In Vitro Approaches To Evaluate Toxicity Induced by Organotin Compounds Tributyltin (TBT), Dibutyltin (DBT), and Monobutyltin (MBT) in Neuroblastoma Cells

Martiña Ferreira, Lucía Blanco, Alejandro Garrido, Juan M. Vieites, and Ana G. Cabado*

Food Safety Division, ANFACO-CECOPESCA, Campus Univ. 16, 36310 Vigo PO, Spain

ABSTRACT: The toxic effects of the organotin compounds (OTCs) monobutyltin (MBT), dibutyltin (DBT), and tributyltin (TBT) were evaluated in vitro in a neuroblastoma human cell line. Mechanisms of cell death, apoptosis versus necrosis, were studied by using several markers: inhibition of cell viability and proliferation, F-actin, and mitochondrial membrane potential changes as well as reactive oxygen species (ROS) production and DNA fragmentation. The most toxic effects were detected with DBT and TBT even at very low concentrations ($0.1-1$ μM). In contrast, MBT induced lighter cytotoxic changes at the higher doses tested. None of the studied compounds stimulated propidium iodide uptake, although the most toxic chemical, TBT, caused lactate dehydrogenase release at the higher concentrations tested. These findings suggest that in neuroblastoma, OTC-induced cytotoxicity involves different pathways depending on the compound, concentration, and incubation time. A screening method for DBT and TBT quantification based on cell viability loss was developed, allowing a fast detection alternative to complex methodology.

KEYWORDS: tributyltin, dibutyltin, monobutyltin, cytotoxicity, neuroblastoma cells

INTRODUCTION

Organotin compounds (OTCs) are manufactured and used by industry in many applications: trisubstituted OTC tributyltin (TBT) and triphenyltin (TPT) have been used extensively as biocides in wood preservatives, in antifouling paints for boats and cooling towers, as preservatives for cotton, textiles, paper, and stain for buildings, as slimicides in industrial processes, as molluscicides to prevent schistosomiasis, and as fungicides in agriculture. Mono- and disubstituted OTCs are generally used in mixtures as polyvinyl chloride (PVC) stabilizers and catalysts and in glass coating.[1] In particular, TBT entered marine and freshwater ecosystems in considerable amounts, with registered values >500 ng L^{-1} in North American and European marinas prior to restrictions,[2] causing extensive damage to nontarget organisms[3] and ecoxicological effects even at very low concentrations, in the range of $1-10$ ng L^{-1},[4,5] and accumulating in sediments and biota.[6−8] For these reasons, restrictions on TBT use were introduced in the mid-1980s; nevertheless, butyltins persist in many areas at levels considered to be chronically toxic to the most susceptible organisms.[5]

As a result, the European Union has included TBT (tributyltin cation) in the list of priority pollutants, subjected to cessation of emissions, discharges, and losses into water, since the year 2001[9] and recently updated,[10−12] to control its fate in natural systems. Present and future restrictions will not immediately remove TBT and its degradation products from the marine environment,[2] because they persist in the sediments. Although the use of antifouling paints containing TBT has been banned in many countries subjected to the Convention on the Control of Harmful Antifouling systems on ships by the International Maritime Organization (IMO),[13] it is likely that these compounds will continue to be produced and used as biocides in other countries. In addition, they continue to be used in material and wood preservatives. Furthermore,

monobutyltin (MBT) and dibutyltin (DBT), among other OTCs, have been detected in drinking water served by PVC pipes.[14]

OTCs are generally lipophilic contaminants sparingly soluble in water, easily adsorbed to particulate matter in the aquatic environment, and absorbed by bacteria and algae.[2,15] Hence, they accumulate in sediments, where they are relatively persistent and can be taken up by benthic organisms and incorporated into filter-feeding zooplankton, grazing invertebrates, and higher organisms such as fish, water birds, and mammals.[6−8,16]

The major sources of organotin compound intake for humans are dietary sources, such as seafood and shellfish, food crops, and drinking water.[17] It has been recognized that organotins used in various consumer products (textiles, wood, etc.) can migrate during normal use and contribute their widespread presence in dusts from the indoor environment.[17] The information on human exposure to butyltin compounds is limited; some studies found TBT, DBT, and MBT levels in human tissues and in wildlife in the range of $3-100$ nM,[18−20] and, in general, DBT was the main butyltin species deposited in human liver.[21] OTCs are rapidly distributed into the body upon ingestion. In rats, daily administration leads to a continuous increase of tributyltin oxide (TBTO) levels in different organs, and it has been estimated that steady state conditions would be reached in $3−4$ weeks, thus indicating the bioaccumulative nature of OTC contamination. Tissues that exhibited highest accumulation of TBTO are liver, kidney, spleen, fat, and testicles, both in rats and in mice.[22,23] Although the

bioaccumulation of OTCs in the brain has been probably less studied because these substances have been mainly considered as immunotoxic compounds, Omura et al. observed that chronic exposure to tributyltin chloride (TBTCl) led to the accumulation of this compound mainly in the brain of rats.[24]

Several animal experiments have suggested that the spectrum of potential adverse chronic systemic effects of organotins in humans is quite broad and includes primary immunosuppressive, endocrinopathic, neurotoxic, metabolic, and enzymatic activities, as well as potential activity affecting many organs and physiological functions, as reproductive/teratogenic/developmental disorders, and possibly carcinogenic activity.[2] Acute toxicity studies in rats revealed that the oral administration of 37 or 75 mg TBT per kg of body weight caused a dramatic decrease in the levels of some neurotransmitters and a inhibition or even suppression of ATPase activity.[25] Alterations in the levels of neurotransmitters have also been observed in mice upon subacute oral exposure at concentrations from 1 to 125 ppm TBTCl for 1 month.[26]

Although the potential toxicity of organotins has been widely reported, the critical target molecules for the mechanisms of toxicity in humans remain unclear.[2] To elucidate the target molecules, conducted in vitro experiments have demonstrated that butyltins exhibit endocrine-disrupting mechanisms.[2] Triorganotins are potent inducers of apoptosis in various cell types including neurons;[27] at low doses, the TBT moiety interacts selectively with critical thiol residues in the adenine nucleotide translocator and opens the permeability transition pore, thereby decreasing mitochondrial membrane potential and releasing cytochrome c, a series of events consistent with established mechanistic models of apoptosis.[17]

TBT has been shown to decrease the viability of human promyelocytic leukemia cells HL-60 in culture in a time- and concentration-dependent manner,[28] inducing cell death in different mammal cell types at submicromolar to micromolar concentrations[29] and even nanomolar concentrations.[30] With respect to the mechanisms of action, several biochemical processes have been identified as targets for TBT, and some of these are involved in fundamental processes such as mitochondrial respiration, ion channels, steroidogenesis, receptor activation, and gene transcription.[2] One of the main TBT and DBT toxicity mechanisms results in perturbation of calcium homeostasis, mainly an elevation of intracellular calcium concentration.[28] Sustained increases in intracellular Ca^{2+} can activate cytotoxic mechanisms, which results in perturbations of cellular structure and function: the stimulation of Ca^{2+}-dependent proteases can result in a disruption of cytoskeletal organization, and Ca^{2+}-mediated phospholipase activation can result in an impairment of mitochondrial function with collapse of membrane potential and cessation of ATP synthesis.[31,32]

