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Interactions of nitromusk parent compounds and their amino-metabolites with the estrogen receptors of rainbow trout (*Oncorhynchus mykiss*) and the South African clawed frog (*Xenopus laevis*)

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Abstract

Nitromusks, musk xylene (MX), musk ketone (MK) and musk moskene (MM) are synthetic fragrances. 4-Amino-MX (4X), 2-amino-MX (2X) and 2-amino-MK (2K) are nitromusk metabolites formed during the sewage treatment process and have been detected in effluent and surface water at concentrations four to 40 times higher than their parent compounds. To date, data to the aquatic toxicity of nitromusk compounds are limited to the parent compounds and the determination of acute and subacute effects in aquatic organisms. No data are available regarding the potential endocrine modulating effects of these compounds and/or their metabolites in aquatic organisms. Therefore, the competitive binding capability of nitromusks and their metabolites to the estrogen receptors (ER) in rainbow trout and xenopus was investigated. No binding of MX, MK and MM to the ER of either species was observed. In contrast, binding to the ER was observed for 4X, 2X and 2K in both species. The IC_{50} (competitive binding at the ER) of 2X in rainbow trout was 1.3 ± 1.1 mM. In contrast, 4X, 2X and 2K bound to the xenopus ER with an IC_{50} of 30.8 ± 28.5, 12.9 ± 10.3 and 70.1 ± 88.3 μM, respectively.

Keywords: Nitromusks; Nitromusk metabolites; Hepatic estrogen receptor; Competitive binding; Fish; Amphibian

1. Introduction

Musk xylene (MX; 1-*tert*-butyl-3, 5-dimethyl-2, 4, 6-trinitrobenzene), musk ketone (MK; 4-acetyl-1-*tert*-butyl-3, 5-dimethyl-2, 6-dinitrobenzene) and musk moskene (MM; 4, 6-dinitro-1, 1, 3, 3, 5-pentamethylindane) are synthetic nitro-aromatic fragrances used in products such as perfumes, cosmetics, lotions, detergents, etc. (Sommer, 1993; Tas and van de Plassche, 1996). The worldwide annual production of nitromusks has been estimated to be approximately 2000 metric tons in 1988 (Gatermann et al., 1998). Nitromusks were

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first detected in 1981 as a new group of environmental pollutants present in the aquatic biota (Yamagishi et al., 1981). Most likely as a result of their lipophilicity (K_{ow}: 4.9 and 4.3 of MX and MK, respectively), planarity of the aromatic ring structure and therefore high particle affinity, between 60 and 80% of the incoming nitromusk mass in the raw sewage can be eliminated during sewage treatment (Tas and van de Plassche, 1996). Consequently, the aquatic environment is continuously exposed to small concentrations of these nitromusks. This is reflected by the fact that the current contamination of surface waters with nitromusks ranges between nanograms and micrograms per liter (Hahn, 1993; Eschke et al., 1994; Gatermann et al., 1995). Nitromusks have been demonstrated to be environmentally persistent (Tas and van de Plassche, 1996). In conjunction with their inherent lipophilicity, it is therefore not surprising that nitromusks (parent compounds) have been detected in aquatic organisms, at concentrations ranging between micrograms to milligrams per kilogram lipid (Hahn, 1993; Rimkus and Wolf, 1993; Eschke et al., 1994).

