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Cytotoxic effects of the synthetic oestrogens and androgens on Balb/c 3T3 and HepG2 cells

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Abstract

The aim of the study was to test and compare the cytotoxic potential of two synthetic oestrogens: diethylstilboestrol (DES) and ethinyloestradiol (EE_2) and two androgens: testosterone propionate (TP) and trenbolone (TREN) on two cell lines. The fibroblast cell line Balb/c 3T3 and the hepatoma cell line HepG2 were selected. To get more insight into the mode of toxic action, four methods were used, which evaluated different biochemical endpoints: mitochondrial activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay), lysosomal activity (neutral red uptake assay), total protein content, and lactate dehydrogenase release. Cytotoxicity was assessed after 24, 48, and 72 h exposure to eight concentrations ranging from 0.78 to 100 µg/mL. Concentration- and time- dependent effects were observed. Depending on the line and assay used, half maximal effective concentration after 72 h ($EC_{50\text{-}72h}$) values ranged as follows: DES 1-13.7 µg/mL (Balb/c 3T3) and 3.7-5.2 µg/mL (HepG2); EE_2 2.1-14.3 µg/mL (Balb/c 3T3) and 1.8-7.8 µg/mL (HepG2); TP-14.9-17.5 µg/mL (Balb/c 3T3), and 63.9-100 µg/mL (HepG2); and TREN 11.3-31.4 µg/mL (Balb/c 3T3) and 12.5-59.4 µg/mL (HepG2). The results revealed that oestrogens were more toxic than androgens and the most affected endpoint was mitochondrial activity. In contrast to oestrogens, for which $EC_{50\text{-}72h}$ values were similar in both lines and by all assays used, Balb/c 3T3 cells were more sensitive than HepG2 cells to TP.

Keywords: synthetic hormones, cytotoxicity, Balb/c 3T3, HepG2.

Introduction

Synthetic hormones are pharmacological agents used in human and veterinary medicine for a variety of purposes such as contraception, cancer treatment, hormone replacement therapy, *etc.* Their residues enter the environment through the effluents of sewage treatment works, causing concern about the environmental exposure of different organisms to them (25). These drugs used in animal therapy, or used illegally as growth promoters, might also be found in food of animal origin (29).

As for most endocrine disruptors, toxicological profiles of the hormones are still incomplete or even lacking. A review has shown that their prolonged use could result in sexual dysfunction and reproductive disorders while less frequently genotoxic, hepatotoxic, neurotoxic, and cardiovascular effects were also observed (2, 5, 10, 16, 19, 20, 26). With this in mind, the question as to the extent these compounds can indeed exert adverse effects on humans is a hot issue.

Assuming that toxic effects seen in a whole organism are due to prior failure of basic cellular function, cytotoxicity studies offer a good source of information about the mechanism of toxicity (8), especially if a battery of tests is used (9, 11, 12, 21).

The aim of this study was to assess and compare the cytotoxic potential of two synthetic oestrogens and two androgens. The oestrogens were diethylstilboestrol (DES) and ethinyl oestradiol (EE_2) and the two androgens were testosterone propionate (TP) and trenbolone (TREN). To get more insight into the mode of toxic action, four assays assessed various biochemical endpoints: mitochondrial activity (17), lysosomal activity (3), cellular protein synthesis (4), and membrane integrity (15). Two cell lines were applied: the mouse fibroblasts Balb/c 3T3, which are most frequently used for the assessment and comparison of general toxicity of chemicals (3) and the human hepatoma HepG2 line, which is preferred over others as this tissue type probably most closely resembles the liver (24). Moreover the prevailing opinion is that the use of human

cell lines increases the predictivity over the alternative of cell lines of animal origin.

