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Antiestrogenic and Antiprogesteronic Activity of Tire Extracts with Yeast-Based Steroid Hormone Receptor Gene Transcription Assay

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It has been known for many decades that chemicals can act as weak hormones. Environmental endocrine disruption in wildlife species and humans has been paid particular attention and great efforts have been undertaken to identify such environmental endocrine disrupting chemicals (EDCs) (Zacharewski, 1997; USEPA, 1997). Bioassays based on genetically modified yeast strains are widely used because of their easy handling, suitability for large-scale screening, high sensitivity and low costs (Arnold et al. 1996; Gaido et al. 1997). The applicability of yeast estrogen bioassay for the analysis of environmental samples has been evaluated by Rehmann et al (1999). Since pollution is presented seldom with pure compounds but complicated mixture of many kinds of contaminants, the application for environmental samples can be very important to estimate actual environmental effects. Those assays could suffer from some disadvantages such as pronounced background activity of the reporter gene product and ignoring bioavailability and metabolism of the compounds. They still serve as a useful component of an *in vitro – in vivo* strategy to assess the effects of chemicals on endocrine function (Gaido et al. 1997; Zacharewski, 1998).

The annual total global production of rubber material was estimated to be 16-17 mt, of which 65% were used for tire production (Holst et al. 1998; Jang et al. 1998). Environmental problems presented by tires are focused on waste treatment because they don't degrade easily but seldom concern the abrasion of tires. The fine dust is spread out into the environment and possibly inhaled by man. In Germany, the abrasion of tires amounts to about 60,000 to 65,000 t per year (Henkelmann et al. 2001). The components of tires include elastomers, carbon blacks, pigments, and other chemicals such as vulcanising agents, accelerators, plasticizers and initiators (USEPA, 1995). Polyaromatic hydrocarbons (PAHs) are contaminants in the tire production (Locati et al. 1979). High concentration of PAHs can also be found in the recycling oil derived from waste tires (Cunliffe et al. 1998). Henkelmann et al (2001) found that the concentration of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) and polychlorinated biphenyls (PCBs) were 0.3 to 181 pg/g WHO-TEQ and 1.3 to 10.1 pg/g WHO-TEQ respectively. Some of the contaminants have been proved to be endocrine disruptors (Joseph, 1997).

In order to investigate the endocrine disrupting characters of tire extracts, tires

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produced by several tire manufactures were collected. Two yeast constructs carrying hormone binding domain of human estrogen receptor (ER) or progesterone receptor (PR) were performed to analyse estrogenic, antiestrogenic, progesteronic and antiprogesteronic activity of the tire extracts.

MATERIALS AND METHODS

17β-estradiol (E_2) and progesterone were obtained from Aldrich, Steinheim, Germany. All other chemicals of the highest purity available were purchased from Merck, Darmstadt, Germany and Fluka, Neu-Ulm, Germany. Yeast Nitrogen Base and Bacto agar were obtained from Difco, Augsburg, Germany. DMSO-dissolved 17β-estradiol and progesterone were stored at 4℃ in a refrigerator in the dark in tightly closed glass bottles.

12 tires with different types were collected. The tires were frozen in liquid nitrogen and pulverized in a rotor mill. Aliquots of 5 g were quantitatively extracted in a Soxhlet apparatus by 200 mL cycle-Hexane:Aceton(1:1) for 24 hr. The extracts were evaporated to dryness using a rotary evaporator at 60℃, 800 mbar for Aceton and then 60℃, 550 mbar for cycle-Hexane. The residues were dissolved in 2 mL DMSO and diluted to a series of concentrations as 1 g/mL, 100 mg/mL, 10 mg/mL till to 10 μg/mL. The samples were stored at 4℃ until required.

