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Cytotoxic Effects of Valsartan Organotin(IV) Complexes on Human Lung Cancer Cells

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Abstract: The organotin(IV) compounds used in chemotherapy due to its lipophilicity, affected by the number of carbon atoms and the cytotoxicity. These are affected by the obtainability of Sn coordination bond and bond stabilization between ligand and tin. Two novel organotin(IV) complexes were synthesized, characterized, and tested against human lung cancer cells (A549). The cytotoxic effect of the prepared organotin(IV) complexes against human lung cancer cells (A549) was investigated using the MTT colorimetric assay. Apoptosis was investigated by flow cytometry. The cytotoxicity assay reveals that the Bu_2SnL_2 complex is more active to inhibit the growth of A549 cells compared to the Ph_2SnL_2, and doxorubicin, nevertheless at high concentration (50 and 100) µg/mL the doxorubicin was more affective to inhibit the viability of A549 cells.

Keywords: organotin(IV); valsartan; anti-cancer; A5493; IC_{50}; MTT; flow cytometry; Apoptosis.

1. Introduction

Tin forms several organic compounds called organotin compounds that have at least one covalent bond between tin and carbon atoms; these compounds possess industrial and pharmaceutical importance [1]. Historically, the first preparation of the organotin compounds' indirect method was in 1849 by Frankland from heated ethyl iodide and tin; the result was crystals of diethyltin diiodide [2]. The organotin(IV) compounds got recognized as chemotherapy based on metallotherapeutic drugs obtained after cis-platin discovered in 1969 [3].

Organotin (IV) compounds showed high antitumor activity against different types of cancer with very low doses, such as ovarian, lung, epidermoid, lymphomas, cervical, bladder, germ cell, and many others. The cancer cells undergo apoptosis, the programmed cell death; the cells also face the lipophilicity, the effect of carbon atoms [4-6]. Clear evidence of this therapeutic activity of di-organotin(IV) complexes (R_2SnL_2 where R are Me, Et, Bu, Ph or Bz and L is 2-phenylmono-methylglutarate), the diethyltin(IV) complex showed a visible activity against human epidermoid cancer (KB cell lines) better than cisplatin [7].

Chemically, the cytotoxic activity of organotin(IV) complexes affected by obtainability of coordination bond at Sn and the bond stabilization between ligand and tin such as Sn-S and

Sn-N in ligands like acrylates, Schiff bases, acetic acid, xylene, etc. The significant role of the complexes played by the alkyl or aryl groups, while ligand represents the secondary role [8].

The malignant cells lead to metastasis, which can happen in practically all the positions of the body so that there are more than 100 different kinds of malignancies. Lung cancer leading to death overall percent exceeds that of the breast, colon, and prostate cancers. Worldwide one million deaths annually as a result of lung cancer, which means it is truly plague. The primary cause is usually attributed to cigarette smoking with a proportion of 80%, while asbestos, other vocational and environmental exposures, and the genetic factors contributed to the rest [9]. In the eukaryotic cell cycle, the first phase of the period is called the G1 phase (Gap 1), which precedes the DNA replication. In this phase, the cell grows in size and increases in the number of proteins and the number of organelles (such as mitochondria and ribosome). Follows that DNA replication, which occurs during the S (synthesis) phase. Next step, the G2 phase (Gap 2) occurs, in which the proteins synthesis and cell growth to prepare the cell to the mitosis (M) phase. In this phase, the chromosomes in the cell nucleus separated into two identical sets in two nuclei. In the G0 phase, the cell stopped dividing and have gone out of the cell cycle [10].

Valsartan chemically is N-{4-[(1-(1H-tetrazole-5-yl)phenyl)benzyl}-N-valeryl-L-valine, and it is a medication that is mainly used to reduce high blood pressure and to treat heart failure cases. It has a high molecular weight (435.5) and a high aromatic content (two aryl groups and a tetrazole ring), and it contains a high proportion (27%) of heteroatoms (nitrogen and oxygen) [11-12]. This study aims to evaluate the cytotoxicity of the valsartan organotin complexes against A549 cells.