Brain alterations caused by exposure to TBT have been observed in vivo in several animal models,[1,25,26,33] but in contrast neurotoxicity is scarcely characterized in in vitro studies.[27,34] One TBT effect at cellular level is the inhibition of mitochondrial ATP-ase,[35] affecting any cell type, neurons, and other nervous cells.[36−38] Recently, some studies have shown that DBT is a potent neurotoxicant in vitro at concentrations that can be found in human tissues,[14] and in vivo in rodents.[39]

In vitro cytotoxic studies show variable results, probably related to the differences in toxicity and action mechanisms from the different compounds and the variety of cell lines used as well as the toxicant concentration range assayed. However,

the mechanism of its cytotoxicity has not been completely established.[27]

Neuroblastoma has been successfully used to study cytotoxicity, cell death, or apoptosis triggered by toxic compounds.[40−44] The aim of this study was to characterize TBT, DBT, and MBT toxicity in neuroblastoma cell line BE(2)-M17, in particular, at the cellular and molecular levels, including features related with cell death and apoptosis. In addition, the suitability of this cell line to develop a new in vitro method for organotin quantification was also evaluated. The rapid response and lower equipment requirements make cellular bioassays an attractive alternative to conventional analytical techniques for environmental monitoring.[45] Particularly, the combination of chromatographic fractionation and in vitro cell-based assay (CBA) offers a valuable tool for initial screening;[46] in addition, it allows an approach to the toxicity of products destined for human consumption.[47]

■ MATERIALS AND METHODS

Reagents. *Cell Culture Reagents.* Ham's F-12 nutrient mixture, minimum essential medium Eagle, fetal bovine serum, nonessential amino acid solution, glutamine, amphotericin B, PBS, and trypsin–EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentamycin was purchased from Biochrom Ltd. (Cambridge, UK).

Organotin Compounds. Monobutyltin trichloride (MBT), di-*n*-butyltin dichloride (DBT), and tributyltin chloride (TBT) were purchased from LGC Standards (Teddington, Middlesex, UK). The structures of the butyltins are shown in Figure 1.
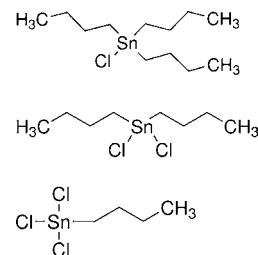

Figure 1. Structures of TBT chloride, DBT dichloride, and MBT trichloride (top to bottom).

Fluorescent Probes for Cell Assays, DNA Extraction, and Other Reagents. The fluorescent probes AlamarBlue, CyQUANT Cell Proliferation Assay Kit, MitoTracker Red CMXRos, Oregon Green 514 phalloidin, propidium iodide, and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate were from Molecular Probes (Life Technologies Co., Carlsbad, CA, USA). The ApoTarget Apoptotic DNA Ladder Detection Kit was from Invitrogen (Life Technologies). Midori Green DNA stain was from Nippon Genetics Europe GmbH (Dueren, Germany). The in vitro Toxicology Assay Kit TOX-7 (lactic dehydrogenase-based) was from Sigma-Aldrich. All other reagents were of analytical grade.

Cell Culture. The human neuroblastoma cell line BE(2)-M17 was purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and seeded on 25 cm² flasks. Culture medium consisted of Ham's F-12 nutrient mixture and minimum essential medium Eagle (1:1) supplemented with 1.2% nonessential amino acid solution, 12% fetal bovine serum (FBS), 50 mg L^{-1} gentamycin, and 50 μg L^{-1} amphotericin B.[41] Cell cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For microplate assays, cells were detached from the flasks by incubation for 5 min with 1 mL of 0.25% trypsin–EDTA and seeded in 96-well microtiter plates containing 200 μL of culture medium. Microtiter plates were incubated for an additional 48 h to achieve 80% of cell confluence and subsequently used for cytotoxicity assays.

Cytotoxicity Assays. MBT, DBT, and TBT stock solutions were prepared by dissolving in sterile DMSO to concentrations ranging from 25 μM to 100 mM. These stock solutions were subsequently diluted (1:1000) in cell culture medium to prepare working solutions of concentrations ranging from 25 nM to 100 μM. For cytotoxicity assays, culture medium was removed from microplate cell cultures and replaced by 200 μL of OTC working solutions. Plates were incubated for an additional period, between 1 and 48 h depending on the assay.

Cell Viability. Cell viability was assessed by the fluorescent reagent AlamarBlue. After 4 h of incubation at 37 °C with OTC dissolved in culture medium, 10% of AlamarBlue was added to cell cultures. Fluorescence was measured in a Spectramax M5 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) at 560 nm excitation and 585 nm emission.

Light Microscopy. Neuroblastoma cells were seeded on plates and treated with different concentrations of OTC. After incubation, cells were visualized with an Olympus optic microscope to observe cell morphology and detachment. Images were recorded with a Leica color digital camera (DFC 480).

Cell Proliferation. A CyQUANT Cell Proliferation Assay Kit was used to quantify cell proliferation. After incubation with OTC, culture plates were centrifuged (3000 rpm, 5 min). Supernatant was discarded, and plates were stored at −20 °C until analysis, to allow efficient cell lysis. On the day of the assay, a 1:20 CyQUANT cell lysis buffer solution was prepared in Milli-Q water, and CyQUANT GR dye was diluted (1:400) in the buffer solution. Then, 200 μL of this preparation was added at each well, and microplates were incubated for 2−5 min at room temperature protected from light. Fluorescence was measured at 485 nm excitation and 520 nm emission.[43]

Integrity of Plasma Membrane. Plasma membrane permeabilization was evaluated by the measurement of two indicators: propidium iodide (PI) uptake and release of lactate dehydrogenase (LDH), as previously described.[43,48] For the determination of PI uptake, cell culture plates were centrifuged, washed once with 200 μL of phosphate buffer saline (PBS) per well, and incubated for 1 min with 100 μL of 15 μM PI per well. After centrifugation and a second wash with 200 μL of PBS per well, fluorescence was measured at 535 nm excitation and 617 nm emission.

Release of LDH from treated cells was assessed by the LDH-based toxicology assay kit (TOX-7). After incubation, 100 μL of cell culture medium was transferred to clean microplate wells. Then, 200 μL of reagent mixture was added to each well, and after incubation in the dark at room temperature for 30 min, absorbance was measured at 490 nm in a Spectramax M5 microplate reader. Background absorbance at 690 nm was subtracted from the absorbance value at 490 nm.[48] In both assays, integrity of plasma membrane was compared to positive controls (100% of plasma membrane damage) consisting of neuroblastoma cells permeabilized with 0.1% Triton X-100 in PBS.

Depolymerization of F-Actin. After treatment with OTCs for 24 h, cells were fixed with 18% formaldehyde and permeabilized with 0.1% Triton-X in PBS to allow the uptake of Oregon Green. After rinsing with PBS, 100 nM Oregon Green in PBS was added to the cell cultures. Cells were then incubated at room temperature for 30 min and rinsed again, and fluorescence was measured at 511 nm excitation and 528 nm emission.[41]

Formation of Reactive Oxygen Species (ROS). A positive control, consisting of cell cultures treated with 2% H_2O_2 in culture medium, was incubated at the same time as cells treated with organotins. After incubation for 24 h, plate cell cultures were centrifuged and washed with PBS. Then, 200 μL of 10 μM 6-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate, di(acetoxymethyl ester), in PBS was added to cultures and incubated at 37 °C for 30 min. Following this period, the reagent was replaced by fresh culture medium, and cells were incubated at 37 °C for 5 min. Fluorescence was subsequently measured at 492 nm excitation and 522 nm emission.

