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Relative binding affinity does not predict biological response to xenoestrogens in rat endometrial adenocarcinoma cells

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Abstract

The possible adverse effects of the so-called environmental estrogens have raised considerable concern. Developmental, endocrine and reproductive disorders in wildlife animals have been linked to high exposure to persistent environmental chemicals with estrogen-like activity (xenoestrogens); yet, the potential impact of environmental estrogens on human health is currently under debate also due to lack of data. A battery of in vitro assays exist for identifying compounds with estrogenic activity, but only a few models are available to assess estrogenic potency in a multiparametric analysis. We have recently established the endometrial adenocarcinoma cell line RUCA-I; it enables us to compare estrogenic effects both in vitro and in vivo as these cells are estrogen responsive in vitro and grow estrogen sensitive tumors if inoculated in syngeneic animals in vivo. Here we report in vitro data concerning (a) the relative binding affinity of the selected synthetic chemicals Bisphenol A, nonylphenol, *p-tert*-octylphenol, and *o,p*-DDT to the estrogen receptor of RUCA-I cells and (b) the relative potency of these compounds in inducing increased production of complement C3, an endogenous estrogen-responsive gene. Competitive Scatchard analysis revealed that xenoestrogens bound with an at least 1000-fold lower affinity to the estrogen receptor of RUCA-I cells than estradiol itself, thereby exhibiting the following affinity ranking, estradiol $>>>$ nonylphenol > bisphenol A ~ *p-tert*-octylphenol > *o,p*-DDT. Despite these low binding affinities, bisphenol A, nonylphenol and *p-tert*-octylphenol increased production of complement C3 in a dose dependent manner. Compared with estradiol, only 100-fold higher concentrations were needed for all the compounds to achieve similar levels of induction, except *o,p*-DDT which was by far less potent. Northern blot analyses demonstrated that the increased production of complement C3 was mediated by an increased transcription. In summary, cultured RUCA-I cells represent a valuable endometrial derived model system to assess the relative potencies and the molecular mode of action of environmental estrogens in vitro. Our results further show that no intimate correlation exists between the relative binding affinity and the biological response of these compounds. Therefore, data obtained from single-parametric analyses may result in misleading conclusions. On the other hand, the presented in vitro data will provide us with tools to study the activity of xenoestrogens in vivo and thus carry risk assessment one step further.

Keywords: Responses; Rats; Xenoestrogens

1. Introduction

The controversy over the potential negative impact on the public health of environmental chemicals with estrogenic activity was initially debated only within the scientific community and has now gained tremendous public attention. Generally, there is agreement that in high doses under laboratory conditions or under extreme exposure at highly polluted sites in the natural environment such compounds certainly could affect the endocrine system thereby causing developmental, repro-

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ductive and oncological effects [1–5]. A serious matter of controversy is the question of risks associated with such compounds under realistic exposure scenarios. This controversy stems particularly from the uncertainty of how to assess the risk originating from exogenous chemicals interacting with such a complex system as the endocrine system and its even more complex hierarchial regulation (for review see [6]). At present, at least for some organism it is apparently impossible to define scientifically sound thresholds (no-effect-levels) for these compounds. It has been seriously questioned whether it will be possible to define such a threshold level at all [7].

Applying the in vitro models only, this complex array of scientific problems can not be solved, however in vitro models are useful tools to assess the relative potency of environmental compounds with estrogenic activity [8,9] or to contribute to the elucidation of their molecular mode of action [10].

The aim of the presented study was to assess the relative potency of the selected industrial chemicals in an endometrial derived in vitro model and to investigate whether or not the relative binding affinity of these substances represent a reliable predictor of the biological potency. We tested Bisphenol A (BPA), the environmentally relevant technical mixture of nonylphenol (NP), a pure *p-tert*-octylphenol (*pt*OP) stock and *o,p*-DDT. We selected these substances because humans have been or are still exposed to these chemicals. Human exposure to BPA, first discussed as being estrogenic in 1938 [11], stems from canned food and beverages, particularly liquors [12]. In addition, considerable exposure is due to some dental resins containing BPA [13]. The technical mixture of NP and the pure preparation of *pt*OP are representative alkylphenolic compounds. These alkylphenol polyethoxylates are contained in industrial detergents, domestic detergents (except in UK and most of Europe), some shampoos, shaving foams and other cosmetics, spermicidal lubricant nonoxynol-9 and in pesticide formulations. In addition, they are used as antioxidants in some clear plastics. Processing within sewage treatment plants leads to breakdown of these substances and to the occurance of free alkylphenols [14,15]. Again they were first suspected to mimic estrogenic responses in 1938 [11], however, it was only in 1991 that publications on effects of nonylphenols in MCF-7 breast cancer cells led to health concerns [16]. *o,p*-DDT has been choosen as an organochloric pesticide with a weak estrogenicity, but a high environmental persistance. Many hormone related wildlife effects have been attributed to those organochlorine pesticides (for review see [17,18]).

