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Organometallic Complexes with Biological Molecules: XIII. Organotin(IV)[*meso*-tetra (4-carboxyphenyl)porphinate]s and the Cell Cycle: A Flow-cytometric Approach

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The cytotoxic derivatives diorganotin(IV) and triorganotin(IV) [*meso*-tetra(4-carboxyphenyl)-porphinates, with stoichiometries $[R_2Sn]_2TPPC$ and $[R_3Sn]_4TPPC$ [R = Me, Bu, Ph; $TPPC^{4-}$ = *meso*-tetra(4-carboxyphenyl)porphinate^{4-}], namely bis[dimethyltin(IV)], bis[dibutyltin(IV)], bis[diphenyltin(IV)], tetra[trimethyltin(IV)], tetra[tributyltin(IV)] and tetra[triphenyltin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinate]s, have been used to investigate their effects on the cultured human kidney cell cycle in order to understand further the origin of cell-growth inhibition induced by the above-mentioned chemicals. The cell-cycle-dependent DNA content distribution of cultured cells exposed to these compounds has been analyzed through flow cytometry, a potent technique capable of probing several aspects of drug-induced cytotoxicity. Cultured human kidney cells have been used as a model system, on the premise of greater physiological similarity to the human situation *in vivo*.

Keywords: cytotoxicity; DNA; cell; diorganotin; triorganotin; carboxyphenylporphinates; flow cytometry

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INTRODUCTION

Organotin compounds are known to be neurotoxic as well as immunotoxic to humans and other species.[1]

Several biological effects of organotin(IV) derivatives have been the focus of some papers and reviews that have been published recently.[2–7] In particular, the cytotoxic properties of several organotin(IV) derivatives towards ascidian embryonic development have been widely studied.[2–5] Reduced fertility, arrest of the mitosis process, mortality of embryos and inhibition of larval movement, which implies reduction of the intrinsic rate of population growth, were major effects.[2–5] Moreover, biological effects of diorgano- and triorganotin(IV) derivatives, such as neurotoxicity and hepatotoxicity, have been reviewed recently by Arakawa.[6,7]

On the other hand, the interaction of several newly synthesized compounds — tetra[trimethyltin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinate, tetra[tributyltin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinate and dialkyl and trialkyltin(IV) thiaminepyrophosphates (alkyl = methyl, butyl), with DNA and eukariotic cells has been the aim of recent research.[8,9] All the complexes induced a clear inhibitory effect on the growth of NIH-3T3 cells, tetra[tributyltin] [*meso*-tetra(4-carboxyphenyl)porphinate and tributyltin(IV) thiaminepyrophosphate being the most active.[8,9] To understand better the origin of this inhibition, the cell-cycle-

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dependent DNA content distribution of cells exposed to the action of bis[diorganotin(IV)] and tetra[triorganotin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinates has been studied and is reported in this paper. Flow cytometry[10] has been chosen because of its potency as a tool of investigation not only for cell-cycle analysis, but also to probe many other aspects of drug-induced cytotoxicity, such as membrane and chromosomal damage, cell viability and morphological alterations.

MATERIALS AND METHODS

Chemicals

The bis[diorganotin(IV)] and tetra[triorganotin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinate] complexes were synthesized, as previously reported,[8] by refluxing, in dry methanol, R_2SnO or R_3SnOH, freshly prepared by hydrolysis of the parent R_2SnCl_2 and R_3SnCl respectively (gifts from Witco GmbH, Bergkamen, Germany), and [*meso*-tetra(4-carboxyphenyl)porphine] (= H_4TPPC) at high purity, from Porphyrin Products (Logan, UT, USA) in the molar ratios 2:1 and 4:1, respectively, for $[R_2Sn]_2TPPC$ and $[R_3Sn]_4TPPC$ derivatives [R = Me, Bu, Ph]. Correspondence between the obtained and the published complexes has been verified by chemical, IR and Mössbauer spectroscopic analysis, in the solid state, and by ^1H and ^{13}C NMR in [D6]DMSO solutions.[8]

In the Results and Discussion section bis[dimethyltin(IV)], bis[dibutyltin(IV)], bis[diphenyltin (IV)], tetra[trimethyltin(IV)], tetra[tributyltin(IV)] and tetra[triphenyltin(IV)] [*meso*-tetra(4-carboxyphenyl)porphinate]s will be denoted as DMTPPC, DBTPPC, DPTPPC, TMTPPC, TBTPPC and TPTPPC, respectively.