Mitochondrial Membrane Potential. Changes in mitochondrial membrane potential were determined using the fluorescent probe MitoTracker Red CMXRos as described in the literature.[48,49] An aliquot of 50 μL of medium was removed from fresh cell cultures, and 50 μL of 4 μM Mitotracker Red CMXRos diluted in PBS was added to

give a final concentration of 1 μM. Culture plates were incubated at 37 °C for 45 min and subsequently centrifuged and washed with PBS. Fluorescence was measured at 579 nm excitation and 599 nm emission.

Determination of DNA Fragmentation. DNA from neuroblastoma cells was extracted using the ApoTarget Quick Apoptotic DNA Ladder Detection Kit. DNA samples (100 ng) were loaded onto a 1.2% agarose gel, and 5 μL of Midori Green as DNA stain was added. Gel was run at 5 V cm^{-2} for 2 h and 20 min using Bio-Rad electrophoresis equipment.

Statistical Treatment of Data. Results are expressed as the mean ± SEM of two or four experiments performed in triplicate. Statistically significant differences among means were assessed by analysis of variance (ANOVA) followed by a Tukey *b* test for post hoc multiple comparisons at a level of significance of $p < 0.05$.

■ RESULTS

Cell Viability. To evaluate the effect of OTC on neuroblastoma, we first studied the loss of cell viability after exposure to these toxic compounds. Viability of neuroblastoma cells was assessed after 24 h of incubation at five different concentrations between 0.1 and 100 μM. In this assay, MBT did not have any significant effect on the viability loss of neuroblastoma cells. However, a concentration of 100 μM of MBT seems to increase cell viability. At present we have no explanation for this unexpected result. On the contrary, DBT and TBT induced significant losses ($p < 0.05$) of cell viability from the lowest concentration tested. In these conditions, minimum values of cell viability, ranging between 32 and 45%, were observed in cultures treated with ≥10 μM, TBT being slightly more toxic than DBT (Figure 2).
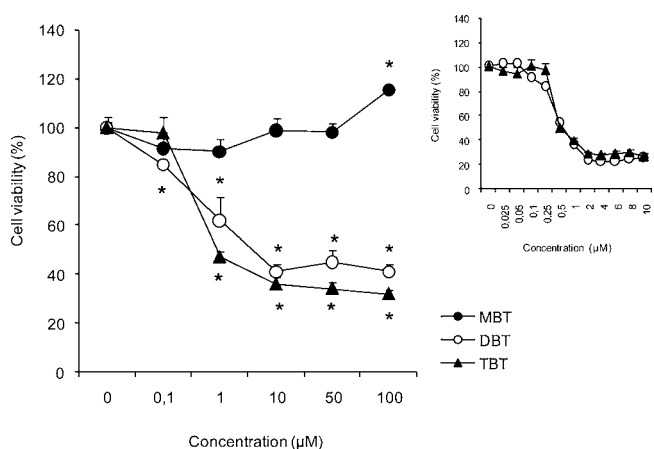

Figure 2. Effect of MBT, DBT, and TBT on viability of neuroblastoma cells incubated for 24 h with different concentrations of organotin compounds. (Inset) Effect of DBT and TBT at very low concentrations (0−10 μM) on viability of neuroblastoma cells treated for 24 h. Means and SEMs represent data obtained from two different experiments with four replicates each. Results are expressed as a percentage of AlamarBlue relative fluorescence with respect to control (untreated cells). *, $p < 0.05$.

A second trial was undertaken to establish a dose−response relationship between DBT or TBT concentrations, from 25 nM to 10 μM, and loss of cell viability (Figure 2, inset). For DBT, loss of cell viability was significant from 100 nM, and almost linear in the range from 100 nM to 2 μM. For TBT, significant loss of viability was observed from 250 nM onward, linearity being found between 250 nM and 2 μM (see also Figure 10).
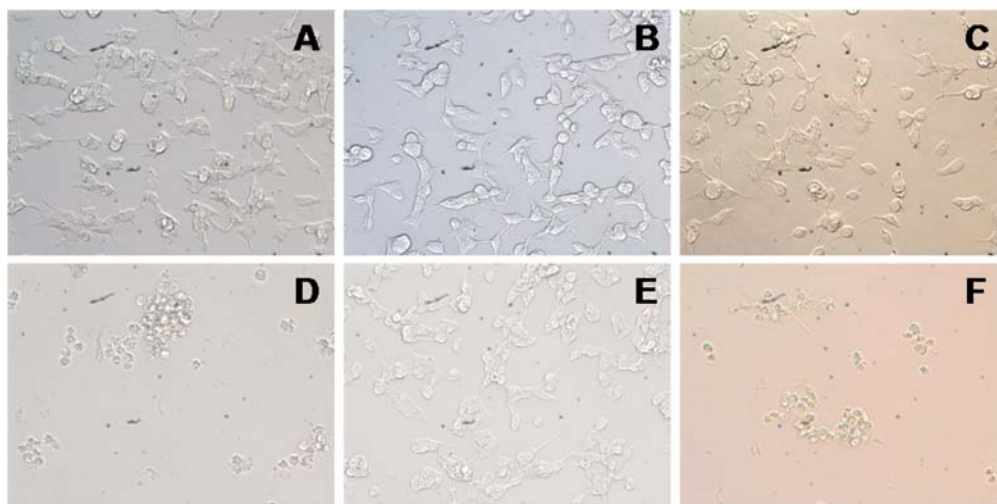
Figure 3. Changes in neuroblastoma cell morphology upon incubation for 24 h with organotin compounds: (A) control cultures; (B) incubation with 100 μM MBT; (C) incubation with 0.1 μM DBT; (D) incubation with 1 μM DBT; (E) incubation with 0.1 μM TBT; (F) incubation with 1 μM TBT.

Cell viability reached minimum values at 2 μM for both compounds.

Cell Morphology and Detachment. Morphological changes and detachment from the substrate are events that normally occur during cell death. Loss of viability was also reflected in changes in cell morphology (Figure 3). Cells incubated with low doses of MBT maintained the typical neuron-like cell morphology, similar to control cells (Figure 3A), and only a slight rounding was observed at treatment with 100 μM MBT (Figure 3B). In contrast, treatments with DBT and TBT caused loss of cell morphology at much lower concentrations. Cells maintained their morphology at low doses of DBT, 0.1 μM (Figure 3C), but appeared as a round mass when incubated with 1 μM DBT (Figure 3D) or 0.1 μM TBT (Figure 3E). At higher concentrations of these compounds, cells completely lost their shape and detached from substrate, forming clumps; moreover, a considerable amount of cell debris was noticeable (Figure 3F).

Cell Proliferation. The influence of OTCs on cell proliferation was studied next. All organotin compounds tested had an adverse effect on neuroblastoma cell proliferation, causing significant decreases after 48 h of incubation at all concentrations tested. Minimum values of proliferation, equivalent to 20% of control cultures, were obtained with concentrations of 1 μM TBT and 10 μM DBT and higher, whereas MBT showed lower toxicity (Figure 4). Exposure of neuroblastoma cells to these pollutants for 24 h triggered almost similar effects (data not shown).

