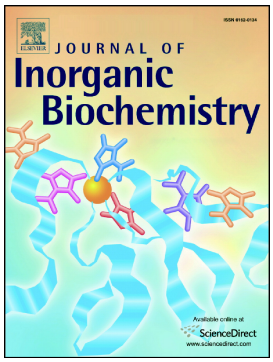

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Synthesis, characterization and *in vitro* biological evaluation of novel organotin(IV) compounds with derivatives of 2-(5-arylidene-2,4-dioxothiazolidin-3-yl)propanoic acid

Nebojša Đ. Pantelić[a,b,c], Bojana B. Zmejkovski[d], Bojan Božić[e], Biljana Dojčinović[d], Nebojša R. Banjac[b], Ludger A. Wessjohann[c], Goran N. Kaluđerović[a,c*]

[a]*Department of Engineering and Natural Sciences, University of Applied Sciences Merseburg, Eberhard-Leibnitz-Straße 2, 06217 Merseburg, Germany;* [b]*Department of Chemistry and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina 6, Belgrade-Zemun, Serbia ;* [c]*Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120, Halle (Saale), Germany;* [d]*Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 14, 11000 Belgrade, Serbia;* [e]*Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia.*

ABSTRACT

Two novel triphenyltin(IV) compounds, [**Ph_3SnL1**] (**L1** = 2-(5-(4-fluorobenzylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate (**1**)) and [**Ph_3SnL2**] (**L2 =** 2-(5-(5-methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate (**2**)) were synthesized and characterized by FT-IR, (^{1}H and ^{13}C) NMR spectroscopy, mass spectrometry, and elemental microanalysis. The *in vitro* anticancer activity of the synthesized organotin(IV) compounds was determined against four tumor cell lines: PC-3 (prostate), HT-29 (colon), MCF-7 (breast), and HepG2 (hepatic) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-12 diphenyltetrazolium bromide) and CV (crystal violet) assays. The IC_{50} values are found to be in the range from 0.11 to 0.50 μM. Compound **1** exhibits the highest activity toward PC-3 cells (IC_{50} = 0.115 ± 0.009 μM; CV assay). The tin and platinum uptake in PC-3 cells showed a threefold lower uptake of tin in comparison to platinum (as cisplatin). Together with its higher activity this indicates a much higher cell inhibition potential of the tin compounds (calculated to ca. 50 to 100 times). Morphological analysis suggested that the compounds induce apoptosis in PC-3 cells, and flow

* Corresponding author e-mail: goran.kaluderovic@hs-merseburg.de

cytometry analysis revealed that **1** and **2** induce autophagy as well as NO (nitric oxide) production.

Keywords: tin(IV), *in vitro*, prostate cancer, apoptosis, autophagy, NO

1. Introduction

Modern medicine fights against cancer with radiotherapy, hormonotherapy, and chemotherapy. In 1970s chemotherapy developed after the discovery of cisplatin [1], and most of the metallodrugs used as treatment are cisplatin analogues [2]. The initial focus, synthesis and evaluation of platinum-based anticancer drugs nowadays is shifted to non-platinum metal-based agents [3–14] with the aim to minimize the well-known side effects of currently used drugs [2–13]. Many organotin(IV) compounds [15–19], especially carboxylato derivatives [20–27], have shown interesting antitumor activities. Additionally, it is possible to examine the influence of varying the coordination mode of the carboxylato ligands, from monodentate or chelate (symmetric or asymmetric) to bridging, on the anticancer properties. Moieties such as $R_nSn^{(4-n)+}$ (n = 2 or 3) may bind to membrane proteins or glycoproteins, or to cellular proteins such as hexokinase, ATPase, acetylcholinesterase of the human erythrocyte membrane, and skeletal muscle membranes [22]; they may also interact directly with DNA [28], causing cell death either by apoptotic or necrotic mechanisms. The potential binding of a tin-based compound to the phosphodiester backbone of DNA [29,30], which changes the intracellular metabolism of the phospholipids of the endoplasmic reticulum, has also been reported [31]. According to published literature there are various pathways described in which tin compounds may interact with the cell membrane or constituents within the cell, and it may be concluded that the mechanism of action of organotin compounds still remains unclear [32–35].

Nevertheless, some tricyclohexyltin(IV) derivates ([$SnCy_3$(DMNI)], [$SnCy_3$(BZDO)], [$SnCy_3$(DMFU)], and [$SnPh_2(BZDO)_2$] (Cy = cyclohexyl, DMNIH = 2,6-dimethoxynicotinic acid, BZDOH = 1,4-benzodioxane-6-carboxylic acid, DMFUH = 2,5-dimethyl-3-furoic acid) exhibit very intriguing properties that were able to overcome the multidrug resistance found for metallodrugs used today as therapeutics [36]. It was also found that cytostatic mechanism of certain organotin compounds depends on the ligands or organic substituents bound to the tin center [36]. It is important to point out that organotin(IV) compounds containing 2-

thiobarbiturato ligands presented a high specific activity against MCF-7 (ER positive) but not against some other breast cancer cells (MDA-MB-231) (ER negative), indicating that estrogen receptors (ER) may play a role in the mechanism of cell death promoted by organotin compounds [37]. In some cases, ligand precursors drugs, which alone possesses ability to target various diseases, can give more higher therapeutic potential triggering different mechanisms of action than drugs which target merely one disease. These drugs with dual or multiple mechanisms can display lesser side-effect than a polypharmaceutical combination of several drugs that target the same disease individually. Thus, these types of multifunctional ligands should be taken in account for preparation of novel organometallic compounds. 2,4-Thiazolidinediones (TZDs) are a class of commercially available antidiabetic drugs with potential multifunctional targeting [38–40]. Moreover, propanoic acid derivatives such as ibuprofen, naproxen or ketoprofen belong to the class of non-steroidal anti-inflammatory drugs (NSAIDs) and also, as TZDs, possess ability to influence on different diseases [41]. The synthesis of combined molecules containing both biologically important chemical chores is very promising in terms of potential cumulative therapeutically effects, considering allo that TZDs and NSAIDs may induce cell apoptosis.