2. Materials and Methods

2.1. Cytotoxicity assay.

The cytotoxic activity of samples stock solution was performed by using MTT assay. Tumor cell line A549 (Human Lung Cancer Cell line) was were grown in DMEM media supplemented with 100 µg/mL streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum in a humidified and 5% (v/v) CO_2 atmosphere at 37 ºC. Cells were seeding twice a week, they were grown in 96 flat well micro-titer plates (10^4-10^6 cells/well), in a final volume of 200 µL complete culture medium per each well. Plates were incubated at 37 °C, 5% CO_2 for 24 h. The media were removed, and the wells were washed once by PBS, and two-fold serial concentrations of the samples under test (0, 6.5, 12.5,25, 50, and 100 µg/ml) were added to the cell monolayer. The assay was conducted in triplicates for each dose and incubated for 48 hours at 37 ºC in a 5% CO_2 incubator. After incubation, 20 µL of MTT solution was added to each well, mix well, and incubated again at 37°C, 5% CO_2 for 4 hours in the dark. To dissolve the formed crystals, 50 µL acidic isopropanol was added to each well and incubated 4 h at 37 ºC in the dark. The absorbance was measured using ELISA Reader-measure OD at 570 nm (background wavelength is 630 nm). The IC_{50} values will be calculated using sigmoidal concentration-response curve fitting models (Sigmaplot software) [13].

2.2. Cell cycle analysis.

The principle of the Annexin V apoptosis detection kit is set up on the perception. When the apoptosis occurs, the cells transfer the membrane phosphatidylserine (PS) from the internal surface of the plasma membrane to the outer cell surface. The moment that the PS appear on the cell surface can be easily discovered via staining with a fluorescent joint of Annexin V,

which the protein has an affinity towards PS. These detections can be tested by flow cytometry (an instrument, characterize the cells as they pass in a flow-through a laser beam. The irradiated cells show several types of signals that are detected, convert to digital data, and analyzed by computer) or by fluorescence microscopy. According to this kit, it can distinguish between apoptosis and necrosis through both Annexin V-FITC and PI staining [14].

Annexin V-FITC assay protocol includes collecting 5×10^5 A549 cells by centrifugation and re-suspend cells in 500 µL of Binding Buffer. After that, 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI 50 mg/ml) were added to the cell suspension. The mixtures were incubated at room temperature for 5 min in the dark. Analyze Annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2).

For detection by fluorescence microscopy, place the cells suspension on a glass slide and cover the cells with a glass coverslip. The cells under a fluorescence microscope Observe using a dual filter set for FITC and rhodamine.

2.3. Statistical analysis.

The Statistical Analysis System- SAS (2012, Statistical Analysis System, User's Guide. Statistical. Version 9.1.th ed. (SAS. Inst. Inc. Cary. N.C., USA) program has been used to estimate the effect of variable factors on the main study. Least Significant Difference (LSD) test (Analysis of Variation-ANOVA) has been used in this study to considerably compare means.

3. Results and Discussion

3.1. Synthesis and Characterization of Organotin(IV) complexes.

The ligand (valsartan) and the synthesized complexes (Ph_2SnL_2 and Bu_2SnL_2) were characterized by elemental analyses, flame atomic absorption spectrophotometer, FT-IR, proton nuclear magnetic resonance (^{1}H-NMR) and tin-119 nuclear magnetic resonance (^{119}Sn-NMR) spectroscopy in recent research [15]. According to the instrumental characterizations, bonding occurs between the tin and the hydroxyl oxygen of the ligand carboxylic acid. The ligand was asymmetry bi-dentate, which reveals that organotin(IV) complexes are hexa-coordinated as shown in Figure 1.

