Toxicology Research

PAPER

View Article Online
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Cite this: *Toxicol. Res.*, 2014, **3**, 254

The effects of tributyltin oxide and deoxynivalenol on the transcriptome of the mouse thymoma cell line EL-4†

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The main goal of this study was to assess the potential of the mouse thymoma EL-4 cell line in screening for chemical induced immunotoxicity. Therefore, EL-4 cells were exposed to two well-known immunotoxicants, organotin compound tributyltin oxide (TBTO, 0.5 and 1 μM for 3 or 6 h) and the mycotoxin deoxynivalenol (DON, 0.25, 0.5 and 1 μM for 3, 6 or 11 h). Previous studies in human Jurkat T cells and mouse thymus *in vivo* showed that the primary mode of action of TBTO is induction of endoplasmic reticulum (ER) stress, T cell activation and apoptosis. DON induces ribotoxic stress and, similarly to TBTO, induces ER stress, T cell activation and apoptosis. In the present study, the effects of TBTO and DON on EL-4 mRNA expression were assessed by whole genome microarray analysis. The microarray data were then compared to those obtained with mouse thymuses *in vivo*, mouse thymocytes *in vitro*, and CTLL-2 cells and human Jurkat cells *in vitro* exposed to TBTO or DON. Analysis at the level of gene sets revealed that part of the previously detected modes of action of TBTO and DON were not observed in the EL-4 cell line. In EL-4 cells, TBTO induced genes involved in calcium signalling and ER stress but did not induce genes involved in T cell activation and apoptosis. DON induced RNA related processes and ribosome biogenesis. Furthermore, DON downregulated ER stress, T cell activation and apoptosis which is opposite to the mechanism of DON observed in the mouse thymus *in vivo* and in Jurkat T cells *in vitro*. Apparently, EL-4 cells lack factors that are necessary to link ribotoxic stress to ER stress. In addition, of the lack of T cell activation response of EL-4 cells to TBTO is likely due to the fact that these cells are in a constitutively activated state already. Based on the results obtained for TBTO and DON, it can be concluded that the EL-4 cell line has limited value for immunotoxicogenomics based screening.

Received 21st November 2013,
Accepted 24th February 2014

DOI: 10.1039/c3tx50100k

www.rsc.org/toxicology

Introduction

REACH, the European regulation community on chemicals and their safe use aims to test all new and existing chemicals for toxicity of which the yearly production exceeds one tonne.[1,2] In immunotoxicity testing the current strategy relies heavily on animal models. Since testing all new and almost all existing chemicals would require enormous amounts of animals, money and time, *in vitro* alternatives are urgently needed.[3] Some recent studies used mainly human Jurkat T cells as a model for immunotoxicity.[4–6] However, because the immune system comprises multiple organs and multiple cell types, a battery of *in vitro* tests with a diversity of cell lines may be useful as screening tool for the prediction of immunotoxicity. In this study we followed a toxicogenomics approach to assess the usefulness of mouse thymoma EL-4 cells as a potential *in vitro* system to screen compounds for immunotoxicity.

EL-4 cells were derived from a thymoma of a C57BL/6 mouse more than sixty years ago.[7] These cells express CD3 but they do not express CD4 or CD8 molecules on their surface.[8,9] All T cells including progenitor cells express CD3 and during development in the thymus they will become double positive thymocytes expressing both CD4 and CD8 at positive selection.[10] Further development into CD4+ or CD8+ T cells occurs in the thymic medulla in a process called negative selection, where T cells with high affinity for the interaction with the

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3tx50100k

Major Histocompatibility Complex (MHC) undergo apoptosis to prevent autoreactivity.[10] Since EL-4 cells contain CD3 molecules but no CD4 or CD8 they can be classified as early thymocytes.[11]

In the present study, EL-4 cells were treated with two well-known immunotoxicants, the mycotoxin deoxynivalenol (DON) and the organotin compound tributyltin oxide (TBTO) and were subsequently subjected to DNA microarray analysis. Both compounds have been used before in human and rodent transcriptomics studies.[4,5,12–18]

TBTO is an organotin compound that has been applied for various industrial purposes. It was for instance utilized as marine anti-fouling agent in ship paint, as a wood preservative and in the production of plastic floor tiles. Because of its extensive use, TBTO is a widely spread environmental contaminant.[19] Humans can be exposed to organotin compounds through inhalation, absorption and consumption of contaminated food and water.[20] In the period before the ban on TBTs, human blood butyltin concentrations ranging from 21 to 155 ng m^{-1} were measured in volunteers in Michigan.[21,22] More recently, a Dutch study in 2004 detected butyltin levels higher than 0.1 ng ml^{-1} in only six out of hundred blood samples.[23] Furthermore, in a Finnish study, none of 300 blood samples contained more than 1 ng butyltin per ml.[24] The differences are probably caused by restrictions in the use of organotin compounds and regional differences.

TBTO causes peripheral T cell depletion in its main target organ, the thymus, by inhibition of proliferation and induction of apoptosis.[25] Recently, it has been shown that TBTO induces ER stress, affects calcium homeostasis, induces T cell activation and apoptosis in human Jurkat T cells.[5,15] *In vivo*, mouse microarray data indicated that TBTO induces oxidative stress and apoptosis in the thymus.[16] In mouse primary thymocytes TBTO upregulates genes that are involved in ER stress, NFκB and TNFα pathways, DNA damage, p53 signalling and apoptosis.[13]

DON belongs to a class of mycotoxins, trichothecenes, and is a common contaminant of wheat and corn.[26–28] DON, which is a hydroxylated form of nivalenol, is produced by the fungus *Fusarium* sp. and is a commonly found mycotoxin in food and feed.[28–31] DON is chemically very stable during food processing[32] and as a result humans are almost continuously exposed to low levels of this mycotoxin in their diet. Although data on human blood levels of DON are lacking, estimations for a tolerable daily intake range from 1 to 1000 μg kg^{-1} body weight per day.[33–35] DON has been shown to cause a series of toxic effects in animals including immunomodulation.[36] The primary action of DON is the interference with the active site of peptidyltransferase on ribosomes,[37–39] leading to inhibition of protein synthesis.[40] This triggers a so called ribotoxic stress response[39,41] that in human Jurkat cells is followed by induction of endoplasmic reticulum stress, T cell activation and apoptosis.[4] A recent microarray study on the thymuses of mice that were fed different concentrations of DON for 3, 6 and 24 hours showed that DON induces cellular effects that also occur during T cell activation.[14] On the basis of this finding it was postulated that these processes led to negative selection of the activated thymocytes and removal of apoptotic cells out of the thymus by phagocytosis.[14]

The microarray data of the present work, where EL-4 cells were exposed to two well-known immunotoxicants TBTO and DON, were compared to transcriptome data from previous studies that examined the effects of TBTO and DON on murine thymus, mouse primary thymocytes and the human Jurkat T cell line.[4,5,13,14,18] These comparisons were performed in order to investigate the feasibility of using EL-4 cells as part of an *in vitro* strategy for immunotoxicity testing. To our knowledge, this is the first study that performed transcriptomics on murine EL-4 cells.