Material and Methods

Reagents. Triton X-100, neutral red (NR), dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), bovine calf serum (BCS), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), Coomassie Brilliant Blue R-250 dye, 0.25% trypsin-EDTA solution, and antibiotic solution (10.000 U/mL of penicillin, 10 mg/mL of streptomycin) were purchased from Sigma Aldrich (Poland). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

Drugs. Analytical standards of 17α-ethinyloestradiol (EE_2 at purity >98% (CAS 57-63-6), diethylstilboestrol (DES at purity >99% (CAS 56-53-1), testosterone propionate (TP at purity >97%, (CAS 57-85-2) were purchased from Sigma Aldrich and trenbolone (TREN at purity 99% (CAS 10161-33-8) from Dr. Ehrenstorfer GmbH (Germany).

Cell lines and culture conditions. The Balb/c 3T3 clone A31 cell line (a gift from the Department of Swine Diseases of the National Veterinary Research of Institute in Pulawy) was cultured in Dulbecco`s Modified Eagle`s Medium (DMEM, Gibco, USA). The HepG2 cell line was purchased from the American Type Culture Collection where it is identified under number ATCC HB-8065. These cells were cultured in Minimum Essential Medium Eagle (MEME) (Sigma Aldrich). The media were supplemented with 10% calf serum (Balb/c 3T3), 10% FBS (HepG2), 1% L-glutamine, and 1% penicillin and streptomycin. The cells were maintained in 75 cm^2 cell culture flasks (NUNC) in a humidified incubator at 37°C and an atmosphere of 5% CO_2. The medium was refreshed every two or three days and after reaching 70%–80% confluence, the cells were trypsinised with 0.25% trypsin–0.02% EDTA. Single cell suspensions were prepared and adjusted to a density of 2×10^5 cell/mL (HepG2) and 1×10^5 cell/mL for 24 h and 48 h exposure or 5×10^4 cell/mL for 72 h exposure (Balb/c 3T3). The cell suspension was transferred to 96-well plates (100 µL/well) and incubated for 24 h before the exposure to drugs.

Compound preparation. All drugs were dissolved in DMSO. The final concentration of DMSO in the medium was 0.1% and the same final concentration of the solvent was used in the corresponding control. The medium used for test solutions and in control preparation did not contain serum or antibiotics. All drug solutions in medium were freshly prepared and protected from light.

Exposure to drugs and cytotoxicity assessment. Each drug was tested in eight concentrations: 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 µg/mL. Each concentration was tested in six replicates. The viability/cytotoxicity was assessed after 24, 48, and 72 h of exposure, using four assays as described below.

MTT reduction assay. The assay is based on the reduction of the tetrazolium salt MTT in live cells to the dark formazan product inside the cell *via* mitochondrial dehydrogenases. The amount of formazan generated is directly proportional to the cell density (17). Then cells were incubated with MTT. MTT was dissolved in phosphate buffered saline (PBS) (5 mg/mL), sterilised by filtration through a 0.22 µm Millipore® filter. A volume of 10 µL was added to every microplate well and cultures were incubated for further 4 h at 37°C in a 5% CO_2 humidified atmosphere. The medium was removed and the intracellular formazan crystals were dissolved in 100 µL of DMSO. The plate was shaken for 15 min at room temperature and transferred to a microplate reader (Multiscan RC, Labsystems, USA) to measure the absorbance at 570 nm, using a blank as a reference. The mean optical density (OD) was used to calculate the percentage of cell survival for each dilution of the drug.

Neutral red uptake (NRU assay). The assay is based on the staining of living cells by neutral red, which readily diffuses through the plasma membrane and concentrates in lysosomes (3). After the incubation, the medium containing the drug was removed and cells were washed with PBS. Then 100 µL/well of NR solution (50 µg/mL) was added for 3 h. After this time, the cells were washed again with PBS. The dye from viable cells was released by extraction with a mixture of acetic acid, ethanol, and water. After 10 min of shaking, the absorbance of the dissolved NR was measured at 540 nm using a blank as a reference.