Transformation of nitromusks during sewage treatment entails a reduction of nitro groups in the *para* and/or *ortho* position (4-amino-MX and 2-amino-MX) and in the *ortho* position (2-amino-MK) of musk xylene and musk ketone, respectively (Gatermann et al., 1998). While these metabolites were not found in raw sewage, they were shown to be present in the sewage effluent and consequently in the surface waters. The fact that metabolite concentrations are four to 40 times higher than parent compound concentrations in surface waters suggests that nitro reduction is a major pathway of nitromusk metabolism during sewage treatment. Nitro reduction to the amine renders the metabolites more polar, thus approximately one order of magnitude less lipophilic (K_{ow} of 4X: 3.8), and therefore more water-soluble. Higher water solubility increases the bioavailability of these metabolites to aquatic organisms. Consequently, contamination analysis of aquatic organisms revealed that in some cases, these metabolites were present at higher concentrations (μg/kg lipid) than their respective parent compounds (Rimkus et al., 1998, 1999). All of the data presented so far demonstrate that nitromusks exert negligible acute toxicity on aquatic organisms even at the highest possible soluble concentrations tested (Tas and van de Plassche, 1996; Chou and Dietrich, 1999). However, no data are available regarding the potential endocrine modulating activity of these compounds and/or their metabolites in aquatic organisms. Indeed, in view of the numerous highly lipophilic aromatic compounds demonstrated as having endocrine modulating capacities (Soto et al., 1995; Klotz et al., 1996; Danzo, 1997; Nagel et al., 1997; Nimrod and Benson, 1997), the fact that nitromusks and their metabolites are lipophilic with an aromatic structure, accumulate in aquatic organisms and represent an important group of environmentally persistent contaminants, demanded investigation of their possible endocrine modulating effects. Therefore, in this study, the competitive binding capability of nitromusks and of their metabolites to the estrogen receptors (ER) of rainbow trout (*Oncorhynchus mykiss*) and the South African clawed frog (*Xenopus laevis*) was investigated.

2. Materials and methods

2.1. Chemicals

MX (CAS # 81-15-2), MK (CAS # 81-14-1) and MM (CAS # 116-66-5) were obtained from Givaudan-Roure (Clifton, NJ). 4-NH_2-musk xylene (4X), 2-NH_2-musk xylene (2X) and 2-NH_2-musk ketone (2K) were generously provided by Dr Rimkus (Neumünster, Germany). ^{3}H-17β-estradiol (^{3}H-E_2, specific activity: 48–52 Ci/mmol) was obtained from Amersham (Braunschweig, Germany). Tris was purchased from ICN Biochemical (OH), sodium molybdate, EDTA, 17β-estradiol (E_2), 2, 2-bis-(4-hydroxyphenyl)-propan (bisphenol A), phenylmethylsulfonyl fluoride (PMSF) and activated charcoal Norit A® (4–7 μm) were obtained from Sigma (St Louis, MO). Monothioglycerol was purchased from Aldrich (Steinheim, Germany), dithiothreitol was obtained from Serva (Heidelberg, Germany) and Dextran T70 was purchased from Pharmacia Biotech (Uppsala, Sweden).

2.2. Animals, housing and handling

2.2.1. Rainbow trout (O. mykiss)

One- and 2-year old female rainbow trout (0.5–0.6 and 0.8–1 kg b.w., respectively) were purchased from a local fish hatchery in Konstanz, Germany. The fish were kept in a 100-l tank with a constant flow of active charcoal filtered dechlorinated tap water, with an average temperature of 14 ± 2°C, a 14:10-h light:dark cycle and fed ad libitum until being killed. All experiments were carried out between April and August, 1998.

2.2.2. Clawed frog (X. laevis)

One-year-old female xenopus (80–100 g b.w.) were taken from the animal research center (TFA) at the University of Konstanz, Germany. All xenopus were kept in large aquaria (1000 l) with a constant flow of active charcoal filtered dechlorinated tap water, with an average temperature of 20 ± 2°C, a 14:10-h light:dark cycle and fed ad libitum until being killed.

2.3. Hepatic estrogen receptor preparation

Animals were killed by blow to the head. The livers of the respective animals were immediately excised, placed into ice-cold Tris–HCl buffer (see below), minced finely with scissors, washed with fresh buffer and homogenized in four volumes (1:4 w/v) of buffer (see below) with a motor-driven teflon-tip pestle in a glass homogenizer. The homogenate was first centrifuged at $10\,000 \times g$ at 4°C for 10 min. The supernatant was removed without lipid layer and centrifuged further at $100\,000 \times g$ at 2°C for 90 min. The resulting supernatant was designated the 'cytosolic' fraction and was composed of the nucleosolic and cytosolic proteins. This fraction was either used immediately (1-year-old trout) in the characterization and/or competition assay or stored at -80°C for later use (2-year-old trout and xenopus).