With these chemicals and under consideration of the experimental restrictions discussed we used the rat endometrial adenocarcinoma cell line RUCA-I, as an experimental model. This cell line, which has been developed in our laboratory, is expressing the ERα only. Further, if cultured on a reconstituted basement membrane we could demonstrate the estrogenic control of the expression of several genes [19–22], amongst them complement C3, a well known estrogen regulated gene in the juvenile rat uterus in vivo [23]. We decided to use estrogenic regulation of this endogenous gene as a molecular endpoint for the determination of the relative potency of the industrial chemicals BPA, NP, *pt*OP and *o,p*-DDT. In parallel, using a competitive binding analysis we assessed the relative binding affinity of these substances to the ERα of RUCA-I cells in order to determine whether we could establish a correlation between the relative binding affinity of these substances and their biological effectiveness in an endometrial model.

2. Materials and methods

2.1. Hormones

The following control substances were used for the treatment of RUCA-I cells, 17-β estradiol (10^{-8}–10^{-7} M, Sigma, Germany) and the pure antagonists ICI 164384 and ICI 182780 (5×10^{-8}–5×10^{-7} M) which were kindly provided by Dr A.E. Wakeling (Zeneca Chemicals, Macclesfield, UK) and by Schering AG, Berlin, Germany. The following xenoestrogens were used as test substances: bisphenol A (BPA; 10^{-8}–10^{-6} M, Bayer AG, Wuppertal, Germany), a technical mixture of nonylphenol (NP; Hüls AG, Marl, Germany), *p-tert*-Octylphenol (*pt*OP; 10^{-9}–10^{-6} M, Hüls AG, Marl, Germany) and *o,p*-DDT (10^{-9}–10^{-6}

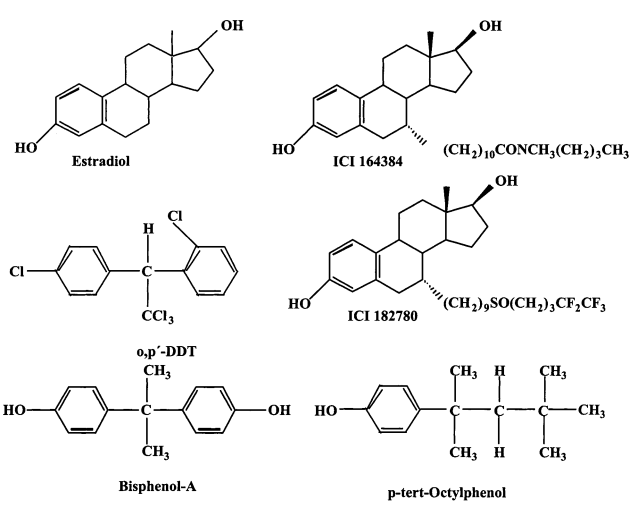

Fig. 1. Substances: structures of the substances used are shown.

M, Sigma-Aldrich, Deissenhofen, Germany). The structure of control substances and of tested industrial chemicals is shown in Fig. 1.

2.2. Cell culture

RUCA-I cells were precultured for 2 days on plastic in the presence of DMEM/F12 medium containing 5% charcoal stripped (DCC) serum. After harvesting 300 000 RUCA-I cells were seeded on top of 230 μl extracellular matrix (Harbour Matrix, TEBU, Germany) per well of a 24-well dish and cultured in the presence of 2 ml of a previously described serum free defined medium (SFDM; [19]) for 80 h, the first 24 h without additional hormonal treatment. Medium was changed daily and hormonal treatment was repeated.

2.3. Hormonal treatment

Following 24-h culture on top of an extracellular matrix RUCA-I cells were treated for 56 h with the estrogenic and antiestrogenic substances described above. Estrogenic and antiestrogenic substances were added from stock solutions in ethanol at a volume of 0.1% of the total culture medium. Controls received ethanol only.