Depolymerization of F-Actin. To study the role of the cytoskeleton in mediating cell detachment and survival, the effect of OTCs on F-actin was evaluated. Exposure to MBT for 24 h induced a slight, although not significant, F-actin depolymerization. On the contrary, the depolymerization caused by DBT and TBT was much stronger, and minimum values of F-actin depolymerization (35−40%, $p < 0.05$) were observed in treatments with concentrations of ≥10 μM (Figure 5).

Plasma Membrane Integrity. Two parameters were evaluated to study plasma membrane damage: PI uptake and LDH release.
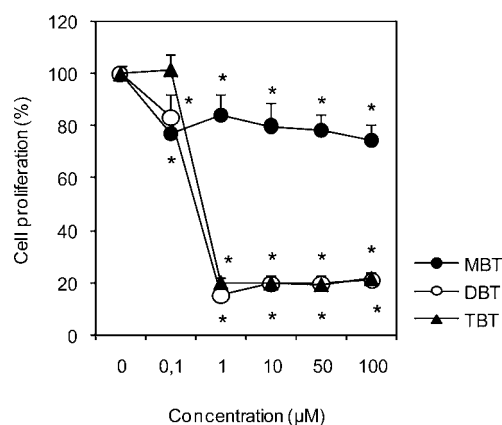

Figure 4. Effect of MBT, DBT, and TBT on neuroblastoma cell proliferation after 48 h of incubation with increasing concentrations of organotin compounds. Results are expressed as percentage of CyQUANT relative fluorescence with respect to controls (untreated cells). Results are means and SEMs from two different experiments with four replicates each. ∗, $p < 0.05$.
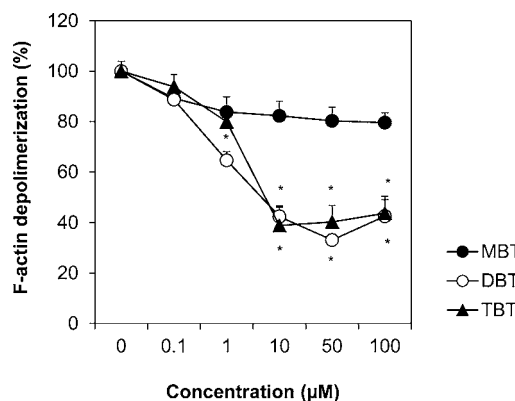

Figure 5. Depolymerization of F-actin in neuroblastoma cells induced by 24 h of incubation with increasing concentrations of MBT, DBT, or TBT. Results are means and SEMs from two different experiments with four replicates each and expressed as percentage of F-actin with respect to controls (untreated cells). ∗, $p < 0.05$.

PI uptake was minimum in cells exposed to organotins for 24 h in comparison with positive controls permeabilized with the surfactant Triton X-100. No differences among treated and control cultures were observed (data not shown). With regard to release of LDH, MBT and DBT did not cause any significant effect, whereas treatment with the most toxic compound, TBT, at 10 μM and higher doses led to a significantly higher ($p <$ 0.05) release of LDH compared with control cultures (Figure 6).
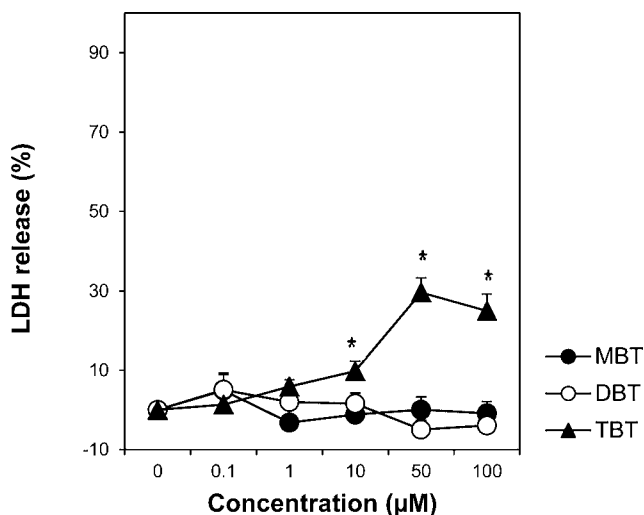

Figure 6. Release of LDH by neuroblastoma cells after 24 h of incubation with increasing concentrations of MBT, DBT, or TBT. Results are means and SEMs from two different experiments with four replicates each. ∗, $p < 0.05$.

ROS Production. The effect of organotins in the formation of ROS inside neuroblastoma cells was studied next, because it is known that ROS can contribute to cellular death. MBT caused a significant increase of the formation of ROS at all concentrations tested after 24 h of incubation (Figure 7). On the contrary, DBT (data not shown) and TBT did not have significant effects on ROS production.

Mitochondrial Membrane Potential. Mitochondria assays allow the detection of changes in the early phase of
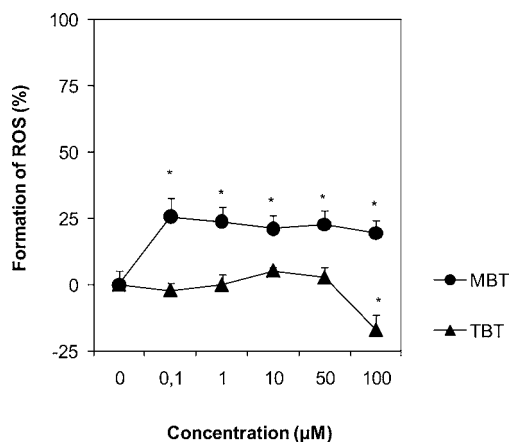

Figure 7. Formation of ROS in neuroblastoma cells after 24 h of incubation with increasing concentrations of MBT and TBT. Results are means and SEMs from two different experiments with four replicates each. ∗, $p < 0.05$.

apoptosis. Alterations in the potential of mitochondrial membrane were monitored at 1, 2, 4, and 24 h of incubation with organotin compounds. No significant changes in mitochondrial membrane potential in cultures incubated with MBT were recorded (Figure 8A). DBT caused slight but
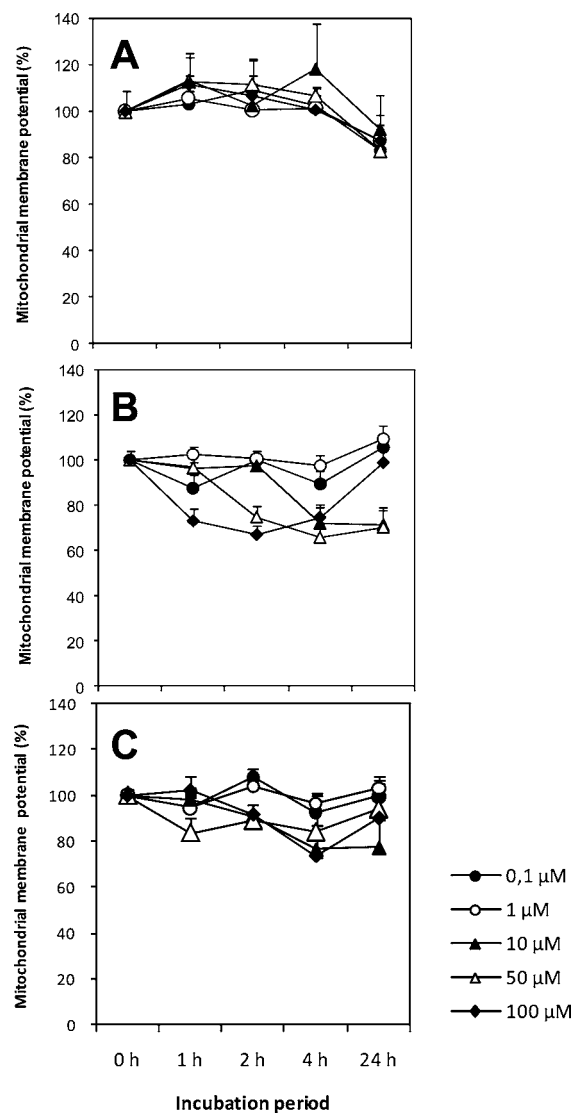

