Higher concentrations of organotin compounds are required to decrease bacterial growth than to kill human cells

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Organotin compounds (OTC) are chemicals that contain one or several carbon tin bonds and have a wide range of industrial and agricultural applications. Their inclusion as antifouling components in paints used to coat the bottom of the ships and their subsequent release in the marine environment caused irreversible sexual masculinization in female gastropods. World-wide pollution persists, despite global ban of harmful organotins in anti-fouling paints since OTC have also been widely used as biocides, catalysts and PVC stabilizers, generating a global contamination of air, soil and water. Many reports have clearly demonstrated now that OTC have neurotoxic, immunotoxic and hormone disruptor potential. OTC water pollution and agricultural applications create another complex scenario of interactions between OTC and microorganisms that may determine bacterial resistance to the same OTC that exert toxicity to humans. Here, we report that the OTC concentrations required to inhibit bacterial growth may be far superior to those required to decrease the viability of human cells. Such contrast in bacterial vs human susceptibility to OTC creates an enormous risk for chronic human exposure to OTC contaminated watered-food that may silently contribute to human health deterioration.

Keywords organotin, bactericide, human exposure

1. Introduction

Organotin compounds (OTC) are persistent, toxic, bioaccumulative organometallic compounds, able to travel long distances through different media [1, 2]. OTC have the capacity to induce the irreversible appearance of a penis and/or vas deferens in female gastropods, a phenomenon known as imposex. Therefore, the International Maritime Organization declared a global ban that entered into force by September 2008, prohibiting the use of harmful organotins in anti-fouling paints for ships [3]. Levels of OTC maritime contamination are now reduced where stringent measures were taken [4, 5, 6]. However, global pollution persists, since imposex is still observed worldwide and the recent detection of hot spots of OTC contamination in some countries strongly suggests both that the OTC usage is uninterrupted and that there is an urgent need for better regulatory strategies [5, 7, 8, 9].

Not all tin compounds are equally toxic. Low toxic tetra-organotins can be metabolized to tri-organotins that are used as catalysts, PVC stabilizers, industrial biocides, antifungal paints, agricultural fungicides, miticides and acaricides [10, 11]. Tri-substituted OTC are widely recognized as having higher toxicity, whereas di-organotins and mono-organotins are reputed with lower and the lowest toxicity, respectively [11, 12]. Their antibacterial activity seems to follow the same order of efficacy. Based on their cytotoxic properties, several investigators have even proposed certain organotin complexes to be used in anticancer therapy [13, 14].

The ubiquitous presence of OTC is caused by their multiple industrial and agricultural applications and the subsequent opportunity to be released into the environment [10, 11]. Triphenyltin (TPT) was used as plant protection product for potatoes in Europe up till 2003 [15]. TPT acetate and TPT hydroxide have increasingly been used worldwide as soil treatment fungicides to treat a variety of crops [11]. Not significant uptake by lettuce or barley has been reported even from heavily OTC contaminated soil and the usage of tributyltin (TBT)-contaminated, land-deposited harbour sludge for plant production has been considered a safe practice [16, 17]. However, OTC remaining on food destined to human consumption create another source of human exposure that should not be lightly disregarded, either they are taken up by the roots or the shoots or they just remain as residual contamination within the harvest.

OTC present in the fields may come from contaminated soil or water or from biocides added to protect the crops. The risk derived from OTC and bacteria simultaneously present in food obtained from plants is determined by the amount and type of OTC and bacteria, as well as the environmental conditions and the interactions between OTC and bacteria that may result in bacterial death or bacterial resistance as well as OTC persistence or biodegradation [18, 19]. Various OTC have a recognized bactericidal effect but numerous resistant bacteria have been reported [20, 21, 22].

2. OTC toxicity to humans

OTC exposure may lead to toxic effects in the nervous, immune and endocrine systems in several organisms, including mammals where OTC may alter lipids, proteins and DNA [23, 24, 25]. Bioaccumulation on molluscs and other marine

organisms continues all the way up into the food chain generating a serious risk for human health, which is difficult to evaluate since it depends on individual dose, frequency and time of ingestion, metabolism and excretion of OTC and its metabolites [25, 26, 27]. Our knowledge on the levels at which the OTC exist in foodstuffs consumed by humans as well as safety levels of exposure to chemicals is limited [11, 12]. The results of experiments demonstrating that TPT, TBT, DBT and other OTC affect *in vitro* viability and functionality of normal and abnormal human cells have generated serious concerns on the damage that can be caused to human health by the environmental contamination with OTC [24, 28, 29, 30].

OTC are immunotoxic and neurotoxic both to invertebrates and humans. The endocrine disruptor ability of OTC may include humans through interference with nuclear receptors activation [25, 31]. Effects are related to frequency, amount and way of exposure, besides individual susceptibility, absorption, metabolism and excretion. Nonetheless, the obesogenic and other endocrine effects could result from early exposure and the possibility to suffer any kind of OTC toxicity will be increased with chronic, inadvertent exposure through dietary intake or environmental contamination [31].

