A novel compound of triphenyltin(IV) with N-tert-butoxycarbonyl-L-ornithine causes cancer cell death by inducing a p53-dependent activation of the mitochondrial pathway of apoptosis

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1. Introduction

Organotin(IV) derivatives have been prepared and tested in the past as possible anticancer agents [1–6] and many of them exhibited interesting activity in specific cancer models. Tin-based drugs represent an excellent alternative to platinum ones as antitumor agents, having the considerable advantage to display a lower toxicity. Triorganotins have demonstrated potential antiproliferative activity in vitro [7] against human tumor cell lines, which has been related to their ability to bind to proteins [8,9]. Special attention is given to organotin(IV) carboxylates with significant cytotoxic properties against different cancer cell lines [10,11]. Some compounds have shown strong apoptosis inducing character in vitro, which can be higher than the corresponding activity of cisplatin or other clinical anticancer drugs [12].

Although the majority of organotin(IV) compounds cause apoptotic cell death, the exact mechanism of action is not yet clearly determined [13]. Several researches have been focused on understanding the binding mode of organotin(IV) compounds to biologically relevant ligands [3,14], often using small peptides, as low-molecular-weight proteins to mimic the metal ion interaction. The organic ligand facilitates the transport of the compounds across the cell membrane, while the antitumor activity is due to dissociated organotin(IV) moieties.

On account of their structural variability, organotin(IV) derivatives of N-substituted amino acids have been extensively studied [14]. Moreover, also organotin(IV) carboxylates of N-protected amino acids have shown interesting pharmacological applications as antitumor agents. Thus, it is of particular interest to examine the structural variations caused by protecting groups on the amino nitrogen of the ligand [15]. One of the most widely used N-terminal protecting groups is the tert-butoxycarbonyl (Boc) group. Boc-amino acids are often used as substrates, substrate analogues or competitive inhibitors of proteolytic enzymes. The study of their conformational preferences is also important for understanding their interactions with enzymes [16].

Several reports have been published concerning structural characterization and bioactivity as antitumor agents of di- and tri-organotin(IV) compounds [17]. Following our previous
investigations on organotin(IV) compounds of l-arginine and N-tert-butoxycarbonyl-l-arginine [18,19], we extended our work to the synthesis, structural characterization and biological activity of a triphenyltin(IV) derivative with N-tert-butoxycarbonyl-l-ornithine (Boc-Orn-OH) (Fig. 1).

Ph3Sn(Boc-Orn-O) has been synthesized and characterized by elemental analysis, FT-IR, ESI mass spectrometry, 1H, 13C and 119Sn NMR spectroscopic techniques and its cytotoxic behavior has been investigated on three human tumor cell lines, HepG2 (hepatocarcinoma cells), MCF-7 (mammary cancer) and HCT116 (colorectal cancer) as well as on non-malignant human-derived hepatic cells (Chang). Moreover, the mechanism of its anti-tumor activity has been evaluated.

2. Experimental

2.1. Materials and physical measurements

Ph3SnOH (Aldrich) and N-tert-butoxycarbonyl-l-ornithine (Fluka) were used without further purification. Elemental microanalyses for C, H and N were performed at the Laboratorio di Microanalisi, University of Padova, Italy. Infrared spectra were recorded with a Perkin-Elmer Spectrum One FT-IR spectrometer, using KBr disc with a resolution of 4 cm⁻¹. All NMR spectra were acquired with an Avance II DMX 400 MHz (9.40 T) spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at 149.20 MHz for 119Sn. One-dimensional 1H and 13C{1H} spectra in CD3OD and DMSO-d6 solutions were acquired at 27 °C with a spectral width (i.e. SW) of 12 ppm and 200 ppm, respectively. One-dimensional 119Sn{1H} NMR spectra in CD3OD and DMSO-d6 solutions were recorded at 27 °C with a SW of 800 ppm by inspecting four spectral windows with SW = 250 ppm at once in the +200 to −600 ppm range. For 119Sn, Me3Sn was employed as external reference (119Sn, δ = 0.00 ppm). 1H and 13C resonances were calibrated on MeSi as external reference (1H, δ = 0.00 ppm; 13C, δ = 0.00 ppm). 119Sn{1H} and 13C{1H} spectra were acquired with broadband proton power-gated decoupling. For all nuclei, positive chemical shift had higher frequencies than the reference. LW in the text is intended as line width at half height. Solutions concentrations were ca. 0.5 M.