Recently, investigations on metal-based anticancer drugs [42–51] with carboxylato ligands which have shown significant anticancer activities, even higher than that of cisplatin, have been reported [27,46,47]. Some of these compounds represent the first examples of triethylammonium salts of triphenyltin(IV) carboxylates $NHEt_3[SnPh_3Cl(L)]$ (L = *N*-phthaloylglycinate (*P*-Gly), *N*-phthaloyl-L-alaninate (*P*-Ala), and 1,2,4-benzenetricarboxylate 1,2-anhydride (BTC)) which have shown an extremely high cytotoxic activity [52]. Due to the outstanding biological activity of tin(IV) compounds with various carboxylate ligands [53], a need has arisen for synthesis and investigation of new compounds of similar type.

Herein, triphenyltin(IV) chloride is reacted with 2-(5-(4-fluorobenzylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate, **L1**, and 2-(5-(5-methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate, **L2**, to form two novel organotin(IV) compounds, **1** and **2,** respectively. The compounds are characterized by standard analytical methods, and their antitumor activity evaluated against tumor cell lines: PC-3 (prostate), HT-29 (colon cancer cells), MCF-7 (breast cancer cells), and HepG2 (hepatic cancer cells) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-12 diphenyltetrazolium bromide) and CV (crystal violet) assays. The

mechanism of action induced by the new compounds on PC-3 cells is further examined *via* cell cycle distribution, caspase activity, autophagy and cell division analysis, as well as investigation of ROS/RNS (reactive oxygen species/reactive nitrogen species) and NO (nitric oxide) production. Furthermore, the metal uptake in PC-3 cells also has been investigated.

2. Experimental

2.1. Materials and instrumentation

The ligands, 2-(5-(4-fluorobenzylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate, **L1**, and 2-(5-(5-methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate, **L2** were obtained after the neutralization of previously synthesized propanoic acid derivatives (**HL1** and **HL2)** according to a published method [54]. Regents and solvents were purchased from Merck and used without further purification.

Elemental analyses were performed on an Elemental Vario EL III microanalyzer. A Nicolet 6700 FT–IR spectrometer and ATR (attenuated total reflection) technique were used for recording mid-infrared spectra (4000-400 cm^{-1}). NMR spectra of compounds were recorded in DMSO-d_6 on a Bruker Avance III 500 spectrometer using internal standard solvent for ^{1}H and ^{13}C NMR. Mass spectrometry was performed using Agilent technologies ion trap LC/MS 6320 mass spectrometer with electrospray positive ionization mode.

2.1.1. Synthesis of compounds

The compounds **1** and **2** were prepared according to the modified procedure described in literature [55]. Following quantities of the compounds and conditions were used: 0.5 mmol of **HL1** (147 mg) or **HL2** (141 mg), 5 mL of distilled water, 1 M LiOH (0.5 mL, 0.5 mmol), 3 h of stirring on 40 °C (clear solution obtained). Afterwards, 5 mL of methanolic solution of Ph_3SnCl (0.193 g, 0.5 mmol) added and stirred for 6 h. A white precipitate was formed, filtered off, washed with 3 mL of distilled cold water, and dried in *vacuum* over silica gel.
(2-(5-(4-Fluorobenzylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoato)triphenyltin(IV) (**1**). White solid; yield: 48 %; Anal. calcd. for $C_{31}H_{24}FNO_4SSn$: C, 57.79; H, 3.75; N, 2.17; S, 4.98%. Found: C, 57.62; H, 3.95; N, 2.06; S, 4.91%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.20 (d, $^{3}J_{(H,H)}$ =

5 Hz, 3H, C*H*$_3$CHCO$_2$), 4.92 (m, 1H, CH$_3$C*H*CO$_2$), 7.27 (m, 6H, *m*-H SnPh$_3$), 7.31 (d, *o*-H F-Ph moiety), 7.39 (m, *m*-H F-Ph moiety), 7.43 (m, 9H, *o*-H SnPh$_3$ and *p*-H SnPh$_3$), 7.68 (s, 1H, C*H*-Ph-F) ppm; ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.7 (*C*H$_3$), 52.2 (CH$_3$*C*H), 117.1 (*m*-C F-Ph moiety), 121.1 (S-*C*=CO), 128.9 (*m*-C SnPh$_3$), 130.0 (*m*-C F-Ph moiety), 133.3 (*p*-C SnPh$_3$), 134.6 (*o*-C SnPh$_3$), 135.7 (*ipso*-C F-Ph), 138.3 (*ipso*-C SnPh$_3$), 162.8 (C-*C*O-N-), 165.5 (-N-*C*O-S), 167.1 (*C*OO-Sn) ppm. FT-IR (ATR): 3054 (m), 2910 (m), 1741 (s), 1680 (s), 1613 (s), 1563 (s), 1513 (w), 1376 (s), 1238 (m), 1063 (w), 448 (m) cm^{-1}. ESI-MS (MeCN): *m*/*z* 646.41 [M+H]$^+$.

(2-(5-(5-Methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoato)triphenyltin(IV) (**2**).

White solid; yield: 51 %; Anal. calcd. for $C_{30}H_{25}NO_5SSn$: C, 57.17; H, 4.00; N, 2.22; S, 5.09%. Found: C, 56.89; H, 4.05; N, 2.01; S, 4.87%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.35 (d, $^3J_{(H,H)}$ = 6 Hz, 3H, C*H*$_3$), 2.39 (s, 3H, C*H*$_3$C$_4$H$_2$O), 4.62 (q, 1H, CH$_3$C*H*CO$_2$H), 6.41 (d, 1H, $^3J_{(H,H)}$= 3 Hz, C$_4$H$_2$O), 7.04 (d, 1H, $^3J_{(H,H)}$ = 3 Hz, C$_4$H$_2$O), 7.35 (br m, 9H, *m*-H and *p*-H SnPh$_3$), 7.62 (s, 1H, OC-C*H*-S), 7.72 (br m, 6H, *o*-H SnPh$_3$) ppm; ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.2 (*C*H$_3$C$_4$H$_2$O), 14.9 (*C*H$_3$-CH-COO), 52.2 (N-*C*H-COO), 110.7 (C$_4$H$_2$O), 119.5 (C$_4$H$_2$O), 121.0 (S-*C*=CO), 128.6 (*m*-C SnPh$_3$), 129.3 (*p*-C SnPh$_3$), 136.7 (*o*-C SnPh$_3$), 142.7 (*ipso*-C SnPh$_3$), 148.4 (C$_4$H$_2$O), 158.0 (C$_4$H$_2$O), 165.7 (C-*C*O-N), 168.2 (N-*C*O-S), 171.3 (*C*OO-Sn) ppm. FT-IR: (ATR): 3049 (m), 2924 (m), 1734 (s), 1680 (s), 1616 (s), 1571 (s), 1515 (w), 1367 (s), 1270 (m), 1025 (m), 449 (m) cm^{-1}. ESI-MS (MeCN): *m*/*z* 632.36 [M+H]$^+$.