3. OTC bactericidal capacity

Although OTC are widely recognized as bactericide, their effect may greatly vary in different strains and under different circumstances. The bactericide effect of TBT, TPT and other OTC, relates not only to different resistance profiles but also to the medium and the environment in which bacteria grow [20, 21, 22, 32]. Environmental factors such as pH and salinity may determine organotin reactivity and OTC-induced selection of resistant microorganisms in aquatic systems where OTC and bacteria coexist. Biotransformation of organotin compounds by debutylation or methylation may influence the toxicity, mobility, and environmental fate of OTC [18]. OTC used as biocides in agriculture may not only be unable to eliminate pathogens but originate a new problem, remaining either as superficial or absorbed contaminant [10, 16, 17]. A small, remaining OTC concentration may still be sufficient to exert toxicity to humans ingesting them when eating contaminated food.

4. Materials and Methods.

4.1. Reagents.

RPMI 1640, Hepes, antibiotics, glutamine, and fetal calf serum were purchased from Gibco BRL (Gaithersburg, MD, USA). Blood agar, MacConkey agar, Salt mannitol agar, Chocolate agar, and Sabouraud's agar were bought to bioMerieux (Hazelwood, MO). Antibiotic medium No. 3 was from BD Diagnostic Systems (Circle Sparks, MD, USA). Triphenyltin chloride (TPT), tributyltin chloride (TBT), Dibutyltin chloride (DBT), trimethyltin chloride (TMT) and dimethyltin chloride (DMT) were acquired from Merck (Darmstadt Germany). Trypan blue was purchased from Sigma (St. Louis, MO, USA).

4.2. Clinical isolates.

Eight pathogenic bacteria strains were isolated of clinical samples taken from external patients that assisted to the Hospital between January and February 2012. Samples were taken from pharyngeal exudates (3), vaginal exudates (2) and urine (3) from 8 patients that were not under treatment with antibiotics at that time. These 5 Gram negative and 3 Gram positive strains were seeded in selective and differential culture media and incubated for 48 h at 37°C. Strains were isolated after incubation and identified both by diagnostic medical criteria and the automated system Vitek II (bioMerieux, Hazelwood, MO).

4.3. Bacterial sensitivity to OTC.

Isolated pure strain were inoculated in Erlenmeyer culture flasks containing 50 ml of antibiotic medium No. 3 and incubated overnight at 37°C. Bacterial mass was harvested from each culture, washed and resuspended again in broth medium No. 3. One hundred and eighty µl from 22.2 x 10^6 UFC/ml of every strain were placed in every well of 96 microwell plates in order to analyse their sensitivity to OTC. Twenty µl of one of each OTC (TPT, TBT, DBT, TMT and DMT) were added at one of 5 different concentrations (1 mM, 100 µM, 10 µM, 1 µM and 100 nM) to each well. Experiments were performed twice by triplicate. Wells receiving DMSO, Ethanol, or medium served as controls. Plates were incubated with agitation and humid atmosphere overnight at 37°C. Optical density was read at 600 nm, at 0, 24 and 48 h and the OD averages were used to calculate the percentages of growth inhibition.

4.4. Cell lines.

NALM-6 is a pre-B leukemia cell line that has low cyclin E protein levels and is induced to enter into apoptosis by exposure to low concentrations of nitric oxide (NO) [33]. NALM-6R is a NO-resistant variant that was obtained after repeated exposure of NALM-6 cells to increasingly higher concentrations of SNAP, a short-life NO chemical donor [34]. Both NALM-6 and NALM-6R were maintained in culture with RPMI medium supplemented with 10 % FCS, 2 mM glutamine, and antibiotics (100 IU penicillin and 100 μg streptomycin/ml). Cell density was adjusted 24 h before the experiments to 1 x10^6/ml in order to maintain the cells in logarithmic phase of growth during culture. Cell suspensions > 95% viable, newly adjusted to 1 x10^6/ml RPMI, were cultured by triplicate in the presence or in the absence of the OTC that were added once. Cells were exposed to OTC (TPT, TBT, DBT, TMT and DMT) at similar concentrations to those used to test bacterial susceptibility. Since OTC were dissolved in DMSO (TPT) or ethanol (TBT, DBT, TMT and DMT), DMSO and ethanol were added at similar volume in control wells. Cell viability was measured by trypan blue exclusion.

5. Results

Isolated bacteria are opportunistic (*K. pneumoniae*, *P. aeruginosa* and *S. haemolyticus*), commensal (*E. faecalis*) or virulent through the Shiga toxin production (*E. coli*) or being coagulase positive (*S. aureus*). Their clinical importance also relates to the fact that they all are often responsible for nosocomial infections.

Table 1 Percentage of growth inhibition by OTC at 1 mM concentration.

