CHAPTER 7

Genotoxicity and Immunotoxicity of Organotins

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Abstract: Leached from various sources, all the organotin compounds have an impact on natural aquatic environments. In both freshwater and seawater ecosystems, they are dangerous in that they can have deleterious effects on biocenoses already at low concentrations. All these compounds are known to be toxic at relatively low levels, not only for aquatic invertebrates, but also for fish and laboratory mammals. Moving easily along the trophic chains, organotins are also rapidly bioaccumulated in the tissues of non-target organisms living in the water-sediment interface, causing severe, long-term toxic effects on local epifauna, with repercussions on biodiversity and human health. Among toxic effects, genotoxicity and immunotoxicity are the most important affecting the capacity for survival of animals. Genotoxicity appearing in the form of chromosomal aberrations, increasing in frequency of micronuclei and induction of cytogenetic damage has recently been reported in mammals, fish and aquatic invertebrates. Organotins interfere selectively with the immune system of vertebrates, causing atrophy of the thymic cortex and lymphoid tissues with a consequent leucopoenia. Short-term *in vitro* exposures of haemocytes of various vertebrate and invertebrate organisms reveal inhibition of phagocytosis, cytolysis and/or apoptosis of leucocytes after inhibition of chemotaxis and respiratory burst, with resulting depression of cell-mediated immune responses. These immunosuppressive effects are dose- and time-dependent, and vary according to the number and type of organic moiety present. Both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms of action have been proposed. They are linked and synergistic in triggering the cascade of secondary events that lead to toxic action.

Keywords: Genotoxicity - mutagenicity - immunotoxicity - lymphoid organ atrophy - apoptosis - calcium-dependent and calcium-independent immunosuppression - organotins.

GENOTOXICITY OF ORGANOTIN COMPOUNDS

The term genotoxicity indicates the ability of a xenobiotic to interact with DNA and induce changes (mutations) in the cell genotype or chromosomal damage; in the case of heritable changes, the term mutagenicity is preferentially used. Since mutations are a pre-requisite for carcinogenicity, genotoxic compounds can contribute to the development of tumours; conversely, heritable mutations can lead to birth defects and genetic diseases.

In the last two decades, *in vitro* assays have mainly been used to assess the extent of genotoxicity of new compounds entering the environment, in order to identify mutagens and their modality of action in terms of dose-response relationships and mutagenic mechanisms. Conversely, today, there is an increasing search for new *in vivo* assays and model organisms for improved predictions of risks associated with the use of xenobiotics.

Until 1990, negative results were obtained in extensive investigations on TBT genotoxicity and there was no convincing evidence for any mutagenic potential of this organotin compound [1]. Negative results were obtained in assessments of i) point mutations in *Salmonella typhimurium*, the yeast *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and mammalian cells (V79 Chinese hamster cells), ii) recombination in *Bacillus subtilis*, iii) mitotic gene conversion in *Saccharomyces cerevisiae*, iv) clastogenic potential in human lymphocytes, v) chromosomal damage in mouse bone marrow *in vivo*. Therefore, organotins were not considered genotoxic. However, some clastogenic potential was suggested for Chinese hamster ovary cells [2], in which an increase in structural aberrations, mainly represented by deletions together with endoreduplication, were observed at the highest concentrations of TBT tested (1.5 $\mu g\ mL^{-1}$), although the interpretation of results was difficult, since the effects were reported only for concentrations associated with high TBT toxicity.

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The first evidences of a change in this outlook occurred when mice treated orally with TBT were exposed to the standard mutagen mitomycin C and the increased frequency of micronuclei in peripheral reticulocytes was observed, suggesting that this compound could cause chromosomal damage [3]. Similarly, in the larvae of the mussel *Mytilus edulis*, an increase of the frequency of sister chromatid exchanges to approximately twice the control value was observed after TBT exposure in the co-presence of mitomycin C [4]. In addition, chromosomal aberrations in bone marrow cells were reported for mice after i.p. injection of TMT [5], for rats in long-term chronic dietary studies with DBT [6] and in cultured Chinese hamster cells exposed to TBT and TPhT [7]. Recently, in Chinese hamster fibroblasts (CHO-9) exposed for 1 h and 24 h to methyltin derivatives at concentrations ranging from 1 μM to 1 mM, only DMT was reported to induce a significant increases in the percentage of micronuclei, chromosome aberrations and sister chromatid exchanges at a cytotoxic concentration of 1 mM, whereas TMT showed weak genotoxic effects, in terms of micronuclei formation, at non-cytotoxic concentrations [8]. In human peripheral blood lymphocytes, organotins cause *in vitro* chromatid and chromosome breaks and gaps, dicentrics, increased sister-chromatid exchanges, altered cell cycle kinetics [9] and induction of aneuploidy at concentrations between 10^{-3} and 10^{-9} M (TBT>DPT>TMT, TPhT) [10] together with the induction of chromosomal supercontraction [11]. TPhT is responsible for chromosomal aberrations after metabolic activation in human lymphocytes [12], causes an increase in micronucleated reticulocytes, and also appears to be clastogenic or co-clastogenic *in vitro* and *in vivo* [13] exceeding the previous interpretation of positivity in two mouse lymphoma mutation assays [14] in terms of the toxicity of this compound to lymphocytes rather than in terms of a specific clastogenic action.

