Triorganotin Isothiocyanates Affect Migration and Immune Check-point Receptors in Human Triple-negative Breast Carcinoma MDA-MB-231 Cells

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Abstract. *Background/Aim:* Triple-negative breast cancer (TNBC) constitutes 15-20% of all breast carcinomas, affecting younger women more often and has a worse prognosis than other types of breast cancer, due to the combination of more aggressive clinical behavior and lack of molecular targets for therapy. This study assessed the effects of non-genotoxic concentrations of tributyltin isothiocyanate (TBT-ITC) and triphenyltin isothiocyanate (TPT-ITC) on MDA-MB-231 cells. *Materials and Methods:* MTT assay, comet assay, kinetic imaging and flow cytometry were used for analysis of MDA-MB-231 cells. *Results:* The results showed that 100 nM concentration of TBT-ITC and TPT-ITC, that did not affect viability or DNA integrity, slowed-down migration by CD44 down-regulation. Moreover, both compounds demonstrated immunomodulatory properties, attenuating PD-L1 expression in MDA-MB-231 cells. *Conclusion:* TPT-ITC was more effective in down-regulating CD44 expression and reducing migration than TBT-ITC, while TBT-ITC was more potent in lowering PD-L1 expression in comparison with TPT-ITC.

Triple-negative breast cancer (TNBC) (estrogen-, progesterone- and HER2-receptor negative) harbors more aggressive clinical

behavior than other types of breast cancer and still lacks targeted treatment options (1). Among them, there is hope that the emerging field of immunotherapy will bring progress. One interesting biomarker related to the immune/tumor interaction is programmed death-ligand 1 (PD-L1) (2).

Among a variety of organotin compounds, triorganotin derivatives have demonstrated marked cytotoxic properties against various tumor cell lines (3-5). They are expected to exert anti-cancer properties through different mechanisms at the molecular level (6, 7). Research on the biological properties of triorganotin compounds thus opens a new research subarea in experimental oncology (4, 8, 9). They act as nuclear retinoid X receptor (RXR) agonists, due to their capacity to bind to the ligand-binding domain of RXR subtypes (10-12), and thus, may function as potent transcriptional activators (4, 10).

Various studies have demonstrated the anticancer effects of isothiocyanates (ITCs). Recent research has been focused on the possible therapeutic benefit of natural (13) or synthetic (14, 15) ITCs in cooperation with standard anticancer drugs. The anticancer efficacy of sulforaphane has been shown to be associated with the reversal of epithelial-mesenchymal transition, and with reduced production of pro-inflammatory cytokines and pro-angiogenic growth factors in MDA-MB-231 cells (16).

In our very recent study, we combined the anticancer/genotoxic properties of two chemically different types of molecules, triorganotins and ITCs, into tributyltin isothiocyanate and triphenyltin isothiocyanate, and showed/compared their genotoxic effects in human breast carcinoma MCF 7 and MDA-MB-231 cells (17). Therefore, the aim of this study was to assess the biological effects of non-genotoxic concentrations of triorganotin isothiocyanates in the more aggressive, triple-negative MDA-MB-231 cells.

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Table I. DNA damage caused in human breast cancer MDA-MB-231 cells by the treatment with TBT-ITC and TPT-ITC expressed either as a mean percentage of tail DNA or as a percentage of StO-induced DNA migration.

Samples	Alkaline comet assay	
	% of mean tail DNA	% of StO-induced DNA migration
Control	6.39±3.23	100.00±6.60
300 μ M H ₂ O ₂	51.01±2.47###	
20 μ M cisPt		58.17±9.85###
100 nM TBT-ITC	3.00±0.74	100.74±8.72
100 nM TPT-ITC	4.71±1.52	87.29±14.95

Data are shown as the means of three independent experiments $\pm$ standard deviation (SD). Significant differences ### p <0.001 in comparison to control. 300 μ M H₂O₂ and 20 μ M cisPt, positive controls for the respective assays.