Cell Culture

Human embryonal kidney cell line 293T (293 cells expressing the simian virus 40 large tumor antigen) cells were grown in monolayer in Dulbecco's modified Eagle's medium, (Gibco BRL, MD, USA) supplemented with 1% penicillin–streptomycin (Gibco BRL), 1% Fungizone (Gibco BRL) and 10% heat-inactivated fetal bovine serum (Gibco BRL) on 100 mm tissue culture dishes (Falcon, NJ, USA). Asynchronous cells were grown at 37 °C in an atmosphere of 5% CO_2 in 95% humid air and were passed every 2–3 days to maintain logarithmic growth. In all experiments log-phase cells were investigated.

After the cells had been allowed to adhere to the surface of the dish, the organometallic solution [in water or dimethyl sulfoxide (DMSO)] to be tested was added to the medium to a final concentration of 10^{-6} or 10^{-5} M. When DMSO was used as a solvent, its final concentration was always kept $\leq$1%. After a 24 or 48 h incubation with the compound, adherent cells were washed with phosphate-buffered saline (PBS) (Gibco BRL) and dispersed with 0.05% trypsin–EDTA (Gibco BRL).

Floating cells contained in the culture medium as well as in the wash medium were pooled with the trypsinized cells and pelleted. Cells were fixed by resuspension in ice-cold 70% ethanol in PBS and stored at -20 °C for at least 12 h prior to staining. Two hours before flow-cytometric analysis, fixed cells were pelleted, washed with PBS, resuspended in staining solution [25 mg ml^{-1} propidium iodide (PI) (Calbiochem, CA, USA) and 100 mg/ml DNase-free RNase A (Sigma, MO, USA) in PBS], sieved through a 35 μm cell strainer cap (Falcon, NJ, USA) and stored in the dark at 4 °C until analysis.

Flow cytometry

PI-stained cells were analyzed with an Epics Profile II flow cytometer (Coulter Corporation, FL, USA) fitted with a 250 mm BioSense flow cell with attached lens and mirror, a 15 mW air-cooled argon-ion laser emitting at 488 nm and a 635 nm band-pass filter. In all samples, a total of 10,000 cells were analyzed. Both forward-angle and orthogonal (side) light-scatter signals were collected as correlated parameters of DNA content. List Mode file format was converted to Flow Cytometry Standard file format using ProFCS version 3.2 (Verity Software House, ME, USA) and data were analyzed using WinList version 3.0 (Verity Software House) and WinMDI version 2.7 (Joseph Trotter, The Scripps Institute, CA, USA) software.

RESULTS AND DISCUSSION

The DNA content of each cell in any living organism is generally highly uniform. In the resting phase of the cell cycle, a human somatic cell contains approximately 6–7 pg of DNA (diploid DNA content). This stage is occupied by non-