Other studies to establish the genotoxicity of organotin compounds have been carried out with the comet assay on nucleated fish erythrocytes. This assay is a valid tool for direct measurement of DNA damage in individual cells, and consists of embedding cells in agarose, followed by lysis, electrophoresis and staining to visualise DNA by fluorescence microscopy. Breaks in the duplex DNA molecule release its complex supercoiling and the liberated DNA migrates towards the anode, so that the nucleus resembles a comet with a brightly fluorescent head and a tail streaming away from it. The extent of DNA damage can be quantified by measuring the displacement of the genetic material between the nucleus - the "head" of the comet - and its "tail". Results obtained with this method show that, in *Salmo irideus*, TBT does have a marked genotoxic effect after a 30 min-exposure at 10 μM, although the effect is less pronounced for DBT and completely absent for MBT at the same concentration [15]. However, the latter compound results in an immediate and stable increase in tail length in *Sparus aurata* [16], indicating that, although data match general alkyltin toxicity, maximum for TBT, fish species behave differently towards pollutants. Recently, in the human mammary carcinoma MCF-7 cell line, organotin(IV) carboxylates are reported to induce elevated micronucleus formation and, as observed with the "comet" assay, significantly elevated levels of DNA single-strand breaks at sub-genotoxic concentrations (0.01 μg mL^{-1}) [17].

Even among marine invertebrates, although TBT was first considered not to be genotoxic for the larvae of the bivalve *M. edulis* [18], its ability to induce cytogenetic damage was reported for the early life stages of this bivalve species [19] and for the embryo-larvae of the polychaete worm *Platynereis dumerilii* [20]. In the latter case, genotoxicity was assessed by cytogenetic endpoints which included the frequency of sister chromatid exchanges and chromosomal aberrations from metaphase spreads, which appeared to be dose-dependent on TBT exposure (from 0.31 to 3.11 μg L^{-1}). In early-developing embryos of the crustacean *Anilocra physodes*, a parasite on gills and fins of various marine fish, chromosome abnormalities were mainly observed at the metaphase stage, with a squash technique, after exposure to a DMT complex in proportion to its concentration and not to the length of exposure. These abnormalities were classified as chromosome fragments, large decondensed chromosome areas involving the centromeric region, and chromosome bridges [21]. The latter effect, which occurs in the anaphase, is commonly thought to be responsible for non-disjunction of daughter chromosomes, thereby inducing the formation of nuclei with chromosome numbers that deviate from the normal diploid complement. Furthermore, presuming that these embryos, which contain a cell mosaicism, can develop into adults, organotin compounds pose a risk to gametogenesis, thus limiting fecundity.

In the Ames or *Salmonella*/microsome mutagenicity test, a rapid biological assay widely used to assess the mutagenic potential of chemical compounds and, when positive, indicating that the compound causing DNA damage may act as a carcinogen, TMT, TBT, DMT, MBT and DBT appear to be positive [22], whereas TCT is generally negative [13]. The Ames assay is easy, very stable and highly sensitive, providing about 10^8 cells in the exposed group and thus detecting slight increases in the mutation rate by test substances. One of its disadvantages is the very limited capability of

bacteria to metabolise exogenous substances, so that the addition of an external microsomal system - S9 mixture - is necessary. In addition, since the structure of the bacterial chromosome differs from the complex eukaryotic one, substances that cause chromosome mutations in the animal cells *via* interactions with proteins associated with DNA are not recognised as mutagen agents in assays with bacteria. No evidence of carcinogenicity of organotin compounds was found in long-term dietary studies in mice and rats [23]. The pattern of tumour incidence throughout both the control and test groups of mice and rats, after dietary treatment with TPhT and TCT, appears to be random and does not suggest a dose-response relationship [24,25]. Tumour types after TBT exposure appear in high and variable incidences and are considered as having questionable biological significance [1]. TBT and FBTO are classified as group E carcinogens ("evidence of non-carcinogenicity in humans") by the U.S. EPA.

TPhT is the exception to this rule and is classified as a B2 carcinogen ("probable human carcinogen") by the U.S. EPA: the value calculated for the unit of cancer potency factor from a cancer risk model (Q_1*) is 18.3 mg kg^{-1} day^{-1}. It is also possible that TBT initiates the multistage process of carcinogenesis by influencing steroid hormonal metabolism and predisposes animals to malignancy after inhibition of NK cells to bind to tumour cells, as observed in mice [26].

