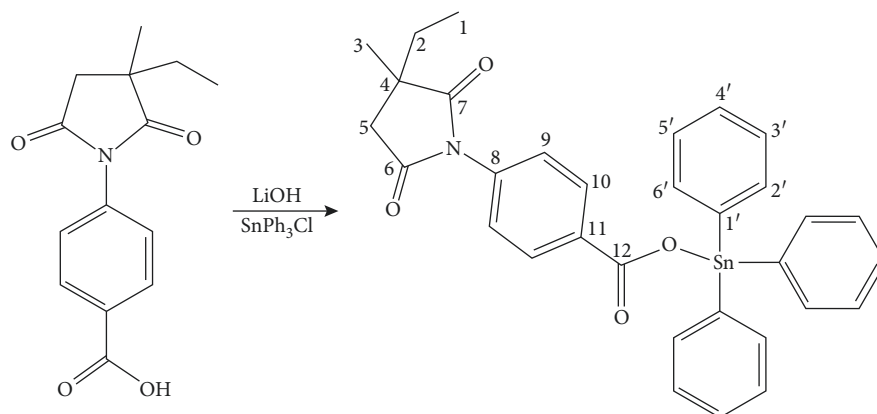
SCHEME 1: Synthesis of complex [Ph$_3$Sn(CEMPD)].
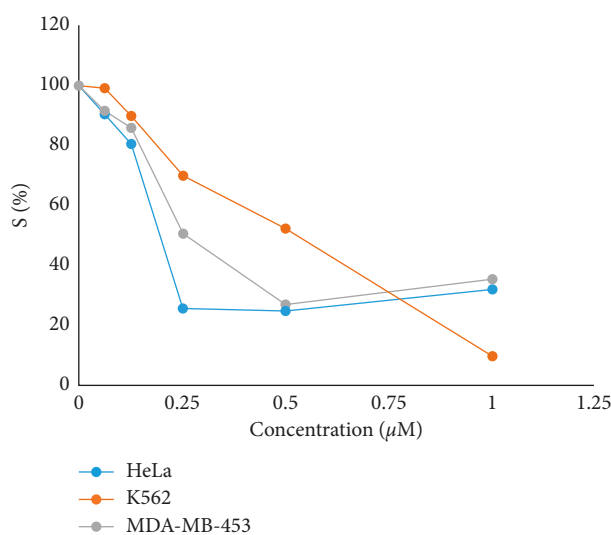
FIGURE 1: Survival of tumor cells as a function of different concentrations of [Ph$_3$Sn(CEMPD)] determined by MTT test, after 72 h incubation.

TABLE 1: IC$_{50}$ (μM) (mean $\pm$ SD) of CEMPDH, [Ph$_3$Sn(CEMPD)], and cisplatin (72 h of action).

Compounds	IC50 (μM)		
	HeLa	K562	MDA-MB-453
CEMPDH	>200	>200	>200
[Ph$_3$Sn(CEMPD)]	0.22 $\pm$ 0.04	0.53 $\pm$ 0.01	0.28 $\pm$ 0.03
Cisplatin	6.90 $\pm$ 1.71	5.82 $\pm$ 0.17	6.73 $\pm$ 0.48

highest against HeLa cells and the lowest IC$_{50}$ value observed against K562 cells. In comparison with cisplatin (Table 1), the IC$_{50}$ value of [Ph$_3$Sn(CEMPD)] ranges from 0.22 to 0.53 μM and novel organotin(IV) compound is 11 up to 31 times more active. Furthermore, the activity of [Ph$_3$Sn(CEMPD)] is in expected range for (carboxylato)triphenyltin(IV) compounds [28, 63]. It is well known that some analogue [Ph$_3$Sn(RCOO)] compounds (e.g., RCOO = 4-methoxyphenylacetato, 2,5-dimethyl-3-furoato, or 1,4-benzodioxane-6-carboxylato) may possess relatively high selectivity towards K562 tumor cells

[28]. Namely, they can be up to four times more active against the K562 tumor cell line than that on normal rested or stimulated peripheral blood mononuclear cells (PBMCs). Thus, further studies should concern the activity of [Ph$_3$Sn(CEMPD)] against normal cells.