2.1.1. Synthesis of Ph3Sn(Boc-Orn-O)

The reaction of Ph3SnOH with Boc-Orn-OH in a 1:1 molar ratio, led to the formation of the organotin(IV) compound according to Eq. (1).

$$\text{Ph}_3\text{SnOH} + \text{Boc-Orn-OH} \rightarrow \text{Ph}_3\text{Sn(Boc-Orn-O)} + \text{H}_2\text{O} \quad (1)$$

A solution of Ph3SnOH (0.734 g, 2 mmol) in dry methanol (15 mL) was added drop wise to a methanol solution (15 mL) of the ligand (Boc-Orn-OH, 0.464 g, 2 mmol) and left to react, under stirring, for 4 h. After cooling at room temperature, the solvent was reduced under vacuum to a small volume (5 mL) using a rotary evaporator; a white solid residual was obtained, which was filtered off, washed three times with a total amount of methanol of 50 mL and dried in vacuo in presence of P2O5. CP-MAS 119Sn NMR (149.20 MHz, CD3OD) of the compound according to Eq. (1).
2.2. Biological assays

2.2.1. Viability assays in vitro

Ph3Sn(Boc-Orn-O) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in culture medium so that the effective DMSO concentration did not exceed 0.1%. HepG2, MCF-7 and HCT116 tumor cell lines and human hepatic Chang liver cells were purchased from American Type Culture Collection, Rockville, MD, USA. All of them were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL) and gentamicin (5 μg/mL). HepG2 culture medium also contained sodium pyruvate (1.0 mM). Cells were maintained in log phase by seeding twice a week at a density of 3 × 10^6 cells/L in humidified 5% CO2 atmosphere, at 37 °C. In all experiments, cells were made quiescent through overnight incubation before the treatment with the compounds or vehicle alone (control cells). No differences were found between cells treated with DMSO 0.1% and untreated cells in terms of cell number and viability.

Cytotoxic activity of Ph3Sn(Boc-Orn-O) against three human tumor cell lines HepG2, MCF-7, HCT116 and human hepatic Chang liver cells was determined by the MTT colorimetric assay based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)bromide-2,5-diphenyl-2H-tetrazolium (MTT) [20] to purple formazan by mitochondrial dehydrogenases of living cells. This method is commonly used to illustrate inhibition of cellular proliferation. Monolayer cultures were treated for 24 h with various concentrations (0.1–5 μM) of the drug. Cisplatin was used for comparison.

Briefly, all cell lines were seeded at 2 × 10^4 cells/well in 96-well plates containing 200 μL RPMI. After an overnight incubation, cells were washed with fresh medium and incubated with the compounds in RPMI. After 24 h incubation, cells were washed, and 50 μL PBS-free medium containing 5 mg/mL MTT was added. The medium was discarded after 2 h incubation at 37 °C by centrifugation, and formazan blue formed in the cells was dissolved in DMSO. The absorbance, measured at 570 nm in a microplate reader (Bio-RAD, Hercules, CA), of MTT concentration at which 50% of cells remained viable relative to the control. Each experiment was repeated at least three times in triplicate to obtain the mean values.