Mechanisms of Action for Organotin Genotoxicity

Organotin compounds (butyltins, phenyltins, methyltins) have high specificity of action and are genotoxic in several test systems, but studies on their genotoxicity are rare compared with reports of other types of toxicity [27]. The mechanisms of action of the genetic damage induced by organotin compounds are still unclear: genotoxicity is probably provoked by several simultaneous cooperative mechanisms and appears to be species-specific. For many authors, genotoxicity is attributed to tin, the heavy metal, contained in these compounds, since heavy metals are well known to be clastogenic in *in vitro* mammalian cell assays and produce a variety of chromosomal aberrations in terrestrial and aquatic animals. Due to their liposolubility, organotin compounds can easily cross membranes and rapidly reach the cell nucleus, where the easily dissociable chelating ligands yield intermediates such as $R_nSn^{(4-n)+}$ (n = 2 or 3) moieties which are able to bind cellular macromolecules, such as DNA and repair enzymes directly forming DNA-protein crosslinks and inducing genetic damage [28,29]. The binding efficiency to DNA of organotin compounds depends on the coordination number and nature of groups bonded to the central tin atom. The phosphate group of the DNA sugar backbone usually acts as an anchoring site and often results in the stabilisation of the tin centre as an octahedral stable species [30]. These pollutants have been shown to induce apoptosis in various species and eukaryotic cell models. They cause not only an increase in cytosolic free Ca^{2+} from intracellular stores [31] leading to the DNA cleavage typical of apoptosis through cytosol calcification [32], but also both generation of reactive oxygen species (ROS) and release of cytochrome c from mitochondria in a time- and dose-dependent manner [33]. Caspase activation occurs after cytochrome c export from its mitochondrial intermembrane location to the cytosol, due to direct interaction of organotins at low concentrations with the vicinal thiols present on the adenine nucleotide translocator opening the permeability transitional pore [34,35]. Conversely, caspase inhibition, with consequent activation of necrotic cell death, occurs at higher organotin concentrations after interaction with vicinal thiols of an unknown caspase inhibitor [36]. The activated caspases finally cleave target proteins leading to irreversible DNA fragmentation *via* endonuclease activation [37,38]. Butyltin compounds but not TET are potent activators of extracellular signal-regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPKs) pathways, where Ca^{2+} mobilised from intracellular stores is an important prerequisite for organotin-induced kinase phosphorylation. Since tributyltin can activate all kinases simultaneously, the activation of transcriptional factors such as ATF-2 and c-Jun may induce the expression of immediate early genes like c-*fos* and c-*jun* and the resulting increase in activator protein-1 (AP-1) activity may regulate the expression of target genes responsible for the cellular damage induced by organotin compounds [39]. Moreover, the TPhT-induced Ca^{2+} increase generates ROS and activates protein kinases, which may contribute to activate the nuclear transcription factor NF-kB. The latter, by interacting with specific elements in gene promoters and enhancers, could in turn generate TNF-α triggering DNA fragmentation [40]. Despite the apoptosis-inducing effect, there is evidence that TBT also exerts an apoptosis-inhibiting effect only when cultured cells are exposed to low concentrations (10^{-12}-10^{-3} g L^{-1}), since both DNA fragmentation and the content of proapoptotic protein Bax are significantly decreased by the treatment: the nucleus-accumulated TBT at low concentrations is thought to play a role in preventing apoptosis [41]. Fig. (**1**) summarises the principal pathways involved in hypothesised mechanisms of genotoxicity at subcellular level.

Alteration in sister chromatid exchanges, chromosomal aberrations in metaphase, irregular anaphase processes, and aneuploidy are all due to alterations of microtubule assembly of the mitotic spindle as a consequence of the direct interaction of organotin compounds with tubulin [42]. These events are deleterious to the cells on account of their capacity to induce the loss of more or less large DNA portions which may contain gene loci of vital importance to the organism.

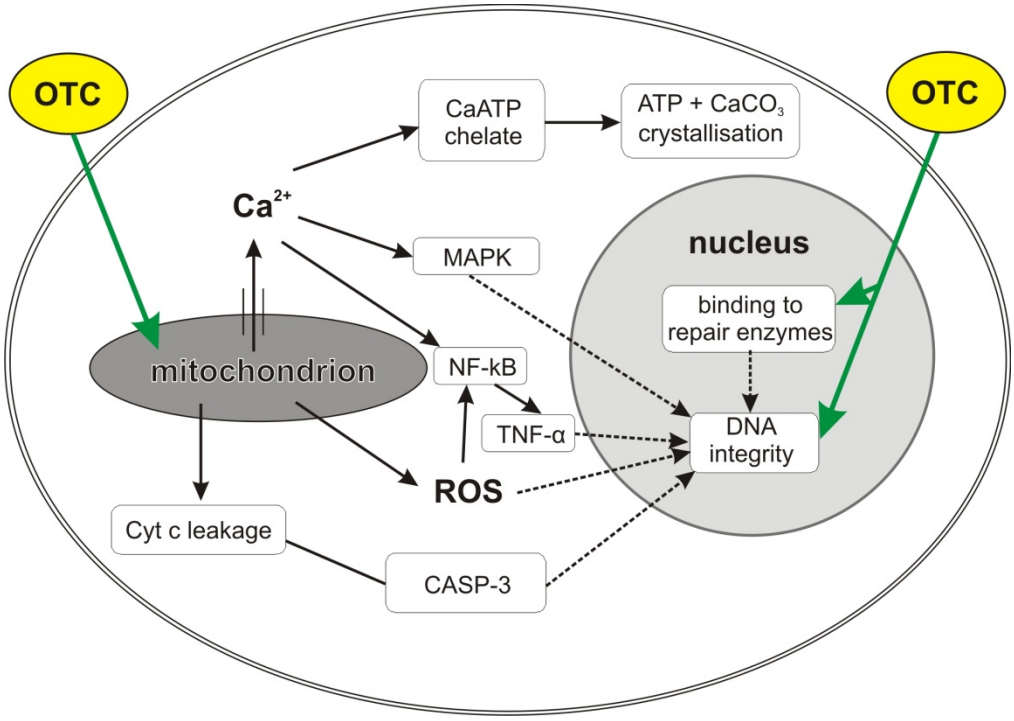

Figure 1: Summary of pathways involved in genotoxic mechanisms of action of organotin compounds (OTC). After crossing cell membranes (green arrows), OTC activates multiple cascades, all culminating in DNA degradation. They can reach the nucleus where they bind directly to DNA and repair enzymes. By interacting with mitochondria, they cause release of i) calcium with consequent cytosol calcification, ii) reactive oxygen species (ROS), and iii) cytochrome c that leads to caspase activation. Both calcium and ROS rise in cytosol can also activate pathways of kinase phosphorylation inducing expression of target genes responsible for DNA damage and nuclear transcription factor NF-kB which, in turn, generates TNF-α. Solid arrows indicate activation; dashed arrows indicate inhibition.

