Validation and Application of a Rapid *in vitro* Assay for Assessing the Estrogenic Potency of Halogenated Phenolic Chemicals

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Objective

The proliferation of human breast cancer cells (MCF-7) was used as a rapid method to determine direct estrogenic effects of environmental chemicals. First, the assay described in the literature was simplified and validated with known xenoestrogens. Secondly, the optimized assay was used to screen some halogenated phenolic chemicals with particular relevance to human exposure for their estrogenic potency.

Introduction

Several investigations conducted in different countries give evidence for an increase in testicular cancer incidence in the last decades, e.g. in Denmark[1]. There is also growing evidence of a substantial decrease in average sperm counts and sperm quality in normal men over the last 20 to 50 years[2,3]. Sharpe and Skakkebaek[4] postulated that environmental contaminants with estrogenic properties could be responsible for these effects. Davis et al.[5] hypothesized that xenoestrogens are a major cause for the steady increase in breast cancer incidence in many industrialized countries over the last decades. Various observations of reproductive disruptions in different wildlife populations of birds, amphibia, and fish support this hypothesis[6].

Although no systematic testing of chemicals for their possible estrogenic properties has been carried out up to now, such estrogenic effects have been shown for more than 40 pesticides, industrial chemicals, and phytoestrogens with a large structural variety[6,7,8]. Thus, it is to be expected that many more xenobiotics with estrogenic properties are present in the environment and biosphere. The rapid screening of a large number of chemicals and samples for their estrogenic potencies requires simple, sensitive, and specific *in vitro* bioassays. Several *in vitro* assays with different endpoints like recombinant reporter gene induction, ligand binding, and cell proliferation have ben developed. However, up to now none of these bioassays has sufficiently been validated in order to be recognized by regulatory authorities.

The primary effect of an estrogen is the stimulation of mitotic activity in tissues of the female genital tract. The MCF-7 cell line, which is derived from a human adenocarcinoma of the breast, is well established as a model of estrogen-responsive cells. Therefore, we simplified in a first step the proliferation assay with the MCF-7 cell line, originally described as "E-screen assay" by Soto et al.[9]. In a second step we could demonstrate for some xenoestrogens that our modified proliferation assay

gives reproducibly similar results like those published by Soto et al.[7], Villalobos et al.[10], and Sonnenschein et al.[13] indicating that the assay could be suitable for the determination of toxicity equivalency factors for environmental estrogens.
We used the proliferation assay for testing some halogenated aromatic chemicals with particular relevance to human exposure due to their amounts of production and/or their spectrum of application, e.g. flame retardants and disinfection agents.

Materials and Methods

Cultivation of the MCF-7 cells and performance of the proliferation experiment was carried out according to the principle method described by Soto et al.[9] with substantial modifications and simplifications. Briefly, the cells are cultivated in DME medium with 5 % fetal calf serum (FCS) in a humidified atmosphere with 5 % CO_2. For starting the proliferation experiment the cells were seeded in 24 well plates with a density of 10,000 cells per well. After 24 hours the medium was changed to the experimental medium, phenolred-free DME medium containing 5 % CD-FCS (FCS treated with charcoal-dextrane to remove all steroids). Four wells per assay are the negative control without hormones. The other wells additionally contain 17ß-estradiol (E2) in concentrations between 10^{-13} M and 10^{-8} M as positive control or the test compounds in final concentrations between 10^{-9} M and 10^{-4} M, respectively. Each concentration of a compound is tested in four wells. The stock solutions of the compounds are prepared in DMSO or ethanol. The final concentration of the solvent in the medium does not exceed 0.1 %.
Five days later the cells in each well are counted during the exponential phase of proliferation. The basic endpoint is the cell number relative to the hormone free control.
The *proliferative effect (PE)* is the ratio of the highest cell number achieved with E2 or the test compound and the cell number of the negative control:

PE = cell number max. (compound) / cell number (negative control)

The estrogenic activity of environmental chemicals is evaluated by determination of:

a) the relative efficacy: the **relative proliferation effect (RPE)**
The RPE compares the maximal proliferation induced by E2 with that induced by a test compound. Thus, full agonists (RPE = 100 %) can be distinguished from partial agonists (RPE < 100 %).