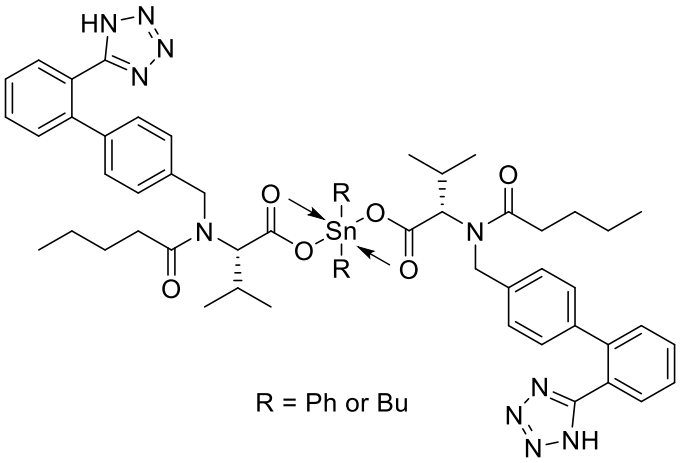

Figure 1. Structure of di-organotin(IV) complexes.

3.2. Cytotoxic effect of Organotin(IV) complexes.

The cytotoxic effect of the di-organotin(IV) complexes against human lung cancer cells (A549) was investigated using MTT colorimetric assay; which is based on the color reduction of 3-(4,5-dimethyl-2-thiazolyl)bromide-2,5-diphenyl-2Htetrazolium from yellow to purple formazan due to the mitochondrial dehydrogenases of the living cells after apoptosis [16,17]. The di-organotin(IV) complexes and doxorubicin were dissolved in acidic isopropanol solvent and diluted with culture medium. Doxorubicin drug used for comparison. The human lung cancer cells (A549) were grown in culture medium and treated with (0, 6.25, 12.5, 25, 50, and 100) µg/mL of the di-organotin(IV) complexes and doxorubicin for 48 hours in an incubator. After that, the MTT solution was added and incubated for 4 hours in the dark. The absorbance measured at 570 nm. The growth inhibition activity of organotin(IV) complexes expression by half-maximal inhibitory concentration (IC_{50}), which elucidates the molar concentration of the compounds that inhibit 50% of cell proliferation compared to untreated cells [18]. Statistical data and IC_{50} values are shown in Table 1, and Figure 2 shows the effect of complexes and their concentrations in the viability of A549 cells.

Table 1. Statistical data and IC_{50} values of complexes and control effect of the viability of A549 cells.

(µg/mL)	**Mean ± SE of the viability of A549 cells**			**LSD value**
	doxorubicin	Ph_2SnL_2	Bu_2SnL_2	
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	0.00 NS
6.25	84.20 ± 4.39	90.65 ± 3.92	15.37 ± 0.72	9.543 *
12.5	54.29 ± 2.41	88.75 ± 3.52	12.99 ± 0.54	8.461 *
25	33.00 ± 1.75	80.82 ± 3.28	8.39 ± 0.46	11.594 *
50	4.69 ± 0.14	46.90 ± 2.71	7.92 ± 0.37	8.955 *
100	3.52 ± 0.08	45.48 ± 1.42	7.76 ± 0.25	7.842 *
LSD value	11.664 *	9.578 *	11.052 *	-
IC_{50}%	14.91	67.88	0.03631	-

*** = (P<0.05) Similar litters mean the absence of significant differences**
LSD = Least Significant Difference

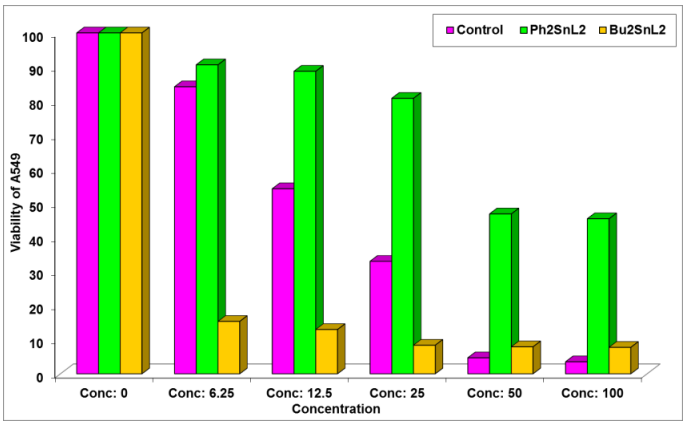

Figure 2. Effect of complexes and their concentrations in the viability of A549 cells.