Lastly, organotin compounds can induce DNA oxidative damage, generating double-strand breaks, base damage and intrastrand crosslinks [43,44], but it is still necessary to elucidate the relative importance of direct genotoxic and indirect effects through alteration of the metabolism of steroid hormones [19] or their metabolites which either directly or through the production of reactive oxygen species are known to induce chromosome damage.

The toxicological potential of organotin species depends on their membrane permeability. As a consequence, the genotoxicity of alkyltin compounds is modulated by cellular uptake and extrusion capability, which differ for the various compounds - generally low (<0.5%) but dose-dependent. Uptake peaks (40%) at the highest applied pollutant concentration, but appears to be inhibited and/or the efflux increases at higher concentrations of organotin compounds in the treatment solution [8].

Different behaviour in genotoxicity can be also explained in some cases, *e.g.* methyltin compounds, by the fact that some cells in culture, for instance mouse fibroblasts, do not express a specific protein (stannin, SNN) which mediates the toxicity of organotin compounds. SNN causes TMT demethylation and binds DMT, increasing the toxicity of the latter [45], and cells which do not express SNN are considerably more resistant to TMT toxicity [46].

However, it must be emphasised that organotin compounds can enhance the potential genotoxicity of other contaminants available in the ecosystem, thereby increasing the complexity of action.

IMMUNOTOXICITY

The main function of the immune system in all metazoans is protection against invading non-self organisms or molecules in order to avoid infections and parasitism. This occurs through the recognition of non-self molecular patterns by an array of receptors which prevents foreign molecules or bodies from entering the organism or allows their clearance from internal fluids or tissues.

The term immunotoxicity indicates any alteration of immune responses following exposure to xenobiotics. It may thus be the consequence either of interference with receptor-mediated non-self recognition or alteration of cellular metabolism. Today, immunotoxicity is a matter of great concern, as the immune system is a potential target for drugs and chemicals. Common environmental contaminants are, for instance, pesticides and antifoulants, the concentrations of which are progressively increasing in the soils in both internal and coastal waters as a consequence of their massive use in agriculture and as protection for artificial submerged structures, such as pipes and piles. Xenobiotics can affect immune functions in both acute and chronic manners, with an adverse impact on immunocyte physiology. This is frequently associated with increased susceptibility to infectious diseases consequent upon the alteration or suppression of basic immunological responses, such as phagocytosis, cytotoxic cell functions or synthesis of humoral components. A direct relationship between the immune status of organisms and the level of environmental contamination is difficult to assess especially in vertebrates as the ability to elicit an immune response is closely connected with the neural and endocrine status of the organism. However the potential of xenobiotics to alter immune responses has been demonstrated in many laboratory studies using vertebrates and invertebrates as reference organisms.

The great concern raised by the increasing amounts of organotins in the environment, especially the aquatic one, has stimulated a great variety of investigations aimed at studying the effects on the immune system in both vertebrates and invertebrates. An overview of the state-of-the-art is given below.

Atrophy of Vertebrate Lymphoid Organs

Despite the absence of specific accumulation of organotin compounds in the thymus in contrast to liver, kidney and brain, these pollutants interfere selectively with lymphoid tissues, causing a dose-dependent but reversible atrophy of thymus, spleen and other lymphoid tissues such as tonsils and lymph nodes. The cell-mediated function is impaired, non-specific resistance is affected and the general effects reported for laboratory animals regard the weight and morphology of lymphoid tissues, peripheral lymphocyte counts and total serum immunoglobulin concentrations. The developing immune system appears to be more sensitive to the effects of organotin compounds than the immune system of adult animals [47].

Generally, organotin compounds exhibit greater lethal potential when administered parenterally, as compared with the oral route, probably because they are only partially absorbed from the gut. Although they efficiently penetrate the skin, acute toxicity *via* the dermal route is low, whereas aerosols are highly toxic.

In rats, which are the most sensitive laboratory species, exposure to TBT aerosol at 2.8 mg m^{-3} leads high mortality, associated with histopathological changes of lymphatic organs [1]. TBT is rapidly adsorbed from the gut of rats - 20-50% depending on the vehicle - and both distribution and dealkylation are also fast: thymus, spleen and blood are among the target organs [48-50].The non-observed-effect level (NOEL) in short-term dietary studies on rats is 5 mg kg^{-1} per day [1]. *In vivo* host resistance studies have shown that TBT is able to compromise specific immune functions after exposure to a daily dietary level between 20 and 80 mg kg^{-1} and both clearance of the Gram-positive bacterium *Listeria monocytogenes* and resistance to the nematode *Trichinella spiralis* decrease. More specifically, oral TBT exposure results in reduction in the weight of the thymus and spleen, loss of T lymphocytes, decreased serum IgG, increased serum IgM and reduced helper activity in antibody formation after immunisation with immunogens like ovalbumin in rats [51], as well as suppression of proliferation from lymph nodes in mice [52]. Analogously, DOT and DBT administered at dietary levels between 50 and 150 mg kg^{-1} for 4-6 weeks or longer causes atrophy of the thymus and thymus-dependent peripheral lymphoid organs, with consequent reduction of cortical thymocyte number and thymus weight, lymphopenia, suppression of T-cell mediated immune responses, delay of allograft rejection - in rats, but not in mice or guineapigs [53,54]. Low doses of both DBT and TBT inhibit