RPE = [PE (test compound) / PE (E2)] * 100 %

b) the relative potency: the **estradiol proliferation equivalent (EE)**
The EE is the quotient of the minimal concentrations of E2 and the test compound required for maximal proliferation.

$$EE = \text{conc. } [E2]_{min} / \text{conc. } [\text{test compound}]_{min}$$

The measurement of total protein content using the sulforhodamine B (SRB) assay[11] and the determination of the mitochondrial metabolic activity using the MTT assay[12] were applied as alternative endpoints as compared to cell counts. Within a certain range both endpoints are proportional to the cell number.

Results

1. Simplification of the proliferation assay
The substitution of human serum by FCS is a simplification and standardization of the proliferation assay. This step does not reduce the sensitivity of the assay. Maximal proliferation is achieved at E2 concentrations of 10^{-10} M. The assay has a detection limit of 10^{-12} M E2 (0.3 pg/ml).
The SRB and especially the MTT assay are much faster and easier to perform than the cell count. The RPE and EE values obtained for a number of tested chemicals did not show significant differences

between the SRB and MTT assay. The use of two different endpoints increases the reliability of the proliferation assay.

2. Validation of the optimized assay

Seven known xenoestrogens were tested in our simplified proliferation assay in three or four independent experiments: 4-tert.-octylphenol 90 % (4-t-OP), technical 4-nonylphenol (4-NP tech.), Bisphenol-A 97 %, Bisphenol-A-dimethacrylate, benzylbutylphthalate 98 % (BBP), di-n-butylphthalate 99 % (DBP), and 3-tert.-butyl-4-hydroxyanisole 98 % (BHA). In Figure 1a the relative efficacy and in Figure 1b the relative potency obtained with our simplified proliferation assay is compared with data recently published in the literature.

Fig. 1a: Relative proliferative effect (RPE) of some xenoestrogens in MCF-7 breast cancer cells compared to literature data

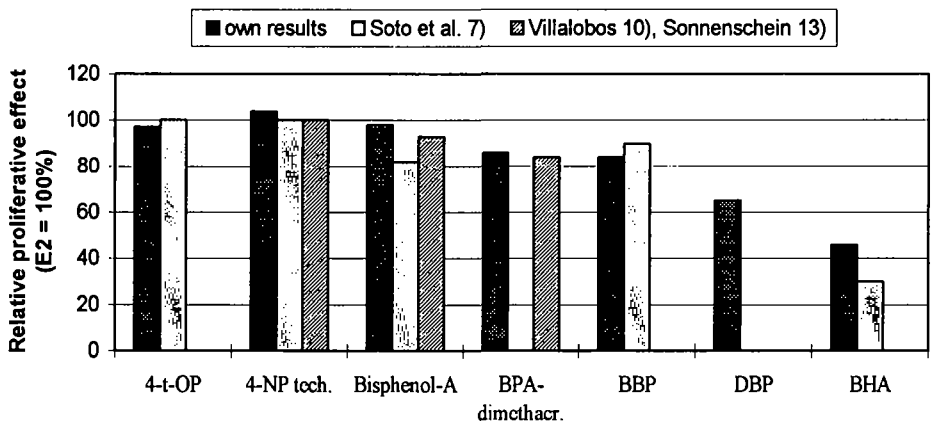
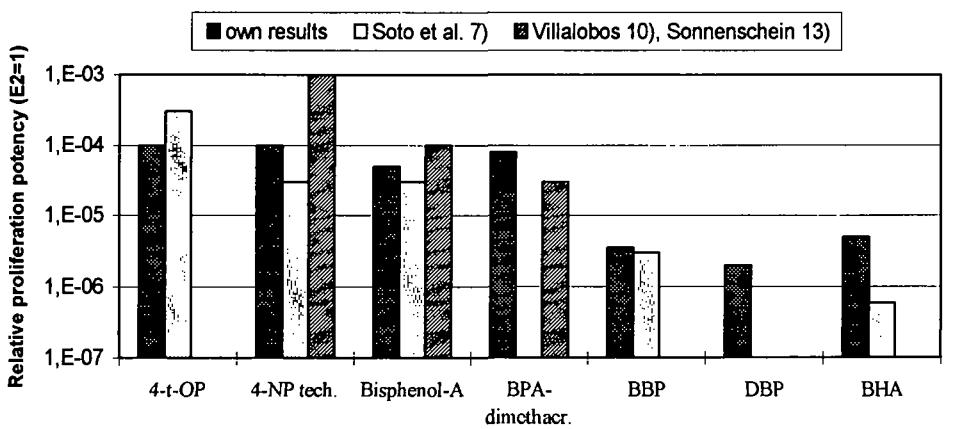