2.4. Metabolic labeling of secretory proteins

After 24–48 h of hormonal treatment secretory proteins were labeled metabolically with ^{35}S-methionine. For this purpose cells were cultured for another 16 h in 150 μl medium per well, whose methionine content was reduced by 90% and which was instead substituted with 200 μCi/ml ^{35}S-methionine. Thereafter, supernatants containing de novo synthesized metabolically labeled secretory proteins were collected and centrifuged (3 min, $3000 \times g$). The supernatant (15 μl) was used to determine the incorporation rate of the radioactive amino acid into TCA precipitable material, the remainder was used for SDS-PAGE electrophoresis.

2.5. Electrophoresis

Metabolically labeled proteins were separated according to the standard protocols using a discontinuous system, nonreducing conditions and 6% polyacrylamide gels. The same amount of radioactive counts of each experimental condition was loaded per lane onto the gel. After electrophoresis gels were fixed in 10% acetic acid, 30% methanol, incubated in En^{3}hance (DuPont, Germany) and dried for 1 h at 80°C under vaccuum. Bands were visualized by autoradiography. Following exposure to a Kodak XAR5 Film, autoradiographs were subjected to semiquantitative evaluation by densitometry.

2.6. Ligand binding assay

The ligand binding affinity of the estrogenic and antiestrogenic substances described above was assessed by a competitive Scatchard analysis as described previously [24,25] using the cytosol of RUCA-I cells. For this purpose RUCA-I cells were cultured on plastic to near confluency in the presence of DMEM/F12 medium containing 5% DCC serum, then trypsinized, pelleted by centrifugation ($800 \times g$, 4°C) and washed twice with PBS. The pellet was resuspended in EORTC buffer [26] and the cells were lysed by three freeze-thaw cycles in liquid nitrogen. The lysed cells were centrifuged for 45 min ($105\,000 \times g$, 4°C) to yield the supernatant (= cytosol). Total binding was assessed by incubation of aliquots (100 μl) of cytosol with eight different concentrations of tritiated estradiol (Amersham, Braunschweig Germany) ranging from 0.05–1 nM for 18 h at 4°C. To compare the binding affinity of tested compounds to that of estradiol 10 pM unlabelled estradiol were additionally added and used to generate a reference. For comparative purposes the ideal concentration of the competitor (either estradiol as a reference or industrial chemicals as test substances) should be titrated to a concentration that competes for 30–70% of the binding activity measured for tritiated estradiol alone. The binding affinity of the unlabelled competitors was determined as described above for unlabelled estradiol except that NP, *pt*OP, BPA or *o*,*p*-DDT were used at concentrations ranging from 10^{-9} to 10^{-9} M to compete for binding of the tritiated estradiol. For the estimation of non-specific binding 200 nM of unlabelled estradiol was added to the incubation mixture. After incubation free steroids were absorbed from samples by treatment with charcoal dextran for 10 min at 4°C. Specific binding was then calculated from total binding minus non specific binding values.

2.7. Northern blot

Northern blotting was performed according to the standard procedures. Cells were cultured and treated as described above. After appropriate culture periods cells from six identically treated wells of a 24-well plate were directly lysed using a guanidinium isothiocyante lysis medium. The cell lysate was subsequently layered on top of a cesium chloride solution and RNA was isolated by centrifugation through the cesium chloride. After UV quantification 10–20 μg of total RNA were separated on a 2.2 M formaldehyde gel, subsequently the RNA was blotted onto a nylon membrane using a vacuum gene pump (Pharmacia, Freiburg, Germany).

Complement C3 mRNA was visualized with a ^{32}P-labelled probe generated from a complement C3 containing plasmid (kindly provided by Dr C.R. Lyttle, Wyeth Ayerst, Radnor, PA). For labeling with radioactivity,

Table 1
Relative binding affinities of xenoestrogens to the ER in cytosolic extracts of RUCA-I cells[a]

Compound	Concentration of the competitor (nM)	Method for evaluation	
		Intersection with the *y*-axis (%)	Slope (%)
Estradiol	0.01	100	100
p-tert-Octylphenol	100–1000	0.013 ± 0.004	0.013 ± 0.005
Nonylphenol	100–1000	0.06 ± 0.019	0.072 ± 0.022
Bisphenol A	100–1000	0.025 ± 0.01	0.027 ± 0.013
o,p-DDT*	10000	0.00017 ± 0.000033	0.00017 ± 0.00053