immature thymocyte proliferation, whereas high doses of TBT cause a depletion of cortical thymocytes by apoptosis [37]. Since TBT is indeed rapidly dealkylated to DBT after oral administration, the atrophy may in fact be induced by the dialkyl metabolite, suggesting that the latter is the toxicologically active compound [55]. Similarly, although the higher trialkyltin compounds such as TCT and TOT are less toxic than the lower ones, further *in vivo* metabolism converts them to their dialkyltin forms, which are highly immunotoxic. DET and DPT have effects similar to those of TBT but less pronounced; MBT does not cause atrophy of lymphoid organs [56]. In orally dosed mice, TPhT and TCT are more active in decreasing spleen weight than TBT or FBTO [57], although TBT is significantly more potent than TPhT in its thymolytic action [31]. A decreased immune response to tetanus toxoid stimulation due to a reduction in the number of both leucocytes and plasma cells is reported for TPhT in the spleen and in lymph nodes of guineapigs, accompanied by atrophy of the white pulp of the spleen [58]. A decrease in the number of leucocytes is also reported in dogs [25], rats [59,60] and chickens [61]. However, the immunosuppression caused by TPhT as a consequence of lymphopenia and lymphocyte depletion in the spleen and thymus seems to be transient and tends to diminish on longer exposure [62].

On the basis of the relative sensitivity of the various species, it would be prudent to base the assessment of the potential hazard of organotin compounds to humans on data from other species since the interpretation of the significance of these data for human risk is controversial.

The presence of organotin compounds as pollutants in the aquatic environment has led to various toxicity studies in marine mammalian and fish species. Dietary TBT is suspected to increase susceptibility to infections and cause death in cetaceans and seals [63,64]. In teleosts, thymus atrophy is reported in carp (*Cyprinus carpio*), flounder (*Platichtys flesus*) and guppy (*Poecilia reticulata*) but not in medaka (*Oryzias latipes*) after dietary exposure to 0.32 μg L^{-1} TBT and 320 μg L^{-1} DBT for 1 month [65,66], indicating some species specificity regarding the histopathological effects of these compounds. Lymphocyte depletion caused after exposures to 0.6-4 μg L^{-1} TBT for 28 days is reported in rainbow trout (*Onchorhynchus mykiss*) [67].

Many *in vitro* studies have been performed in order to explain the mechanisms that are responsible for the toxicity of organotin compounds in whole organisms. TBT has been reported to cause apoptosis in cultured thymocytes [31, 68-70] and in leucemia T-cell lines [36, 71] and an anti-proliferative effect has been suggested for both rat and fish lymphocytes [72,73] probably causing a decrease in circulating antibodies. DBT also decreases the survival rate of rat and human thymocytes [54]. Organotins have deleterious effects on survival, proliferation and differentiation of human B lymphocytes derived from tonsil tissue at concentrations of 35 μg L^{-1} [74]. TPhT causes cytolysis of mouse thymocytes [75]. The synthesis of IL-2, a cytokine involved in early thymocyte activation and proliferation, is down-regulated at the mRNA level by DOT, supporting the hypothesis that organotin compounds induce a blockade of intrathymic T-cell maturation.

Mechanisms of Action for Lymphoid Tissue Atrophy

The subcellular mechanisms for this selective type of cytotoxicity are still debated and only partially known. Toxic effects have been attributed to disruption of macromolecular synthesis and mitochondrial energy metabolism, reduction of DNA synthesis, activation of purine metabolic enzymes, direct interaction with cell membranes, and sustained increase in the cytosolic free calcium concentration, with subsequent activation of endogenous endonucleases. They all lead to the apoptosis of the thymic cortex with a consequent loss in weight, depletion in T lymphocytes in peripheral blood, and suppression of T-cell mediated immune response in mammals [51, 55, 57, 60, 76], also demonstrated in fish spleen [73]. Although the disruption of the function of 11β-hydroxysteroid dehydrogenase type 2 (11 β-HSD2) by organotin compounds is responsible for enhanced glucocorticoid concentrations, there is no glucocorticosteroid-mediated mechanism of cytotoxity to thymocytes, since adrenalectomy has no effect on organotin-induced thymic involution.

Impairment of Cell-Mediated Immune Responses

Organotin compounds can give rise to inhibition of chemotaxis, phagocytosis and the respiratory burst, and, in vertebrates, also trigger extensive cytolysis of circulating leucocytes. Early observations were carried out *in vitro* on polymorphonuclear leucocytes (PMNs) in rabbit [77,78]. After exposure to TBT at concentrations between 0.1 and 10 μmol L^{-1}, suppression of the chemotactic response towards formyl-methionyl-leucyl-phenylalanine (FMLP) and

the almost complete inhibition of both uptake of opsonised zymosan and release of lysozyme were observed. The parallel cell lysis suggests a damage to the plasma membrane. Mammalian macrophage phagocytosis is also affected by TBT, DPT, DOT and TPhT [60]. Short-term TBT exposure of cultured fish macrophages isolated from toadfish (*Opsanus tau*), hogchoker (*Trinectes maculatus*) and Atlantic croaker (*Micropogonias undulatus*) suppresses phagocytic function [79,80] and chemotaxis [81].