Bacteria	TPT		TBT		DBT		TMT		DMT	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Klebsiella pneumonia	24	41	31	23	39	20	16	26	0	0
Klebsiella pneumonia	64	39	56	71	51	63	26	8	0	0
Escherichia coli	63	64	61	62	62	67	18	25	9	0
Escherichia coli	70	40	70	65	30	6	0	0	0	0
Pseudomona aeruginosa	80	60	72	60	0	0	0	0	0	0
Staphylococcus aureus	61	61	55	56	61	60	22	20	75	0
Staphylococcus haemolyticus	63	64	58	67	57	57	21	29	12	0
Enterococcus faecalis	71	72	65	66	62	67	47	43	17	0

As expected, bacterial growth inhibition achieved by OTC greatly varied. TPT and TBT were more effective, since they achieved higher growth inhibition of all tested bacteria. DBT failed to inhibit the growth of *P. aeruginosa* and was less effective inhibiting the growth of one strain of *E. coli* but it was otherwise almost as effective as TPT and TBT (Table 1).

Although DMT was the less effective OTC, it did significantly inhibit growth of *S. aureus* and, at low extent, also inhibited growth of *S. haemolyticus* and *E. faecalis* at 24 h. However, all three strains were fully recovered at 48 h showing similar growth to control cultures (Table 1). All OTC were ineffective for bacterial growth inhibition at < 1 mM concentration (not shown).

Two cell lines from human origin were similarly exposed to various concentrations of 5 OTC and cell viability was measured by trypan blue exclusion. The results of two experiments by triplicate are presented in Table 2. While most of the cells remained alive after 24 and 48 h incubation with 100 nM OTC, there was a significant mortality when incubated with 10 μM TPT, TBT and DBT, particularly during 48 h. NALM-6R cells were somehow more resistant but only the first 24 h, since almost all the NO-sensitive NALM-6 and the NO-resistant NALM-6R cells were dead after 48 h incubation with 10 μM TPT, TBT or DBT. In addition, all tested OTC, except TMT, killed nearly 100 % of the cells, when present at 1 mM concentration during 24 h. Later, 1 mM TMT also caused 100 % cell mortality after 48 h.

Table 2 Percentage of viability from NALM-6S and NALM-6R during 48 h *in vitro* culture. Cells incubated with solvent as control were always > 95% viable.

Cells + OTC concentration		TPT		TBT		DBT		TMT		DMT	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
NALM-6	100 nM	100	86	92	91	100	95	100	86	91	85
	10 μM	33	6	8	4	94	8	93	89	85	80
	1 mM	0	0	4	1	6	0	47	0	9	10
NALM-6R	100 nM	100	95	85	81	100	86	85	84	94	84
	10 μM	82	6	88	29	75	8	83	86	93	81
	1 mM	7	0	8	0	0	0	79	0	8	14

6. Discussion

OTC may be released from paints, plastics and biocides into the environment [24, 35]. The persistence of OTC in polluted ecosystems is related to OTC deposit and bioaccumulation as well as physical, chemical and biological removal mechanisms [12, 36]. Humans may be exposed to OTC not only by eating seafood and drinking contaminated water, but also by absorbing them through the skin and the respiratory track [10, 11, 26, 37]. Several groups have reported that OTC concentrations in seafood and estimated intakes of the consuming populations do not exceed safety values [4, 38, 39]. However, TPT and DBT have been detected at nM concentrations in human blood, urine, liver and breast milk, in support of the notion that the contamination in humans may be worldwide spread [11, 24, 37, 40]. Although the actual levels of contamination are largely unknown and difficult to evaluate, OTC concentrations already detected in human tissue may be quite sufficient to induce various kind of toxic effects to the immune, nervous and endocrine systems, especially after long time exposure.

OTC are widely used as biocides in agriculture [10, 11]. However, there are many OTC resistant bacteria [18, 20, 21]. In addition, bacteria require concentrations in the mM rank to be killed, whereas µM and even nM concentrations might be enough to affect the function and viability of human cells from primary cultures [28, 29, 30] and human cell lines [41, 42]. Since the OTC concentrations required to kill bacteria may be 1000 times greater than those concentrations that are sufficient to kill human cells, we should question again the practice of using OTC as bactericides and fungicides in agriculture. If 1/1000 fraction of OTC applied for crops protection is retained or taken up, it may be still big enough to inadvertently affect the health of human consumers. The concentrations required to induce imposex are very low [6, 43] and imposex is still observed in various countries [5, 9]. TBT is a recognized obesogen, and it is conceivable that TBT, its metabolites, and other OTC may affect the human endocrine function in various ways [31, 43]. Very low concentrations of OTC exert toxicity both in invertebrates and mammals, rising serious concerns on the biological effects that OTC may have in chronically exposed people [24, 28, 43]. To determine the risk that OTC pollution generates to human health must be considered in first place when already known OTC continue being used and new OTC are being proposed for old or new applications that may contribute to maintain or to increase environmental pollution [13, 22, 44].

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