Materials and methods

Cell culture and chemicals

EL-4 cells (ATCC; TIB-39) were cultured in RPMI 1640 medium (Invitrogen Life Science, Breda, The Netherlands) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Invitrogen Life Science). Medium was refreshed three times a week. TBTO and DON (Sigma-Aldrich, Zwijndrecht, the Netherlands) were dissolved in absolute DMSO, diluted with standard medium and added to the culture wells in different concentrations. The final DMSO concentration in the culture wells was 0.1%, which had no effect on the cell viability.

Cell viability assay

Cell viability was determined using the water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Mannheim, Germany). This assay is based on the reduction of WST-1 to formazan that is mainly dependent on NADH and NADPH produced by viable cells. The reaction induces a colour change proportional to the mass of living cells in the culture medium. Cells were seeded into 96-well microtiter plates at a concentration of 5×10^5 cells per well and in triplicate per condition. Solutions of TBTO, DON or DMSO or medium (controls) were added to a total volume of 100 μl. Cells were incubated for 24 h and 10 μl WST-1 assay mix was added during the last 2 h. The amount of WST-1 converted to formazan was quantified at 450 nm using a microplate reader (Synergy™ HT Multi-Detection Microplate Reader, Bio-teck instruments). Relative viability was measured by comparing the mean optical density of the TBTO and DON exposed cells with the mean optical density of the control cells. Subcytotoxic concentrations were defined as those concentrations leading to a decrease of 20% viability or less after 24 h. These concentrations were then used in exposure experiments that were subsequently used for microarray hybridisations.

Exposures

EL-4 cells were seeded in six-well plates at a concentration of 5×10^5 cells per well and in triplicate per condition. Twenty hours after seeding, exposure was initiated by adding DON

(0.25 μM, 0.5 μM for 3, 6 and 11 h, and 1 μM for 6 h) or TBTO (0.5 μM and 1 μM for 3 or 6 h) to the EL-4 cells. The maximum DMSO concentration did not exceed 0.1%, which had no effect on the viability of EL-4 cells. These exposures were performed with three different passages of EL-4 cells.

RNA isolation

After exposure, cells were harvested and washed with PBS (10 min, 1200 rpm, 4 °C). The cell pellets were resuspended in 600 μl RLT lysis buffer containing 1% β-mercaptoethanol (Qiagen, Venlo, the Netherlands). The samples were stored at −80 °C until RNA isolation. Total RNA was isolated using RNeasy mini kits including DNase treatment (Qiagen) according to the manufacturer's instructions. RNA concentrations were determined by measuring absorbance at 260 and 280 nm and purity was estimated by 260/280 nm absorbance ratio (Nanodrop technologies, Wilmington, DE).

Microarray hybridizations

RNA was amplified and purified using the Agilent low RNA input fluorescent amplification kit protocol (Agilent Technologies, Palo Alto, CA). 1 μg of each of the linearly amplified cRNA preparations was labelled by incorporation of Cy5-CTP (Perkin-Elmer/NEN Life Sciences, Boston, MA). Stratagene Universal Mouse Reference RNA (Agilent Technologies, La Jolla, CA) was used and labelled with fluorescent Cy3 dye (Perkin-Elmer/NEN). Each of the Cy5 labelled experimental cRNA samples was combined with an equal amount of the Cy3 labelled reference cRNA and hybridized on 44 K whole mouse genome oligo microarrays (Agilent Technologies) following the Agilent two-colour microarray-based gene expression analysis protocol. The microarrays were hybridized for 17 h at 65 °C in Agilent microarray hybridization chambers. Upon hybridization, the microarrays were washed and dried at room temperature following the instructions of the supplier. Arrays were scanned using an Agilent microarray scanner (G2565B). The fluorescent readings from the scanner were converted to quantitative files using Feature Extraction 9.1 software (Agilent Technologies). Quality check of the arrays was performed using software package LimmaGUI in R version 2.3.1. Data were imported in GeneMaths XT 1.5 (Applied Maths, St. MartensLatem, Belgium) and signals below two times background were excluded from subsequent analysis.

Data analysis

As a first step in the microarray data analysis, data were log transformed and normalized as described by Pellis.[42] In short, the Cy5 values were first corrected using the values of the Cy3 labelled internal standard to correct for possible differences in hybridization conditions between slides. Second, the median of the adjusted Cy5 signals was used to correct for possible differences between experiments with respect to the efficiency of probe labelling and amount of probe labelled.

Hierarchical clustering of the data was performed using the programs Cluster and Treeview.[43] An online software suite MetaCore (GeneGo Inc., St. Joseph, MI) was used to identify statistically significantly affected pathways for the subclusters of genes identified in the hierarchical clustering.

Comparative data analysis

Gene Set Enrichment Analysis (GSEA) is a statistical analysis tool for microarray data and is used to detect the affected biological processes and to provide insight into the affected molecular mechanisms. GSEA uses predefined gene sets that are based on literature or previous experimental results. GSEA has the advantage over other statistical tools that no initial filtering is applied to the dataset to select for significantly differentially expressed genes. GSEA first ranks all the genes of the microarray data on their expression ratios and then determines whether a particular gene set is significantly enriched at the top or the bottom of the ranked list, or whether genes are randomly distributed.[44] This enables detection of significantly affected gene sets, while the fold change of expression of the individual genes can be relatively modest.[44]

In addition, gene sets were manually created based on the present EL-4 microarray data. Spots that were up- or downregulated with a fold change of >1.6 in at least two out of three arrays were included. This selection was performed for up- and downregulated spots separately per time point.

We then tested whether these EL-4 responsive gene sets were also affected in previous microarray studies on mouse thymuses exposed *in vivo* to TBTO[18] or DON,[14] mouse thymocytes exposed to TBTO *in vitro*,[13] mouse CTLL-2 cells exposed to TBTO and DON *in vitro*[12] and human Jurkat T cells exposed to TBTO and DON.[4,5] Results of these comparisons are visualized in heat maps of gene sets in which red and green indicate up- and downregulation, respectively. Brighter colours represent a higher significance level. A full green or red colour indicates $p < 0.0001$. Gene sets used to identify biological pathways and processes affected by TBTO and DON were derived from:

1. Lymphocyte database, containing genes upregulated during T cell activation.[45,46]

2. Gene Ontology: gene sets were downloaded from the Gene Ontology consortium (http://www.geneontology.org/) including molecular function and biological process.