Figure 8. Changes in mitochondrial membrane potential of neuroblastoma cells after incubation for 24 h with organotin compounds: (A) MBT; (B) DBT; (C) TBT. Signs (∗) of statistical significance are omitted for clarity.

significant ($p < 0.05$) decreases in mitochondrial membrane potential at high concentrations, 10, 50, and 100 μM (Figure 8B). In those cases, depolarization of mitochondrial membrane was observed after 2 or 4 h of incubation. At 100 μM, mitochondrial membrane potential recovered at 24 h. In cells incubated with TBT, significant depolarization ($p < 0.05$) was observed only after 4 h of incubation with $\geq$10 μM concentrations (Figure 8C).

DNA Fragmentation. DNA laddering is used to visualize the endonuclease cleavage products of apoptosis. Incubation for 24 h with TBT at 50 and 100 μM and DBT at 100 μM caused the fragmentation of DNA of neuroblastoma cells, which could be observed as a "smear" in the electrophoresis gel (Figure 9). On the contrary, lower concentrations of those contaminants or

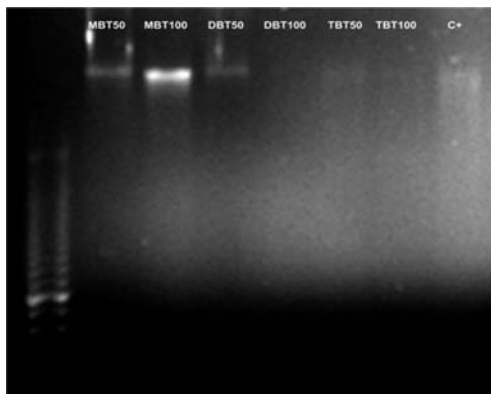MBT at any of the concentrations tested did not cause DNA fragmentation.

Figure 9. Effect of organotin compounds in DNA fragmentation on neuroblastoma cells after 24 h of incubation. From left to right: DNA ladder marker; treatment with 50 μM MBT, 100 μM MBT, 50 μM DBT, 100 μM DBT, 50 μM TBT, and 100 μM MBT, respectively; and positive control (cells incubated at 100 °C for 15 min to induce DNA fragmentation).

In Vitro Screening Method Development. Linearity plots obtained for DBT and TBT at different concentrations and loss of viability in neuroblastoma cells after 24 h of incubation were represented, as shown in Figure 10. Correlation coefficients >0.9 indicated a promising linear regression adjustment of the obtained data, in the concentration range from 0.1 to 2 μM. These preliminary results indicate that the cell viability assay applied in the present study could provide the bases for a suitable screening method.

■ DISCUSSION

In vitro model systems have been incorporated into neurotoxicity screens and mechanistic studies, because they focus on changes at the cellular and molecular level rather than on whole-animal morbidity. This allows the isolation and culturing of cells specifically targeted by neurotoxicants and the determination of cell viability following treatment with a toxic compound.[50] The butyltins are thought to target the immune system, but in recent years more evidence supports the hypothesis that butyltins are also potent neurotoxicants for the adult and the developing nervous systems of vertebrates, not only in experimental setups[25,26,39] but also at environmental levels through natural exposure in fish.[33]

In the present work we studied different cell markers of cell death, including necrosis and apoptosis, to evaluate the cytotoxicity triggered by MBT, DBT, and TBT.

Cell Viability. A reliable in vitro cell viability assay is a critical component of any drug or neurotoxicant study.[50] Our results on cell viability agree with those previously obtained in immortalized cell lines neuroblastomas, T- and B-cell lines,[34] with a TBT 50% toxic concentration (TC_{50}) ranging from 1 to 11 μM and in rat microglial cells and astroglial-microglial cocultures,[51] ranging from 0.7 to 1.1 μM. Minimum viability was reached at 2 μM for TBT and DBT in the present study (Figure 2), agreeing with other authors observing that neurons from rat hippocampal slice cultures died with 5 μM after 24 h of incubation with TBT, with severe damage to the cytomembrane.[52] Nevertheless, differences in sensitivity among the diverse cell cultures and exposure times have been already published in the literature.[27,34] In our hands, similar TC_{50} values, around 0.5 μM, were obtained with DBT and TBT. DBT effect on cell viability was previously described in an in vitro model of neuronal development in PC 12 cells, with significant cell death at 0.3 μM,[39] and in rat primary cultures of cerebellar granule cells, with 50% cell viability at 0.3 μM.[14] Our data are concordant with the literature, showing that DBT is clearly neurotoxic in vitro, with a reported potency equal to or greater than that of the well-known neurotoxic organotin trimethyltin (TMT).[14] By contrast, MBT was not toxic at any of the concentrations tested in this study, up to 100 μM. Furthermore, the increased cell viability observed at the highest MBT tested concentration might be correlated to the observation that MBT induced a significant increase of ROS, taking part in stimulating cell growth, as suggested by others.[53,54]

Cell Proliferation. With regard to cell proliferation, a correlation was noted between the suppression of the cellular energy state and the inhibition of macromolecular synthesis and cell proliferation caused by trialkyltin compounds.[38] Antiproliferative effects of trialkyltin compounds were observed in many cellular types and organisms and are consistent with our results (Figure 4). Concentrations of ≥0.1 μM decreased cell proliferation in a significant way. It was previously reported that

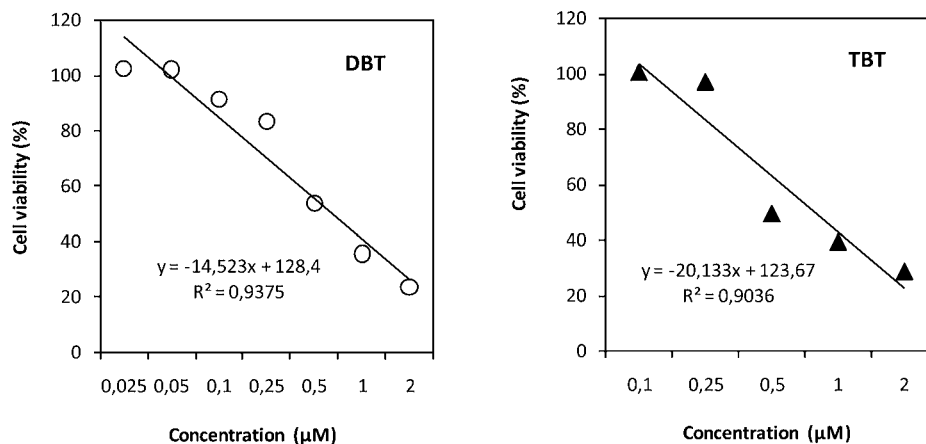

Figure 10. Linearity plots obtained for different concentrations of DBT (left) and TBT (right) and loss of viability in neuroblastoma cells after 24 h of incubation, represented as percentage of negative control (100%).