2.2. Cell lines and IC$_{50}$ determination

Four different cell lines: PC-3 (prostate cancer cells), HT-29 (colon cancer cells), MCF-7 (breast cancer cells), and HepG2 (hepatic cancer cells) were used for determination of the antitumor activities of novel synthesized compounds. The cell lines were grown in T75 flasks containing 10 mL of complete medium and maintained at 37 °C and 5% CO_2 in order to detach the cells. For PC-3 and MCF-7 cells, 7×10^5 cells, whereas for HT-29 and HepG2 3×10^6 cells were transferred in 96 well-plate and used for CV and MTT assay.

Each compound was tested on the four cell lines in 9 different concentrations (100, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 μM). The working solutions were prepared diluting stock solutions

of investigated compounds (20 mM in DMSO) using the complete medium. Three technical replicates were performed for each concentration. The treated cells were incubated for 48 h at 37 °C and 5% CO_2. The viability of the cells was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-12 diphenyltetrazolium bromide) and CV (crystal violet) assays [56–58]. IC_{50} (50% inhibitory concentration) values were calculated with a four-parameter logistic function and presented in a mean. The assays were performed in three biological replicates.

2.3. Metal accumulation

The tin and platinum uptake studies were performed using PC-3 cells. The cells were seeded in T25 flasks and allowed to grow. After 24 h, the exponentially growing cells were treated with 10 mL of novel synthesized organotin(IV) compounds and cisplatin in IC_{50} concentration for 24 h, and afterwards trypsinized and collected by centrifugation at 1000 rpm for 10 min. The cells were washed with 5 mL of ice-cold PBS (Phosphate Buffered Saline). The uptake concentration of elements in prepared samples was determined by inductively coupled plasma mass spectrometry (ICP-MS) according to previously described procedure [59].

2.4. Morphological analysis (AO and DAPI staining)

The morphological characteristics of PC-3 cell death triggered by tested compounds were determined using AO (acridine orange) [60] and DAPI (4',6-diamidino-2-phenylindole) [61] staining, and followed by a fluorescence microscope. Acridine orange can cross the cell membrane and viable and early apoptotic cells can be identified. Chromatin condensation, seen as dense green areas, or membrane blebbing, both appearing in apoptosis, is easily proven by AO staining. Moreover, orange to red autophagosomes might be detected by AO pointing out autophagic process in the cells. PC-3 cells were seeded overnight on coverslips (150,000 cells/coverslip) in 2 mL of complete medium. The following day, cells were treated with IC_{50} and 2 × IC_{50} of the tested compounds for 24 h, then stained with acridine orange (3 μg/mL AO in PBS), and viewed under a fluorescence microscope (Fluorescence microscope - Invitrogen EVOS FL 2 Auto 2 Cell Imaging System). For DAPI staining, PC-3 cells were treated with IC_{50} and 2 × IC_{50} of the tested compounds and after fixing the cells with 0.5 mL of 4%

paraformaldehyde (PFA) for 8 min, 0.5 mL of Triton (0.1% in PBS) was added. Afterwards, the cells were stained with 0.5 mL of DAPI solution and stand for 5 min in a dark condition [61]. The morphological changes were visualized under a fluorescence microscope (Fluorescence microscope - Invitrogen EVOS FL 2 Auto 2 Cell Imaging System).

2.5. Cell cycle analysis

The PC-3 cells were prepared in a 6 well plate. The cells were treated with IC_{50} of novel organotin(IV) compounds and cisplatin as referent compound, and then incubated for 48 h at 37 °C and 5% CO_2. The cells were detached with 0.5 mL of trypsin and incubated for 3 min at 37 °C and 5% CO_2. The Falcon tubes were centrifuged for 3 min at 1000 rpm. The supernatant was discarded, the cells were washed with 1 mL of PBS, centrifuged for 3 min at 1000 rpm and then the supernatant was discarded. The cells were resuspended in 300 μL of ice-cold PBS and then fixed by added dropwise to 700 μL of ice-cold absolute ethanol. The cells were stored for 24 h at 4 °C, then centrifuged for 3 min at 1000 rpm, washed with PBS and stained with 1 mL of DAPI (4',6-diamidino-2-phenylindole) working solution [58]. The prepared cells were incubated at room temperature for 10 min and analyzed by flow cytometry.

2.6. Cell division analysis

The PC-3 cells were detached by applying 1 mL of trypsin, incubated for 3 min and then the trypsin was deactivated by applying 9 mL of complete medium. The cells were collected in 15 mL Falcon tube, centrifuged at 1000 rpm for 3 min and the supernatant was discarded from the tube. The cells were resuspended in 10 mL of complete medium and the required number of cells for seeding 6 wells plate was transferred to a new Falcon tube. The Falcon tube was centrifuged at 1000 rpm for 3 min. and the supernatant was discarded. The cells were stained by applying 1 mL of CFSE (carboxyfluorescein succinimidyl ester) working solution and incubated at 37 °C and 5% CO_2 for 10 min. The staining process was stopped by applying 4 mL of complete medium, centrifuging for 3 min at 1000 rpm and discharging the supernatant. The cells were resuspended in complete medium (1 mL/well) and seeded in a 6 well plate. After 24 h incubation, the cells were treated with IC_{50} of tested compounds and incubated for 48 h at 37 °C

and 5% CO_2. The cells were detached by using 0.5 µL of trypsin and incubation for 3 min at 37 °C and 5% CO_2. The trypsinization was stopped by adding 1 mL of complete medium in each well, and cells were centrifuged at 1000 rpm for 3 min, washed with 1 mL of PBS, centrifuged, and then resuspended in 1 mL of PBS. The prepared cells were analyzed by flow cytometry [57].