3. Tox action (self-made).

4. Genes affected by TBTO or DON in other studies.[4,5,12–14,18]

Genes involved in ribosomal function, ER stress, T cell activation and apoptosis

We then investigated how individual genes that play a role in the modes of action of TBTO and DON respond in EL-4 cells. Genes involved in RNA biosynthesis were taken from Reactome (http://www.reactome.org), genes involved in ER stress were taken from Kyoto Encyclopaedia of Genes and Genomes (KEGG), Biocarta and from literature mining and a set of apoptosis-related genes was taken from Gene Ontology. Genes upregulated during T cell activation were taken from the lymphocyte database.[45,46] Separately for TBTO and DON, the effects on expression of genes involved in the mechanisms of

action in EL-4 cells were compared to the expression of those genes in CTLL-2 cells, Jurkat T cells and for TBTO also in primary mouse thymocytes. This was performed using the programs Cluster and Treeview.[43] Red and green indicate up- and down-regulation *vs.* the average of the control samples. The threshold for up- and downregulation was set on a 2log ratio of 0.7 (numerical ratio 1.6).

Results and discussion

Time and dose selection

The viability of EL-4 cells was determined using the WST-1 assay which is based on formazan formation by living cells. The viability of EL-4 cells was increased to 180% and 140% when exposed to 0.25 or 0.5 μM TBTO for 6 h, while it was decreased to 83 and 102% after 24 h exposure, respectively (Fig. 1a). This might be an example of stimulation of various processes at lower concentrations of the toxicants, which is not unexpected. In previous experiments using TBTO, the viability of Jurkat and CTLL-2 cells also increased after 6 h and then decreased after 24 h, although it did not increase to levels higher than 120%.[12,47] Such stimulatory effects were also observed with respect to the generation of reactive oxygen species after *in vitro* exposure of oyster blood phagocytes to tributyltin at lower concentrations.[48] Possibly, the early, increased proliferation might be induced by the T cell activation response while at later time points the proliferation is reduced due to ribotoxic stress or ER stress response leading to apoptosis. Exposure to 1 and 2 μM TBTO for 6 h resulted in a viability of 67 and 43%, respectively. Exposure of EL-4 cells to 1 and 2 μM TBTO reduced the viability to less than 25% after 24 h.

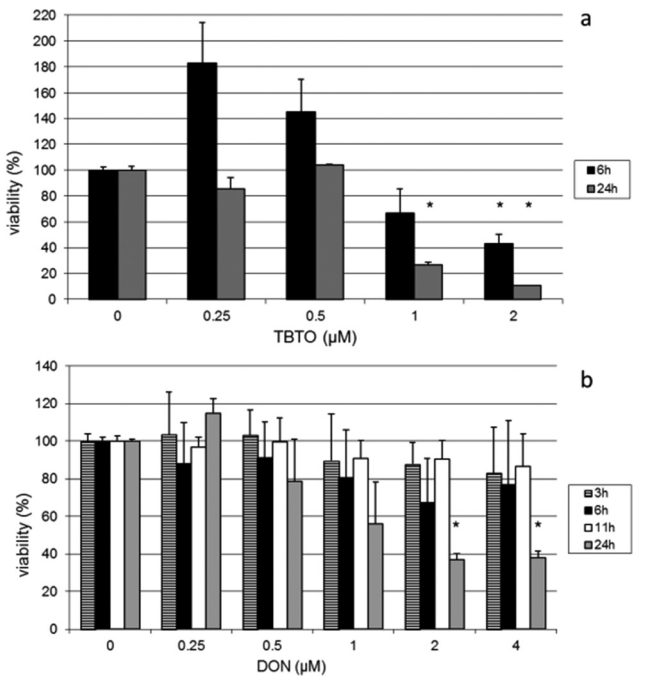

Fig. 1 Average viability of EL-4 cells exposed to (a) TBTO and (b) DON. Viability results reflect the average ± SD of three independent measurements presented as percentage relative to solvent control DMSO at the same time point. *$P \leq 0.01$ compared to solvent control DMSO (Student's *t* test).

The viability of EL-4 cells exposed to DON was almost unchanged by 0.25 μM at any time point and gradually decreased from 0.5 μM onwards (Fig. 1b). Exposure for 24 h to 0.5 and 1.0 μM DON reduced the viability to 78% and 57% respectively. 24 h exposure to 2 or 4 μM DON resulted in 38% viability.

The criterion for selecting doses for microarray exposures was the same as used before.[5,12,14] Doses were selected that resulted in a decrease in viability of less than 20% after 24 h exposure. Furthermore, for both compounds one dose resulting in more than 20% reduction in cell viability was selected to assess the gene expression profile at cytotoxic conditions. For TBTO, 0.5 μM and 1 μM were chosen as subcytotoxic and cytotoxic doses, respectively. These doses were 2.5 to 5 times higher than the highest concentrations found in human blood samples.[21,22] For DON, 0.25 and 0.5 μM were selected as subcytotoxic and 1 μM was selected as cytotoxic doses. No data could be obtained on human blood concentrations of DON. The tolerable daily intake (TDI) of DON in humans varies between 1 and 1000 μg kg^{-1} bodyweight per day (0.003375 to 3.37 μM).[33–35] The concentrations of DON as used in the present study are thus within the range of the TDI of DON.

To study the effects of TBTO and DON in time, EL-4 cells were exposed for 3 and 6 h. The effect of DON was also examined upon 11 h exposure, since it was shown in a DON *in vivo* study that the number of differentially expressed genes decreased with longer exposure times.[14]

Hierarchical clustering and pathway analysis

Transcriptomics was performed on RNA from triplicate exposures. However, for 3 h and 6 h exposure to 1 μM TBTO and 6 h exposure to 1 μM DON only duplicates were obtained since three samples did not meet the quality control criteria.