Total protein content (TPC assay). The assay is based upon staining total cellular protein (proliferation) (4). After the incubation, the medium containing the drug was removed and 100 µL of Coomassie Brilliant Blue R-250 dye was added to each well. The plate was shaken for 10 min. Then the stain was removed and the cells were rinsed twice with 100 µL of washing solution (glacial acetic acid/ethanol/water). After that, the washing solution was replaced with 100 µL of the desorbing one (1 M potassium acetate) and the plates were shaken again for 10 min. The absorbance was measured at 595 nm in the microplate reader using a blank as a reference.

LDH release assay. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in all cells. It is rapidly released into the cells upon damage to the plasma membrane (15). The leakage of LDH from the cytoplasm into the surrounding culture medium was monitored using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Poland). Medium in the volume of 100 µL without cells was transferred into corresponding wells of an optically clear 96-well flat bottom microplate and 100 µL of reaction mixture was added to each well. Then, the plates were incubated for 30 min at room temperature in the dark. After that time, 50 µL/well of 1 M HCl was added to stop the reaction. The absorbance was measured at 492 nm in the microplate reader using a blank as reference. The

positive control constituted wells with cells and culture medium with 2% Triton X-100.

Data analysis. The experiments were performed in three independent replications. The results of the tests were expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Dunnett`s *post-hoc* test was applied. The values indicating cytotoxicity concentration (EC_{50}) at three time points (24, 48, and 72 h) were calculated according to Hill's equation (sigmoidal model of concentration-response curve) and expressed as mean ± standard error of the mean (SEM) (n = 3). Statistical evaluation was performed using ANOVA followed by Tukey`s *post-hoc* test. $P \leq 0.05$ were considered statistically significant.

Comparison of cell viability. The results of cell viability were expressed as a % cell viability score (%CVS). The $\%CVS_{50}$ was determined by the number of measurements showing a viability ≥50%. The $CVS_{40/80}$ was calculated as the number of measurements showing viability >80% minus the number of measurements showing viabilty <40% (13).

Results

Figs 1 and 2 present the concentration and time dependent response curves for the drugs tested by four assays (MTT, NRU, TPC, and LDH) on two cell lines (Balb/c 3T3 and HepG2). Concentrations ranged from 0.78 to 100 µg/mL and times were 24, 48, and 72 h. In general cell viability decreased with an increase in concentration and exposure time.

The earliest significant signs of toxicity (after 24 h exposure) and after relatively low doses were observed for DES and EE_2 on both lines when measured by MTT and then NRU assays and HepG2 cells were more sensitive than Balb/c 3T3 cells to both oestrogens in the LDH leakage assay at 72 h (Fig. 1 and Table 1).

In the case of androgens, Balb/c 3T3 cells were more sensitive to TP than HepG2 cells in all assays used but higher concentrations and longer exposure time were required (Fig. 2). Up to the highest TP dose used (100 µg/mL), no effects were observed in the TPC assay in the study on HepG2 cell cultures (Fig. 2). The LDH assay was the most sensitive to TREN. A significant effect at low doses was noted in both cell lines after 72 h exposure (Fig. 2).

For more transparent imaging of the results, EC_{50} values of the drugs were compared at three time points (24 h, 48 h, and 72 h) against both lines (Fig. 3). The values confirmed that the MTT and NRU assays are the most sensitive in the case of DES, EE_2, and TP in the Balb/c 3T3 line and at all time points. These two assays are also the most sensitive in the case of DES in the HepG2 line and at all time points. The lowest cytotoxic effects were observed for TP in the study on HepG2 cells by all methods used (Fig. 3).

To compare cytotoxic potential, the most useful $EC_{50\text{-}72h}$ value was applied for each drug and line (Table 1). Depending on the assay used, $EC_{50\text{-}72h}$ values ranged as follows: DES 1-13.7 µg/mL (Balb/c 3T3) and 3.7-5.2 µg/mL (HepG2); EE_2 2.1-14.3 µg/mL (Balb/c 3T3) and 1.8-7.8 µg/mL (HepG2); TP 14.9-17.5 µg/mL (Balb/c 3T3) and 63.9-100 µg/mL (HepG2); and TREN 11.3-31.4 µg/mL (Balb/c 3T3) and 12.5-59.4 µg/mL (HepG2).