[a] For all substances values for the highest concentration of the competitor are given which means 1 μM for all substances except *o,p*-DDT (10 μM).

the entire Complement C3-fragment was released by restriction digestion, purified by agarose gel electrophoresis and labeled by random priming using a DNA labeling kit (GIBCO; Eggenstein, Germany). The filter was always first hybridized at 42°C using the ^{32}P-labelled DNA probe. After hybridization filters were washed in two steps. Following hybridization with DNA probes, membranes were first washed for 20 min at room temperature using $3 \times$ SSC containing 0.1% SDS. Thereafter, filters were washed for 20 min at 55°C using $0.5 \times$ SSC containing 0.1% SDS. After stripping the filter was rehybridized with a 18S rRNA probe (kindly provided by Dr N. Schütze, University of Würzburg, Germany) which was used as control. Following exposure to a Kodak XAR5 Film, autoradiographs were subjected to semiquantitative evaluation by densitometry.

3. Results

3.1. Relative binding affinity of selected xenoestrogens to the estrogen receptor of RUCA-I cells

The relative binding affinity of NP, *pt*OP, BPA and *o,p*-DDT to the ERα of RUCA-I cells was measured by a competitive ligand binding assay according to Scatchard. Following linearization of the binding curve by the Scatchard transformation, the apparent binding affinity can directly be read from either the slope of the straight line or by its intersection with the *y*-axis of the graph. The competition of radiolabeled estradiol obtained by 10 pM of unlabelled estradiol was set to 100% and the relative binding affinities of the xenoestrogens at the indicated concentrations were calculated accordingly and expressed as percent binding affinity of estradiol. All xenoestrogens tested exhibited a more than 1000-fold lower binding affinity if compared to natural ligand estradiol (Table 1). Finally, the following affinity ranking estradiol (100%) $>>>$ NP (0.06%) $>$ BPA (0.025%) $\sim$ *pt*OP (0.013%) $>$ *o,p*-DDT (0.00017%) could be established.

3.2. Effects of xenoestrogens on the production of complement C3

Complement C3 is the major estradiol-inducible secretory protein of the juvenile rat uterus in vivo. This feature is preserved in the RUCA-I rat endometrial adenocarcinoma cell line used in this study and can be used as a marker for an estrogenic response. Estradiol at a concentration of 10^{-8} M induces an increase of production of this protein to a value of 300% above levels found in untreated controls, whereas treatment with pure antiestrogen ICI 182780, a positive control for potential antiestrogenicity, reduces complement C3 levels even below that of untreated controls (Fig. 2). In parallel to this control experiment RUCA-I cells were treated with BPA, NP, *pt*OP and *o,p*-DDT in doses ranging from 10^{-9} to 10^{-6} M. Despite the very low relative binding affinities of BPA and ptOP concentrations as low as 10^{-7} M were sufficient to significantly increase production and secretion of complement C3 (Fig. 2). In the case of *o,p*-DDT concentrations of 10^{-6} M induced a significant 30% increase in complement C3 production. At this concentration BPA, NP and *pt*OP levels of secreted complement C3 were elevated to more than 200% above control values, however, levels measurable following estradiol treatment could never been reached completely (Fig. 2).

Mechanistically, the increased production of complement C3 protein is regulated on a transcriptional level by the increase of steady state mRNA levels of complement C3. Like 10^{-8} M estradiol, BPA, NP and *pt*-OP at 10^{-6} M concentrations stimulate a several-fold induction of steady state mRNA-levels of complement C3 (Fig. 3), whereas *o,p*-DDT at a concentration of 10^{-6} M only stimulates a two-fold increase in complement C3 mRNA (Fig. 3). Stimulation of complement C3 production by either of the chemicals tested could be inhibited by a simultaneous treatment of cells with the pure antiestrogen ICI 182780 (Fig. 4), thus clearly demonstrating that induction of gene expression by xenoestrogens is mediated by the ER.

4. Discussion

Exposure of humans to chemicals with potential endocrine disrupting capacity has raised considerable concerns. The number of identified compounds increases almost weekly due to the availability of efficient screening assays like the yeast ER-assay [9]. Yet the potential impact of environmental estrogens on human health is currently under debate. The general uncertainty partially stems from the lack of data, since in contrast to screening assays, only a very limited number of experimental models is available which allow to address either questions towards the understanding of the mechanism of action of environmental estrogens or even enable researcher to conduct a careful risk assessments.