2.3.1. Fresh receptor preparations — single animal preparations (rainbow trout)

Hepatic ER fractions were prepared from individual 1-year-old female rainbow trout (500–600 g, b.w.). The preparation followed the procedures described above and the buffer was composed of 20 mM Tris–HCl, 250 mM sucrose, 10 mM sodium molybdate and 5 mM dithiothreitol (pH: 7.4).

2.3.2. Frozen receptor preparations — pooled animal preparations (rainbow trout and xenopus)

Livers of 12 2-year-old female rainbow trout (800–1000 g w.w.) and of ten female xenopus, respectively, were excised, pooled, minced and washed in ice-cold buffer composed of 10 mM Tris–HCl, 1.5 mM EDTA, 20 mM sodium molybdate, 10% glycerol (pH 7.4). The preparation followed the procedures described above and the wash buffer supplemented with monothioglycerol (1 ml/l) and saturated ethanol solution of PMSF (1 ml/l) was used for homogenization.

2.3.3. Stripped versus non-stripped receptor preparations (rainbow trout)

Due to the fact that preliminary experiments indicated a low K_d for the ER of rainbow trout, the question was asked whether charcoal-dextran stripping of the ER preparations would increase the K_d and thus enhance detection of xenobiotic competitive binding. Charcoal-dextran pellets were prepared via centrifugation of a Tris–HCl solution containing 0.375% dextran and 3.75% charcoal followed by decanting the supernatant. The supernatant of fresh ER preparations from individual 1-year-old trout after $10\,000 \times g$ was added to the charcoal-dextran pellet, the mixture vortexed, incubated for 15 min at 4°C, and finally centrifuged at $100\,000 \times g$ for 90 min at 2°C.

Charcoal-dextran stripped and non-stripped ER fractions were incubated parallel to determine respective K_ds and N_{max}s.

2.4. Cytosolic fraction protein concentrations

The protein content of the receptor preparations was determined using a kit employing a modified Bradford method (Bio-Rad Laboratories, Munich, Germany).

2.5. Estrogen receptor characterization-determination of K_d and N_{max}

Prior to characterization of the ER, initial experiments were carried out to determine the optimal incubation conditions. For this, rainbow trout hepatic cytosol was incubated with 5 nM ^{3}H-E_2 at 4°C and the specific binding was determined after different incubation time points using liquid scintillation spectrometry. The incubation time at which maximum specific binding was achieved (20 h) was subsequently considered optimal and was used routinely for all subsequent experiments. In order to determine the K_d and N_{max}, a modification of the routine procedure (Nimrod and Benson, 1997) was employed. Briefly, cytosolic fractions with an approximate protein content of 1 mg in a final volume of 280 μl were incubated in duplicate with ^{3}H-E_2 at final concentrations ranging between 1 and 20 nM both in the presence and absence of 1000-fold excess E_2 at 4°C for 20 h in order to determine non-specific binding (NB) and total binding (TB), respectively. Unbound ^{3}H-E_2 was removed from the solution by adding 300 μl of dextran (0.375%)-coated charcoal (3.75%) suspension to each sample tube. The tubes were vigorously mixed, incubated on ice for 5 min and then centrifuged at $4000 \times g$ for 7 min. Three hundred microliters of the resulting supernatant was mixed with 3 ml scintillation fluid (Ready Safe, Beckman) and quantified by liquid scintillation spectrometry. Specific binding (SB) was determined from the difference between TB and NB. The equilibrium dissociation constant (K_d) and the maximum number of binding sites (N_{max}) were calculated using Scatchard analysis (Scatchard, 1949).

The quantification of estrogen binding to the xenopus hepatic ER followed the same method described above except that the absolute amount of protein used and the final concentrations of ^{3}H-E_2 used for incubation ranged between 0.67–0.75 mg and 1–70 nM, respectively.