2.2.2. Measurement of phosphatidylserine exposure

HepG2 cells were seeded in triplicate in 24-wells culture plates at a density of 5.0 × 10^3 cells/cm². After overnight incubation, the cells were washed with fresh medium and incubated with the compounds in RPMI. After 24 h, cells were harvested by trypsinization, collected by centrifugation and submitted to Annexin V-FITC/PI double staining (eBioscience, San Diego, CA) as previously reported [21]. The immunoblot was incubated over-night at 4 °C with blocking solution (5% skim milk), followed by incubation with anti-PARP monoclonal antibody (clone D-1, Cat No. SC-365315, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-p53 (FL-393, Cat No SC-6243, Santa Cruz Biotechnology) for 1 h at room temperature. Blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti-IgG antibody (Dako Denmark, Glostrup, Denmark) for 1 h at room temperature. Blots were again washed five times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.). Immunoreactions were also performed using β-actin antibody as loading controls.

2.2.3. Acridine orange and ethidium bromide morphological fluorescence dye staining

Dual staining with acridine orange (AO)/ethidium bromide (EB) allows enumeration of populations of viable non apoptotic, viable (early) apoptotic, nonviable (late) apoptotic, and necrotic cells [21]. Briefly, after HepG2 cells were treated with Ph3Sn(Boc-Orn-O) at 1–2 μM concentration for 24 h, the medium was discarded. Cells were washed with saline 5 mM phosphate buffer (PBS) and then incubated with 100 μL PBS containing 100 μg/mL of EB plus 100 μg/mL of AO. After 20 s, EB/AO solution was discarded and cells immediately visualized by means of fluorescent microscope equipped with an automatic photomicrograph system (Leica, Wetzlar, Germany). Multiple photos were taken at randomly selected areas of the well to ensure that the data obtained are representative.

2.2.4. Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential (ΔΨm) was assayed by flow cytometry, using the cationic lipophilic dye 3,30-dihexyloxocarbocyanine iodide [DiOC6(3)] (Molecular Probes,Inc.) which accumulates in the mitochondrial matrix. Changes in mitochondrial membrane potential are indicated by a reduction in the DiOC6-induced fluorescence intensity. Cells were incubated with DiOC6(3) at a 40 nmol/L final concentration, for 15 min at 37 °C. After centrifugation, cells were washed with PBS and suspended in 500 μL PBS. Samples of at least 1 × 10^4 cells for each sample were subjected to FACs analysis by Epics XL™ flow cytometer as above reported.

2.2.5. Measurement of cytosolic calcium

Intracellular Ca2+ concentration in a single cell was measured using fluo-3/AM as a fluorescent Ca2+ probe, whose intensity is directly representative of cellular concentration of the ion. Fluo-3/AM, at 2 mM final concentration, was added into the cell medium 40 min before the end of the treatment. After centrifugation, cells were washed with PBS and suspended in 500 μL PBS. The fluorescent intensities were analyzed by fluorescence-activated cell sorting analysis in at least 1 × 10^4 cells for each sample.

2.2.6. Measurement of intracellular reactive oxygen species (ROS)

ROS level was monitored by measuring fluorescence changes that resulted from intracellular oxidation of dichloro-dihydro-flur-escin-diacetate (DCFH-DA). DCFH-DA, at 10 mM final concentration, was added to the cell medium 30 min before the end of the treatment. The cells were collected by centrifugation for 5 min at 2000 rpm at 4 °C, washed, suspended in PBS and immediately subjected to fluorescence-activated cell sorting analysis. At least 1 × 10^4 cells were analyzed for each sample.

2.2.7. Western blot analysis

After treatment with the compound, protein extracts were pre- pared and equal amounts of protein samples (80 μg/lane), sub- jected to SDS-PAGE and transferred to nitrocellulose membrane as previously reported [21]. The immunoblot was incubated overnight at 4 °C with blocking solution (5% skim milk), followed by incubation with anti-PARP monoclonal antibody (clone D-1, Cat No. SC-365315, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-p53 (FL-393, Cat No SC-6243, Santa Cruz Biotechnology) for 1 h at room temperature. Blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti- IgG antibody (Dako Denmark, Glostrup, Denmark) for 1 h at room temperature. Blots were again washed five times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.). Immunoreactions were also performed using β-actin antibody as loading controls.