The results show that the Bu_2SnL_2 has a higher activity to inhibit the growth of A549 cells compared to the Ph_2SnL_2, and doxorubicin. Although, at high concentrations (50 and 100) µg/mL the doxorubicin more affective to inhibit the viability of A549 cells. The IC_{50} value of Bu_2SnL_2 in A549 cells higher than those of doxorubicin, Ph_2SnL_2 and also for investigated di- and tri-organotin compounds (R_2SnL_2 and R_3SnL, where R are Me, Bu or Ph, and L is 3-(1H-indol-3-yl)propanoate) in human ovarian carcinoma cells line (A2780) by Shaheen *et al*. [19]. The activity of organotin(IV) compounds to inhibit the growth cells effected by lipophilicity, van der Waals volume, and bulky specialties of an organic group that attaches to the tin atom

as reported by Ullah *et al.* [20]. The lipophilicity of the n-butyl group is larger than the phenyl group. The presence of Sn-O bond and amino group in organotin(IV) complexes significantly improve antitumor activity against several types of cell lines. The nature of the substituent ligand (valsartan) is useful to transport the organotin moiety cross the cellular membrane and hydrolyzed [21,22].

3.3. Cell cycle phase distribution of A549 cells.

Cell cycle phase distribution of the A549 cells was investigated by the flow cytometer to detect the DNA content after cells staining with annexin-V FITC and propidium iodide. Annexin V-Fluorescein isothiocyanate (Annexin V-FITC) binds to phosphatidylserine (PS) that found in the inner membrane of normal cells. Propidium iodide (PI) is a dye combines with annexin-V in order to estimate the apoptosis and necrotic cells. Normal cells are negative for both markers, apoptosis cells are positive for annexin-V, and necrotic cells are positive for both markers. From the results, as shown in Figures 3 and 4, and tabulated in Table 2, early apoptotic cells Annexin V-FITC positive and PI negative. In contrast, late apoptotic and necrotic cells are V-FITC positive and PI-positive. DNA fragmentation occurs during the late stage, while in the early stage apoptosis, the mitochondrial membrane loses it is potential [23,24].

Table 2. Effect of Bu_2SnL_2 complex in apoptosis of A549 cells.

Group	Apoptosis			Necrosis
	Viable Cell	Early	Late	
A549 Cells+ Bu_2SnL_2	83.79 ± 1.05	4.36 ± 0.23	10.13 ± 0.38	1.72 *
A549 Cells	97.83 ± 0.07	0.75 ± 0.04	0.36 ± 0.02	1.06 *
T-Test	3.482 *	1.597 *	3.047 *	---

*** = (P<0.05) Similar litters mean the absence of significant differences**

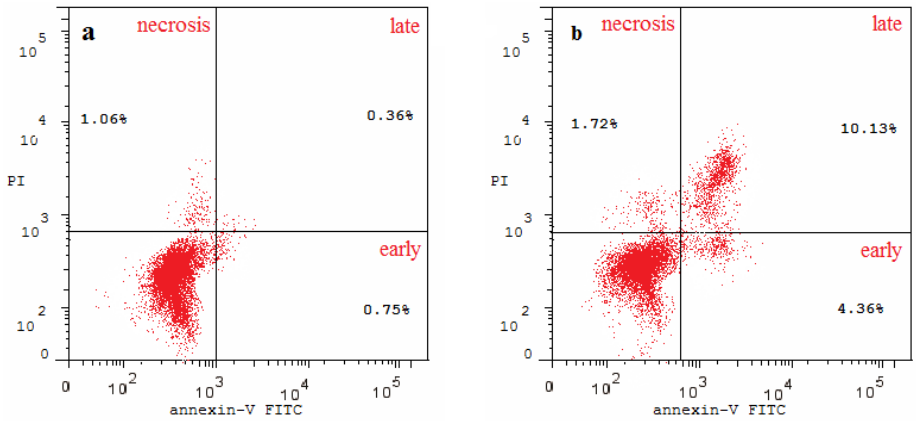

Figure 3. Viable cells, apoptosis, and necrosis of (a) A549 cells (untreated cells) and (b) Bu_2SnL_2 complex in A549 cells measured by flow cytometry (Annexin-V FITC/PI staining).