micromolar concentrations of DBT effectively decreased DNA and protein precursors, whereas the incorporation of uridine into RNA was increased.[38] DBT also affected the proliferation of cultured rabbit chondrocytes. At the micromolar level, these compounds inhibited the incorporation of DNA precursor, whereas at lower concentrations, a stimulation of DNA synthesis was noted.[38]

F-Actin Depolymerization. Changes in cell shape and anchorage are associated with reorganization of actin filaments, mostly depending on their intrinsic ability to rapidly assemble and disassemble, indicating a key role of the actin cytoskeleton in the complex network that engages membrane-related events and signal transduction cascades.[41] In this context, we observed that DBT and TBT caused a significant degree of F-actin depolymerization at concentrations from 1 to 10 μM, consistent with changes in cell morphology (Figures 2 and 3), agreeing with complete loss of shape and detachment from substrate. Moreover, these results correlate with a decrease of cell viability and cell proliferation. There is an emerging view in the literature indicating that cytoskeletal components are involved in downstream signal transduction pathways closely related to cell survival/cell death and one form of apoptosis, termed anoikis, mediated by different signal transduction pathways.[41]

Plasma Membrane Integrity. In relation to plasma membrane integrity, we did not obtain significant effects of toxicants MBT, DBT, and TBT on PI uptake in the range of 0.1−100 μM. In contrast, other authors obtained significant PI uptake after exposure to 5 μM TBT for 24 h, which indicated that chronic TBT treatment resulted in severe cell damage in hippocampal slice cultures.[52] The same authors observed that TBT exerted a regionally different neurotoxicity in different hippocampal subregions, with varying vulnerability.[52] Also, these differences might be related to the cell type, as previously described.[38] This result is consistent with an apoptotic cell death as was reported for some OTCs, proving that apoptosis occurs without loss of membrane integrity and, hence, with no uptake of PI.[14] More studies suggest that the dead or damaged cells after TBT exposure occur by an apoptosis-like process.[52] With regard to release of LDH, other authors observed that extracellular LDH activity increased 24 h after exposure to concentrations of TBT as low as 2.5 μM[55] in primary astrocytes, whereas in our cell model a significant effect of TBT was triggered at $\geq$10 μM. TBT was also reported to produce dose−response curves with discernible cytotoxicity occurring at a concentration of 2.5 μM and maximal cell death at 10 μM,[55] agreeing with our results on cell viability. A longer exposure to contaminant TBT produced significant increases in LDH release and complete cell death at a lower TBT concentration, 5 μM.[55] In the present study, only TBT caused a significant release of LDH from concentrations of $\geq$10 μM showing that, although cells are still viable, because there is lack of PI uptake, plasma membrane is injured, as shown by the experiments of LDH release.

ROS Production. The process of cell injury or cell death often involves free radical generation, and TBT toxicity was suggested to be mediated by ROS, which might contain H_2O_2 more predominantly than O_2^-.[52] In our results formation of ROS is detected only with MBT; however, it was reported that TBT neurotoxicity may require endogenous H_2O_2 and O_2^- rather than TBT itself producing harmful ROS.[52] In this context, the involvement of other types of radicals, including nitric oxide, in TBT toxicity is possible.[52] Also, some studies

reported that ROS generation in OTC-induced toxicity is dependent on the toxicant, the concentration, and the exposure time.[56] On the other hand, the effect of MBT on the increase in both ROS and cell viability, is remarkable with a possible connection related to the beneficial effects at low−moderate concentrations of ROS, as suggested above in the present discussion. It has been described that cells generate ROS to be used in the induction and maintenance of signal transduction pathways involving cell growth and differentiation.[53]

Mitochondrial Membrane Potential and DNA Fragmentation. Several concentrations were tested to determine the involvement of mitochondria in OTC-induced cytotoxicity. Only DBT and TBT at the higher concentrations used triggered a slight decrease in mitochondrial membrane potential, statistically significant at 4 h of incubation. Similar results were found in relation to DNA laddering because only DBT and TBT at high doses are able to produce a ladder-like pattern of DNA after a 24 h exposure. These findings indicate that the mitochondrial pathway is activated as well as DNA fragmentation, but they are not the main mechanisms responsible for the cell death induced by these compounds.

In Vitro Screening Method Development. In vitro cytotoxicity assays are an alternative to analytical methods, time-consuming and expensive, for a rapid screening of the compounds of interest based on their mechanism of action. Human neuroblastoma cells have been successfully applied for the sensitive detection of toxic substances, based on functional activity.[42,57,58] Our preliminary results point out at a linear relationship between the fluorescence obtained in the assay AlamarBlue, indicative of cell viability and TBT and DBT concentration in the range of 0.1−2 μM. This could provide the bases for a suitable screening method, alternative to long and expensive analytical techniques, such as gas chromatography coupled to mass detection (GC-MS). An analytical method employed for producing extracts suitable for both biological testing with cell cultures and quantitation of OTC compounds by GC-MS is being developed at present in our laboratory.

In summary, in our cell model DBT and TBT can induce cell death by necrosis and apoptosis because these mechanisms are not mutually exclusive and this depends on the compound, concentration, and exposure time. Other authors have reported similar findings.[14,29] In fact, this result could be due to the correlation between cytotoxicity and lipophilicity,[14,39,59] which may involve interaction between the butyltins and the surface of the cell membrane.[60] A higher lipophilicity of TBT (octanol/water partition ratio, log K_{ow} = 3.89)[61] compared to DBT (log K_{ow} = 1.49)[61] and MBT (log K_{ow} = 0.41)[62] was previously described. On the basis of log K_{ow}, TBT is more lipophilic and may penetrate the cell membrane and adhere to and denature membrane proteins.[60] The toxic effects of OTC are connected to high bioavailability (given by lipophilicity) and a slow biodegradation; the toxicity varies depending on the length of the alkyl and aryl groups and increases in the order MBT < DBT < TBT for certain end points, for example, endocrine disruption.[62]

At present TBT is included as a priority substance within the European Union, but taking into account that DBT is one of the most common OTCs found in some geographical locations,[62] this compound should be also considered in future risk assessment. More experiments are being done in our laboratory to further explore cytotoxicity induced by OTCs in neuroblastoma cells.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 00 34 986 469 303 (344). Fax: 00 34 986 469 269. E-mail: agcabado@anfaco.es.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs. *EFSA J.* **2004**, *102*, 1–119.

(2) Antizar-Ladislao, B. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. *Environ. Int.* **2008**, *34* (2), 292–308.

(3) Smith, B. S. Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus = Ilyanassa obsoleta. J. Appl. Toxicol.* **1981**, *1* (3), 141–144.

(4) Ruiz, J. M.; Bryan, G. W.; Gibbs, P. E. Chronic toxicity of water tributyltin (TBT) and copper to spat of the bivalve *Scrobicularia-plana* – ecological implications. *Mar. Ecol.: Prog. Ser.* **1994**, *113* (1–2), 105–117.

(5) Fent, K. Ecotoxicological effects at contaminated sites. *Toxicology* **2004**, *205* (3), 223–240.