2.2.8. Statistical analysis

Results are given as mean ± SD. Three independent observations were carried out for each experiment replicated three times. Com- parison between matched-paired samples was by Student’s t test. In all cases, significance was accepted if the null hypothesis was rejected at the P < 0.05 level.

3. Results and discussion

3.1. Spectroscopic data

The IR bands of compound were assigned by comparison with the IR spectra of the free amino acid (N-tert-butoxycarbonyl-1-
ornithine) [22,23] and of the related organotin(IV) compound (Ph3SnOH). The vibration at 3379 cm⁻¹ in the IR spectra of the ligand attributed to the ν(N–H), remains unaltered in the compound. Ph3SnOH exhibits a ν(OH) absorption band of medium intensity at 3617 cm⁻¹, which is not observed in the Ph3Sn(Boc-Orn-O) due to the loss of the OH group on binding to the ligand. The infrared spectrum of the free ligand shows a band at ca. 1631 cm⁻¹ corresponding to the vibrational stretching mode νasym(CO₂⁻) of the carboxylate moiety. After deprotonation and binding to tin atom, this band is replaced by a strong absorption at 1654 cm⁻¹, while the corresponding νsym(CO₂⁻) at 1413 cm⁻¹ remains unshifted. The considerable shift of the νasym(CO₂⁻) vibration upon coordination in Ph3Sn(Boc-Orn-O) is due to the binding to the metal through the carboxyl oxygen atom. The criterion of the separation between the symmetric and asymmetric stretching wavenumbers of the carboxylate moiety, Δν = |ν(COO⁻)sym−ν(COO⁻)asym|, has been applied to ascertain its bidentate/chelation or monodentate coordination type [24]. Although a clear line between the two categories cannot be drawn, the observed value of Δν (241 cm⁻¹) strongly points to a monodentate coordination of the carboxylate group of the ligand to the tin atom [25]. For a bridging or chelating carboxylate group, Δν would be expected to be <150 cm⁻¹, as widely observed in the infrared spectra of triorganotin(IV) carboxylates [26]. A medium intensity band corresponding to the Sn-O stretching mode of vibration appears at 447 cm⁻¹, while the bands at 280 and 227 cm⁻¹ are attributed to νs(Sn–O) and νas(Sn–O) stretching modes, respectively. Thus, the presence of both asymmetric and symmetric stretching vibrations in the infrared spectra of the compound in the solid state rules out planar arrangement of the three Sn–Ph bonds. The appearance of two new bands of strong intensity in the IR spectra of Ph3Sn(Boc-Orn-O) at 693 and 722 cm⁻¹ may be assigned to the C–H out-of-plane deformation vibrations [27]. The compound also shows the characteristic Whiffen Y-mode absorption at 450 cm⁻¹, typical of the covalent phenyl-tin bonds [28]. The measured ¹H, ¹³C and ¹¹⁹Sn chemical shifts, in ppm, in CD₃OD and DMSO-d₆ solutions, are given in the experimental section. ¹³C NMR resonances for the free ligand in solution could not be recorded due to its limited solubility. The resonances for the organotin(IV) compound are compared with the free ligand L-ornithine [29]. In the ¹H NMR spectra of Ph3Sn(Boc-Orn-O), proton resonances experience a shift towards higher frequencies for H-5, H-6 and H-7, while H-2 and Boc’s protons remain almost unchanged. The resonances ascribed to the aromatic protons of the phenyl groups, appeared as two well separated sets of multiplets in the regions centered around 7.78 and 7.42 ppm in CD₃OD, and 7.79 and 7.38 in DMSO-d₆. In addition to these signals, ¹H NMR spectra of Ph3Sn(Boc-Orn-O) showed one signal at 1.41 ppm in CD₃OD and 1.38 ppm in DMSO-d₆ corresponding to the protons of the Boc group. The signal of the carboxylic carbon (C-1) is observed at 173.4 ppm in CD₃OD and 170.1 ppm in DMSO-d₆ solutions. Ph3Sn(Boc-Orn-O) exhibits the signals of the ipso-carbon atoms of the phenyl ring at 140.8 and 140.9 ppm in CD₃OD, while in DMSO-d₆ these signals are found at 142.05 and 141.98 ppm: the presence of these two peaks also indicate two different symmetries around the central Sn atom. In CD₃OD, the ¹J(¹¹⁹Sn,¹³C) value of 738 Hz, is closer to the low limit of the typical ¹J(¹¹⁹Sn,¹³C) range (750–850 Hz) [30–32] for penta-coordinated triphenyltin(IV) compounds rather than to the range (550–650 Hz) of tetra-coordinated ones. In CD₃OD solution, the measured ¹¹⁹Sn chemical shift of -166.8 ppm (LW ≈ 380 Hz) for organotin(IV) compound, lies at the boundary between tetra- and penta-coordinated tin [30,31], even if it is more shifted towards a penta-coordinated tin arrangement. In addition, the large measured LW for the tin signal indicates the occurrence of a dynamic process conceivably due to solvent-tin exchange. In order to check the effect of the solvent upon the δ (¹¹⁹Sn), a further ¹¹⁹Sn NMR spectrum has been acquired in DMSO-d₆ solution. Two signals, with LW values smaller than in methanol solution, were found at -125.3 (LW ≈ 120 Hz) and -137.0 ppm (LW ≈ 120 Hz), again pointing to penta-coordination on the tin center. The two similar δ(¹¹⁹Sn) values indicate a slight symmetry variation around the tin center. Moreover, despite the greater coordinating ability of DMSO-d₆ relative to CD₃OD, in DMSO-d₆ the observed signals were more deshielded than in CD₃OD solution, most likely due to the occurrence of weaker Sn–DMSO interaction. These findings highlight the existence of...
monomeric units of the compound in both solution phases where the metal center is also involved in tin-solvent exchange.