2.6. Competition binding studies

One- and, 2-year-old rainbow trout and xenopus cytosolic fractions with a protein content of approximate 1 mg were incubated with ^{3}H-E_2 at the final concentration of 2, 3 and 30 nM ^{3}H-E_2, respectively, both in the presence (NB) and absence (TB) of 1000-fold excess E_2.. Competitive binding capacity of xenobiotic were determined using the same procedure as described for non-specific binding except that increasing concentrations of xenobiotic (dissolved in ethanol) were added instead of excess E_2. Each concentration of the competitor was assayed in duplicate and each experiment included both estradiol (E_2) and bisphenol A (BA) as positive controls. The resulting data were plotted as percentage of control (specific binding in the absence of competitor) versus the log of the competitor concentrations. The IC_{50} values were defined as the concentration of competitor that inhibited ^{3}H-E_2 specific binding by 50%.

2.7. Statistics

The IC_{50} values were calculated by logit-log transformation of the data derived from competition curves and the comparison of the results were analyzed by ANOVA followed by Tukey's multiple comparison test (Toxstat 3.3, University of Wyoming, 1991). Shapiro–Wilk's and Bartlett's tests were used to assess the normality of the data distribution and the homogeneity of the variances, respectively.

3. Results

3.1. Stripped versus non-stripped receptor preparations (rainbow trout)

Comparable K_d values (1.4 nM, mean of three replicates) were obtained for non-stripped and stripped rainbow trout hepatic cytosolic fractions. On the other hand, stripping resulted in a significant decrease of N_{max}, $N_{max,\ stripped} = 56 \pm 4$ fmol/mg protein versus $N_{max,\ non\text{-}stripped} = 88 \pm 2$ fmol/mg protein, most likely the result of receptor adherence to charcoal-dextran and thus reduction of absolute numbers of receptors present in the incubation. Based on the latter results, no charcoal-dextran stripping of hepatic cytosol was car-

ried out for subsequent rainbow trout or xenopus receptor preparations.

3.2. Estrogen receptor characterization-determination of K_d and N_{max} in fresh and frozen cytosolic fractions

3.2.1. Fresh cytosolic fractions of rainbow trout and xenopus

Maximal specific binding of ^{3}H-E_2 (5 nM) to rainbow trout hepatic cytosolic fractions was achieved upon incubation at 4°C for 20 h (Fig. 1). Typical binding curves and Scatchard analyses for rainbow trout and xenopus hepatic cytosolic fraction incubated with varying concentrations of ^{3}H-E_2 are shown in Figs. 2 and 3, respectively. The K_ds and N_{max}s determined for 1- and 2-year-old rainbow trout were 1.9 ± 0.2 and 2.5 ± 0.6 nM and 102 ± 12.5 and 103 ± 16.4 fmol/mg protein ($n = 3$), respectively. The K_d and N_{max} determined for xenopus were 32 ± 12 nM ($n = 4$) and 286.5 ± 110.3 fmol/mg protein, respectively.

3.2.2. Comparison of fresh receptor fractions with fractions kept at − 80°C

Preliminary studies with three individual 1-year-old female rainbow trout indicated K_d and N_{max} were 1.4 ± 0.07 nM; 74 ± 20 fmol/mg protein versus 1.2 ± 0.5 nM; 73 ± 23 fmol/mg protein for fresh and frozen-thawed ER preparations. No significant differences in binding activity, i.e. K_d and N_{max} were observed between fresh cytosolic fractions and fractions stored at − 80°C for varying time periods. Frozen fractions were stored for up to 1 month.