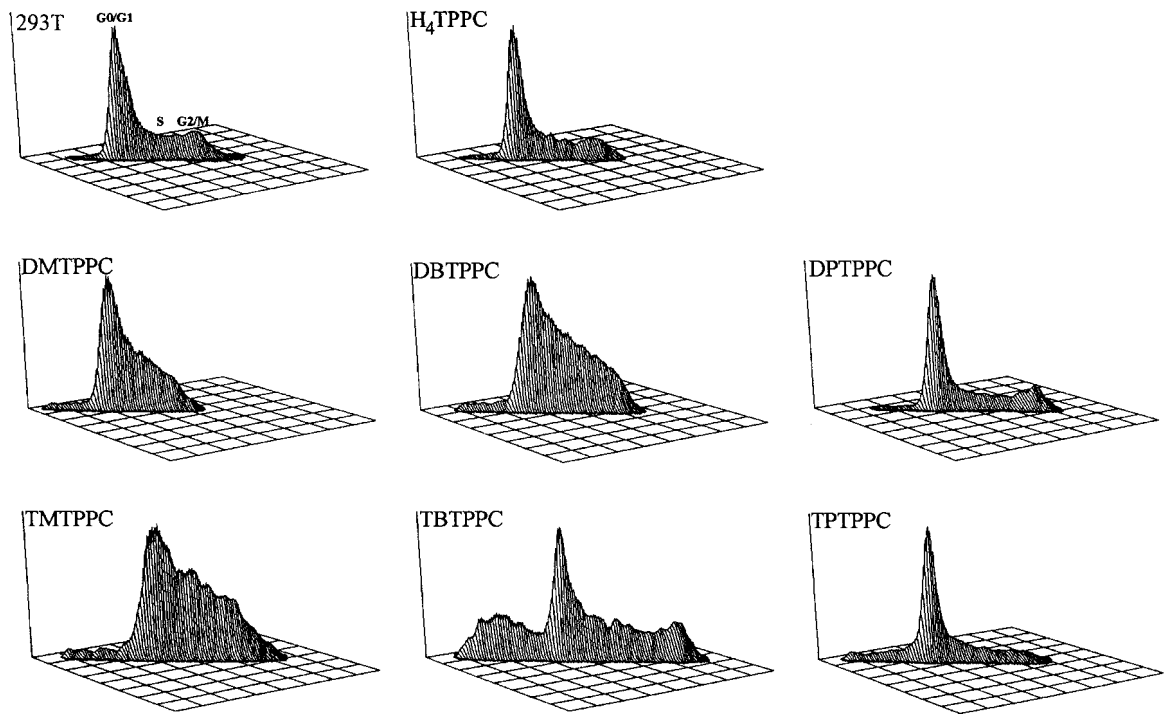

Figure 1 DNA content distributions of 293T cells exposed to various organotins at 10^{-5} M in water for 24 h. 293T, 293T cells grown in organotin-free DMEM. See text for full compound names and definition of G0/G1, S and G2/M phases of the cell cycle. *x*-axis: Pl fluorescence area; *y*-axis, Pl fluorescence peak; *z*-axis, cell number.

cycling cells (G0 phase) as well as those recovering from the previous division or preparing for the next cycle (G1 phase). When beginning the process of replication, a cell enters DNA synthesis (S phase). As DNA synthesis proceeds, cellular DNA content progressively increases until replication is complete and the cell enters the G2 phase with a DNA content twice that of G1 (tetraploid). After repairing DNA damage and organizing the chromosomes, the cell enters mitosis (M phase) dividing into two diploid daughter cells and completing the cycle.[10]

The DNA flow histograms presented in this study represent *snapshots* of the distribution of nuclei in the various phases of the cell-cycle at the time of fixation.[11]

Because organotin toxicity in cell cultures evolves over 24 to 48 h,[12] these were the chosen incubation times at which cells were harvested. By comparing the normal cell-cycle DNA content distribution of 293T cells with that of cells incubated with the various potential toxicants at 10^{-5} M for 24 h (Fig. 1), it immediately emerges that, apart from a moderate flattening of the histogram areas corresponding to the S and G2/M fractions, DPTPPC and TPTPPC are the compounds which yield the least dramatic effects on the distributions. Moreover, periodic microscopic analysis revealed no evident alterations of morphology or cell growth. DMTPPC and DBTPPC-exposed cells show an increase in the S-phase population and this effect is particularly enhanced by TMTPPC. This S-phase blockade is suggestive of an inhibitory effect on replication. Indeed, it has been shown that organotins can affect DNA synthesis.[13–16] DNA synthesis can also be inhibited as a direct as well as an indirect mechanism of chromosome aberrations,[17] which have also been detected as a consequence of exposure to organotins.[18–21]

Moreover, it has been recently shown that persistent DNA damage leads to S-phase inhibition.[21] Interestingly, the genotoxicity of dibutyltin(IV) and dioctyltin(IV) dichloride has been ascribed to complex formation with DNA.[22,23] Bearing in mind that several modes of interaction between porphyrin and DNA have been proposed,[24]