The data on the RUCA-I adenocarcinoma model presented here, demonstrate that we have a sensitive endometrial derived experimental model available that allows to assess the molecular mechanism of action of xenoestrogens in whole cell assay using endogenous parameters. Using this endometrial derived model we are able to assess xenoestrogen function in a tissue specific manner. We measured relative binding affinities or NP, *pt*OP, BPA and *o*,*p*-DDT and correlated them to the relative potency of these substances to induce increased expression of complement C3. This correlation of our data provides some mechanistic clues as to where some of the uncertainties about controversial discussion of a potential risk about xenoestrogens may come from. We provide clearcut evidence for the neces-

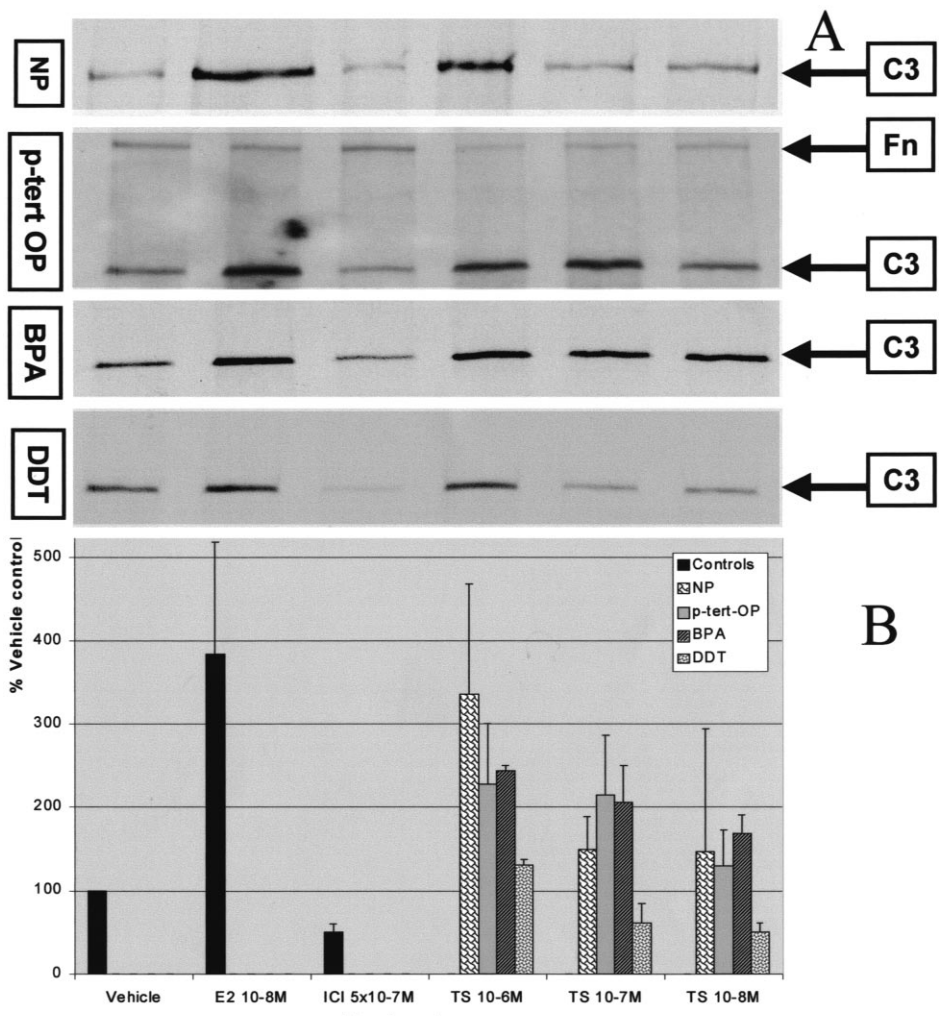

Fig. 2. Regulation of complement C3 protein expression by xenoestrogens: RUCA-I cells were cultured on matrigel in the presence of SFDM. Following treatment of cells with the indicated concentrations of test and control substances de novo synthesized proteins were metabolically labeled with ^{35}S-methionine, cell culture supernatants were collected and subjected to SDS gel electrophoresis under non-reducing conditions. Protein bands were visualized by fluorography (part A) and bands representing complement C3 were subjected to semiquantitative densitometry. Mean values and standard deviations are given in part B of the figure. Abbreviations: E2, estradiol; ICI, ICI182780; C3, Complement C3; Fn, fibronectin; NP, nonylphenol; *p-tert* OP, *p-tert*-octylphenol; BPA, Bisphenol A; DDT, *o*,*p*-DDT; TS, Test substance.