2.7. Activation of caspases

The PC-3 cells were prepared in a 6 well plate. The cells were treated with IC_{50} of novel organotin(IV) compounds and cisplatin as referent compound and incubated for 48 h at 37 °C and 5% CO_2. The medium from the treated wells was collected into separate 15 mL Falcon tubes and the cells were washed with 1 mL of PBS. The cells were detached by using 0.5 µL of trypsin and incubation for 3 min at 37 °C and 5% CO_2. The previously collected medium was used to deactivate the trypsin while for the untreated wells, 1 mL of complete medium was added and the cells were collected by pipette tip harvesting technique from each well in a new Falcon tube. The Falcon tubes were centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the cells were washed with 1 mL of PBS, centrifuged for 3 min at 1000 rpm and then the supernatant was discarded. The cells were stained with 100 µL of the apostat working solution and then incubated for 30 min at 37 °C and 5% CO_2. The staining process was deactivated with the addition of 1 mL of PBS, centrifuged at 1000 rpm for 3 min and then the supernatant was discarded. The cells were resuspended in 1 mL of PBS and then analyzed with flow cytometry [57].

2.8. Autophagy analysis

The PC-3 cells were prepared in a 6 well plate. The cells were treated with IC_{50} of novel organotin(IV) compounds and cisplatin as referent compound, and incubated for 48 h at 37 °C and 5% CO_2. The medium from the treated wells was collected into separate 15 mL Falcon tubes and the cells were washed with 1 mL of PBS. The cells were detached by using 0.5 µL of trypsin and incubation for 3 min at 37 °C and 5% CO_2. The previously collected medium was used to deactivate the trypsin while for the untreated wells, 1 mL of complete medium was added and the cells were collected by pipette tip harvesting technique from each well in a new Falcon tube.

The Falcon tubes were centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the cells were washed with 1 mL of PBS, centrifuged for 3 min at 1000 rpm and then the supernatant was discarded. The cells were stained with 500 µL of AO (acridine orange) working solution. The cells were incubated for 15 min at 37 °C and 5% CO_2. The staining process was deactivated with the addition of 1 mL of PBS, centrifuged at 1000 rpm for 3 min and the supernatant was discarded. The cells were resuspended in 1 mL of PBS and then analyzed with flow cytometry [57].

2.9. Investigation of ROS/RNS and NO production

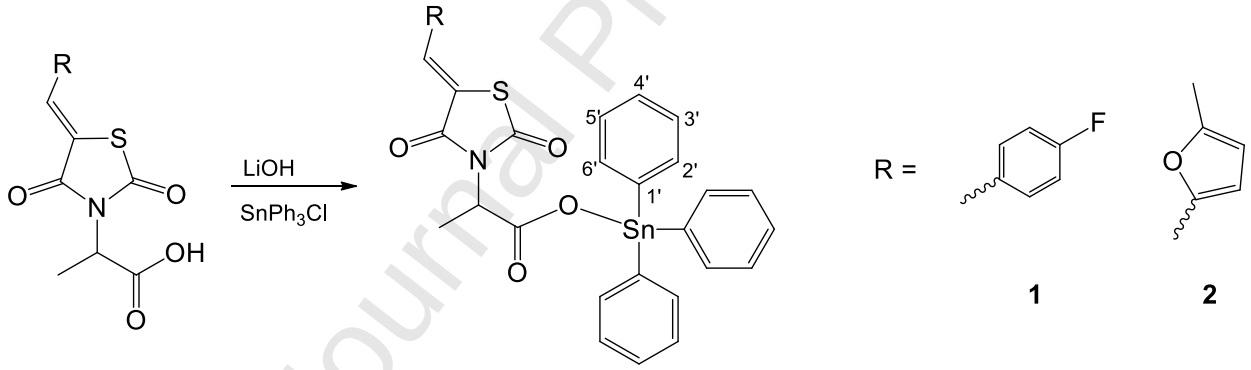
For the investigation of ROS/RNS production the cells were detached by applying 1 mL of trypsin, incubated for 3 min and then the trypsin was deactivated by applying 9 mL of complete medium. The cells were collected in 15 mL Falcon tube, centrifuged at 1000 rpm for 3 min and the cells were resuspended in 10 mL of complete medium. The required number of cells for seeding in 6-well plates was transferred to a new Falcon tube. The Falcon tube was centrifuged at 1000 rpm for 3 min. and the supernatant was discarded. The cells were stained by applying 1 mL of DHR (dihydrorhodamine) working solution and incubated at 37 °C and 5% CO_2 for 10 min. The staining process was stopped by applying 4 mL of complete medium, centrifuged for 3 min at 1000 rpm and the supernatant was discarded. The cells were resuspended in complete medium (1 mL/well) and seeded in a 6 well plate. After 24 h incubation, the cells were treated with IC_{50} of tested compounds and incubated for 48 h at 37 °C and 5% CO_2. The cells were detached by using 0.5 µL of trypsin and incubation for 3 min at 37 °C and 5% CO_2. The trypsinization was stopped by adding 1 mL of complete medium in each well, and cells were centrifuged at 1000 rpm for 3 min, washed with 1 mL of PBS, centrifuged, and then resuspended in 1 mL of PBS. The prepared cells were analyzed by flow cytometry [56]. The cells for NO (nitric oxide) production were prepared in 6 a well plate. The cells were treated with IC_{50} of tested compounds and incubated for 48 h at 37 °C and 5% CO_2. The cells were washed with PBS and then they were treated with 1 mL of DAF-FM (4-amino-5-methylamino-2,7'-diflurofluorescin diacetate) working solution. The treated cells were incubated for 1 hour at 37 °C and 5% CO_2. The stain was deactivated by incubation with a medium for 15 min then the cells were washed with 1 mL of PBS. The cells were detached by using 0.5 µL of trypsin and

incubation for 3 min at 37 °C and 5% CO_2, centrifuged at 1000 rpm for 3 min, washed with 1 mL of PBS. The prepared cells were analyzed by flow cytometry [58].