In summary, the results showed that oestrogens were more toxic than androgens and their $EC_{50\text{-}72h}$ values were <10 µg/mL as measured by MTT, NRU, and TPC assays in both lines and by LDH assay in HepG2 cells. As regards TP, there was a great difference between the results obtained in Balb/c 3T3 cells and HepG2 cells. Fibroblasts were much more sensitive than HepG2 cells, represented in the results for $EC_{50\text{-}72h}$ values of 14.9–17.5 µg/mL for the fibroblasts and ≥63.9 µg/mL) for the hepatoma cells (Table 1). For TREN the differences between the lines were less pronounced and the most sensitive assay was LDH release.

To rank the cytotoxic potential of the drugs, cell viability scores (CVSs) were calculated and compared (Table 2). Based on the $\%CVS_{50}$ and $\%CVS_{40/80}$, the order of cytotoxic potential, from the highest to the lowest, was as follows: DES > EE_2 > TP > TREN (Balb/c 3T3) and DES > EE_2 > TREN > TP (HepG2).

Table 1. Comparison of $EC_{50\text{-}72h}$ (µg/mL) for drugs tested on two cell lines and by four assays

Cell line	Assay	DES	EE_2	TP	TREN
Balb/c 3T3	MTT	1.0 ± 0.8	5.6 ± 1.4	14.9 ± 1.7	29.4 ± 0.8
	NRU	4.7 ± 1.4	2.1 ± 0.4	16.7 ± 1.2	26.8 ± 3.6
	TPC	4.7 ± 2.1	5.2 ± 2.2	17.5 ± 1.2	31.4 ± 2.5
	LDH	13.7 ± 2.8	14.3 ± 1.0	15.9 ± 1.9	11.3 ± 2.8
HepG2	MTT	3.9 ± 0.7	6.2 ± 0.9	63.9 ± 5.6	37.4 ± 1.0
	NRU	4.8 ± 0.2	1.8 ± 0.5	72.4 ± 1.8	16.9 ± 4.7
	TPC	5.2 ± 0.4	7.8 ± 1.0	-	59.4 ± 5.5
	LDH	3.7 ± 0.2	1.4 ± 0.1	73.7 ± 2.3	12.5 ± 3.0

DES: dietylostilboestrol; EE_2: 17α- etinyloestradiol; TP: testosterone propionate; TREN: trenbolone. Data are expressed as mean ±SEM (n = 3)