Materials and Methods

Reagents. Tributyltin isothiocyanate (TBT-ITC) and triphenyltin isothiocyanate (TPT-ITC) were prepared by refluxing alcohol solutions of the respective tin chlorides with KSCN used in excess (1.5 equiv.), as previously described (18, 19). IR spectra were recorded on a SmartMIRacle ATR Zn/Se for Nicolet Impact 410 FT-IR (Thermo Scientific, Langensfeld, Germany). NMR spectra were measured on a JEOL ECZR-400 MHz spectrometer (Jeol Ltd., Akishima, Tokyo, Japan). The purities of the prepared triorganotin compounds (tributyltin isothiocyanate and triphenyltin isothiocyanate) were better than 97%, as determined by NMR (17).

Cells and treatment. The MDA-MB-231 human breast cancer cell line was routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 μ g/ml penicillin and 50 μ g/ml streptomycin. Cells were plated at $3\text{--}6 \times 10^4$ cells/cm² density one day before treatment and exposed to 100 nM concentration of TBT-ITC and TPT-ITC for the indicated time periods. Stock solutions of tested compounds were dissolved in ethanol and an equal volume of it (final concentration <0.02%) was added to the control cells.

Cytotoxicity test. The effect of tested compounds on survival of cells was determined by the MTT assay (20). Cells were seeded at $1\text{--}2 \times 10^3$ cell density in 96-well culture plates. Each dose of tested compounds (added in the volume of 50 μ l) was tested in triplicate or quadruplicate. After 48 h, the cells were incubated with 50 μ l of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/ml; Sigma-Aldrich Chemicals Co., Schnelldorf, Germany) and left in the dark at 37°C for an additional 4 h. Thereafter, medium was removed, the formazan crystals were dissolved in 200 μ l of DMSO, and the absorbance was measured at 540 and 690 nm in an xMark™ Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., Vienna, Austria).

Alkaline single cell gel electrophoresis (alkaline comet assay). For the evaluation of DNA damage in MDA-MB-231 cells the alkaline

Figure 1. Cytotoxicity of triorganotin isothiocyanates (TBT-ITC and TPT-ITC) in MDA-MB-231 cells. Relative viability was measured by the MTT test following treatment with various concentrations of triorganotin isothiocyanates for 48 h.

comet assay described by Gabelova *et al.* (21) was precisely followed. The positive control in this conventional assay was treated with hydrogen peroxide (300 μ M H₂O₂; Sigma-Aldrich Chemicals Co.) in PBS at 4°C for 5 min in a fridge. To verify that the compounds tested (24 h treatment) induce crosslinks, the cells were exposed to styrene oxide (StO; Sigma-Aldrich Chemicals Co.) before the lysis step according to Hunakova *et al.* (13). The positive control was treated for 24 h with crosslinking cisplatin (20 μ M cisPt; Lachema, Czech Republic). At least one hundred of ethidium bromide (EtBr; Sigma-Aldrich Chemicals Co.)-stained nucleoids per three parallel slides per sample in one experiment were examined using a Carl Zeiss AxioImager.Z2 fluorescence microscope attached to a computerized image analysis Metafer 5 (MetaSystems GmbH, Germany). The mean percentage of DNA in the tail was used as a parameter for the measurement of DNA damage and for the presentation of the conventional comet assay data. Results from the detection of crosslinks are shown as percentages of StO-induced DNA migration calculated using the formula suggested by Hunakova *et al.* (13). The obtained results underwent analysis using the SPSS 23.0 software. The Shapiro-Wilk test was used to test the normality of data distribution. Differences between more than two groups were assessed by one-way analysis of variance (ANOVA) and by the Bonferroni or Tamhane's test for multiple comparisons. Non-normally distributed data were tested by Kruskal-Wallis H test and by multiple comparisons. Differences of p <0.05 were considered as significant.

Flow cytometry. Surface antigen expression was determined by multicolour flow cytometry using CANTO II, Becton Dickinson flow cytometer equipped with 3 lasers (violet 405 nm, blue 488 nm and red 633 nm). Cells were harvested by trypsinization, adjusted to 10^6 cells/ml and washed with PBS. Isotype-matched non-reactive monoclonal antibodies (eBioscience, San Diego, CA, USA), mouse monoclonal fluoro-isothiocyanate (FITC)-labeled anti-CD44v6 (BD Pharmingen, San Jose, CA, USA), phycoerythrin (PE)-labeled anti-CD44 (Exbio, Prague, Czech Republic) or PE-labeled anti-PD-L1 (kindly provided by Jana Jakubikova, PhD, Cancer Research Institute, Biomedical Research Center, University Science Park for

Figure 2. Migration assay. Relative wound closure was measured after creating the scratch wound by wound making tool in MDA-MB-231 cells treated with 100 nM TBT-ITC, 100 nM TPT-ITC, or culture medium (control). Images were taken every 2 h for the next 70 h using the IncuCyte ZOOM™ Kinetic Imaging System and evaluated by IncuCyte ZOOM™ 2013A software.