Unsupervised hierarchical clustering was performed to visualise genes that were affected by TBTO (Fig. 2a) or DON (Fig. 2b). Genes were selected on >1.6 fold up- or downregulation in ≥3 arrays. This resulted in two large subclusters for TBTO and three subclusters for DON. These subclusters of genes were uploaded to MetaCore for identification of the most significantly represented pathways and biological processes in these clusters. Genes that were upregulated by TBTO corresponded to the processes of apoptosis and stress response (Fig. 2a). The apoptosis-related genes included AP1, CHAC1, GADD34, GADD45, NUR77 and PUMA. The genes downregulated by TBTO were related to regulation of molecular function, signal transduction and metabolic process. These downregulated genes are thus involved in some common processes that are not specific for immunotoxicity or the mechanism of TBTO. One of the three subclusters affected by DON consisted of genes that were specifically upregulated

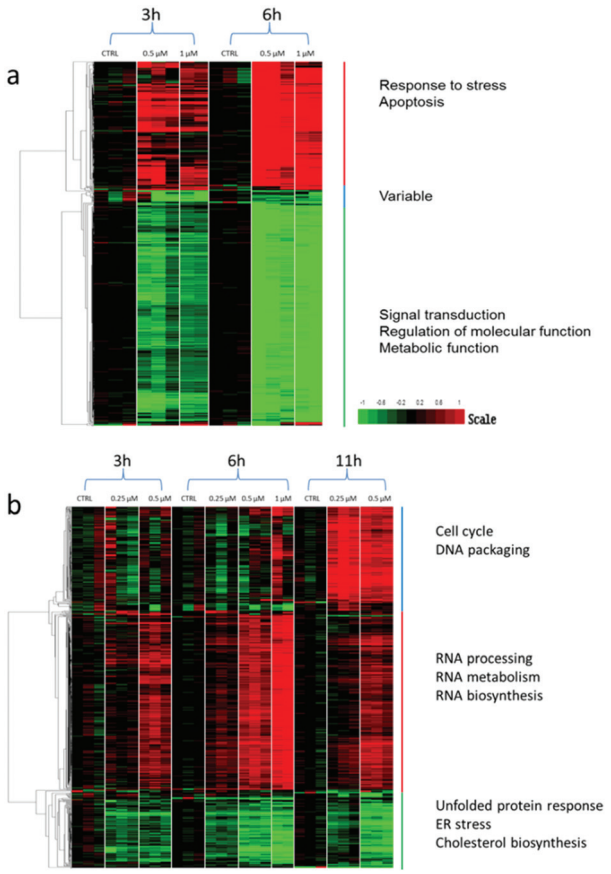

Fig. 2 Unsupervised hierarchical clustering of spots altered by TBTO or DON exposure in EL-4 cells. (a) This heat map contains 708 spots that were >1.6 times up- or downregulated by TBTO in EL-4 cells in at least 3 out of 16 arrays. (b) This heat map contains 717 spots that were >1.6 times up- or downregulated by DON in EL-4 cells in at least 3 out of 29 arrays. For each of the subclusters the corresponding biological processes are indicated on the right based on MetaCore analysis. Scale is displayed in bottom right corner. Green represents downregulation, red represents upregulation and black represents no effect.

by DON after 11 h and a significant number of genes of this cluster are involved in cell cycle and DNA packaging (Fig. 2b). The second subcluster contained genes that were upregulated at all time points at DON concentrations of 0.5 μM or higher and these genes are related to RNA processing, metabolism and biosynthesis, which is in line with the reported primary mechanism of action of DON.[4,14] The third subcluster contained genes that were downregulated upon DON exposure and these genes are related to unfolded protein response (UPR), ER stress and cholesterol biosynthesis. The genes per cluster including the expression data for both TBTO and DON can be found in ESI Tables 1 and 2,† respectively. A clustering on >1.6 fold up- or downregulation in ≥2 arrays is also visualized as ESI Fig. 1.† Spots that were up- or downregulated by 1 μM TBTO were also up- or downregulated by 0.5 μM TBTO. Spots that were affected by 1 μM DON were similarly affected by 0.5 μM but with a lower ratio.

Gene set enrichment analysis (GSEA)

GSEA was used as a complementary tool, next to hierarchical clustering and MetaCore analysis, to identify which pathways and processes were affected by TBTO and DON in EL-4 cells. GSEA enabled us to compare the present EL-4 microarray data to gene sets that are publicly available (see Methods section, 'comparative data analysis'). GSEA was performed separately for TBTO and DON and the results were compared to the time matched controls. The GSEA output was then converted to a heat map for the gene sets that were significantly ($P \leq 0.01$ and FDR ≤ 0.25) up- or downregulated (Fig. 3). Genes involved in calcium signalling were upregulated by exposure for 3 h to 0.5 and 1 μM TBTO and 6 h to 1 μM TBTO and downregulated after exposure for 3 h to 0.5 μM DON. The increase in calcium signalling by TBTO agrees with the results obtained in previous experiments using Jurkat cells.[15] However, TBTO and DON also induced T cell activation and apoptosis in Jurkat cells.[5,15] GSEA does not provide evidence for T cell activation or apoptosis induction after TBTO or DON exposure in EL-4 cells. Genes involved in the process of apoptosis and programmed cell death were downregulated by 1 μM TBTO (3 and 6 h) and 3 h DON exposure (0.25 and 0.5 μM). Since genes involved in T cell activation were not affected, this is not shown in Fig. 3. Genes involved in the cell cycle and RNA related processes were downregulated by TBTO, while these were upregulated by DON after 6 or 11 h. Genes involved in the ER stress response were upregulated by TBTO exposure to 0.5 μM and downregulated at all time points and by all, except

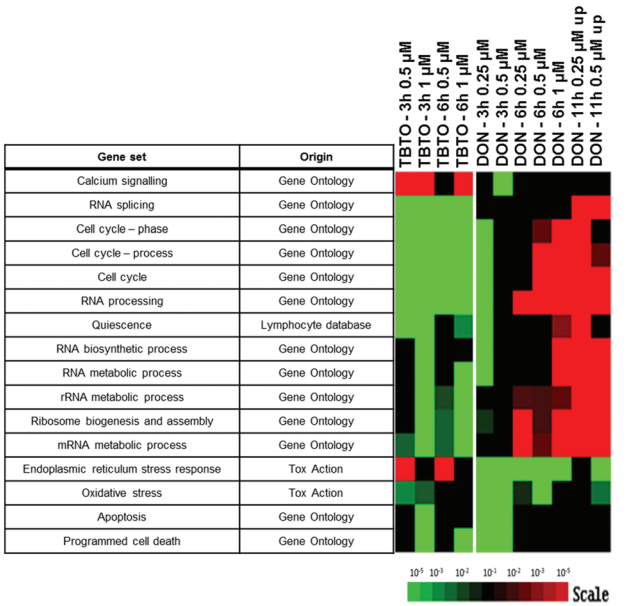

Fig. 3 Heat map of processes significantly affected (p value ≤ 0.01, FDR ≤ 0.25) in the EL-4 cell line showing an overview of the effects of TBTO and DON exposure on a selection of gene sets that were run in GSEA. Each line represents one gene set. Scale indicates statistical significance (p-value) of the gene sets. Green represents downregulation, red upregulation and black no effect.