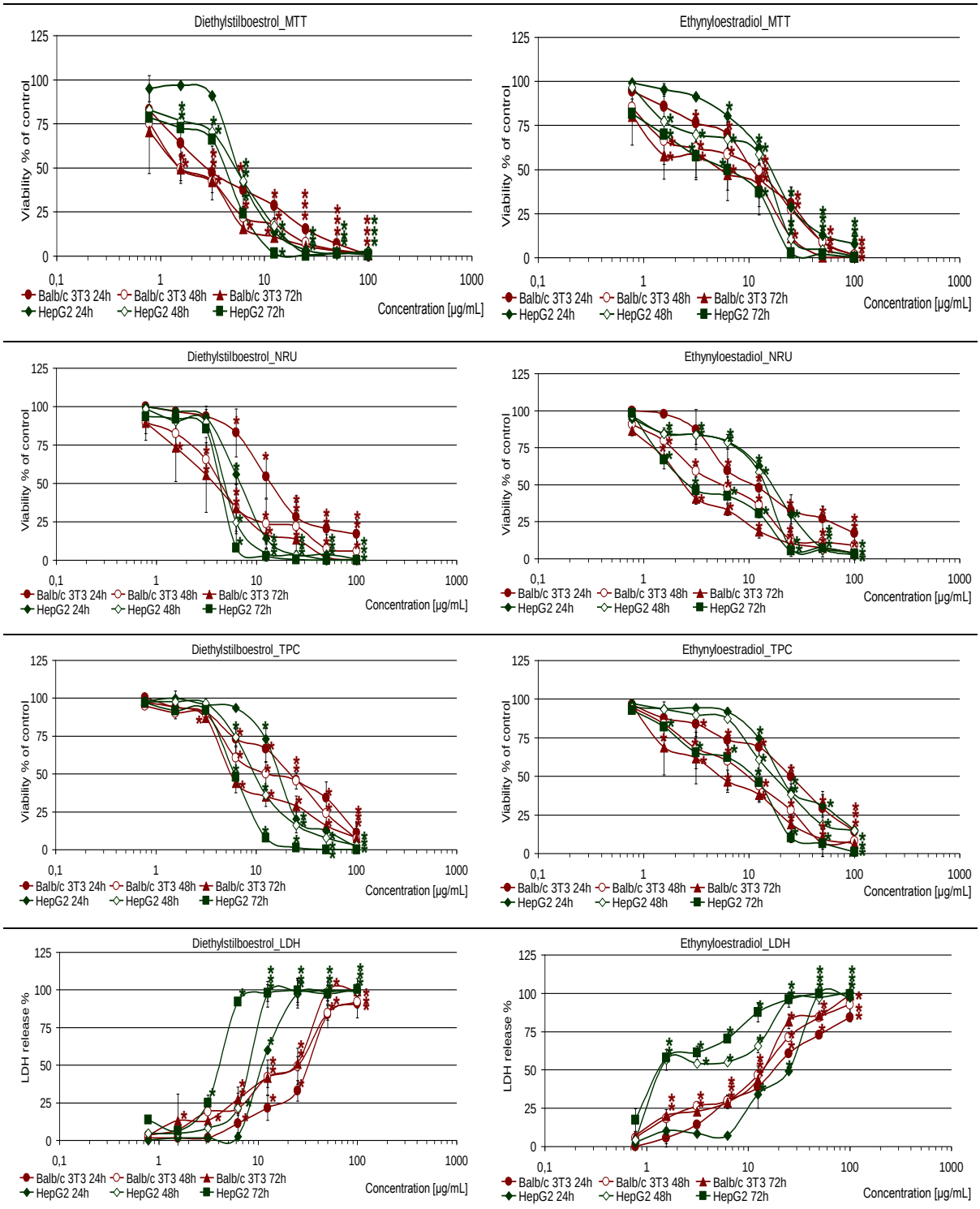


Fig. 1. Concentration- and time-dependent response curves for DES and EE_2 assessed by four assays (MTT, NRU, TPC, LDH release) on Balb/c 3T3 and HepG2 cell lines. Each point represents the mean (±SD) of three independent experiments. *P < 0.05 Dunnett's test