Among invertebrates, the most sensitive non-target organisms are filter-feeding species such as ascidians, bryozoans and bivalve molluscs. Sublethal concentrations of butyltin derivatives and TPhT impair innate cell-mediated immunity compromising many functions in marine invertebrates. In the bivalve *Crassostrea gigas*, *in vitro* chemotaxis and aggregation of haemocytes is altered by exposure to TBT, suggesting impairment of haemocyte functions in internal defence [82]. In the colonial ascidian *Botryllus schlosseri,* the presence of organotin compounds at sublethal concentrations ranging from 0.1 to 10 μM significantly impairs the ingestion of foreign particles (yeast cells) by cultured haemocytes. This effect on phagocytosis is irreversible, dose- and time-dependent. The order of inhibition is DBT > TBT > MBT and TPhTC > TPhTA > TPhTH [83,84]. In the solitary ascidian *Ciona intestinalis,* the percentage of *in vitro* yeast phagocytosis is significantly inhibited by 1.5 μM TBT, whereas TPhT, DPhT and DBT do not cause significant effects [85], suggesting the lower sensitivity to these xenobiotics of solitary species with respect to colonial ones. Haemocytes taken from specimens of the solitary ascidian *Styela plicata* treated for 3 days with 10 μg L^{-1} TBT in aquaria show lower phagocytic activities than those of non-treated controls. This alteration in phagocytosis is transitory since phagocytic activity returns to normal levels after 8 days, suggesting that TBT has become detoxified or that adaptive mechanisms reducing the effect of the toxicant have been activated [86]. Organotin compounds also have inhibitory effects on *in vitro* yeast phagocytosis by haemocytes of sipunculids [87] and bivalves [88-91]. Bivalves appears to be the most sensitive invertebrates owing to their capacity to accumulate high levels of trialkyltin compounds due to the absence of any cytochrome-P450-dependent system. In addition, increased oxygen consumption, *i.e.*, the respiratory burst with ROS production, associated with yeast phagocytosis in *B. schlosseri,* is significantly inhibited by both TBT and TPhT [83,84]. Also in bivalve haemocytes exposed *in vitro* to 0.05 μM TBT, superoxide dismutase (SOD) activity significantly decreases [92]. It has been hypothesised that enhanced progression of infection by the pathogenic protozoan *Perkinsus marinus* in bivalves exposed to environmentally dangerous TBT levels is not due to effects on cell-mediated responses, but to inhibited production of reactive oxygen intermediates [93]. Also in fish, decreased resistance to infections has been found at the lowest-effect concentrations of TPhT, as well as to TBT and DBT after continued exposure [94]. However, in bivalves, a dose-dependent stimulation of ROS formation was paradoxically seen after exposure of cultured macrophages to TBT [95]. Conversely, sublethal exposures to TBTO but not TBTC induce a transient increase in the activity of nitric oxide synthase (NOS), with a corresponding increase in nitric oxide (NO) production in mammalian macrophages. This was explained as either a localised inflammatory response or a stress-induced increase in circulating hormone levels since, although the concentration of NO produced is too low to exert effective bactericidal activity, it may combine with superoxide to yield the more reactive species peroxynitrite [96].

One remarkable consequence of vertebrate and invertebrate haemocyte exposure to organotin compounds is change in morphology. Phagocytes lose their typical amoeboid shape, withdraw their cytoplasmic projections, and assume a spherical shape, indicating that these pollutants can interfere with cytoskeletal components, as first reported for mammalian neutrophils [97] and thymocytes [98]. Both actinic and tubulinic cytoskeletons are affected by TBT. F-actin is dramatically reorganised, from a diffuse cytoplasmic location to limited clusters along the peripheral cytoplasm, indicating massive breakdown of microfilaments. Microtubules are not recognisable, although their organising centre does not disappear [99]. Disaggregation of actin filaments and inhibition of tubulin polymerisation, to which cytoskeletal modifications have been ascribed, hinder chemotactic movements and particle adhesion by leucocytes, negatively affecting phagocytosis [77].

Long-term *in vitro* exposition of both mammalian thymocytes and invertebrate haemocytes at the higher sublethal concentration of TBT induce apoptosis [32, 100]. Nuclear changes, detected as chromatin condensation and DNA fragmentation, together with cytoplasmic blebbing and surface alterations take place. Detrimental effects on membrane permeability and consequent haemocyte mortality significantly increase only after a longer time of exposure to TBT but, unlike the polymorphonuclear leucocytes (PMNs) of mammals [78] no cytolytic response has been reported in invertebrate leukocytes. In the haemolymph of the bivalves *M. edulis* and *Crassostrea virginica* grown for 60 days on 0.7 μg L^{-1}-TBT-painted panels, lysozyme activity and DNA content revealed no difference

with respect to controls, suggesting that no lysis of the haemocytes had occurred and there was no increase in their number [101].

Another toxic effect observed *in vitro* in bivalves is the activity inhibition of hydrolytic enzymes. TBT exposure of cultured haemocytes of *Ruditapes philippinarum* affects the lysosomal compartments which play an important role in the immune responses: upon phagocyte stimulation, lysosomal hydrolases are released from cells to degrade foreign materials or into phagosomes, thus participating in the degradation of internalised foreign particles. Low TBT concentrations cause structural alterations of lysosomal membranes with the consequent formation of enlarged lysosomes, whereas at high TBT concentrations lysosomal membrane integrity is altered profoundly, causing undesired release of hydrolases into the cytosol, with consequent damage for the cells themselves [92].

Alterations in cytotoxic responses are also reported. TBT and TPhT inhibit the tumour-killing activity of human NK lymphocytes at submicromolar concentrations *in vitro* [62, 102]. This effect may be related to a B2 class carcinogen property ("probable human carcinogen") of TPhT, although chronic dietary studies at comparable dose rates in mice and rats resulted negative for carcinogenicity [14]. In channel catfish (*Ictalurus punctatus*), *in vitro* exposure to TBT suppresses non-specific cytotoxic cell activity and the humoral immune response to heat-killed *Edwardsiella ictaluri* [103,104]. In solitary ascidians, *in vitro* exposure of haemocytes to TBT causes a significant decrease in the activity of phenoloxidase, an enzyme involved in inflammatory responses present in the cytotoxic line of ascidian blood cells [105]. This ability may reflect inhibitory effects on calcium-dependent signalling systems, such as those involved in the exocytosis of prophenoloxydase-containing granules, altering tubulin-associated cytoskeletal events. A significant decrease in the activity of lysozyme (bacteriolytic enzyme) is observed in haemocytes of the bivalve *R. philippinarum* and the worm *Sipunculus nudus* exposed *in vitro* to 0.05 μM TBT [87, 92]. Since this lysosomal enzyme is secreted by haemocytes in the haemolymph during phagocytosis and participates in inactivating invading pathogens, its reduced activity suggests immunosuppression, resulting in lowered resistance to bacterial challenge.