3. Results and discussion

3.1. Synthesis and characterization

In the reaction of Ph_3SnCl and equimolar amount of ligand precursors: 2-(5-(4-fluorobenzylidene)-2,4-dioxothiazolidin-3-yl)-propanoic acid, **HL1**, or 2-(5-((5-methylfuran-2-yl)methyl)propanoic acid, **HL2**, previously deprotonated with LiOH, the novel triphenyltin(IV) compounds, **1** and **2,** were obtained as white solids in good yields (**Scheme 1**). Products were soluble in dimethyl sulfoxide, chloroform, dichloromethane, chloroform, and acetonitrile. The synthesized compounds were characterized *via* elemental microanalysis, FT-IR and NMR (^{1}H and ^{13}C) spectroscopy, and mass spectrometry.



Scheme 1. Synthesis of compounds **1** and **2**

In general **1**/**2** in comparison to **HL1**/**HL2** show changes in absorption bands in the IR spectra [54], for the characteristic carboxylic/carboxylato moieties, where the most significant changes were as expected. Explicitly, ligand precursors gave wide bands around 3400 cm^{-1} due to hydrogen bonding and dimerization [27]. However, after deprotonation and coordination to the tin center, these bands disappeared indicating that coordination of the carboxylic oxygen atom occurred [27]. As expected, asymmetric ν(–CH_3/–CH_2) vibrations of moderate intensities were found in the range of 3200–2800 cm^{-1}. Furthermore, strong

v(C=O) and *v*(C–O) absorption stretching bands are observed in both complexes, as they appear at 1741, 1376 cm^{-1} (**1**) and 1734, 1367 cm^{-1} (**2**). It should be noted that, in the spectra of the ligand precursors, these bands were found at 1719, 1376 cm^{-1} (**HL1**) and 1725, 1368 cm^{-1} (**HL2**), thus suggesting monodentate coordination because this results in a non-equivalence of the two oxygen atoms [52]. Specifically, strong bands were found at 1613 and 1376 cm^{-1} for **1** and 1616 and 1367 cm^{-1} for **2**, which correspond to the asymmetric and symmetric vibrations of carbonyl group from the COO moiety, respectively. 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 carbon-oxygen bond lengths are affected, a pseudo-ester configuration is obtained [62], and with the electron withdrawing covalently bound metal, the more pronounced properties of the double C=O bond moves this bond to higher wavelength values. In addition, the medium absorptions corresponding to the Sn–O stretching mode of vibration appear at 448 and 449 cm^{-1} for **1** and **2**, respectively [53]. ESI-MS were recorded in positive ion mode, and in both cases, the $[M+H]^+$ mass peak was detectable.

NMR spectra of ligand precursors and tin(IV) compounds were fairly different due to their structures. Resonances of aliphatic protons and carbons were found at expected positions at lower ppm values. In ^{1}H NMR spectra, methyl protons from 2-propanoate moiety in **1** and **2** resonated as doublets at 1.20 and 1.35 ppm, respectively, while the CH protons belonging to the same moiety gave chemical shifts at 4.92 and 4.62 ppm. Methyl hydrogen atoms from the furan ring in **2** can be found as a singlet at 2.39 ppm. Hydrogen atoms from the furan and fluorophenyl together with Ph from $SnPh_3$ moieties brought resonances in the range of 6–8 ppm. Coupling with tin nucleus through three bonds can be observed as satellites nearby resonances of *o*-H atoms from the $SnPh_3$ moiety. Similar findings apply also for ^{13}C NMR spectra. Aromatic carbon atoms bonded to the tin center can easily be assigned due to visible coupling through one (*ipso*-C) and two (*o*-C) bonds. All carbonyl carbon atoms have chemical shifts at values higher than 160 ppm (Experimental part, section 2.1.1) which is consistent with proposed structures (Scheme 1).

The stability of anticancer drugs is also important features which may influence the antiproliferative activity of medicaments in the body. Therefore, the stability of synthesized compounds has been investigated by ^{13}C NMR spectroscopy. Namely, immediately after

dissolution of organotin(IV) compounds, after 2, and 24 h in DMSO stability study was performed. The results have shown that at investigated time points there are neither changes in chemical shifts of carbon atoms nor appearance of new resonances which may be assigned to decomposition products in the applied medium.

3.2. In vitro cytotoxicity

The cytotoxic potential of the two described newly synthesized organotin(IV) compounds, **1** and **2,** were studied against four malignant cell lines: PC-3 (prostate cancer cells), HT-29 (colon cancer cells), MCF-7 (breast cancer cells), and HepG2 (hepatic cancer cells) by using MTT and CV assays, with 48 h incubation. The results are summarized in **Table 1**. Both organotin(IV) compounds, **1** and **2**, exhibit very significant antiproliferative activity against all tested cell lines, with IC_{50} values in the range from 0.11–0.5 μM. In contrast to these findings, the investigated non-ionized ligands, **HL1** and **HL2**, have no influence on cell proliferation at the investigated concentrations.

Table 1. IC_{50} values (mean ± SD, μM) of **1**, **2** and cisplatin using MTT and CV assays (48 h incubation)

	PC-3	**HT-29**	**MCF-7**	**HepG2**
HL1/HL2 (MTT/CV)	> 100	> 100	> 100	> 100
1 (MTT)	0.147 ± 0.009	0.137 ± 0.010	0.111 ± 0.010	0.111 ± 0.024
1 (CV)	0.115 ± 0.009	0.136 ± 0.056	0.176 ± 0.019	0.211 ± 0.075
2 (MTT)	0.412 ± 0.031	0.267 ± 0.036	0.152 ± 0.019	0.125 ± 0.036
2 (CV)	0.304 ± 0.028	0.497 ± 0.032	0.491 ± 0.006	0.361 ± 0.041
cisplatin (MTT)	14.30 ± 0.26	14.18 ± 0.73	15.16 ± 1.04	3.47 ± 0.46
cisplatin (CV)	12.95 ± 0.45	15.97 ± 0.95	15.83 ± 0.55	3.65 ± 0.35

In comparison to cisplatin, compound **1** is over 100 times more active against the investigated cell lines. These outstanding results are similar to values gathered for compound **2**, with emphasis on the fact that **1** is overall more active. Furthermore, the synthesized compounds have demonstrated comparable or even better antiproliferative activity in comparison to other organotin(IV) compounds that were incubated even for a longer time [27,63,64]. According to

the results obtained by MTT assays, both compounds have shown the highest antiproliferative activity toward HepG2 cells, while the exhibited results obtained by CV assay indicate that both compounds demonstrated the lowest IC_{50} values against PC-3 cells (**Table 1**). Further examination of the compounds was conducted on PC-3 cells with the aim to investigate the mechanism of their action.