Biomedicine, Slovak Academy of Sciences, Bratislava, Slovak Republic) were added to tube containing a 50 µl aliquot of cells according to the instructions of the manufacturer. Cells were incubated at room temperature for 30 minutes. 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Chemicals Co.) fluorescence was used to exclude dead cells from the analysis. Data were exported and analyzed in FCS Express 4.0 (De Novo Software). Mean of fluorescence intensity of each positive parameter (CD44, CD44v6 and PD-L1) was acquired after exporting statistics in an individual projection. Only viable (DAPI negative) cells were analyzed by FCS Express 4.0 (De Novo Software).

Migration assay. Forty thousand MDA-MB-231 cells per well were plated in triplicates in ImageLock 96-well plates (Essen BioScience, Welwyn Garden City, UK) and let to adhere for 16 h. Confluent monolayers were wounded with wound making tool (Essen BioScience), washed twice, and supplemented with 100 nM TBT-ITC, 100 nM TPT-ITC, or culture medium. Images were taken every 2 h for the next 70 h by the IncuCyte ZOOM™ Kinetic Imaging System and evaluated by IncuCyte ZOOM™ 2013A software (Essen BioScience) based on the relative wound density measurements.

Results

Based on the MTT analysis (Figure 1), 100 nM concentration of the studied compounds were selected for *in vitro* experiments. At this concentration, viability of cells was over 93% for TBT-ITC and over 95% for TPT-ITC.

Conventional comet assay (determination of DNA breaks) and its slight modification used for the detection of crosslinks

revealed the presence of neither of them (Table I) in the population of MDA-MB-231 cells after 24 h treatment with both ITC derivatives. These results indicated that 100 nM of TBT-ITC or TPT-ITC have no genotoxic effects.

Under these conditions both tested compounds were able to slow-down the migration of highly invasive MDA-MB-231 cells (Figure 2) as shown by the migration assay using the IncuCyte ZOOM™ Kinetic Imaging System. Relative wound density, measured 22 h after creating the scratch wound, returned to 97% in control cells, and to 90% in TBT-ITC-treated (100 nM) and 85% in TPT-ITC-treated (100 nM) cells.

Flow cytometry revealed that the anti-migration effect of triorganotin isothiocyanates (TBT-ITC and TPT-ITC) was associated with down-regulation of CD44 cell surface antigen, but not of its CD44v6 variant (Figure 3), expression of which was only negligible in our setting. Similarly to migration reduction, TPT-ITC was more effective than TBT-ITC.

The immunomodulatory properties of the tested derivatives were demonstrated again by flow cytometry. Incubation of cells with 100 nM concentration of both drugs for 24 h resulted in down-regulation of PD-L1 (Figure 4). Our results showed higher efficacy of TBT-ITC in comparison with TPT-ITC.

Discussion

Better understanding of mechanisms that guide tumor growth and invasion and quest for agents that could inhibit these

Figure 3. CD44 and CD44v6 staining, as determined by flow cytometry. Representative histograms (number of cells vs. fluorescence intensity) of CD44 (PE) and CD44v6 (FITC) expression, in MDA-MB-231 cells incubated with triorganotin isothiocyanates (TBT-ITC and TPT-ITC) for 24 h. Isotype-matched monoclonal antibodies were used as negative controls.

processes are important in combating dissemination of cancer. We explored the effects of non-toxic and non-DNA-damaging doses of triorganotin ITCs on various features of metastatic breast cancer MDA-MB-231 cells.