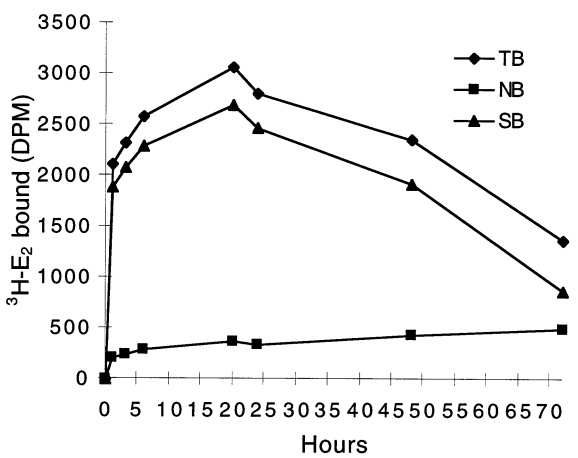

Fig. 1. Time courses of ^{3}H-E_2 specific binding to the ER of 1-year-old female rainbow trout. ^{3}H-E_2 (5 nM) was incubated alone or together with 5 μM E_2 with hepatic extract (1 mg protein).

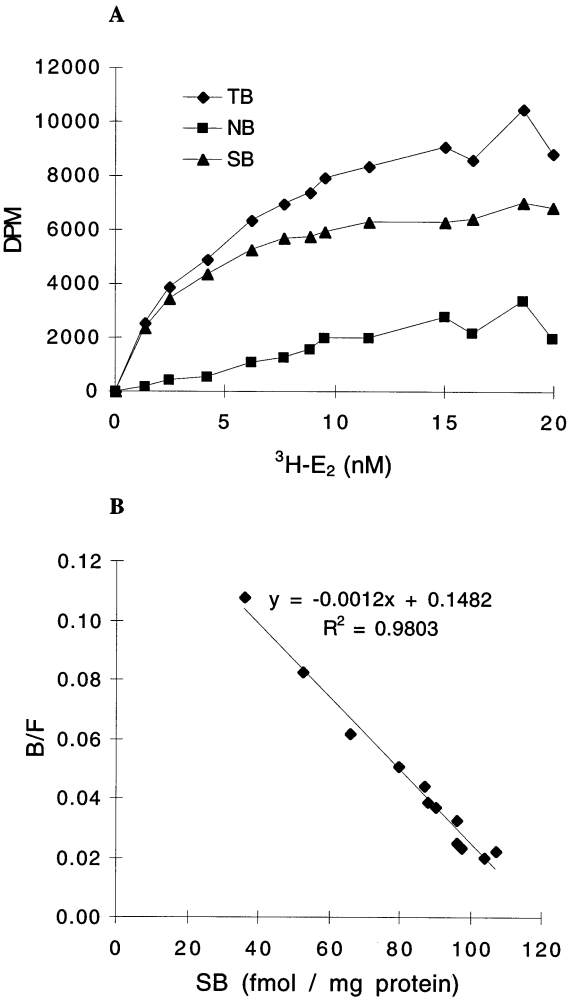

Fig. 2. (A) ^{3}H-E_2 binding saturation curve with female rainbow trout hepatic extract. The hepatic extract was incubated with ^{3}H-E_2 (1–20 nM) in the absence or presence of 1000-fold excess of E_2 for 20 h at 4°C. (B) Scatchard plot derived from the data presented in (A). Line fitted by linear regression. $K_d = 3.3$ nM, $N_{max} = 124$ fmol/mg protein. B, bound; F, unbound.

3.3. Competitive binding experiments

3.3.1. Nitromusk parent compounds (musk xylene, musk ketone, musk moskene)

No competitive binding of nitromusk parent compounds was observed with either rainbow trout or xenopus ER preparations. In contrast, both E_2 and BA competitively inhibited ^{3}H-E_2 binding to the ER of both species. The respective