Fig. 1b: Relative proliferation potency (EE) of some xenoestrogens in MCF-7 breast cancer cells compared to literature data



3. Estrogenic potency of halogenated phenolic compounds

The flame retardant Tetrabromo-Bisphenol-A 97 % (Br4BPA) and the three chlorocresols 4-chloro-3-methylphenol 99 % (p-chloro-m-cresol), 4-chloro-2-methylphenol 97 % (p-chloro-o-cresol), and 2-chloro-4-methylphenol 97 % (o-chloro-p-cresol) were tested in our simplified proliferation assay. p-chloro-m-cresol is widely used as an disinfection agent.

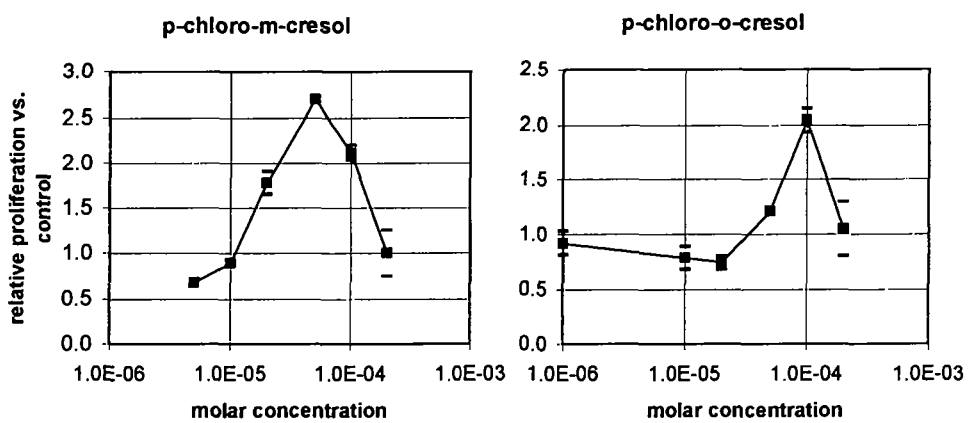
While o-chloro-p-cresol did not show any estrogenic activity, the other three chemicals clearly stimulated proliferation of the MCF-7 cells. The quantitative results are summarized in Table 1. Figure 2 shows the dose-proliferation-curves of the three active compounds compared with that of 17ß-estradiol.

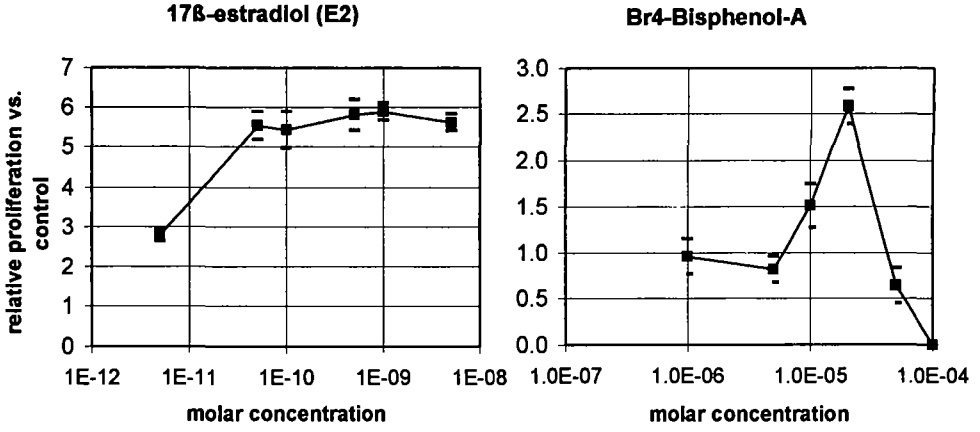
All three active chemicals were tested together with the antiestrogen Tamoxifen in order to determine whether the proliferation is estrogen receptor-mediated or not. Tamoxifen acts via binding to the estrogen receptor. Cotreatment with 5*10-6 M Tamoxifen completely antagonized the proliferative effect of all three compounds.