3.3. Metal uptake

Metal uptake studies of **1**, **2** and cisplatin were done by using inductively coupled plasma mass spectrometry (ICP-MS) analysis. The tin and platinum intracellular uptake studies were performed by the treatment of PC-3 cells for 24 h with concentrations corresponding to their IC_{50} values (CV assay).

Treatment of cells with **1**, **2** and cisplatin resulted in significantly different metal uptake, where platinum accumulation was up to 3 times higher than that of tin (**Figure 1**). Nevertheless, both complexes show similar accumulation in the PC-3 cell line. Accordingly, the results suggest that lower concentrations of tin have more powerful effects on killing cancer cells in comparison to cisplatin. It could be concluded that, considering the threefold lower amount of tin taken up by PC-3 cells, will calculatorily result in a *ca*. 50 to 100 times higher cytotoxic potential compared to platinum.

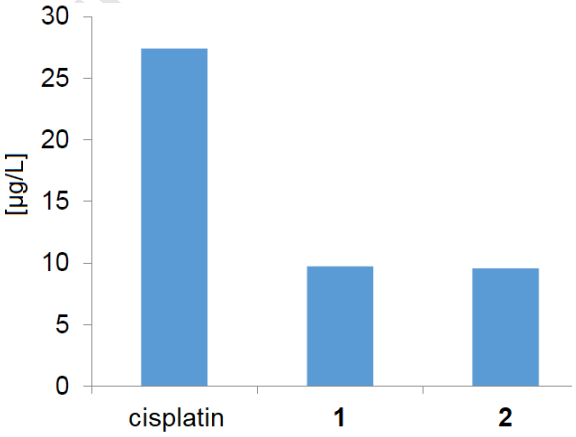


Figure 1. Metal uptake (Pt and Sn) in PC-3 cells treated with cisplatin, **1** or **2** (IC_{50}) after 24 h of action.

3.4. In vitro investigations

The impact of **1** and **2** on the morphological changes of PC-3 cells induced was determined by using AO (acridine orange) [59] and DAPI (4',6-diamidino-2-phenylindole) [57] stainings. The selected cell line was treated at the IC_{50} and 2 × IC_{50} concentrations with cisplatin and the new tin compounds for 24 h. The results from fluorescence microscopy are shown in **Figure 2**. Both triphenyltin(IV) compounds, as well as cisplatin (at IC_{50} or 2 × IC_{50} concentrations) induced nuclear condensation, a typical morphological feature of apoptosis, which was detected by the permeable dye AO. Furthermore, DAPI staining also confirmed nuclear condensation corroborating the apoptotic morphological changes in treated PC-3 cells with all compounds.

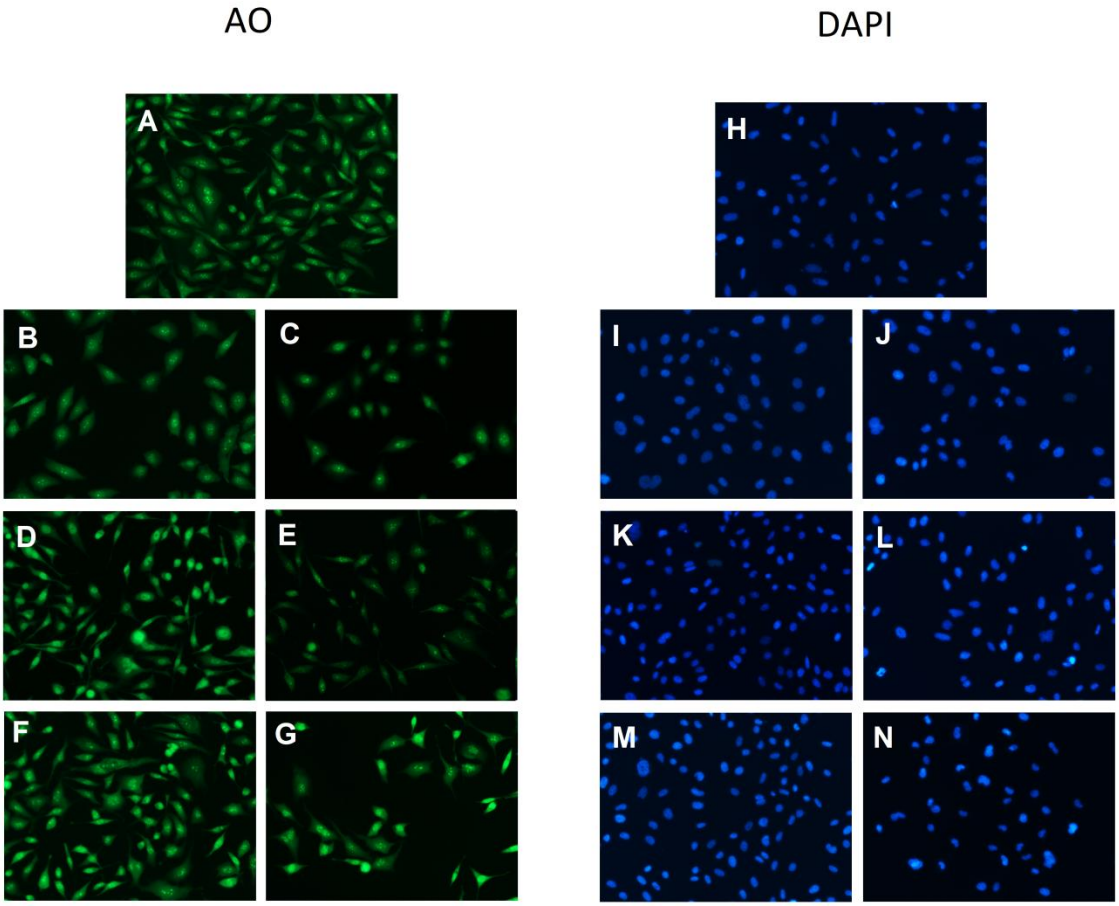


Figure 2. Fluorescent micrographs of PC-3 cells. A, H - control, B, I - cisplatin (IC_{50}), C, J - cisplatin (2 × IC_{50}), D, K – **Ph_3SnL1** (IC_{50}), E, L - **Ph_3SnL1** (2 × IC_{50}), F, M - **Ph_3SnL2** (IC_{50}), G, N - **Ph_3SnL2** (2 × IC_{50}); AO = acridine orange; DAPI = 4',6-diamidino-2-phenylindole.