Mechanisms of Action in Cell-Mediated Immunosuppression

A number of mechanisms have been proposed to explain the immunotoxic effects of organotin compounds, which affect a variety of biochemical and physiological systems. Their mode of action may vary according to the compound, its dose, and the species and route of administration. Organotin lipophilicity plays a key role in the mechanism of action, since the general sequence of toxicity is trisubstituted organotins > disubstituted organotins > monosubstituted organotins ≥ tetrasubstituted organotins. Their major action is associated with interference with mitochondrial energy production, including interruption of oxidative phosphorylation, changes in permeability of outer mitochondrial membrane and suppression of enzyme activity [106,107]. Other mechanisms of toxicity include interference with the glycolytic pathway, decreased cAMP production, inhibition of both RNA and protein synthesis, and involvement of lipophilic and sulphydryl groups [1]. In addition, the calcium flux is increased from mitochondria [108]. Most of their toxic effects of organotins are irreversible, dose- and time-dependent, and can be divided into Ca^{2+}-independent and Ca^{2+}-dependent mechanisms, but they are linked and synergistic in triggering the cascade of secondary events which lead to toxic action. Fig. (**2**) summarises the principal pathways involved in hypothesised mechanisms of immunotoxicity at subcellular level.

Basic knowledge regarding the mechanism of action of organotin-induced humoral immunosuppression is still lacking and most information regards subcellular targets in cell-mediated responses.

Phagocytosis is an energy-dependent process and thus sensitive to intracellular energy levels. Motility requires significant amounts of ATP for cytoskeletal rearrangement through the actomyosin system, intracellular protein synthesis, and transport and membrane insertion of receptors are compromised by reduced intracellular energy levels. All organotin compounds are able to inhibit mitochondrial oxidative phosphorylation by inhibiting the fundamental energy processes of the cell system in different ways, although it is still not clear which of them predominates as the ultimate cause of the toxic effects observed *in vitro*. The compounds provoke oligomycin-like inhibition of coupled phosphorylation - higher in the case of TBT than TPhT [83, 109]; alteration of Cl^-/OH^- exchange across lipid membranes, producing a reduction in the intramitochondrial substrate and phosphate concentrations, followed by structural damage [25]; an opening effect of the mitochondrial permeability transition pore, leading to rapid and severe mitochondrial swelling [110]; inhibition of ATP synthesis due to a reaction of the

phenyl groups of TPhT with the thiols of the lipoic acid, followed by enzymatic inhibition of lipoic acid (acetyltransferase and lipoamide dehydrogenase) [111]; and interaction with haem proteins such as cytochromes, which are inhibited [112].

Organotin compounds inhibit protein synthesis through the formation of coordination bonds with amino acids [113] and can also directly interact with proteins, giving rise to conformational changes. The activities of many enzymes are thus deranged. Sulphydryl groups are pivotal in membrane-related functions such as secretion, phagocytosis, transport, cytoskeletal functions in cell-cell contact, and transmembrane signalling. Several important thiol-containing and GSH-dependent enzymes are inhibited by organotins. These enzymes include aromatase, Na^+,K^+-ATPase, caspases, glutathione S-transferase (GST) and glutathione peroxidase (GPX), SOD and catalase. Organotin compounds significantly decrease the cytosolic GSH content [114].