3.3. Morphological Analysis of HeLa Cell Death. To determine the mode of cell death induced by [Ph$_3$Sn(CEMPD)] in HeLa cell line, AO/EB double staining assay was performed. Selected cell line was treated for 24 h with the concentrations of the investigated compound corresponding to 2 $\times$ IC$_{50}$ and 4 $\times$ IC$_{50}$ values obtained in MTT assay. The results from fluorescence microscopy of treated HeLa cells are showed in Figure 2.

Treatment of HeLa cells for 24 h with [Ph$_3$Sn(CEMPD)] (2 $\times$ IC$_{50}$) induced nuclear condensation detected by freely permeable dye AO, which is a typical morphological feature of apoptosis. Higher concentration of [Ph$_3$Sn(CEMPD)] (4 $\times$ IC$_{50}$) induced a significantly higher level of apoptosis, primarily nuclear fragmentation. Furthermore, a typical feature correlated to late apoptosis, damage of the plasma membrane, could be detected by the entry of the EB into the cell and binding to the DNA (red fluorescence).

3.4. Cell Cycle Analysis. Based on the results described above, to further examine the mechanisms of action of [Ph$_3$Sn(CEMPD)] in HeLa cells, determination of cell cycle distribution was performed on the basis of DNA content in HeLa cell line, after exposure for 24 and 48 h by flow cytometry analysis (PI staining). As shown in Figure 3, after exposure of HeLa cells to [Ph$_3$Sn(CEMPD)] in concentration corresponding to 2 $\times$ IC$_{50}$, number of cells in sub-G1 increases significantly in comparison with control cells (24/48 h: 1.62/3.68%, control; 9.0/10.11%, [Ph$_3$Sn(CEMPD)]). After 48 h of incubation, there is a small increase in the number of cells in the sub-G1 phase (relative to 24 h), but also a decrease in the number of cells in the S and G2/M stages relative to the control cells. It could be that cells in the S and G2/M phase are more susceptible to apoptotic stimuli than those in the G1 phase.
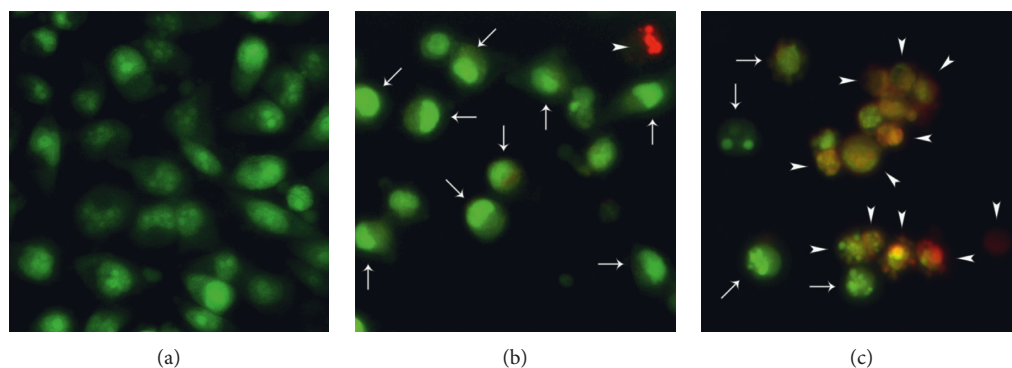
(a)   (b)   (c)