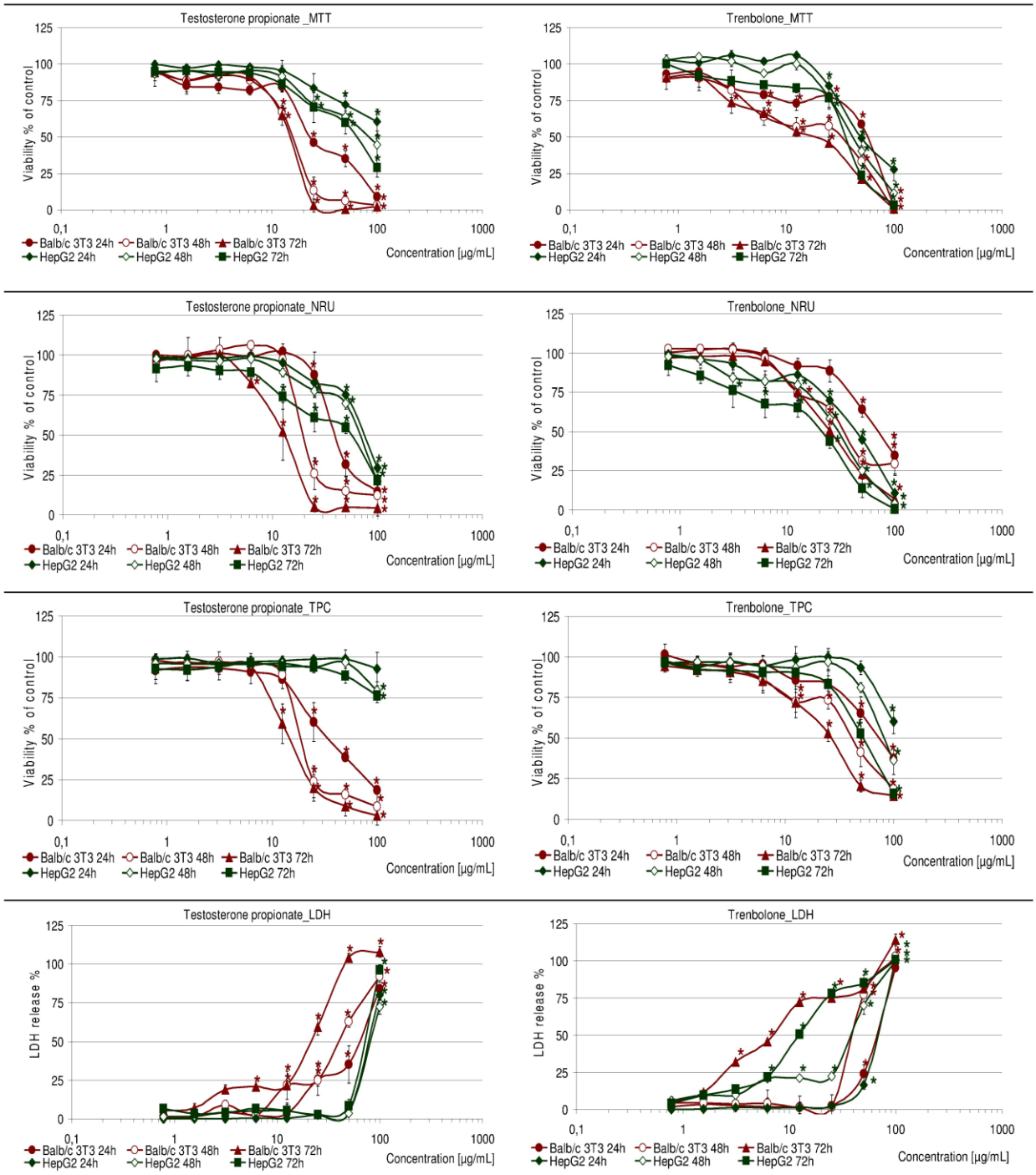


Fig. 2. Concentration- and time-dependent response curves for TP and TREN assessed by four assays (MTT, NRU, TPC, LDH release) on Balb/c 3T3 and HepG2 cell lines. Each point represents the mean (±SD) of three independent experiments. *P < 0.05 Dunnett's test

Table 2. CVS_{50} and $CVS_{40/80}$ percentage of drugs tested on Balb/c 3T3 and HepG2 cell lines

Balb/c 3T3			HepG2		
Drug	% CVS_{50}	% $CVS_{40/80}$	Drug	% CVS_{50}	% $CVS_{40/80}$
DES	45.6	-29.0	DES	50.9	-19.1
EE_2	56.4	-23.0	EE_2	60.2	-7.7
TP	71.5	23.3	TREN	89.6	51.9
TREN	86.3	34.5	TP	92.3	78.9

The results are expressed as a percentage of total number of measurements (288) for each drug (eight concentrations, three exposure times, four assays, three experiments)