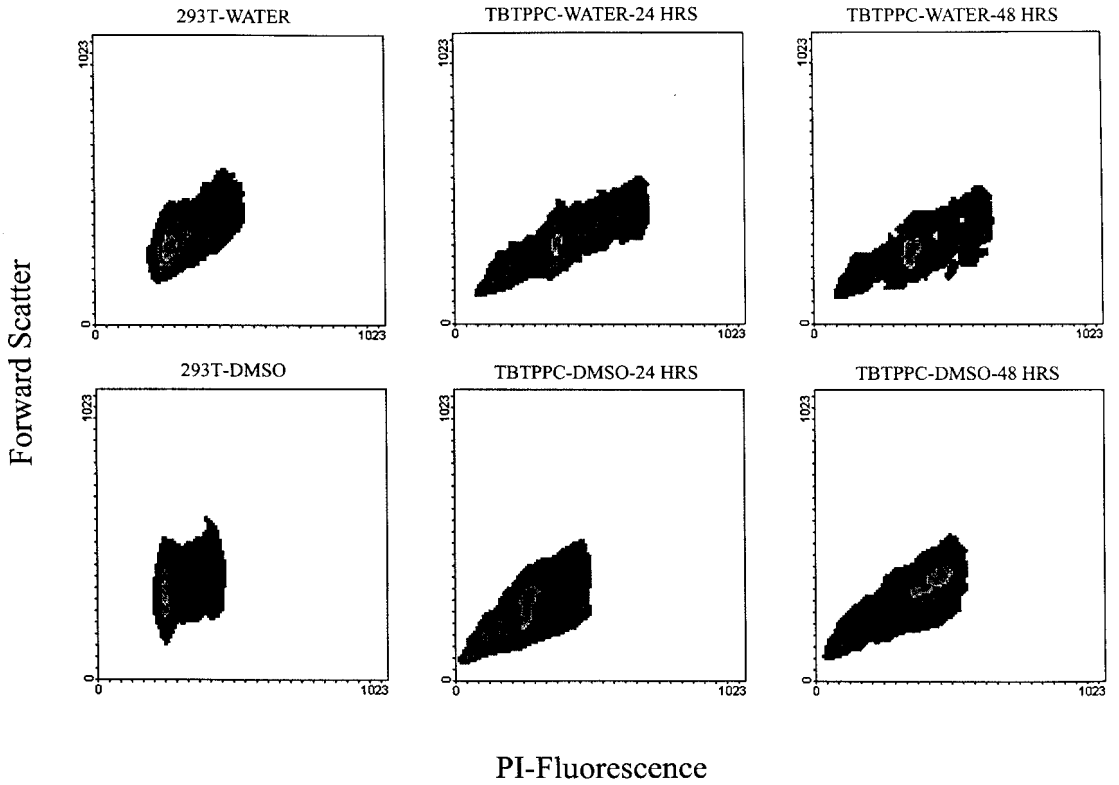

Figure 2 Forward scatter (cell size) versus Pl fluorescence (DNA content) density plots of 293T cells exposed to 10^{-5} M TBTPPC solubilized in water or DMSO for 24 hours and 48 hours.

it is tempting to speculate that the complexes might synergistically interact with DNA, the porphyrin ligand and the organometallic moieties acting in a concerted fashion and the complex behaving as a pseudo-bifunctional adduct. TBTPPC is the most toxic of the tested compounds, causing cell death, as indicated by the appearance of a sub-G1 peak.

The appearance of distinct subpopulations characterized by reduction of PI fluorescence (DNA content) and forward angle (1–19 °) light scatter (cell size) (Fig. 2) is indicative of apoptotic cells. Indeed, tributyltin is known to be capable of inducing programmed cell death[12,24–26] and immunotoxic organotins have been proposed as possible model compounds in studying apoptosis.[27,28] This result is currently being validated by using more specific apoptotic assays in order to rule out the possibility that the observed sub-G1 peak is due to necrotic cells which can present similar parameters. The toxic effects after 48 h of exposure appear enhanced but qualitatively similar (Fig. 3), apart from an increase of the S and G2/M fractions in addition to the cell death already induced by TBTPPC after 24 h. Furthermore, lowering the concentration of toxicants to 10^{-6} M did not result in qualitative differences, although TBTPPC-induced cell death was substantially decreased (data not shown). A previous study[29] reported G2/M arrest following exposure of murine erythroleukemic cells to tributyltin(IV) dissolved in ethanol. Interestingly, when 293T cells are exposed to DBTPPC solubilized in DMSO, a marked G2/M arrest is observed (Fig. 4).

Moreover, after 48 h, the S and G2/M phase accumulation is much more enhanced by exposure to TBTPPC DMSO solutions in comparison with the corresponding aqueous solutions (compare Fig. 4 with Fig. 3).

We chose not to use ethanol as a solvent because of its intrinsic ability to alter the cell cycle,[30] while water possesses greater similarity to the