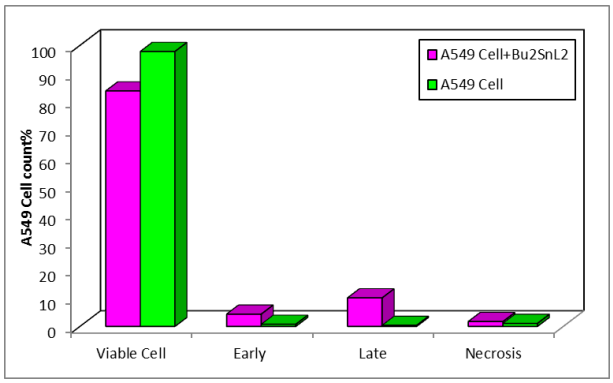

Figure 4. Effect of Bu_2SnL_2 complex in apoptosis results.

The early and late apoptotic A549 cells identify by Annexin V-FITC conjugation with PI [25]. The presence of the Bu_2SnL_2 complex increases the percentage of early and late apoptotic cells, and decrease the percentage of the viable cells. Bu_2SnL_2 complex has proven its activity to damage 10% of the DNA and disrupt 4% of mitochondrial membrane potential. In a recent study, organoselenium-tin complexes with R= di-n-Bu (n-$Bu_2Sn(O_2CCH_2SeC_6H_4F$-p)$_2$) damaged 0.43% of DNA, while disrupting 21% of mitochondrial membrane potential in human breast cancer cell lines (MDA-MB-231) which have higher cytotoxicity than organoselenium-tin complexes with R= tri-n-Bu or di- and tri-Me [26].

The development of anticancer drugs depends on the induction of cell cycle arrest and apoptosis. Restraining the cell cycle, in the case of tumor cells will inhibit the cell proliferation and lead to programmed cell death. Figure 5 shows the fluorescence microscopy histogram of A549 cells in the absence and presence of the Bu_2SnL_2 complex, which explains the cell cycle stages, including G0/G1, S, G2/M, and pre G1. The results revealed that the percentage of A549 cells in G2/M and G1 phases dramatically increased in the presence of the Bu_2SnL_2 complex, the accumulation of treated A549 cells with the Bu_2SnL_2 complex suggested that the cell cycle in the G2/M and pre G1 phases were blocked. The results improve that the organotin moieties after hydrolyzed might be interacted with protein kinases and DNA or binding to DNA through phosphate group, as reported by Kaluđerović *et al.* for triphenyltin(IV) chloride carboxylate complexes in human colon adenocarcinoma cells line (DLD-1) [27], While in S and G0/G1 phases, A549 cells significantly decrease, which denotes the cell growth inhibited in these phases [28]. Table 3 and Figure 6 show the A549 cells percentage content in each phase. The mitochondrial membrane potential (MMP) in the presence of Bu_2SnL_2, several substrates, may not fully be oxidized by the mitochondrial tri-carboxylic acid (TCA-cycle); these substrates observed as the accumulation of α-keto acids, pyruvic acid, and α-ketoglutarate. The Bu_2SnL_2 suggested inhibiting the α-ketoglutarate and pyruvate dehydrogenase in mitochondria [29].

Table 3. DNA content % in the presence of the Bu_2SnL_2 complex in A549 cells.