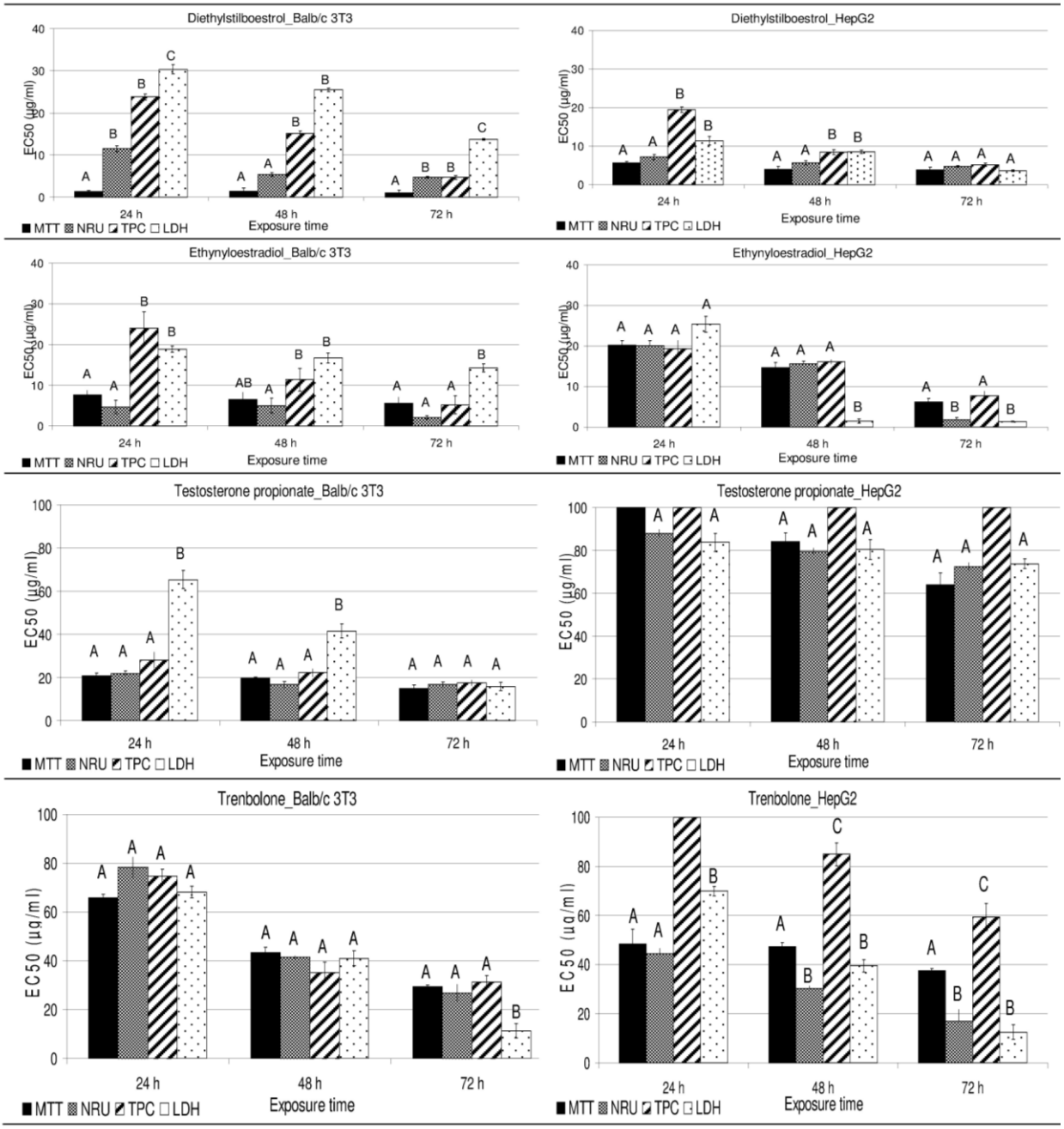


Fig. 3. EC_{50} (µg/mL) values of drugs calculated at 3 time points (24 h, 48 h, and 72 h) when studied on Balb/c 3T3 and HepG2 cell lines by MTT, NRU, TPC and LDH assays. Different letters (A,B,C) denotes significant differences ($P < 0.05$ Tukey's test) between assays at the corresponding time point

Discussion

Among the several biochemical parameters examined in this study, two (mitochondrial and lysosomal activity) were the earliest affected by the cytotoxic concentrations of diethylstilboestrol and ethinyl oestradiol. Comparison of their $EC_{50\text{-}72h}$ values, which ranged from 1 to 5.2 µg/mL, showed almost equal sensitivity of both cell lines in the MTT, NRU, and TPC assays. When the LDH assay was performed, HepG2 cells were approximately threefold (DES) and tenfold (EE_2) more sensitive than Balb/c 3T3.