(6) Kannan, K.; Senthilkumar, K.; Loganathan, B. G.; Odell, D. K.; Tanabe, S. Elevated accumulation of tributyltin and its breakdown products in bottlenose dolphins (*Tursiops truncatus*) found stranded along the U.S. Atlantic and Gulf coasts. *Environ. Sci. Technol.* **1997**, *31* (1), 296–301.

(7) Borghi, V.; Porte, C. Organotin pollution in deep-sea fish from the northwestern Mediterranean. *Environ. Sci. Technol.* **2002**, *36* (20), 4224–4228.

(8) Berge, J. A.; Brevik, E. M.; Bjorge, A.; Folsvik, N.; Gabrielsen, G. W.; Wolkers, H. Organotins in marine mammals and seabirds from Norwegian territory. *J. Environ. Monit.* **2004**, *6* (2), 108–112.

(9) European Parliament. Decision No. 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC. *Off. J. Eur. Communities* **2001**, *L331*, 1–5.

(10) European-Parliament. Directive 2006/11/EC of the European Parliament and of the Council of 15 February 2006 on pollution caused by certain dangerous substances discharged into the aquatic environment of the Community. *Off. J. Eur. Union* **2006**, *L64*, 52–59.

(11) European-Parliament. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. *Off. J. Eur. Union* **2008**, *L348*, 84–97.

(12) EC. *European Communities Environmental Objectives (Surface Waters) (Amendment) Regulations 2012; S.I. No. 327 of 2012;* Stationery Office: Dublin, Ireland, 2012; p 8.

(13) International Maritime Organization (IMO). International Convention on the Control of Harmful Antifouling Systems on Ships, 2001; http://www.imo.org/about/conventions/listofconventions/pages/international-convention-on-the-control-of-harmful-anti-fouling-systems-on-ships-(afs).aspx.

(14) Mundy, W. R.; Freudenrich, T. M. Apoptosis of cerebellar granule cells induced by organotin compounds found in drinking water: involvement of MAP kinases. *NeuroToxicology* **2006**, *27* (1), 71–81.

(15) Mimura, H.; Sato, R.; Sasaki, Y.; Furuyama, Y.; Taniike, A.; Yoshida, K.; Kitamura, A. Accelerator analysis of tributyltin adsorbed onto the surface of a tributyltin resistant marine *Pseudoalteromonas* sp. cell. *Int. J. Mol. Sci.* **2008**, *9* (10), 1989–2002.

(16) Harino, H.; Ohji, M.; Wattayakorn, G.; Adulyanukosol, K.; Arai, T.; Miyazaki, N. Accumulation of organotin compounds in tissues and organs of stranded whales along the coasts of Thailand. *Arch. Environ. Contam. Toxicol.* **2007**, *53* (1), 119–125.

(17) Nath, M. Toxicity and the cardiovascular activity of organotin compounds: a review. *Appl. Organomet. Chem.* **2008**, *22* (10), 598–612.

(18) Kannan, K.; Senthilkumar, K.; Giesy, J. P. Occurrence of butyltin compounds in human blood. *Environ. Sci. Technol.* **1999**, *33* (10), 1776–1779.

(19) Nielsen, J. B.; Strand, J. Butyltin compounds in human liver. *Environ. Res.* **2002**, *88* (2), 129–133.

(20) Takahashi, S.; Mukai, H.; Tanabe, S.; Sakayama, K.; Miyazaki, T.; Masuno, H. Butyltin residues in livers of humans and wild terrestrial mammals and in plastic products. *Environ. Pollut.* **1999**, *106* (2), 213–218.

(21) Appel, K. E. Organotin compounds: toxicokinetic aspects. *Drug Metab. Rev.* **2004**, *36* (3–4), 763–786.

(22) Hümpel, M.; Kühne, G.; Täuber, U.; Schulze, P. E. Studies on the kinetics of bis(tris-*n*-butyl-113tin)oxide (TBTO). In *Toxicology and Analytics of the Tributyltins: The Present Status*, Ortepa Workshop; Verlag Ortepa Association: Berlin, Germany, 1986; pp 122–142.

(23) Evans, W. E.; Cardarelli, N. F.; Smith, D. J. Accumulation and excretion of (1–14C) bis(tri-*n*-butyltin) oxide in mice. *J. Toxicol. Environ. Health* **1979**, *5*, 871–877.

(24) Omura, M.; Shimasaki, Y.; Oshima, Y.; Nakayama, K.; Kubo, K.; Aou, S.; Ogata, R.; Hirata, M.; Inoue, N. Distribution of tributyltin, dibutyltin and monobutyltin in the liver, brain and fat of rats: two-generation toxicity study of tributyltin chloride. *Environ. Sci.* **2004**, *11* (2), 123–132.

(25) Elsabbagh, H. S.; Moussa, S. Z.; El-Tawil, O. S. Neuro-toxicologic sequelae of tributyltin intoxication in rats. *Pharmacol. Res.* **2002**, *45* (3), 201–206.

(26) Tsunoda, M.; Konno, N.; Nakano, K.; Liu, Y. Altered metabolism of dopamine in the midbrain of mice treated with tributyltin chloride via subacute oral exposure. *Environ. Sci.* **2004**, *11* (4), 209–219.

(27) Yamada, J.; Inoue, K.; Furukawa, T.; Fukuda, A. Low-concentration tributyltin perturbs inhibitory synaptogenesis and induces neuronal death in immature but not mature neurons. *Toxicol. Lett.* **2010**, *198* (2), 282–288.

(28) Ade, T.; Zaucke, F.; Krug, H. F. The structure of organometals determines cytotoxicity and alteration of calcium homeostasis in HL-60 cells. *Anal. Bioanal. Chem.* **1996**, *354* (5–6), 609–614.

(29) Zaucke, F.; Zöltzer, H.; Krug, H. F. Dose-dependent induction of apoptosis or necrosis in human cells by organotin compounds. *Fresenius' J. Anal. Chem.* **1998**, *361* (4), 386–392.

(30) Umebayashi, C.; Oyama, Y.; Chikahisa-Muramatsu, L.; Nakao, H.; Nishizaki, Y.; Nakata, M.; Soeda, F.; Takahama, K. Tri-*n*-butyltin-induced cytotoxicity on rat thymocytes in presence and absence of serum. *Toxicol. in Vitro* **2004**, *18* (1), 55–61.

(31) Orrenius, S.; McCabe, M. J., Jr.; Nicotera, P. Ca^{2+}-dependent mechanisms of cytotoxicity and programmed cell death. *Toxicol. Lett.* **1992**, *64–65* (0), 357–364.

(32) Stridh, H.; Fava, E.; Single, B.; Nicotera, P.; Orrenius, S.; Leist, M. Tributyltin-induced apoptosis requires glycolytic adenosine trisphosphate production. *Chem. Res. Toxicol.* **1999**, *12* (10), 874–882.

(33) Zhang, J.; Zuo, Z.; Chen, R.; Chen, Y.; Wang, C. Tributyltin exposure causes brain damage in *Sebastiscus marmoratus. Chemosphere* **2008**, *73*, 337–343.

(34) Thompson, T. A.; Lewis, J. M.; Dejneka, N. S.; Severs, W. B.; Polavarapu, R.; Billingsley, M. L. Induction of apoptosis by organotin compounds in vitro: neuronal protection with antisense oligonucleotides directed against stannin. *J. Pharmacol. Exp. Ther.* **1996**, *276* (3), 1201–1216.