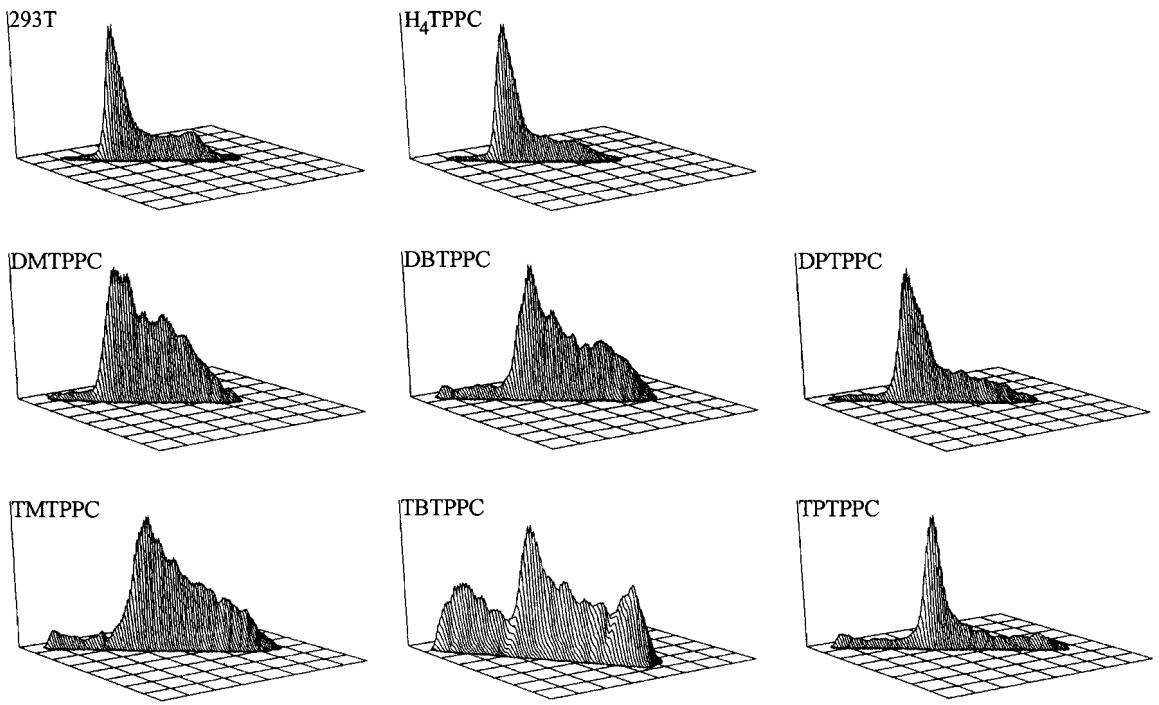

Figure 3 DNA content distributions of 293T cells exposed to various organotins at 10^{-5} M solubilized in water for 48 h. For details, see text and caption to Fig. 1.

environmental distribution of organotins and physiological conditions *in vivo*. However, the difference in behavior between the aqueous solutions and the DMSO ones could be related to hydrolytic reactions which occur in solution and which are enhanced in water compared with DMSO, due to the well-known coordinating properties of the latter solvent.

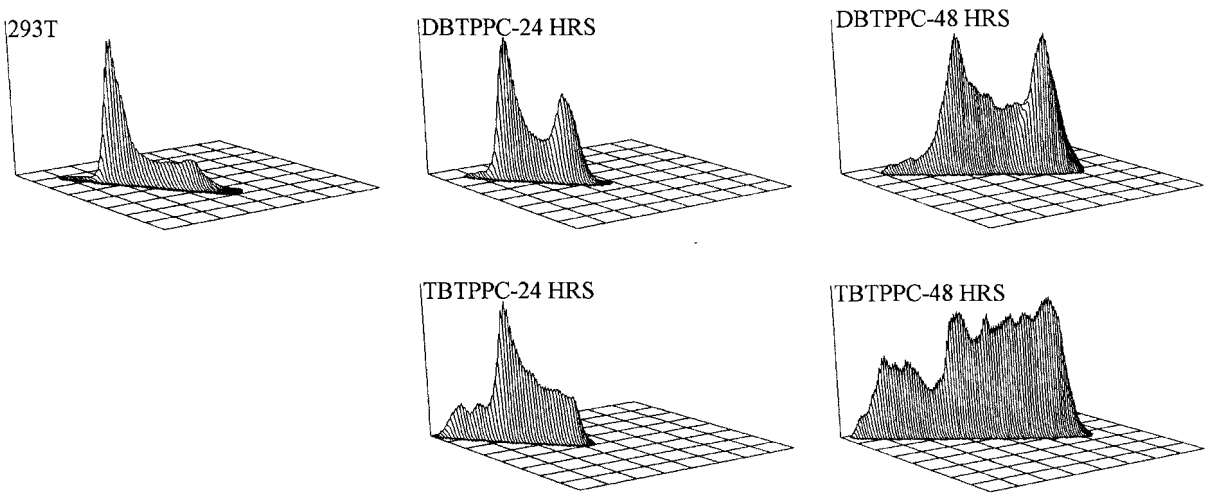

Figure 4 DNA content distributions of 293T cells exposed to 10^{-5} M DBTPPC or TBTPPC in DMSO for 24 h and 48 h.

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