The mechanism of action is further examined by analyzing cell cycle distribution by flow cytometry of PC-3 cells treated with cisplatin, **1** or **2** after exposure for 48 h. The cells were treated at concentrations corresponding to the test compounds IC_{50} values and afterwards stained with DAPI (4',6-diamidino-2-phenylindole). As shown in **Figure 3**, the number of cells treated with cisplatin decreases in G1 phase. Alongside, an increase of cells in sub-G1 and S phases was detected. On the other hand, **1** and **2** did not induce significant changes in the cell cycle distribution of PC-3 cells. The ability of cisplatin and the novel triphenyltin(IV) compounds to affect cell division was investigated using a CFSE assay. The obtained results indicate that only cisplatin was able to notably induce inhibition of PC-3 cell division, while the effect of **2** was slight, and **1** did not show any influence on cell division (**Figure 4.**A).

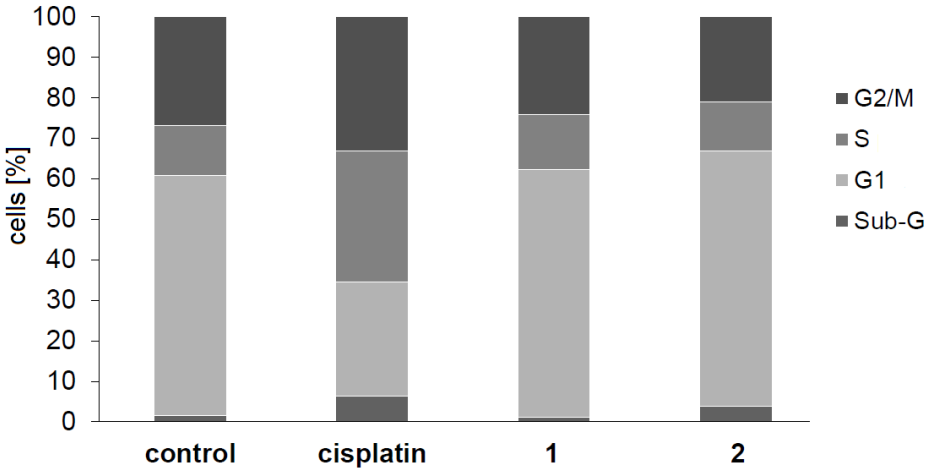


Figure 3. Effect of cisplatin, **1** and **2** on cell cycle phase distribution: PC-3 cell lines were exposed to IC_{50} doses.

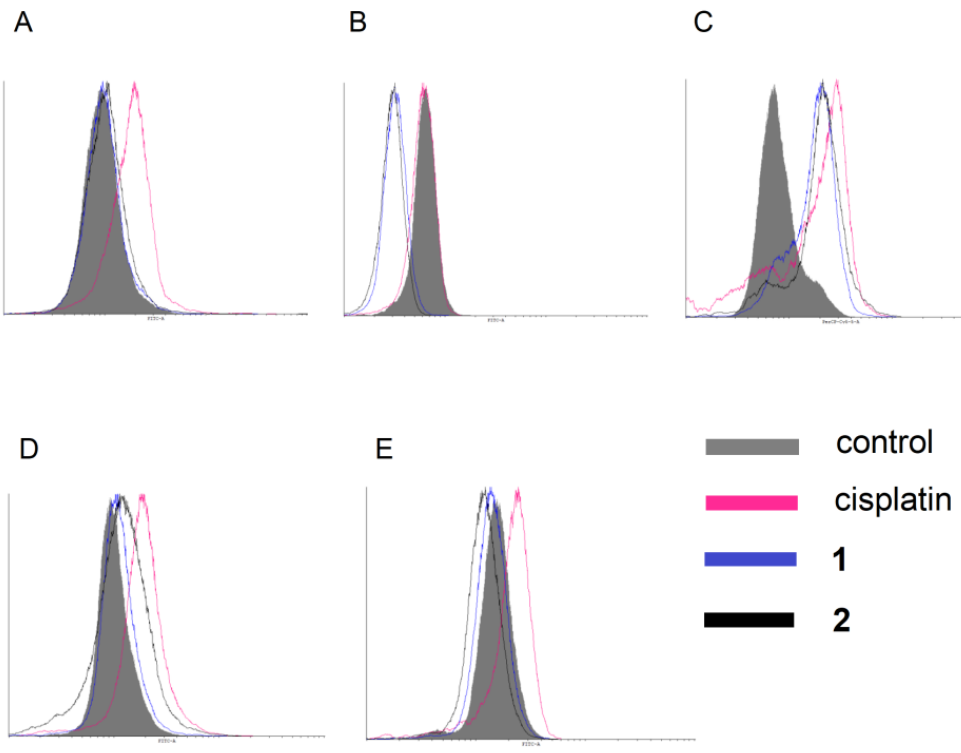


Figure 4. Flow cytometry results: A – cell division inhibition (CFSE), B – caspase activation (apostat), C – autophagy (AO), D – NO activation (DAF-FM), E - ROS/RNS activation (DHR).

A distinctive feature of the early stages of apoptosis is the activation of caspases, which participate in the cleavage of protein substrates and in the subsequent disassembly of the cell. To assess if the caspases are involved in apoptosis activation, the PC-3 cells were treated for 48 h with the novel organotin(IV) compounds, as well as cisplatin as reference compound at IC_{50} concentrations. Subsequently, collected samples were stained with apostat and analyzed by flow cytometry (**Figure 4.**B). The outcomes show that neither cisplatin nor **1** and **2** caused caspase activation. Moreover, **1** and **2** downregulated caspases expression. Accordingly, the cell death occurs through caspase independent apoptosis.