Group	**DNA content %**			
	G0/G1	S	G2/M	Pre G1
A549 Cell+ Bu_2SnL_2	35.66 ± 1.27	24.18 ± 1.36	40.16 ± 2.05	16.21 ± 0.46
A549 Cell	49.71 ± 2.08	33.09 ± 2.54	17.20 ± 0.87	2.17 ± 0.07
T-Test	6.522 *	5.742 *	6.835 *	4.279 *

*** = (P<0.05) Similar litters mean the absence of significant differences**

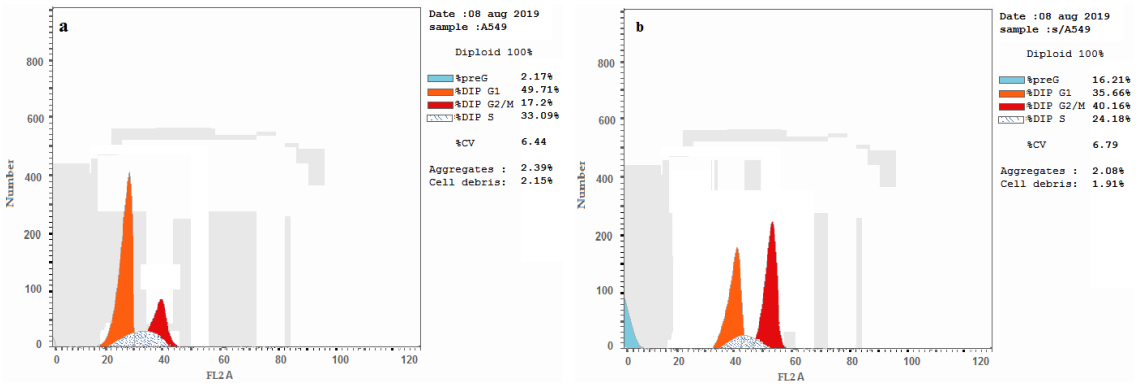

Figure 5. Fluorescence microscopy histogram of (a) A549 cells and (b) A549 cells in presence of Bu_2SnL_2 complex.

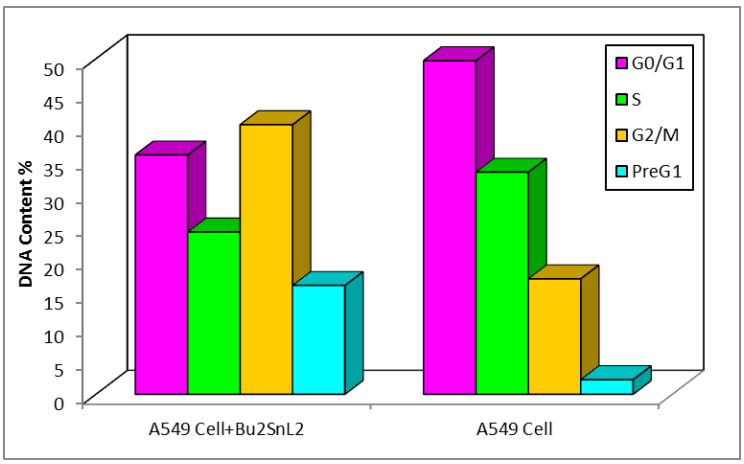

Figure 6. A549 cells content % in the presence of the Bu_2SnL_2 complex in A549 cells.

4. Conclusions

The cytotoxic effect of the organotin(IV) complexes was investigated by using MTT colorimetric assay. Results showed that the Bu_2SnL_2 was more active in inhibiting the growth of A549 cells compared to the Ph_2SnL_2. Cell cycle phase distribution for the A549 cells was investigated by flow cytometer since Bu_2SnL_2 prove its activity to damage the DNA in 10% percentage and disruption of mitochondrial membrane potential in 4% percentage. The results revealed that the percentage of A549 cells in G2/M and pre G1 phases dramatically increased in the presence of the Bu_2SnL_2 complex. At the same time, the existence of these cells in S phase was significantly decreased and also decreased in the G0/G1 phase. These results improve that the Bu_2SnL_2 complex blocked the cell cycle in the G2/M phase and inhibit the cell growth in S and G0/G1 phases.

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Conflicts of Interest

The authors declare no conflict of interest.

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