4202

dx.doi.org/10.1021/jf3050186 | *J. Agric. Food Chem.* 2013, 61, 4195–4203

(35) Aldridge, W. N. The influence of organotin compounds on mitochondrial functions. In *Organotin Compounds: New Chemistry and Applications*; American Chemical Society: Washington, DC, 1976; Vol. 157, pp 186−196.

(36) Snoeij, N. J.; Punt, P. M.; Penninks, A. H.; Seinen, W. Effects of tri-*n*-butyltin chloride on energy-metabolism, macromolecular-synthesis, precursor uptake and cyclic-AMP production in isolated rat thymocytes. *Biochim. Biophys. Acta* **1986**, *852*, 234−243.

(37) Snoeij, N. J.; van Rooijen, H. J. M.; Penninks, A. H.; Seinen, W. Effects of various inhibitors of oxidative phosphorylation on energy metabolism, macromolecular synthesis and cyclic AMP production in isolated rat thymocytes. A regulating role for the cellular energy state in macromolecular synthesis and cyclic AMP production. *Biochim. Biophys. Acta (BBA)* − *Bioenergetics* **1986**, *852*, 244−253.

(38) Snoeij, N. J.; Penninks, A. H.; Seinen, W. Biological-activity of organotin compounds − an overview. *Environ. Res.* **1987**, *44* (2), 335−353.

(39) Jenkins, S. M.; Ehman, K.; Barone, S., Jr. Structure-activity comparison of organotin species: dibutyltin is a developmental neurotoxicant in vitro and in vivo. *Dev. Brain Res.* **2004**, *151*, 1−12.

(40) Leira, F.; Cabado, A. G.; Vieytes, M. R.; Roman, Y.; Alfonso, A.; Botana, L. M.; Yasumoto, T.; Malaguti, C.; Rossini, G. P. Characterization of F-actin depolymerization as a major toxic event induced by pectenotoxin-6 in neuroblastoma cells. *Biochem. Pharmacol.* **2002**, *63* (11), 1979−1988.

(41) Cabado, A. G.; Leira, F.; Vieytes, M. R.; Vieites, J. M.; Botana, L. M. Cytoskeletal disruption is the key factor that triggers apoptosis in okadaic acid-treated neuroblastoma cells. *Arch. Toxicol.* **2004**, *78* (2), 74−85.

(42) Valverde, I.; Lago, J.; Vieites, J. M.; Cabado, A. G., Neuroblastoma cells as a cell model to study BADGE (bisphenol A diglycidyl-ether) and BFDGE (bisphenol F diglycidyl-ether) toxicity. In *Focus on Neuroblastoma Research*; Fernandes, J. A., Ed.; Nova Science Publishers: Hauppauge, NY, 2007; pp 119−147.

(43) Valverde, I.; Lago, J.; Reboreda, A.; Vieites, J. M.; Cabado, A. G. Characteristics of palytoxin-induced cytotoxicity in neuroblastoma cells. *Toxicol. in Vitro* **2008**, *22* (6), 1432−1439.

(44) Ferreira, M.; Cabado, A. G.; Chapela, M. J.; Fajardo, P.; Atanassova, M.; Garrido, A.; Vieites, J. M.; Lago, J. Cytotoxic activity of extracts of marine sponges from NW Spain on a neuroblastoma cell line. *Environ. Toxicol. Pharmacol.* **2011**, *32* (3), 430−437.

(45) Campbell, C. G.; Borglin, S. E.; Green, F. B.; Grayson, A.; Wozei, E.; Stringfellow, W. T. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: a review. *Chemosphere* **2006**, *65* (8), 1265−1280.

(46) Caillaud, A.; Cañete, E.; de la Iglesia, P.; Giménez, G.; Diogène, J. Cell-based assay coupled with chromatographic fractioning: a strategy for marine toxins detection in natural samples. *Toxicol. in Vitro* **2009**, *23* (8), 1591−1596.

(47) Rossini, G. P. Functional assays in marine biotoxin detection. *Toxicology* **2005**, *207* (3), 451−462.

(48) Lago, J.; Santaclara, F.; Vieites, J. M.; Cabado, A. G. Collapse of mitochondrial membrane potential and caspases activation are early events in okadaic acid-treated Caco-2 cells. *Toxicon* **2005**, *46* (5), 579−586.

(49) Leira, F.; Alvarez, C.; Vieites, J. M.; Vieytes, M. R.; Botana, L. M. Study of cytoskeletal changes induced by okadaic acid in BE(2)-M17 cells by means of a quantitative fluorimetric microplate assay. *Toxicol. in Vitro* **2001**, *15* (4−5), 277−282.

(50) Thompson, T. A. Viability assays for cells in vitro: the ethidium/calcein assay and the immunofluorescence combination assay. *Methods Mol. Med.* **1998**, *22*, 145−155.

(51) Röhl, C.; Grell, M.; Maser, E. The organotin compounds trimethyltin (TMT) and triethyltin (TET) but not tributyltin (TBT) induce activation of microglia co-cultivated with astrocytes. *Toxicol. in Vitro* **2009**, *23* (8), 1541−1547.

(52) Mizuhashi, S.; Ikegaya, Y.; Matsuki, N. Cytotoxicity of tributyltin in rat hippocampal slice cultures. *Neurosci. Res.* **2000**, *38* (1), 35−42.

(53) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39* (1), 44−84.

(54) Marengo, B.; Raffaghello, L.; Pistoia, V.; Cottalasso, D.; Pronzato, M. A.; Marinari, U. M.; Domenicotti, C. Reactive oxygen species: biological stimuli of neuroblastoma cell response. *Cancer Lett.* **2005**, *228* (1−2), 111−116.

(55) Karpiak, V. C.; Eyer, C. L. Differential gliotoxicity of organotins. *Cell Biol. Toxicol.* **1999**, *15* (4), 261−268.

(56) Nakatsu, Y.; Kotake, Y.; Ohta, S. Concentration dependence of the mechanisms of tributyltin-induced apoptosis. *Toxicol. Sci.* **2007**, *97* (2), 438−447.

(57) Louzao, M. C.; Vieytes, M. R.; de Sousa, J.; Leira, F.; Botana, L. M. A fluorimetric method based on changes in membrane potential for screening paralytic shellfish toxins in mussels. *Anal. Biochem.* **2001**, *289* (2), 246−250.

(58) Leira, F.; Alvarez, C.; Cabado, A. G.; Vieites, J. M.; Vieytes, M. R.; Botana, L. M. Development of a F actin-based live-cell fluorimetric microplate assay for diarrhetic shellfish toxins. *Anal. Biochem.* **2003**, *317* (2), 129−135.

(59) Borenfreund, E.; Babich, H. In vitro cytotoxicity of heavy-metals, acrylamide, and organotin salts to neural cells and fibroblasts. *Cell Biol. Toxicol.* **1987**, *3* (1), 63−73.

(60) Han, G. C.; Cooney, J. J. Effects of butyltins and inorganic tin on chemotaxis of aquatic bacteria. *J. Ind. Microbiol.* **1995**, *14* (3−4), 293−299.

(61) Donkin, P.; Widdows, J.; Evans, S. V.; Staff, F. J.; Yan, T. Effect of neurotoxic pesticides on the feeding rate of marine mussels (*Mytilus edulis*). *Pestic. Sci.* **1997**, *49* (2), 196−209.

(62) Karlsson, C. *Risk Assessment of Compounds That Could Impair the Aquatic Environment*. Uppsala University, p 55.