To some extent our results are in line with the literature data from *in vitro* studies on fish and mammalian cells. The cytotoxic concentrations ($IC_{50\text{-}72h}$) for DES in Channel catfish ovary (CCO) cells were 4.5 µg/mL by MTT assay and 6.5 µg/mL by NR assay and for EE_2 they were 7.2 and 6.8 µg/mL respectively (22). In Chinese hamster ovary (CHO-K1) cells, cytotoxic concentrations ($IC_{50\text{-}72h}$) for DES were 4.2 µg/mL by MTT assay and 6.0 µg/mL by NR

assay and for EE_2 they were 6.2 and >10 µg/mL respectively (22). The mean cytotoxic concentration ($IC_{50\text{-}MTT\text{-}72h}$) of DES against prostate cancer cell lines (I-LN, DU145, PC-3, and LNCaP) was approximately 21 µM (5.6 µg/mL) (23). In murine leukaemia (L1210) and human epithelial carcinoma (KB) lines exposed to DES, 50% inhibition of cell viability was 5.6 µM (1.5 µg/mL) and 9.7 µM (2.6 µg/mL) respectively (18). Konac *et al.* (14) investigated the relationship among the viability, necrosis, and apoptosis rates on human lymphocytes, which were treated with different DES concentrations ranging from 1 to 20 µM (0.26-5.2 µg/mL) for 24, 48, and 72 h. A drastic decrease in the viability rates of the cells for all times of exposure was recorded at the concentration of 5 µM (1.3 µg/mL). When isolated rat hepatocytes were exposed to 17α-ethinyloestradiol, the cytotoxic concentration (LC_{50}) assessed microscopically after a short time (2 h) was 150 µM/L (44.4 µg/mL) (27).

In contrast to oestrogens, for which $EC_{50\text{-}72h}$ values were similar in both lines and in three out of four assays used, TP was approximately fourfold more toxic to Balb/c 3T3 than HepG2 cells. The LDH assay was the most sensitive to TREN, and $EC_{50\text{-}72h}$ were almost the same in both lines, *i.e.* 11.3 and 12.5 µg/mL.

A review of the literature shows that there are few *in vitro* studies concerning TP and TREN. The reduction of mitochondrial and lysosomal activity in myocardial cells was observed after 4 h exposure to TP at the concentration of 100 µM (34.5 µg/mL) (28). In the study on V79 hamster lung fibroblast cells, cytotoxic concentrations ($IC_{50\text{-}18h}$) of TREN and testosterone were 230 µM (62.2 µg/mL) and 285 µM (82.0 µg/mL) respectively (6).

Following the above results the conclusion could be drawn that in the assessment of *in vitro* cytotoxicity, many factors play a role and can influence the rank of toxicity. A series of assays under multiple conditions, including different drug concentrations, exposure times, and different cell lines, gives a useful but staggering volume of information. To summarise the data, the idea of the cell viability score was recently developed by Iwasawa *et al.* (13). This new approach is very useful for comparison of the cytotoxic potential of different drugs. Based on the $\%CVS_{50}$ and $\%CVS_{40/80}$, the oestrogens' and androgens' cytotoxic potential from the highest to the lowest was as follows: DES > EE_2 > TP > TREN (Balb/c 3T3) and DES > EE_2 > TREN > TP (HepG2).

As regards mode of action, the most sensitive endpoint was mitochondrial activity, albeit with minor exceptions. Mitochondria are known to be an important target of oestrogen action (8) and mitochondrial dysfunction is considered a fundamental mechanism in the pathogenesis of several significant toxicities in mammals (1). However, for proper risk assessment, further studies are needed upon this mechanism of pathogenesis, considering different biomarkers.

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