The yeast strain for estrogen bioassay was a kind gift of D. Picard (Louvion et al. 1993). The yeast strain for progesterone bioassay was a kind gift of K. W. Gaido (Gaido et al. 1997). They both grew at 30℃, 130 rpm in Erlenmeyer flasks with one notch. The medium for yeast with ER is SC-medium. For yeast with PR the SC-medium without histidine and leucine, plus tryptophane should be used. Stock cultures were prepared from exponentially growing cultures by adding DMSO up to a final concentration of 15%(v/v) and stored in Eppendof reaction vessels (ERVs) at –80℃.

The exponentially growing overnight cultures were diluted with medium to OD_{600} of 0.75 for estrogen bioassay and 0.15 for progesterone bioassay. Aliquots of 10 mL were distributed into 100 mL-Erlenmeyer flasks with one notch and received 100 μL of DMSO (negative controls), 100 μL of DMSO-dissolved E_2 (a concentration series from 10 μM to 1 nM) and 100 μL of DMSO-dissolved progesterone (a concentration series from 0.1 M to 10 nM) for the respective strains (positive controls) or 100 μL of DMSO-dissolved samples. For antiestrogen and antiprogesterone bioassay, the positive controls were 100 μL of DMSO dissolved E_2 (500 nM) and 100 μL of DMSO-dissolved progesterone (3 μM) and the samples were 50 μL of DMSO-dissolved samples mixed with 50 μL of E2 (1 μM) or progesterone (6 μM) for respective bioassays. Test cultures were incubated at 30℃, 130 rpm. For positive and anti bioassays the cultures were incubated for 30 min and 2 hr for samples. Triplicates of each test cultures should be done to obtain statistically reliable results. Growth was determined afterwards

by measuring the OD_{600} of the test cultures (for estrogen strain the test cultures should be diluted fivefold before measurement). The determination for β-galactosidase activity has been described (Rehmann et al. 1999). In brief: The ERVs containing the 200 µL aliquots of respective test cultures received 620 µL of Z-buffer ($Na_2HPO_4.7H_2O$) mixed with SDS solution (3.5 mM) and 50 µL chloroform. The carefully mixed, closed ERVs (25 sec, Vortex mixer, highest speed) are pre-incubated in the shaking water bath at 30℃ and medium speed for 5 min. The enzyme reaction was started by adding 200 µL of o-nitrophenyl-β-D-galacto-pyrannoside (13.3 mM, dissolved in Z-buffer). Then the carefully mixed ERVs (heavy up side down shaking for a few seconds) were returned to the water bath shaker. The assays were incubated as same as pre-incubated until the positive controls turn significantly yellow within 30 min. Sample induced assays should be continued until the samples turn significantly yellow but maximally five times longer than the positive controls. For antiestrogen and antiprogesterone bioassay the incubation time was as same as the positive controls. After stopping the reaction by adding 500 µL of Na_2CO_3 (1 M), cell debris was pelleted by centrifugation (25,500 g, 15 min) and the absorbance of the supernatants at 420 nm (OD_{420}) was measured.

The dose-response data were fitted by the following equation using the least squares method:

$$y = \frac{A - D}{1 + (C/x)^B} + D$$

The four parameter logistic equation delivers a dose response curve fitted to the measured values as well as the opportunity to calculated EC_{50} values (the concentration reducing half of the maximum effect) for anti assays.

RESULTS AND DISCUSSION

The sensitivities of the yeast constructs were investigated by recording dose response curves for E_2 and progesterone respectively. 5 nM E_2 and 30 nM of progesterone were selected for anti activity tests because those concentrations induced 80% to 100% of the maximum responses (83% and 93% respectively) and any of the anti activity could be detected from the decline of the response. To avoid the effects of toxicity of the tire extracts, OD_{600} of cultures were compared to evaluate the growth of the yeast. No statistically difference (95% confidence interval) could be found if the concentration was less than 1 mg/mL.

There was no detectable estrogenic or progesteronic activity for the tire extracts with those bioassays below a dose of 10 mg/mL but all of them showed obvious both anti-estrogenic and anti-progesteronic activity (Figure 1 and Figure 2). The EC_{50}-values of anti-estrogenic activity ranged from 0.07 to 9.90 mg/mL and 0.07 to 0.38 mg/mL for anti-progesteronic activity (Table 1).