one (11 h 0.25 μM), concentrations of DON. Oxidative stress was downregulated by 3 h TBTO exposure, and 3 or 6 h DON exposure. The induction of the gene sets 'ribosome biogenesis and assembly', 'RNA processing' and 'mRNA metabolic process' by DON is visualised in heat maps showing the expression levels of the individual genes (ESI Fig. 2a–c†). These heat maps convincingly show the upregulation of genes involved in the structure and function of ribosomes and RNA related processes. This holds true for subcytotoxic concentrations after 6 h (0.5 μM) and 11 h (0.25 and 0.5 μM) as well as 6 h exposure to 1 μM DON, a concentration that is cytotoxic after 24 h (Fig. 1). The upregulation of genes involved in ribosome biogenesis and assembly, RNA processing and metabolism and mRNA metabolic process was expected since these are known ribotoxic stress responses.[38,41]

Comparative data analysis

We investigated whether the genes that were affected by TBTO and DON in the present EL-4 study were similarly affected in the mouse thymus *in vivo* (TBTO and DON), mouse primary thymocytes *in vitro* (TBTO), and the mouse CTLL-2 and human Jurkat cell lines *in vitro* (TBTO and DON). To that end, we used microarray data obtained from experiments performed before.[4,5,12–14,18] GSEA statistics was then used to test whether genes affected in the current study using EL-4 cells were similarly affected in previous experiments.

For TBTO, a heat map of this comparison is depicted in Fig. 4a. The sets containing genes that were downregulated by TBTO in EL-4 cells were also downregulated by TBTO in (1) the mouse thymus *in vivo*, (2) mouse thymocytes exposed *in vitro*, (3) Jurkat cells exposed for 6 h to 0.5 μM TBTO, and (4) CTLL-2 cells exposed for 6 h to 0.2 μM TBTO. Gene sets that were upregulated by TBTO in EL-4 cells were not significantly affected by TBTO in the mouse thymus *in vivo* (Fig. 4a). This is likely a result of the depletion of thymocytes out of the thymus after exposure to TBTO in combination with a relatively long exposure time of three days.[25] Upregulation of these gene sets occurred in mouse thymocytes exposed to TBTO *in vitro*, except for those exposed for 3 or 11 h to 0.1 μM TBTO. Gene sets upregulated by TBTO in EL-4 cells were also upregulated by TBTO in Jurkat cells exposed to 0.2 μM for 3 h and 0.5 μM for 3 and 6 h, but not 0.2 μM for 6 h. Genes upregulated in EL-4 cells were also upregulated in CTLL-2 cells exposed to 0.2 μM TBTO and oppositely regulated in CTLL-2 cells exposed to 0.1 μM TBTO. Overall, there is a significant overlap in genes that are up- or down-regulated by TBTO exposure in EL-4 cells to genes that are up- or downregulated in other mouse and human models.

Genes downregulated by DON in EL-4 cells were also downregulated in DON exposed mouse thymus *in vivo*, in Jurkat cells and in CTLL-2 cells (Fig. 4b). Genes that were upregulated by DON in EL-4 cells were also upregulated after 3 and 6 h, but not 24 h, exposure *in vivo*. Thus, with longer *in vivo* exposure

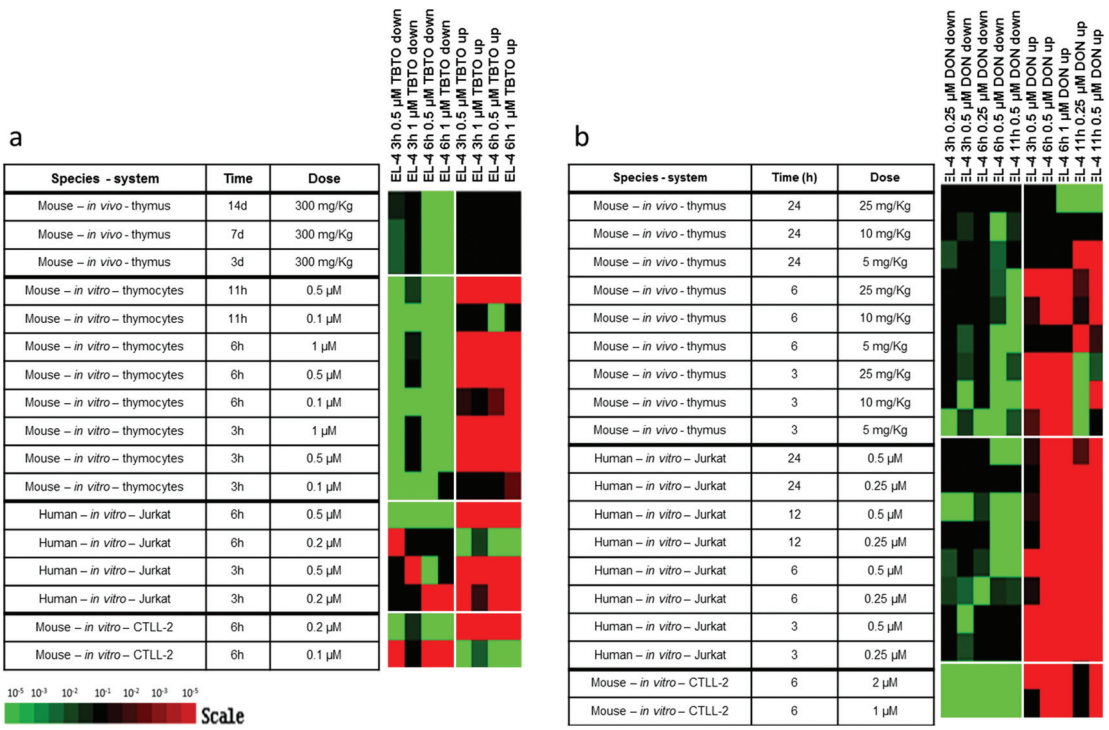

Fig. 4 A significant proportion of genes affected by TBTO or DON in EL-4 cells are also affected by TBTO and DON in the mouse thymus, mouse CTLL-2 cells and in human Jurkat T cells. Sets of genes up- or downregulated by TBTO (a) and DON (b) in EL-4 cells were selected from the present study. Thereafter, GSEA statistics was used to assess whether these gene sets were significantly affected by TBTO or DON in the mouse thymus *in vivo* or *in vitro*, mouse CTLL-2 cells and Jurkat cells *in vitro* using microarray data of previous studies.[4,5,12–14,18] Scale indicates statistical significance (*p*-value) of the gene set. Green represents downregulation, red upregulation and black no effect.

the overlap with genes affected in EL-4 cells decreased. This is likely due to the fact that the number of genes affected by DON in the mouse thymus *in vivo* is much lower after 24 h than after 6 h which is related to the rapid excretion of DON and the recovery of the thymus.[14] Genes upregulated in EL-4 cells were also upregulated in mouse thymus, Jurkat T cells and CTLL-2 cells.