Recently, these compounds underwent investigation of their cytotoxic effects in both human estrogen receptor positive MCF 7 and triple-negative MDA-MB-231 breast carcinoma cells (17). We showed that the DNA damage induced by TBT-ITC and TPT-ITC was the main cause of their cytotoxicity in breast cells studied. Previously, we have studied selected Sn- and Ge-triorganometallic compounds and have reported different cytotoxic and migration modulatory effects on triple-negative breast cancer MDA-MB-231 cells (7).

In this work, we demonstrated that triorganometallic isothiocyanates inhibited migration of these cells more effectively than Sn- and Ge-triorganometallic compounds. Comparing to tributyltin chloride (TBT-Cl) and triphenyltin chloride (TPT-Cl), their isothiocyanate derivatives slowed-down the migration of metastatic MDA-MB-231 cells

differently; TBT-ITC was less effective and TPT-ITC was more effective than TBT-Cl and TPT-Cl, respectively (5).

Modulation of migration potential was found to be associated with CD44 (receptor for hyaluronic acid) down-regulation. The CD44 glycoproteins play multifaceted roles in tumor progression and metastasis, being associated with stem-like breast cancer cells (22). Among the CD44 spliced variants, one of the v6 exon-containing isoforms confers metastatic potential to non-metastatic cells (23). Recently, it has been reported that the CD44v6 isoform had the highest expression levels in non-metastatic breast cancer cells (MCF 7) in comparison to metastatic breast cancer cells (MDA-MB-231). Indeed, in our setting, we also found only negligible expression of CD44v6 isoform on the surface of MDA-MB-231 cells. However, we worked in normoxic conditions and the expression of CD44 and its variant isoforms (CD44v6, CD44v7/8) is known to be increased in triple-negative breast cancer cells under hypoxic conditions (22). Our data are in agreement with the finding of others who have also indicated that the expression of CD44 was

Figure 4. PD-L1 expression determined by flow cytometry. Representative histograms of PD-L1 expression modulated by triorganotin isothiocyanates (TBT-ITC and TPT-ITC) after 24 h incubation of MDA-MB-231 cells.

involved in triple-negative breast cancer cells migration (24).

In the last part of our study, we addressed modulation of immune checkpoint proteins such as PD-L1. Recently, PD-L1 expression was related to poor prognosis in patients with gastric cancer (25). Research in PD-L1 in TNBC has, so far, given diverse and sometimes contradictory results. Programmed death 1 receptor (PD-1) is expressed in activated T-lymphocytes, B-lymphocytes, mononuclear cells, NK cells and some DCs (26). When PD-1 binds to its ligand PD-L1, it serves to down-regulate T-cell activity. Herein, we reported down-regulation of PD-L1 molecule on the surface of MDA-MB-231 cells after the 24 h TBT-ITC and TPT-ITC treatment. In contrast to modulation of migration, TBT-ITC was more potent in down-regulating PD-L1 than TPT-ITC. This suggests that the tested compounds could prevent the undesirable PD-1/PD-L1 interaction which can suppress the

anticancer tumor response. In a study including 35 triple-negative, non-basal-like tumors and 69 basal-like tumors, high expression of PD-L1 was found in 31% and 33%, respectively (27). Mittendorf *et al.* (28) have found higher expression of PD-L1 in TNBC, than in other cancer types. However, the impact of high expression of PD-L1 on overall prognosis of breast cancer is still debated (29, 30). Preliminary results of ongoing clinical trials targeting the PD-1/PD-L1 pathway to treat TNBC patients have been promising (31, 32).

To summarize our present results, we demonstrated that TBT-ITC was more potent in down-regulating PD-L1, an immune regulatory molecule that suppress the anticancer tumor response, while TPT-ITC was more effective in down-regulating CD44 expression and reducing migration compared to TBT-ITC. Interest in organotin (IV) novel compounds are undoubtedly increasing due to their possible medical uses and thorough investigation of both their genotoxic, as well as non-genotoxic anticancer actions is required.

Conflicts of Interest

None of the Authors have any conflict of interest to declare regarding this study.

Authors' Contributions

L.H., E.H., and P.G. conceived, designed and performed the experiments and analyzed the data; J.O. and P.B. synthesized TBT-ITC and TPT-ITC; J.B. drafted the paper and assisted with the final processing of the manuscript.

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