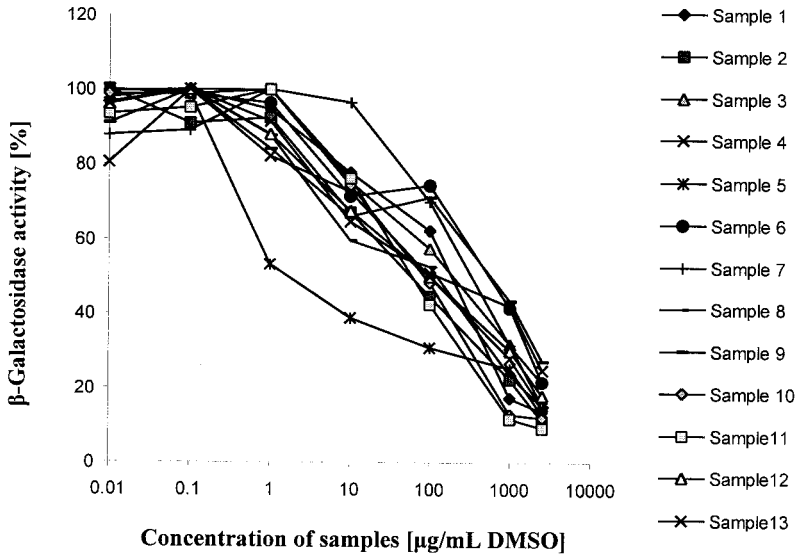

Figure 1. Dose response curves for the DMSO-dissolvable extracts of tire in the yeast anti-estrogen bioassay. Values are presented as the rate of β-galactosidase activity divided by the maximum response.

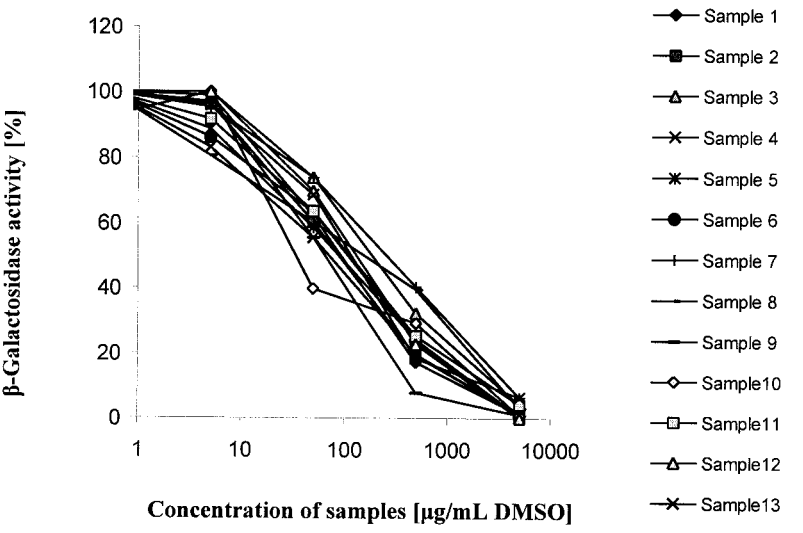

Figure 2. Dose response curves for the DMSO-dissolvable extracts of tire in the yeast anti-progesterone bioassay. Values are presented as the rate of β-galactosidase activity divided by the maximum response.