In general, genes affected by TBTO and DON in EL-4 cells are affected in the same direction in other mouse (*in vitro*) and human lymphocytes and thymocytes.

Genes involved in ER stress, T cell activation and apoptosis

The mode of action of TBTO, as identified in human Jurkat T cells[5,15] and primary mouse thymocytes[13] includes induction of ER stress, T cell activation and apoptosis. The mode of action of DON as identified in mouse thymus *in vivo*[14] and Jurkat T cells includes the induction of ribotoxic stress and RNA related processes followed by ER stress, T cell activation and apoptosis.[4,36,38] As summarized in Table 1, TBTO induces ER stress and DON induces ribotoxic stress in EL-4 cells but neither compound induces the downstream processes. It is therefore of interest to assess the effects of TBTO and DON on the individual genes of these processes in EL-4 cells, and to compare these to the effects in CTLL-2, human Jurkat T cells and for TBTO also in primary mouse thymocytes.

As shown in Fig. 5 and 6, TBTO affects less genes involved in ER stress, T cell activation or apoptosis in EL-4 cells than in Jurkat cells and primary thymocytes. However, those genes that are affected in EL-4 cells are often affected in the same direction in Jurkat cells and primary thymocytes although most often with a higher induction or repression value. TBTO upregulated the early ER stress marker HERPUD1[49] in EL-4, CTLL-2 and Jurkat cells, as well as in primary mouse thymocytes (Fig. 5, panel 1). Other ER stress genes that were convincingly upregulated by TBTO in each of the four cell types were TRIB3, DNA damage inducible transcript 3 (DDIT3, also involved in apoptosis), EIF2AK3, EGR1 and heat shock protein HSPA4L. Other ER stress genes, like ATF3 and GADD45B, were upregulated in CTLL-2, Jurkat and primary mouse thymocytes but not in EL-4 cells. Only a limited number of genes related to T cell activation were upregulated in EL-4 cells (BCL6, GEM, FOS, JUN, FOSL2, DUSP1, CD69, CCL4, KLF6, REL, CXCL10 and EGR1) of which CD69 is considered to be an early response T cell activation marker.[50] More genes were upregulated in CTLL-2 and Jurkat cells while TBTO affected the highest number of T cell activation genes in primary thymocytes. In the process of apoptosis, the number of genes upregulated by TBTO in EL-4 cells was limited to five (NFKBIA, DDIT3, TRIB3, CHAC1 and TNFAIP3). These genes were also upregulated in CTLL-2 (0.2 μM), Jurkat (0.5 μM) and primary thymocytes (0.5 and 1 μM). Similar to ER stress and T cell activation, TBTO affected more apoptosis-related genes in Jurkat and primary thymocytes than in EL-4 and CTLL-2 cells. The response of the different cell types to TBTO is however more alike than the response to DON.

EL-4, CTLL-2 cells and Jurkat cells differ much more in their response to DON than to that of TBTO (Fig. 6 *vs.* 5). The effect of DON on the expression of genes involved in RNA biosynthesis was limited in EL-4 as compared to Jurkat (Fig. 6, panel 1). Two genes that were convincingly upregulated in EL-4 cells are DDX20 and NUPL2. DDX20 was upregulated in all cell models and is involved in alteration of RNA secondary structure.[51] Gene NUPL2 is mostly upregulated in EL-4 cells and functions as exporter of mRNA from nucleus to cytoplasm.[52] Genes involved in ER stress were mostly downregulated in EL-4 and CTLL-2 cells. Among the downregulated genes are HERPUD1 and XBP1, that are also downregulated in Jurkat cells. Both HERPUD1 and XBP1 are known to be induced by the unfolded protein response.[53–55] This indicates that the induction of ER stress by DON in Jurkat cells is independent of the unfolded protein response. DDIT3 is the only ER stress related gene that was upregulated by DON in EL-4 cells. In contrast to EL-4 and CTLL-2 cells, multiple ER stress genes were upregulated in Jurkat T cells. Two genes with the highest upregulation in Jurkat cells were Activating Transcription Factor 3 (ATF3) and Early Growth Factor 1 (EGR-1). ATF3 is induced after ER stress induction and then blocks the cell cycle.[56] EGR-1 encodes for a transcription factor that is transiently induced after ER stress.[57] This activation then results into cell death by apoptosis.[58]

Of the genes involved in T cell activation, only two, KLF10 and TSC22D3, were induced by DON in EL-4 cells. In marked contrast, DON induced approximately half of the genes related to T cell activation in Jurkat cells. The early T cell activation

Table 1 Overview of processes affected by TBTO and DON in this study and previous transcriptomics studies

TBTO	EL-4 (this study)	CTLL-2 [12]	Jurkat[4,5]	Primary thymocytes[13]
ER stress	+	+	+	+
T cell activation	0	+	+	+
Apoptosis	0	+	+	+
DON	**EL-4 (this study)**	**CTLL-2 [12]**	**Jurkat[15]**	**Thymus *in vivo*[14]**
Ribotoxic stress	+	+	+	+
ER stress	−	−	+	+
T cell activation	0	−	+	+
Apoptosis	−	−	+	+

+ upregulated, − downregulated, 0 not regulated.