FIGURE 2: Fluorescent micrographs of acridine orange and ethidium bromide stained HeLa cells, untreated (a) or treated with [Ph$_3$Sn(CEMPD)] for 24 h (b) $2 \times$ IC$_{50}$ and (c) $4 \times$ IC$_{50}$. Arrows indicate apoptotic cells with condensed chromatin and/or fragmented nuclei while arrowheads mark necrotic cells.
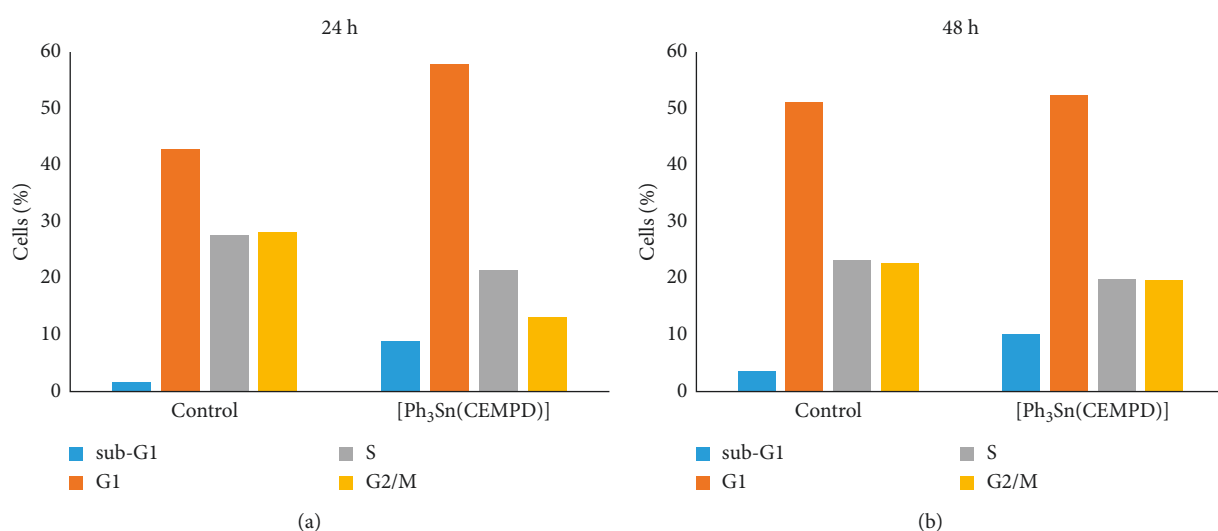

(a)   (b)

FIGURE 3: Effect of [Ph$_3$Sn(CEMPD)] on cell cycle phase distribution: HeLa cell lines were exposed to IC$_{50}$ doses of organotin(IV) compound.

4. Conclusion

The synthesis of a new triphenyltin(IV) complex with 1-(4-carboxyphenyl)-3-ethyl-3-methylpyrrolidine-2,5-dione, [Ph$_3$Sn(CEMPD)] is described. The compound was characterized by elemental analysis, IR, ^{1}H, ^{13}C NMR spectroscopy, and mass spectrometry. [Ph$_3$Sn(CEMPD)] along with appropriate acid CEMPDH was tested against human adenocarcinoma (HeLa), human myelogenous leukemia (K562), and human breast cancer (MDA-MB-453), by MTT assay. The results have shown that [Ph$_3$Sn(CEMPD)] expressed excellent cytotoxicity against all mentioned cancer cell lines with the IC$_{50}$ range from 0.22 to 0.53 μM which is 11 to 31 times higher in comparison with clinically used anticancer drug cisplatin. Nevertheless, the ligand precursor did not show cytotoxic activity (IC$_{50}$ > 200 μM). Additionally, the mode of HeLa cell death induced by synthesized complex was apoptosis, seen as condensation and fragmentation of nuclei, which is consistent with the results of flow cytometry and increase of number of cells in the sub-G1 phase of the cell cycle after 24 h treatment scheme. The effects of [Ph$_3$Sn(CEMPD)] towards cancer cells indicate the necessity for further studies with *in vitro* and/or presumably *in vivo* tests.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Supplementary Materials

Figure S1. ^{1}H NMR spectrum of [Ph$_3$Sn(CEMPD)]. Figure S2. ^{13}C NMR spectrum of [Ph$_3$Sn(CEMPD)]. Figure S3. coupling of protons with tin observed in ^{1}H NMR spectrum of [Ph$_3$Sn(CEMPD)]. Figure S4. coupling of carbon atoms with tin observed in ^{13}C NMR spectrum of [Ph$_3$Sn(CEMPD)]. Figure S5. IR spectrum of [Ph$_3$Sn(CEMPD)]. Figure S6. HR ESI mass spectrum of [Ph$_3$Sn(CEMPD)]; [M+Na]$^+$ (634.10257). Figure S7. HR ESI mass spectrum of [Ph$_3$Sn(CEMPD)]; [M+K]$^+$ (650.07690). (*Supplementary Materials*)

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