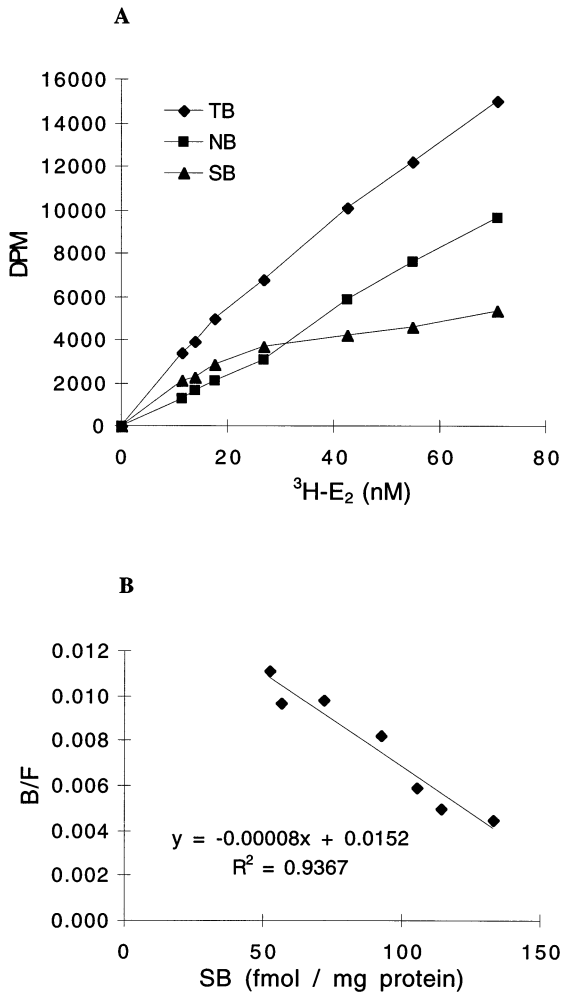

Fig. 3. (A) ^{3}H-E_2 binding saturation curve with female xenopus hepatic extract. The hepatic extract was incubated with ^{3}H-E_2 (1–70 nM) in the absence or presence of 1000-fold excess of E_2 for 20 h at 4°C. (B) Scatchard plot derived from the data presented in (Fig. 4A). Line fitted by linear regression. $K_d = 30$ nM, $N_{max} = 190$ fmol/mg protein. B, bound; F, unbound.

Table 1
Effects of nitromusk metabolites on ^{3}H-E_2 binding to rainbow trout and xenopus ER

Compound	Testing concentration (M)	IC_{50}
Rainbow trout		
Estradiol	10^{-10}–10^{-6} M	5.3 ± 1.2 nM
Bisphenol A	10^{-7}–10^{-3} M	8.8 ± 1.8 μM
4-NH_2-MX	10^{-6}–10^{-3} M	–
2-NH_2-MX	10^{-6}–10^{-3} M	1.3 ± 1.1 mM
2-NH_2-MK	10^{-6}–10^{-3} M	–
Xenopus		
Estradiol	10^{-10}–10^{-6} M	187 ± 76 nM
Bisphenol A	10^{-7}–10^{-3} M	441 ± 247 nM
4-NH_2-MX	10^{-7}–10^{-3} M	30.8 ± 28.5 μM
2-NH_2-MX	10^{-7}–10^{-3} M	12.9 ± 10.3 μM
2-NH_2-MK	10^{-7}–10^{-3} M	70.1 ± 88.3 μM

IC_{50} values determined for E_2 and BA in rainbow trout were 5.3 ± 1.2 nM and 8.8 ± 1.8 μM, respectively, whereas for xenopus these were 187 ± 76 and 441 ± 247 nM (Table 1).

3.3.2. Amino-metabolites of musk xylene and musk ketone (4X, 2X, 2K)

All three nitromusk metabolites, 4-NH_2-musk xylene (4X), 2-NH_2-musk xylene (2X) and 2-NH_2-musk ketone (2K), competitively inhibited ^{3}H-E_2