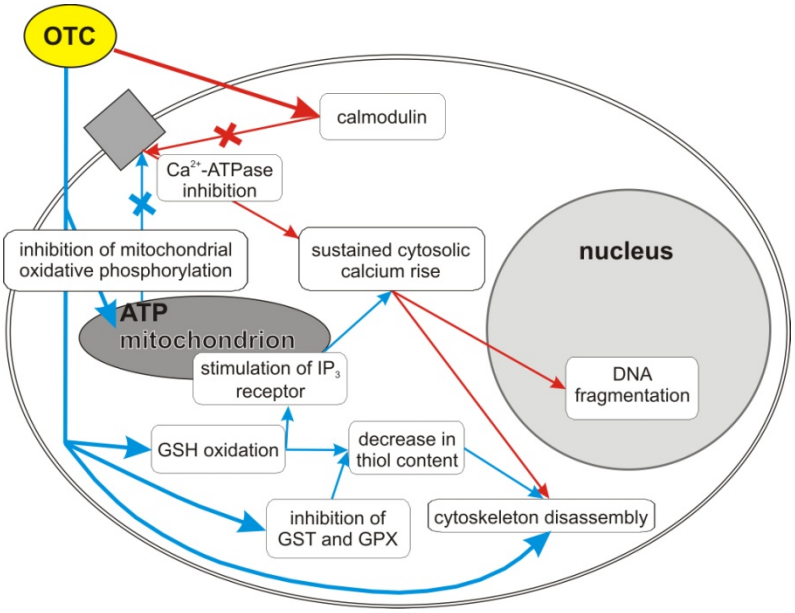

Figure 2: Summary of pathways involved in immunotoxic mechanisms of action of organotin compounds (OTC) conventionally divided in Ca^{2+}-dependent (red arrows) and Ca^{2+}-independent (blue arrows). In Ca^{2+}-dependent mechanisms, OTC directly interact with calcium-activated calmodulin causing a complex formation preventing the regulative activity of calmodulin on the calmodulin-dependent Ca^{2+}-ATPase that leads to sustained increase of cytosolic calcium ion concentration. This takes part in a multifactorial apoptotic process, involving endonuclease activation. In Ca^{2+}-independent mechanisms, OTC impair mitochondrial oxidative phosphorylation, oxidise glutathione (GSH) and inhibit several thiol-containing and GSH-dependent enzymes like glutathione S-transferase (GST) and glutathione peroxidase (GPX), making exposed organisms more vulnerable to oxidative stress. These mechanisms are synergistic since increase in cytosolic calcium is provoked by the presence of high amount of cytosolic oxidised glutathione that causes stimulation of calcium-releasing property of InsP3 receptor. Cytoskeletal protein depolymerisation is due to both cytosolic calcium increase and extensive oxidation of their thiol groups causing severe morphological change, chemotaxis failure, and phagocytosis inhibition.

The first mechanism described in immunosuppression is the induction of alterations in the calcium homeostasis of cells, with increasing concentrations of internal calcium ions [108, 115]. This effect was described as a multifactorial process, involving release of Ca^{2+} from intracellular stores due to an increase in the membrane Ca^{2+} permeability of cell compartments, and a decrease in Ca^{2+}-ATPase activity, which represents the main Ca^{2+} extrusion system [100]. It has been hypothesised that the significantly higher inhibition of phagocytosis by DBT than TBT observed in ascidians and bivalves is due to competition for the active sites of Ca^{2+}-ATPase usually occupied by Ca^{2+}. DBT appears to be a better competitor for these sites than TBT, because the ionic form of DBT is a double-charged cation, like Ca^{2+}, and DBT is much smaller than TBT, which carries only one positive charge. In addition, since TBT is more active than DBT at the membrane level due to its insertion and retention within lipidic bilayers, a low amount of free TBT is available for interacting directly with Ca^{2+}-ATPase [91]. Membrane-bound Ca^{2+}-ATPases are calmodulin-dependent enzymes, so that calmodulin represents one important Ca^{2+}-dependent intracellular target of organotin compounds. Much evidence supports the hypothesis that the severe damage to

immunocytes in the presence of organotin compounds is mediated mainly by the direct interaction of these compounds with calmodulin which, in turn, inactivate Ca^{2+}-ATPases, with a consequent diffuse, delayed cytosolic calcium rise upon cell stimulation. The interaction between butyltins and calmodulin, measured as a change in circular dichroism spectra, is non-covalent, and hydrophobic in nature between the aliphatic chains of butyltins and the hydrophobic regions of Ca^{2+}-activated calmodulin, following a saturation pattern which leads to the formation of a complex preventing the regulatory activity of calmodulin on the membrane-bound calmodulin-dependent Ca^{2+}-ATPase [116]. Reversals of Ca^{2+}-ATPase inhibition, cell shape and cytoskeletal organisation in haemocytes co-exposed to TBT and calmodulin have also been observed. When added together with TBT, increasing concentrations of exogenous calmodulin can totally restore both cytoskeleton organisation and cell shape, although microfilaments appear to be more sensitive than microtubules [117]. The addition of calmodulin to cultured cells exposed to TPhT is also able to reverse the inhibition of Ca^{2+}-ATPase [90].

Altered Ca^{2+} homeostasis may lead either to apoptosis in the case of prolonged exposure, or to inhibition of the respiratory burst and depolymerisation of cytoskeletal components. The latter effect is responsible for the remarkable changes in cell shape and loss of motility shown by organotin-treated phagocytes. Both TBT and TPhT hinder the *in vitro* polymerisation of G-actin in F-actin, followed by disaggregation [118], and inhibit polymerisation of tubulin with a mechanism of action similar to that of heavy metals [42, 119]. In cell cultures, microfilaments assemble in clusters around the peripheral cytoplasm, indicating massive disassembly, with the exception of unaltered adhesion plaques. Microtubules reveal extensive disaggregation, being scattered in the cytoplasm and not recognisable as single filaments, whereas the microtubule-organising centre is still visible [98].

However, since phagocytic capability can never be totally recovered in the presence of calmodulin, other synergistic Ca^{2+}-independent mechanisms of action have been postulated, such as inhibition of oxidative phosphorylation and ATP synthase and irreversible interactions with the thiol groups of many proteins and peptides. Cytoskeletal protein depolymerisation is also due to extensive oxidation of their thiol groups, causing alterations in cell morphology and affecting chemotaxis. The increase in cytosolic Ca^{2+} concentration is not only due to the direct interaction of organotin compounds with calmodulin and to inhibition of Ca^{2+}-ATPase activity, but also to direct interaction with GSH, since the redox state of GSH represents a control system for the activity of the $InsP_3$ receptor, the preferential molecular target of organotin compounds inside cells. Indeed, although organotin compounds do not interact directly with GSH-reductase [114], the activity of this enzyme is insufficient to restore the reduced state of GSH. The cytosolic presence of high amounts of GSSG causes stimulation of the Ca^{2+}-releasing activity of the $InsP_3$ receptor. The resulting calcium mobilisation and inhibition of calmodulin-dependent Ca^{2+}-ATPase increase the intracellular Ca^{2+} content. Therefore, organotin compounds are powerful xenobiotics capable of oxidising thiol groups directly and reacting with antioxidant enzymes GST and GPX [120-122], affecting both the defence ability of organisms against oxidative stress caused by environmental xenobiotics and their resistance to disease.

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