In order to distinguish between the spectroscopic data relating to a monomeric or to a long-chain polymeric structure, the mass spectral data for the organotin(IV) compound were recorded. Tin-containing ions are known to form dimeric and polymeric species [33] and indeed dimeric ions and adducts with sodium and hydrogen were found. All the ions containing tin were observed with the expected isotopic distribution and the given attributions were done after comparison with the simulated spectra obtained by the native software of our instrument. In the positive mode, all the ions can be related with the mass of our compound M or with the dimeric cluster 2 M and fragmentations from these species. All the principal ions found can be attributed as follows: m/z 1185.0 and 1163.0 [2 M + Na]⁺ and [2 M + H]⁺ respectively; m/z 953.0 and 931.0 [M-H + Ph₃Sn + Na]⁺ and [M + Ph₃Sn]⁺ respectively; m/z 819.0 [M + C₁₀H₁₈N₂O₃ + Na]⁺; m/z 716.8 [(Ph₃Sn)₂O + H]⁺ found also by others [34]; m/z 604.9 and 582.9 [M + Na]⁺ and [M + H]⁺ respectively; m/z 450.9 [2C₁₀H₁₈N₂O₃ + Na]⁺; m/z 236.9 [C₁₀H₁₈N₂O₃ + Na]⁺, where the C₁₀ residue could be originated from the fragmentation of Ph₃Sn(Boc-Orn-O) according to Scheme 1. It is worthy to note that no peaks derived from the free Boc-Orn-OH were detected, and that the C₁₀ residue found in the analysis of Ph₃Sn(Boc-Orn-O) was not observed during the analysis of the free Boc-Orn-OH in similar conditions. Our organotin(IV) compound was also investigated applying a negative voltage, again revealing ions originated from the presumed structure as deprotonated species or as adducts with formiate such as: m/z 812.9 [M + BocOrn-O]⁻, m/z 760.7 [(Ph₃Sn)₂O + HCOO]⁻; m/z 626.8 [M + HCOO]⁻, m/z 580.9 [M-H]⁻ only present in traces, and m/z 231.1 [Boc-Orn-O]⁻.