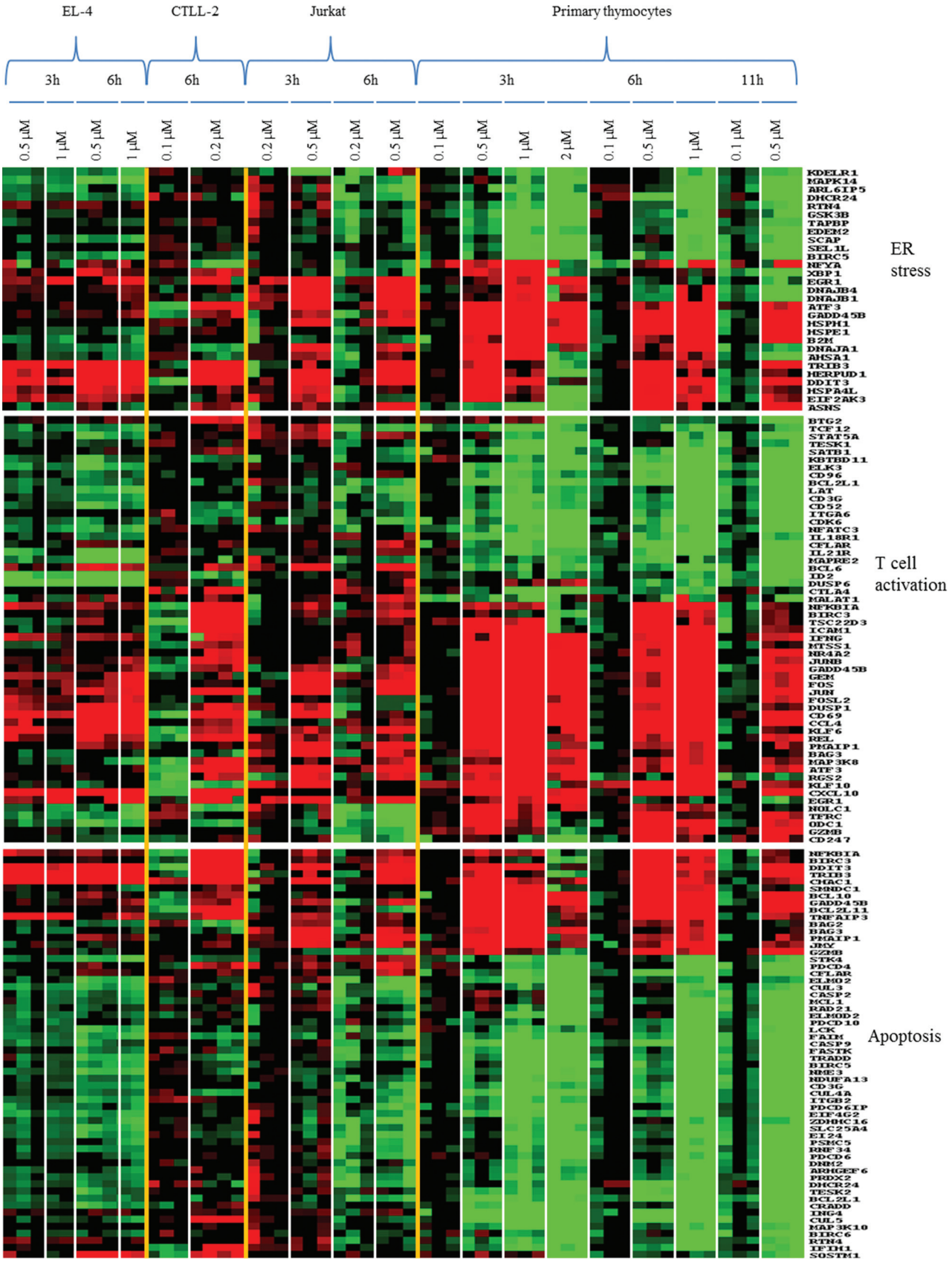

Fig. 5 Overview of the expression of genes involved in the modes of action of TBTO and DON. Effect of TBTO on mRNA expression of genes involved in ER stress, T cell activation and apoptosis in mouse EL-4, CTLL-2, human Jurkat, and mouse primary thymocytes. Scale is displayed in bottom right corner of Fig. 6; green: downregulation, red: upregulation, black: no effect.

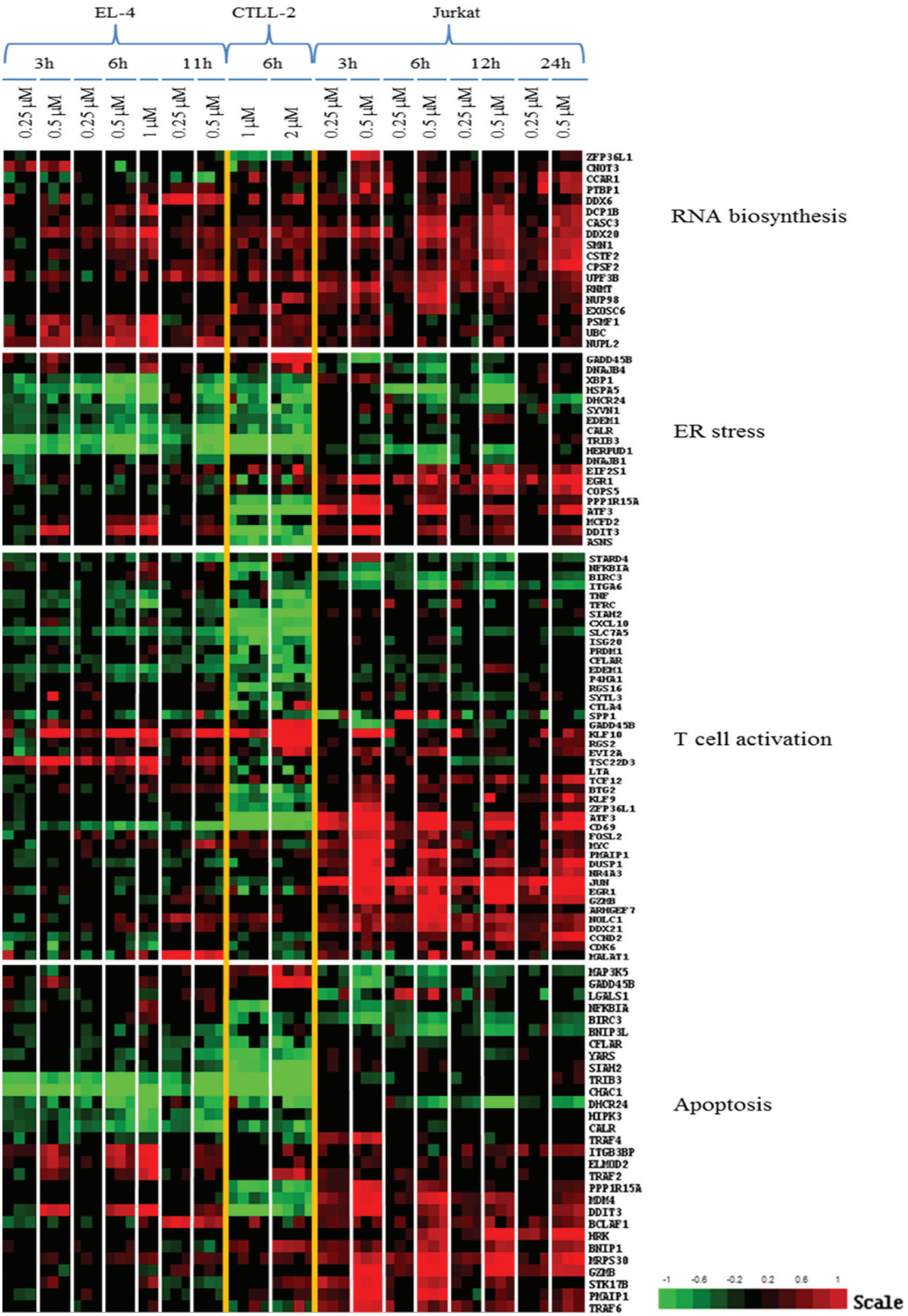

Fig. 6 Overview of the expression of genes involved in the modes of action of TBTO and DON. Effect of DON on mRNA expression of genes involved in RNA biosynthesis, ER stress, T cell activation and apoptosis in mouse EL-4, CTLL-2 and human Jurkat T cells. Genes were selected based on a 2 fold up- or downregulation in at least nine arrays (TBTO) or on a 1.6 fold up- or downregulation in at least three arrays (DON). Scale is displayed in bottom right corner; green: downregulation, red: upregulation, black: no effect.