Table 1. EC_{50}s of anti-estrogenic and anti-progesteronic activity of tire extracts for yeast-based estrogen and progesterone receptor assay

Sample no	Anti-estrogenic EC_{50} (mg/mL DMSO)	Anti-progesteronic EC_{50} (mg/mL DMSO)
Sample 1	9.90	0.13
Sample 2	2.85	0.29
Sample 3	6.82	0.11
Sample 4	6.37	0.14
Sample 5	0.07	0.16
Sample 6	3.67	0.38
Sample 7	1.51	0.23
Sample 8	3.06	0.08
Sample 9	0.99	0.07
Sample 10	0.79	0.17
Sample 11	2.19	0.19
Sample 12	1.99	0.15
Sample 13	1.93	0.34

Several classes of chemicals can present antiestrogenic or antiprogesteronic activity in this screen system. These include:

(a) declining endogenous estrogen or progesterone level by reacting with them,
(b) competitive antagonists that can bind to the estrogen or progesterone receptor without activating them, and simultaneously prevent binding of endogenous estrogen and progesterone,
(c) inhibitors of β-galactosidase transcription or synthesis.

A mere yeast-based bioassay can't find which one causes the anti activities. Further binding and *in vivo* bioassays are needed to determine which one is the reason for the anti activity. If they are induced by (a) or (b) the tire extracts can be defined as EDCs.

Besides PAHs, we have also detected PCBs, PCDD/F at reasonable high levels (Henkelmann et al. 2001). They have been considered as persistent organic pollutants (POPs) and EDCs. So the tire dust is a kind of potential environmental endocrine disruptors. This kind of chemicals causes antiestrogenic effects indirectly through the aryl hydrocarbon receptor (AhR) (Safe et al. 1995). The antiestrogenic effects of them would not be detected in the yeast-based steroid hormone receptor gene transcription assay. So the effects may be underestimated.

The occurrence of concentration of tires high enough for eliciting effects is seldom in the actual environment. But for the occupation such as in rubber or tire industry and so many components and contaminants are belong to POPs, The effects should arouse our attention and further research should be performed.

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REFERENCES

Arnold SF, Robinson MK, Notides AC, Guillette LJ, McLachlan JA, 1996. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environ Health Perspect 104:544-548

Cunliffe AM, Williams PT, 1998. Composition of oil derived from the batch of pyrolysis of tires. J Anal Appl Pyrol 44: 131-152

Gaido KW, Leonard LS, Lovell S, Gould JC, Babaï D, Portier CJ, McDonnell DP, 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143:205-212

Henkelmann B, Wottgen T, Kotalik J, Kettrup A, Schramm KW, 2001. Concentrations of Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in tyres. Organohalogen Compounds 50:363-365

Holst O, Stenberg B, Christiansson M, 1998. Biotechnological possibilitites for waste tyre-rubber treatment. Biodegradation 9:301-310

Jang JW, Yoo TS, Oh JH, Iwasaki I, 1998. Discarded tyre recycling practices in the United States, Japan and Korea. Resour Conserv Recy 22:1-14

Joseph S, 1997. Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: Relationship to carcinogenicity. Chemosphere 34:835-848

Locati G, Fantuzzi A, Consonni G, Li GI, Bonomi G, 1979. Identification of polycyclic aromatic hydrocarbons in carbon black with reference to cancerogenic risk in tire production. American Ind Hyg Assoc J 40:644-652

Louvion J, Havaux-Copf B, Picard D, 1993. Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. Gene 131:129-134

Rehmann K, Schramm K, Kettrup A, 1999. Applicability of a yeast oestrogen screen for the detection of oestrogen-like activities in environmental samples. Chemosphere 38:3303-3312

Safe S, Krishnan V, 1995. Chlorinated hydrocarbons: estrogens and antiestrogens. Toxicol Lett 82/83:731-736

USEPA, 1995. Rubber and miscellaneous plastic products, US Environmental Protection Agency, Office of Compliance, Office of Enforcement and Compliance Assurance, Washington, D.C., EPA/310-R-95-016

USEPA, 1997. Special report on environmental endocrine disruption: an effects assessment and analysis. US Environmental Protection Agency, Office of Research and Development, Risk Assessment Forum, Washington, D.C., EPA/630/R-96/012

Zacharewski T, 1997. In vitro bioassays for assessing estrogenic substances. Environ Sci Technol 31:613-623

Zacharewski T, 1998. Identification and assessment of endocrine disruptors: Limitations of in vivo and in vitro Assays. Environ Health Perspect 106:577-582