It is well known that autophagy may be involved in different processes in the cells such as differentiation, survival, cell death [65–67]. To elucidate if autophagy was triggered in the presence of the investigated compounds and cisplatin, treated cells were stained with AO and

analyzed by flow cytometry (48 h). The results clearly show that all compounds caused a high autophagic response in PC-3 cells (**Figure 4**.C).

Reactive oxygen (ROS) and nitrogen species (RNS) such as nitric oxide (NO) are natural products of any cellular oxidative metabolism and have significant influence in cell signaling and homeostasis [68–70]. It is well-known that uncontrolled generation of these species can lead to oxidative stress which is responsible for an increased damage of biomolecules such as proteins, lipids and nucleic acids and therefore may cause cytotoxicity, inflammation, and the formation of cancer cells [71–74]. In this study, DAF-FM diacetate and DHR stainings were employed for detection of NO and ROS/RNS species in PC-3 cells treated with **1**, **2** and cisplatin for 48 h. The obtained results have shown that all compounds tested triggered the formation of NO species in PC-3 cells (**Figure 4.**D). On the other hand, only cisplatin caused ROS/RNS production, while **1** and **2** reduced the detectable amounts of these highly active species in PC-3 cells (**Figure 4.**E).

4. Conclusions

In summary, two novel triphenyltin(IV) compounds containing 2-(5-(4-fluorobenzylidene)- or 2-(5-(5-methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate (**1** and **2**, respectively) were synthesized. Structures of compounds were confirmed *via* standard techniques: FT-IR, NMR (^{1}H and ^{13}C) spectroscopy, mass spectrometry, and elemental analysis. *In vitro* their cytotoxicity was determined against four tumor cell lines: PC-3 (prostate cancer cells), HT-29 (colon cancer cells), MCF-7 (breast cancer cells), and HepG2 (hepatic cancer cells) using MTT and CV assays. A high anticancer activity was observed, ranging from 0.11 to 0.50 μM. Compound **1** exhibits the highest activity against PC-3 cells (IC_{50} = 0.115 ± 0.009 μM; CV assay) which even can be more than 100-fold higher than the activity of cisplatin considering the reduced uptake of tin as found for PC-3 cells. Triphenyltin(IV) compounds **1** and **2** upregulated autophagosome formations along with NO induction. Beside activation of autophagy, cisplatin was able to accumulate cells in the sub-G1 and S phases alongside inhibiting PC-3 cell division and triggering formation of ROS/RNS species. These very promising results indicate that novel organotin(IV) compounds presented can be considered as good candidates for further *in vitro*/*in vivo* studies with the aim to achieve significant improvements in antiproliferative drug development.

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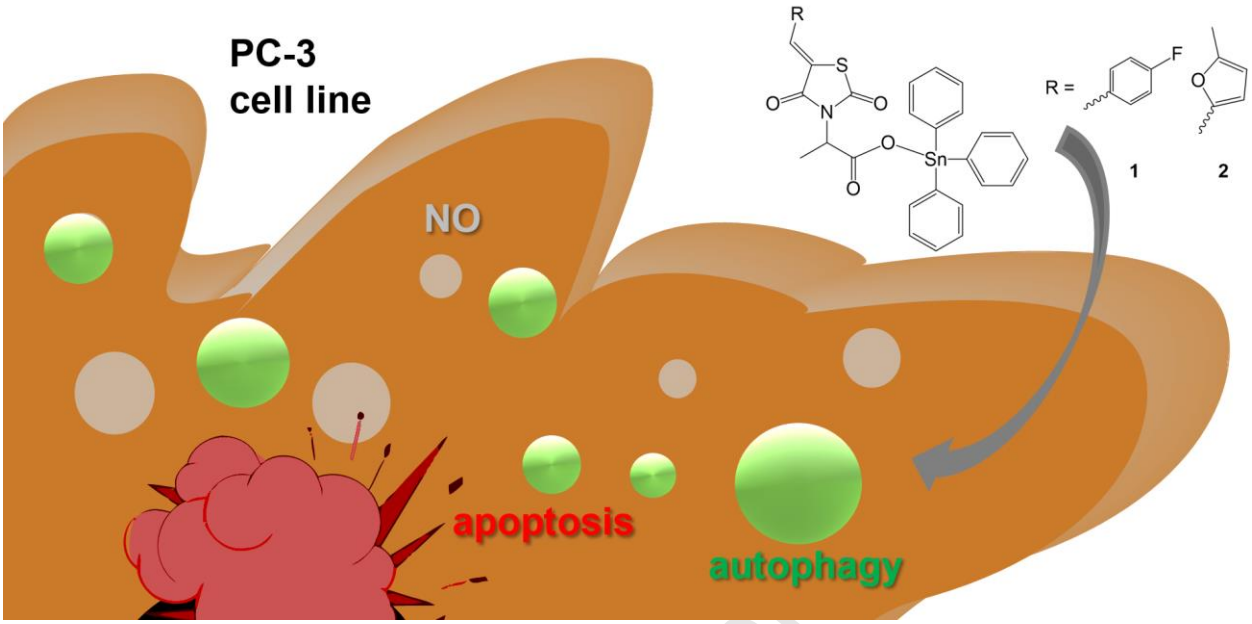
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Triphenyltin(IV) compounds containing 2-(5-(4-fluorobenzylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate (**1**) and 2-(5-(5-methyl-2-furfurylidene)-2,4-dioxotetrahydrothiazole-3-yl)propanoate (**2**) were prepared and characterized. IC_{50} concentrations of **1** and **2** against PC-3 (prostate), HT-29 (colon), MCF-7 (breast), and HepG2 (hepatic) cells are found from 0.11 to 0.50 µM. Mechanism of action of **1** and **2** has been investigated on PC-3 cells.

Highlights

- Two novel (carboxylato)triphenyltin(IV) compounds prepared and characterized
- Both organotin(IV) compounds showed high cytotoxic potential.
- 50-100 times higher cell inhibition potential of organotin(IV) compounds than cisplatin
- Compounds induce apoptosis, autophagy as well as NO production in PC-3 cells