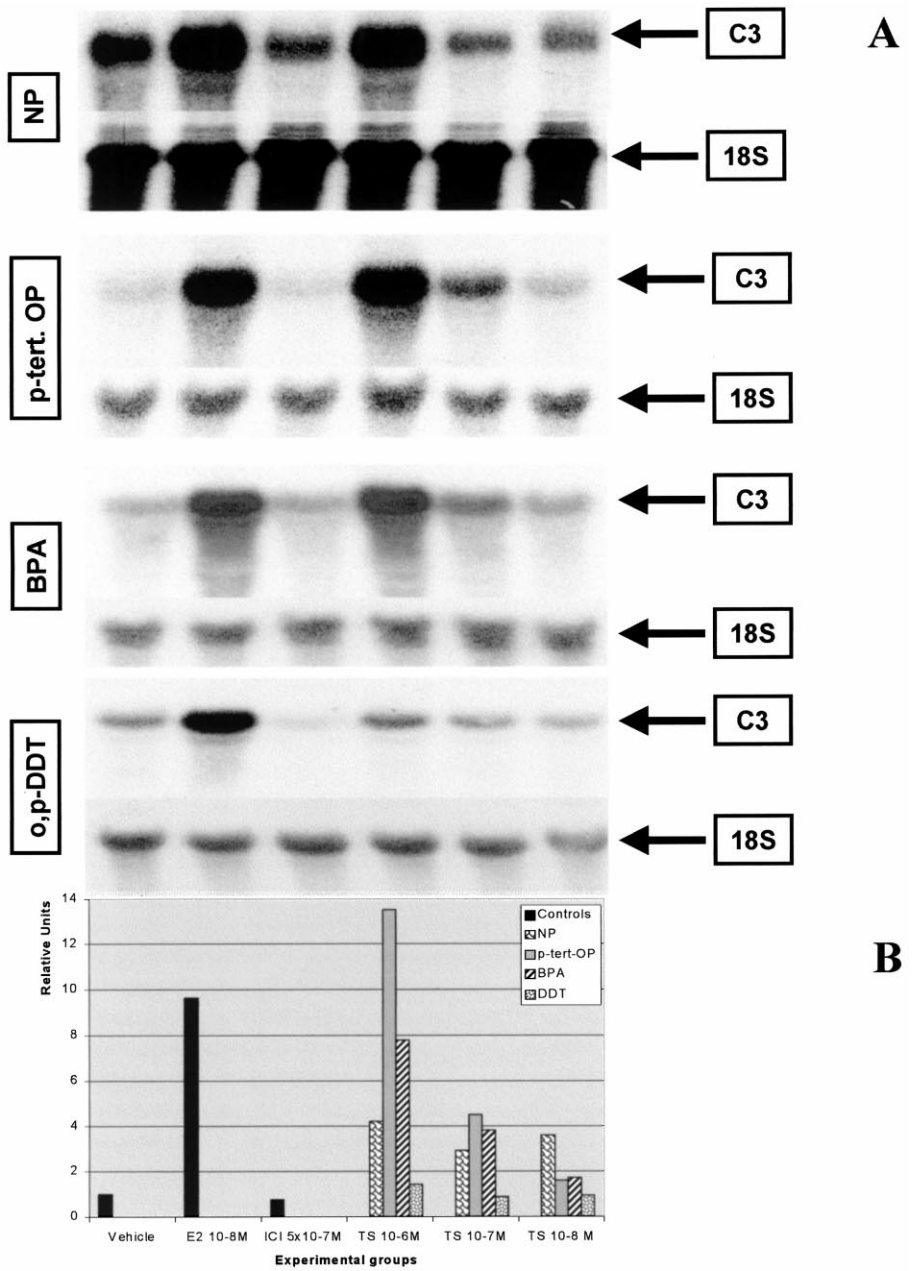

Fig. 3. Regulation of complement C3 mRNA expression by xenoestrogens: RUCA-I cells were cultured on matrigel in the presence of SFDM. Following treatment of cells with the indicated concentrations of test and control substances mRNA was prepared, subjected to denaturing agrarose gel electrophores and blotted. Blots were consecutively hybridized with a complement C3 mRNA and a 18S rRNA specific ^{32}P-labeled probe. Bands were visualized by autoradiography (part A) and bands representing mRNA for complement C3 or 18S rRNA were subjected to semiquantitative densitometry (Part B). Abbreviations are given in the legend of Fig. 2.

sity of a multiparametric analysis as in our hands a biological response of an endometrial adenocarcinoma-cell to a given xenoestrogen can not necessarily be predicted from its relative binding affinity to the ER of the same cell.