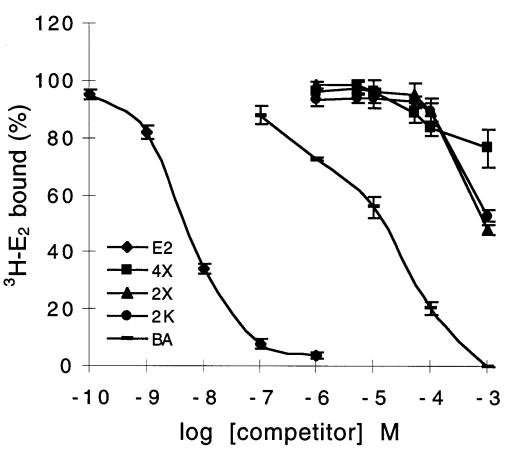

Fig. 4. Competitive binding of E_2, BA, 4X, 2X and 2K to the rainbow trout ER. The incubation concentrations were 10^{-10}–10^{-6} M for E_2, 10^{-7}–10^{-3} M for BA and 10^{-6}–10^{-3} M for amino metabolites. IC_{50}s were 5.3 ± 1.2 nM for E_2, 8.8 ± 1.8 μM for BA and 1.3 ± 1.1 mM for 2X.

binding to the rainbow trout ER (Fig. 4). However, only 2X achieved more than 50% competitive binding within the concentration range of competitor tested (10^{-6}–10^{-3} M), thus allowing logit-log transformation of the data to determine the IC_{50} (Table 1). The IC_{50} values of 4X and 2K could have been extrapolated from the logit-log transformed data. However, in view of the uncertainty involved in predicting the competitive binding behavior of 4X and 2K at concentrations of competitor greater than 10^{-3} M and the irrelevance of said high concentrations in the environment, such extrapolated IC_{50} values would be considered purely theoretical and thus of little environmental consequence.

All three nitromusk metabolites also demonstrated competitive inhibition of ^{3}H-E_2 binding to the xenopus ER (Fig. 5). The concentrations necessary to inhibit 50% of ^{3}H-E_2 binding (IC_{50}), however, were two orders of magnitude lower than those observed with the rainbow trout ER (Table 1).

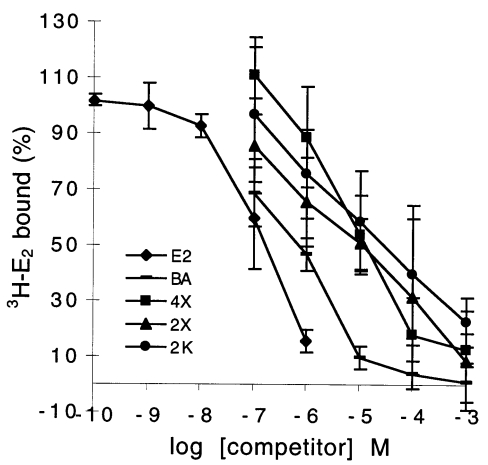

Fig. 5. Competitive binding of E_2, BA, 4X, 2X and 2K to the xenopus ER. The incubation concentrations were 10^{-10}–10^{-6} M for E_2, 10^{-7}–10^{-3} M for BA, 4X, 2X and 2K. IC_{50}s were 187 ± 76 nM for E_2, 441 ± 247 nM for BA, 30.8 ± 28.5 μM for 4X, 12.9 ± 10.3 μM for 2X and 70.1 ± 88.3 μM for 2K.

4. Discussion

The preparatory procedures used for obtaining fractions containing the hepatic estrogen receptor of rainbow trout and xenopus followed previously published protocols (Nimrod and Benson, 1997). The saturation binding curves and corresponding Scatchard analyses obtained from data with rainbow trout cytosolic fraction indicated a homogeneous binding component in the cytosolic fraction, this component having the characteristic to bind E_2 in a specific and saturable manner. The K_ds and N_{max}s of 1- and 2-year-old rainbow trout (1.9 ± 0.2 nM, 102 ± 12.5 fmol/mg protein and 2.5 ± 0.6 nM, 103 ± 16.4 fmol/mg protein), respectively, are comparable to those reported by Campbell et al. (1994) in rainbow trout and similar to those for other teleost species (Lazier et al., 1985; Smith and Thomas, 1990; Nimrod and Benson, 1997).

The K_d value of female xenopus (32 ± 12 nM) determined in the experiments presented here is in agreement with the value reported by Lutz and Kloas (1999). These values are approximately 60-fold greater than the majority of the values reported in the literature (Westley and Knowland, 1978; Wright et al., 1983; Perlman et al., 1984). However, it should be noted that these previously reported values were all obtained in male xenopus of unknown age as well as in primary cell cultures of xenopus hepatocytes. In view of the vast sex differences reported in the K_d and N_{max} values of rainbow trout, females having approximately four- and seven-fold higher values than males, respectively (Campbell et al., 1994), it may be assumed that similar sex differences may occur in amphibian species.