Fig. 4. Ph₃Sn(Boc-Orn-O) induces dose-dependent apoptosis in HepG2 cells. (A) Percentage of Annexin V/propidium iodide (PI) double-stained HepG2 cells, as determined by flow cytometry. (B) Fluorescence micrographs of ethidium bromide/acridine orange double-stained HepG2 cells in 200× magnification. (C) Poly(ADP-ribose) polymerase cleavage by immunoblotting with densitometric analysis of the immunoblot. Data of the densitometric analysis are means and standard deviations. Control, cells treated with vehicle. Representative images of three experiments with comparable results.
3.2. Biological results

3.2.1. Cytotoxic activity

The in vitro cytotoxicity of Ph$_3$Sn(Boc-Orn-O) against human tumor cell lines HepG2, HTC116 and MCF-7 was evaluated using MTT metabolic assay. After 24 h of incubation, a dose dependent antiproliferative effect at very low micromolar concentrations of the compound toward all the studied cancer cell lines was measured (Fig. 2). Table 1 reports the concentration of Ph$_3$Sn(Boc-Orn-O) required to inhibit 50% of cell proliferation when compared to untreated cells (IC$_{50}$).

In addition, cytotoxicity of cisplatin, one of the most widely used antitumor drugs in clinical practice, has been included. On direct comparison with cisplatin, the antiproliferative activity of synthesized organotin(IV) derivative is from 20 to 60 times stronger, being the highest triphenyltin(IV) compound/cisplatin activity ratio observed, under the investigated conditions, in the HepG2 cells.

In order to determine the selectivity in the in vitro cytotoxicity of organotin(IV) compound, some additional experiments were conducted on non-malignant human-derived hepatic cells Chang. As shown in Fig. 3, at the concentrations effective to totally inhibit the growth of HepG2 liver tumor cells, Ph$_3$Sn(Boc-Orn-O) shows only a modest effect on the viability of normal-like Chang cells, indicating selectivity towards cancer cells.

Fig. 5. Effects of Ph$_3$Sn(Boc-Orn-O) on the activation of mitochondrial dysfunction in HepG2 cells. (A) The Δψm was detected by fluorescence intensity of 3,30-dihexyloxacarbocyanine iodide-treated cells, as determined by flow cytometry. (B) Ca$^{2+}$ levels were assayed after cell loading with fluo-3/AM followed by flow cytometry analysis. (C) p53 level by immunoblotting with densitometric analysis of the immunoblots. Data of the densitometric analysis are means and standard deviations. Control, cells treated with vehicle. Representative images of three experiments with comparable results.
3.2.2. Induction of apoptosis in HepG2 cells

To determine whether the antiproliferative effect of Ph$_3$Sn(Boc-Orn-O) was due to apoptosis, HepG2 cells were treated with the compound for 24 h, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. The chosen concentrations were selected on the basis that they represented IC$_{50}$ and twice the IC$_{50}$ value. As shown in Fig. 4A, the control cells stained negative for both Annexin V-FITC and PI. On the other hand, HepG2 cells treated with increasing doses, showed increased proportions of Annexin V-positive cells, indicating that they were at an early stage of apoptosis. The double positive staining of particular cells also increased, which revealed that these cells were at a late apoptotic stage. To confirm apoptotic mechanism of cytotoxicity of Ph$_3$Sn(Boc-Orn-O), we carried out morphological evaluation of HepG2 cells using AO and EB double staining. After 24 h of treatment with 1 $\mu$M of Ph$_3$Sn(Boc-Orn-O), fluorescent microscopy revealed the appearance of cells containing bright green patches in the nuclei as a consequence of chromatinn condensation and nuclear fragmentation, which are typical features of apoptosis. Moreover, after treatment with the compound at 2 $\mu$M concentration, fluorescing orange cells owing to increase of cell permeability to ethidium bromide, cell shrinkage and nuclear fragmentation were also evident as observed for cells in late apoptosis (Fig. 4B).