The relative binding affinities measured for NP, *pt*OP, BPA and *o*,*p*-DDT are in the order of magnitude of data published in the literature, predominantly on binding to the ER of MCF-7 cells [8,27–29]. However, two specific features of RUCA-I cells have to be taken into consideration when ligand-binding data are discussed. The RUCA-I cell line only expresses the ERα and has, therefore, to be regarded as a pure ERα system, since it is well known, that some xenoestrogens differ in their ability to bind to ERα and ERβ [28,29]. Therefore, no conclusions should be drawn for cells or tissues containing the ERβ. Further, the dissociation constant of estradiol to the ERα of RUCA-I cells is at least one order of magnitude lower than that of estradiol to the ERα of MCF-7 cells [10,25], therefore, lower

concentrations of the ligand may be needed in RUCA-I endometrial adenocarcinoma cells to induce a biological response if compared to doses needed in MCF-7 mammary carcinoma cells.

An estrogenic response traditionally used to assess estrogenicity is the stimulation of proliferation in MCF-7 cells. For NP and BPA this response correlates comparatively well with the relative binding affinity, although the maximal response can already be achieved at concentrations below 50% ligand saturation of the receptor [8,27,30]. Minimal concentrations needed for maximal response in the E-Screen are around 1–3 μM, which in turn is the same order of magnitude of the minimal concentration to obtain half maximal response in yeast-based ER assay [9,27]. In our study we needed 1 μM concentrations to obtain maximal stimulation of complement C3 expression in RUCA-I cells in response to BPA, NP and *pt*OP, however the extend of this stimulation was still below that of estradiol, which was used as a control. However, care has to be taken to discuss any data in comparison to the E-Screen for two reasons, first, the minimal effective concentration described for estradiol in the E-Screen differs more than one order of magnitude (3×10^{-11} M; [8,30,31]). Second, the stimulation of proliferation in MCF-7 cells in an ER dependent pathway requires modulation of several signal transducing molecules [32–34]. Since most of the xenoestrogens exhibit side activities, it may well be that in addition to their action on the ER those molecules interfere with signal transduction cascades which are involved in the regulation of proliferation.

The overall correlation of results from different test systems appears to be more scattered for *o*,*p*-DDT. Whereas relative binding affinity correlates at least to some degree to the response in the E-Screen [8,27], much higher concentrations are needed to obtain half maximal stimulation in the ER yeast based assay [9,27]. In our in vitro test system, to avoid problems of solubility of substance, the highest concentration of *o*,*p*-DDT used was 10^{-6} M. This concentration evoked a 30% increase in complement C3 protein, a response although significant which was far below the responses of estradiol or the other xenoestrogens tested.

In our experiments, the impact of xenoestrogens on gene expression was by far more pronounced as would have been predicted from their relative binding affinity, a feature that has also been described previously for sex hormone binding globulin expression in Hep G2 cells [35]. Alkylphenols and BPA bound with a 1000–4000-fold lower affinity to the ER of RUCA-I cells than estradiol, but only 100-fold higher concentrations were needed to stimulate complement C3 protein to a slightly lower extend than estradiol. The same holds for steady state mRNA levels of complement C3. Significant stimulation could already be detected for 10^{-7} M concentrations of the xenoestrogens NP, *pt-OP* and BPA. Effects detectable for DDT were notably lower meaning that the low binding affinity correlated to the weak biological response. If these data are compared with in vivo experiments, in which an uterotrophic assay has been performed with ovariectomized female