marker CD69 was upregulated in Jurkat cells and downregulated in EL-4 and CTLL-2 cells. Most of the genes involved in apoptosis were not affected in EL-4 cells. Exceptions were TRIB3 and CHAC1 that were downregulated and DDIT3 that was upregulated in EL-4 cells. In CTLL-2 cells more apoptosis-related genes were downregulated and only GADD45B was upregulated. The highest number of apoptosis-related genes was upregulated in Jurkat cells. Of these genes HRK, MRPS30 and GZMB are induced by the highest factors. HRK encodes for a protein that induces apoptosis and interacts with the BCL2 survival gene.[59] MRPS30 is a mitochondrial ribosomal protein that is associated with programmed cell death.[60–62] Granzyme B (GZMB) encodes for a serine proteinase that activates cell death pathways.[63]

ESI Fig. 3† shows the effects of TBTO on genes involved in ER stress, T cell activation and apoptosis using the same selection criteria as used for DON (3 arrays ≥2log 0.7). It is evident that TBTO affects a larger number of genes involved in these processes as compared to DON. In a similar way as is shown in Fig. 5, the effect on genes involved in ER stress, T cell activation and apoptosis remains limited in EL-4 cells also when using a less stringent cut-off value.

Overview of the mode of action of TBTO and DON in EL-4 cells

As shown in Fig. 4, a significant proportion of the genes up- or downregulated by TBTO and DON in EL-4 cells were often also up or downregulated by these compounds in the mouse thymus *in vivo*, mouse thymocytes *in vitro* and human Jurkat cells. However, pathways that were affected by TBTO in mouse thymus *in vivo*, mouse thymocytes *in vitro* and human Jurkat cells were not similarly affected in EL-4 cells. ER stress, oxidative stress, T cell activation and apoptosis were induced by TBTO in mouse thymus and Jurkat T cells *in vivo*, but not in EL-4 cells, except the induction of ER stress after 0.5 μM TBTO (Fig. 3).[4,14] GSEA showed that DON induces genes that are involved in ribosome biogenesis and assembly and RNA processing in EL-4 cells (Fig. 3, ESI Fig. 2†), which fits with the classification of DON as a ribotoxic stress inducer.[12,14,38,41] The induction values of RNA biosynthesis genes is still less convincing as compared to Jurkat cells (Fig. 6).

The difference between GSEA and individual gene presentation is due to the detection of more subtle effects by GSEA since no initial data filtering is applied. Also, heat maps of GSEA results show the relative expression of the genes without presenting information on the magnitude of the response. The lack of T cell activation and apoptosis induction for both TBTO and DON, and the lack of ER stress induction in DON exposed EL-4 cells is contrary to previous findings in Jurkat cells and mice experiments.[4,5,13,14,36] Apparently, DON exposure in EL-4 cells does not induce ER stress related genes, which might be due to a lack of molecular hubs that link ribotoxic stress to ER stress. However, it cannot be excluded that DON and TBTO induce ER stress in EL-4 cells at the (post-) translational level. In this regard it is noteworthy that DON induces degradation of HSPA5 protein (alias GRP78) in mouse macrophages.[64] HSPA5 triggers the UPR that would prevent ER stress.[65] We found HSPA5 to be downregulated as well but at the mRNA level by DON exposure in EL-4 cells, but not by TBTO exposure (Fig. 5). However, HSPA5 mRNA is also downregulated by DON in CTLL-2 and Jurkat cells (Fig. 6) and can thus not explain the fact that ER stress was upregulated in DON exposed Jurkat cells, whereas it was downregulated in EL-4 exposed cells. It can be envisaged that due to this absence of ER stress, the intracellular calcium level does not raise and T cell activation is averted after exposure to DON. In contrast, TBTO does induce ER stress in EL-4 cells which is due to a direct effect on the ER.[47] However, the ER stress did not proceed into T cell activation and apoptosis in EL-4 cells. This is very likely due to the fact that the T cell activation response is constitutively activated in EL-4 cells due to a mutation in the calcineurin gene.[66] The T cell activation response is also found to be constitutively activated in CTLL-2 cells but this can be attributed to the presence of ConA and IL-2 in the medium. Under this culture condition, this cell line is also unable to induce a T cell activation response upon exposure to DON or TBTO.[12]

Conclusions

In conclusion, the experiments performed in the present study indicated that there is a substantial overlap in genes affected by TBTO and DON in EL-4 cells with genes affected by these compounds in human Jurkat T cells, mouse CTLL-2 cells and thymocytes that were exposed *in vivo* or *in vitro* (Fig. 4). Nevertheless, more detailed pathway analysis indicated that TBTO and DON induced less processes in EL-4 cells than in Jurkat cells and primary mouse thymocytes. Similar to Jurkat cells and mouse primary thymocytes, TBTO upregulated genes involved in calcium signalling and ER stress in EL-4 cells. However, oxidative stress and apoptosis were downregulated which is contrary to the effects of TBTO in Jurkat cells and primary mouse thymocytes.

DON upregulated cell cycle, RNA related processes and ribosome biogenesis in EL-4 cells, which is indicative for ribotoxic stress induction, and downregulated ER stress, oxidative stress and apoptosis related pathways. The EL-4 results are very similar to those obtained in DON exposed CTLL-2 cells. Before,[12] we hypothesized that CTLL-2 cells might lack genes that play a role in the connection between ribotoxic stress and ER stress. Likely, EL-4 cells lack these genes as well. In addition, both cell lines have a constitutively activated T cell activation response.

Since the immune system consists of multiple organs and cell types, it is of relevance to assess the immunotoxicity of new and existing compounds with a battery of complementary cell systems. Based on the data presented in this study it can be postulated that the mouse thymoma EL-4 cell line model has limited value for *in vitro* immunotoxicity testing. However, to make a definite decision on the use of EL-4 cells in immunotoxicity testing, more compounds with different modes of action should be screened.

Acknowledgements

The study is supported by a grant (MFA 6809) that the University of Maastricht received from the Dutch Technology Foundation STW and the Netherlands Toxicogenomics Centre (grant number 05060510).

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