One of the key executioners of the apoptotic cell death is caspase 3, responsible for proteolytic cleavage of many key proteins, including poly(ADP-ribose) polymerase-1 (PARP-1). In comparison with control cells, high levels of the 89 kD cleaved product from PARP-1, with a decrease of the 116 kD native protein, were observed in HepG2 cells after 24 treatment with Ph$_3$Sn(Boc-Orn-O) (Fig. 4C). Taken together, these findings provided strong evidence that the synthesized Ph$_3$Sn(Boc-Orn-O) induced apoptosis in HepG2 cells.

3.2.3. Mitochondrial damage

In many systems, apoptosis is associated with the loss of mitochondrial inner membrane potential ($\Delta$$\psi$m) which is responsible for the release of some pro-apoptotic factors from the organelle. We investigated the involvement of mitochondria in apoptosis induced by the tin compound using DiOC$_6$(3), a fluorescent mitochondria-specific and voltage-dependent dye. The results, reported in Fig. 5A, indicate that treatment of HepG2 cells with 1 $\mu$M of Ph$_3$Sn(Boc-Orn-O) for 24 h, induced a marked dissipation of $\Delta$$\psi$m as indicated by decreased mitochondrial DiOC$_6$-red.

Owing to mitochondrial dysfunction, perturbation of the intracellular calcium level is expected. A fluo-3/AM staining followed by flow cytometry analysis, gave evidences that, in comparison with control cells, treatment of HepG2 cells with Ph$_3$Sn(Boc-Orn-O) also caused a net elevation of cellular Ca$^{2+}$ content, evident by the increment of fluorescence of the ion probe (Fig. 5B).

To understand more about the apoptosis induction mechanism caused by Ph$_3$Sn(Boc-Orn-O), the levels of p53 protein was assessed by Western blot. p53 is a key suppressor of tumor regulators in the apoptotic process and possesses pro-apoptotic activity through modulation of the BCL-2 family proteines that control the mitochondrial membrane permeability. As shown in Fig. 5C, 24 h treatment of HepG2 cells with 1 $\mu$M of organotin(IV) compound, caused threefold increase of p53 levels. These findings provide evidence that the cytotoxicity of the compound is related to the induction of a p53-dependent activation of the mitochondrial pathway of apoptosis.

3.2.4. ROS generation

Since the generation of intracellular ROS may be related to mitochondrial dysfunction and induction of apoptosis in various cell types, we explored whether our organotin(IV) compound could stimulate ROS generation in HepG2 cells. As illustrated in Fig. 6, the generation of ROS, cytofluorimetrically detected by the fluorescent dye DCFH-DA, dramatically increased in tin compound-treated cells compared with that of the control group, with a clear dose-response mechanism.

4. Conclusions

Ph$_3$Sn(Boc-Orn-O) showed strong cytotoxic and anticancer effects. Compared to cisplatin, organotin(IV) compound exhibited much higher antiproliferative activity in vitro with IC$_{50}$ values for cell growth proliferation 20–60 times larger than those of cis-platin toward all the investigated cancer cell lines (HepG2, HTC116 and MCF-7).

Both oxidative damage and increased concentration of intracellular calcium ions seem to be the main events contributing to triorganotin-induced apoptosis in different panels of human cancer cell lines [35] and promotion of oxidative and DNA damage in rats tributyltin has been detected [36]. There are multiple DNA damage detection and repair systems in a cell but every type of DNA damage is reported to the p53 protein and its pathway [37–39], so that p53-triggered apoptosis plays an important role in regulating cell survival. Similarly, our findings show that apoptosis triggered by our organotin(IV) compound in human hepatocarcinoma cells involves a p53-dependent activation of the mitochondrial pathway of apoptosis, ROS formation and Ca$^{2+}$ influx in the cytosol.
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