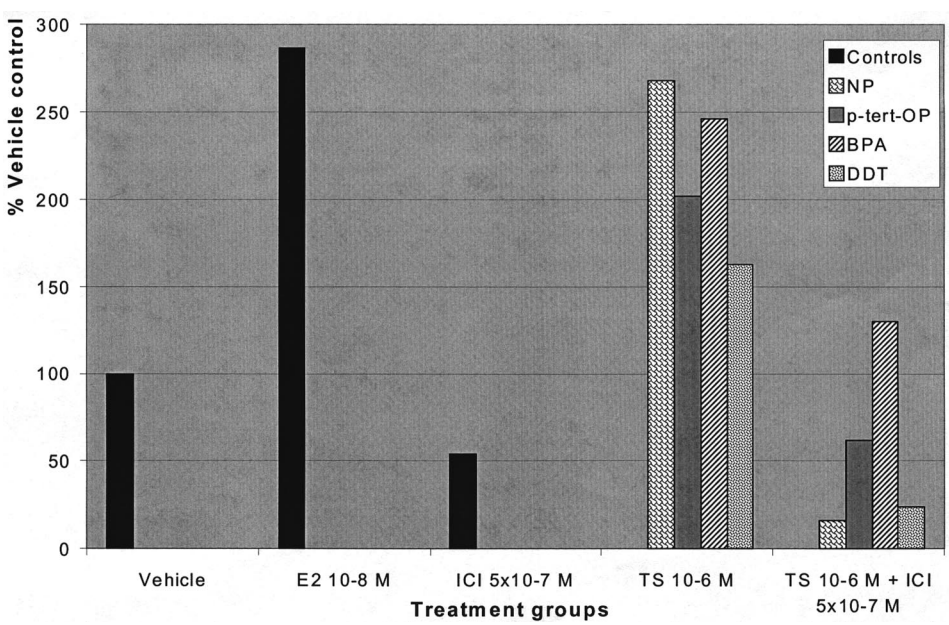

Fig. 4. Inhibition of xenoestrogen-induced expression of complement C3 protein by antiestrogens: RUCA-I cells were cultured on matrigel in the presence of SFDM. Following treatment of cells with the indicated concentrations of test and control substances or combinations thereof, de novo synthesized proteins were metabolically labeled with ^{35}S-methionine, cell culture supernatants were collected and subjected to SDS gel electrophoresis under non-reducing conditions. Protein bands were visualized by fluorography (part A). Bands representing complement C3 were semiquantitatively evaluated by densitometry. Abbreviations are given in the legend of Fig. 2.

rat animals and has been combined with a semiquantitative gene expression analysis, it again turned out that analysis of gene expression is by far more sensitive than uterine wet weight [36]. Oral treatment of animals with BPA did not evoke a significant uterotrophic response, but a significant induction of complement C3 expression. *pt*OP in vivo, as would have been predicted from in vitro studies, behaved as an weak estrogen on both uterine wet weight and on gene expression. Whereas *o,p*-DDT was almost ineffective in the endometrial adenocarcinoma model in vitro, it was the most potent xenoestrogen in vivo and significantly stimulated uterine wet weight in ovariectomized rats, as well as expression of complement C3 mRNA [36]. The power of a gene expression analysis in the endometrium or in our endometrial adenocarcinoma cell line can also be demonstrated by testing phytoestrogens. As previously reported [10,37] daidzein is a weak binder of the ERα of RUCA-I cells but triggers a strong increase in complement C3 expression. This feature is exactly mimicked in the uterotrophic assay in vivo, in which daidzein was inffective to evoke any significant increase in uterine wet weight but most potent stimulated complement C3 gene expression. This finding provides strong evidence to support the validity of the RUCA-I cell line for picking up estrogenicity of xenobiotics. At least in terms of stimulation of gene expression results correlated with those on gene expression in the uterus in vivo. The exception is *o,p*-DDT which exhibited a rather weak estrogenicity in vitro, but very potent stimulated any estrogenic parameter analyzed in vivo [36]. We assume that this is due to the high bioaccumulative capacity of *o,p*-DDT.

In our study in vitro, the relative binding affinity of xenoestrogens did not necessarily correlate with the biological response (complement C3 gene expression). This feature was already apparent in previous studies in our own laboratory on functions of phytoestrogens in RUCA-I endometrial adenocarcinoma cells, where Daidzein and Genistein stimulated complement C3 gene expression to the same extent, although differing 20-fold in their ability to bind to the ERα of RUCA-I cells [10]. This finding suggests that gene expression analysis is by far more sensitive than ligand binding analyis. Similarly, gene expression analysis particularly of complement C3 in vivo turns out to be more reliable and more sensitive predictor for estrogenicity than the investigation of potential estrogens on uterine wet weight. The finding of varying potency of xenoestrogens on different experimental parameters is not unexpected since it is known that the sterical conformation and external accessability of helix 12 of the ERα, harboring several sites for co-enhancer and co-repressors, is depending on the chemical nature of the ligand which occupies the ligand binding domain [38].

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