Tab. 1: Relative proliferative effect (RPE) and relative proliferation potency (EE) of four tested halogenated phenolic compounds in MCF-7 breast cancer cells compared to 17ß-estradiol.

Compound, purity	RPE in % (E2 = 100 %)	EE (E2 = 1)
Br4-Bisphenol-A, 97 %	59	$1*10^{-5}$
p-chloro-m-cresol, 99 %	56	$3*10^{-6}$
p-chloro-o-cresol, 97 %	42	$1*10^{-6}$
o-chloro-p-cresol, 97 %	no effect	no effect

Fig. 2: Dose-response curves of the proliferative effect of E2 and three halogenated phenolic chemicals on MCF-7 breast cancer cells. The diagrams represent mean values and standard deviations of one experiment with four wells per concentration.





Discussion

Irrespective of our simplifying modifications to the MCF-7 proliferation assay the results obtained for seven tested estrogenic chemicals were overall in good agreement with those published by Soto et al.[7], Villalobos et al.[10], and Sonnenschein et al.[13]. The deviations for some substances may mainly be due to different quality of the compounds, as we used chemicals from other distributors or different charges. Thus, it is to be expected that the use of a standardized protocol will deliver satisfying results in an interlaboratory comparison which has not yet been performed.

As the MCF-7 cell line is easy to cultivate and possesses a stable estrogen dependancy compared to other cell lines the proliferative response is a sensitive endpoint suitable for screening chemicals and environmental samples for their direct estrogenic potency. Cotreatment with Tamoxifen or another antiestrogenic compound which binds to the estrogen receptor allows fast confirmation whether the cell proliferation triggered by a chemical is estrogen receptor-mediated or not. The detection limit of the MCF-7 cell line is more than one order of magnitude lower than a recombinant receptor/reporter gene *in vitro* assay using a human estrogen receptor chimeric construct (0.3 vs. 5 pg E2/ml)[14]. The sensitivity is high enough to detect weak estrogenic compounds with potencies six orders of magnitude lower than that of E2.

The estrogenic potencies of the chemicals tested up to now are four to six orders of magnitude lower than that of the endogenous 17ß-estradiol. However, for an appropriate risk assessment the cumulative potency of many of these xenoestrogens has to be taken into account. Further, because of their lipophilicity these compounds are able to cross the placental barrier and thus reach the embryo during the highly sensitive period of organogenesis. In contrast, endogenous steroids are present in the body as bound to proteins and thus barely cross the placental barrier. Recently, Sharpe et al.[15] found that gestational and lactational exposure of rats to 4-octylphenol and benzylbutylphthalate resulted in reduced testicular size and sperm production.

For many known xenoestrogens little or no data are available about the external and especially internal exposure of humans. As no systematic screening of chemicals for their estrogenic and other hormonlike effects has ever been carried out, it is to be expected that more xenoestrogens will be discovered in the future. Therefore, at present a combined risk assessment of xenoestrogens is not yet possible. Our results that out of four tested halogenated phenolic compounds three expressed clear estrogenic activity underline the need for a systematic screening of old and new commercially produced chemicals for their estrogenic properties.

As normally complex mixtures of estrogenic compounds occur in the environment and in organismns toxicity equivalency factors are necessary to assess the estrogenic potency of environmental and biological samples. The estradiol equivalents (EE) obtained from the MCF-7 proliferation assay represent such toxicity equivalency factors for environmental estrogens.

Acknowledgements

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