The competition assay with the positive control E_2 in rainbow trout yielded an IC_{50} value (Table 1) highly concurrent with values reported in the literature (Hewitt et al., 1998). It is interesting to note that the E_2 IC_{50} values in rainbow trout and some mammalian systems are comparable (Shelby et al., 1996; Perez et al., 1998). Unfortunately no BA IC_{50} values were found in the literature for the rainbow trout ER. Literature data, however, were available for the interaction of BA with mammalian ER's. BA has demonstrated a 1000–

10 000-fold lower affinity for the mammalian ER than E_2 (Nagel et al., 1997; Perez et al., 1998). A 1000-fold lower affinity of BA than E_2 for the ER of rainbow trout was observed in this study (Table 1), suggesting concordance between mammalian and rainbow trout ER with regard to binding of E_2 and BA.

In contrast, little literature data were available as to the affinity of E_2 or BA binding to the ER of xenopus. Lutz and Kloas (1999) have recently reported IC_{50} values of 42 ± 9 nM and 30 ± 13 μM for E_2 and BA, respectively. These values are at variance with those observed in our study (187 ± 76 and 441 ± 247 nM, respectively). However, certain differences between the two studies exist including variation of age, sex and size of the animals used. These may, at least partially, account for the discrepancies noted and stress the need for further investigation. Furthermore, additional variation in the K_d values may have resulted from the fact that due to poor solubility of E_2 saturation was not achieved in the xenopus saturation assay.

In order to put the xenopus data into perspective, they were compared to the data obtained in rainbow trout (Table 1). This comparison indicated that E_2 and BA bound with a 30-fold lower and 20-fold higher affinity respectively, to the xenopus ER than to the ER of rainbow trout.

While MX, MK, and MM were found to be unable to inhibit ^{3}H-E_2 binding to the ER of rainbow trout or xenopus, all three nitromusk metabolites (4X, 2X and 2K) demonstrated competitive capacity in both species (Table 1, Figs. 4 and 5). With an approximate IC_{50} of 1.3 ± 1.1 mM in rainbow trout, 2X was found to be the most potent metabolite tested, while no IC_{50} values for 4X and 2K in the concentration range tested. For comparison, the percent inhibition at the highest concentration of the latter two competitors tested (1 mM) demonstrated 19.5 ± 2.1 and $47.0 \pm 2.0\%$ inhibition for 4X and 2K, respectively.

All three nitromusk metabolites, however, were found to compete with ^{3}H-E_2 for binding to the xenopus ER. No statistical differences between the IC_{50} values of the respective metabolites were observed, indicating similar affinities of 2X, 4X, and 2K for the xenopus ER. In comparison to the rainbow trout ER, the xenopus ER appeared to be approximately 100-fold more susceptible to competitive inhibition of E_2 binding. This may be due to the fact that the affinity of E_2 to the xenopus ER (K_d: 30 nM) was a ten-fold lower than to the rainbow trout ER (K_d: 3 nM). These results suggest that xenopus (amphibians) could generally be more susceptible to the effects of xenobiotics with potential endocrine modulating activity.

The affinity of binding to the ER of both rainbow trout and xenopus appears to be highest for 2X, this may be associated with the nitro-reduction at the *ortho* position of musk xylene (Table 1, Figs. 4 and 5). This trend was also apparent for the musk ketone metabolite 2K (Table 1), although lack of significant statistical differences between the IC_{50} values of the three metabolites in xenopus prevents confirmation. Nitro-reduction at the *para* position, maintains the affinity for the ER, however the binding activity appears decreased possibly due to the lack of a stabilizing hydrophobic group. The data presented suggest that reduction of the nitro- to an amino-substituent enhances binding to the ER. This is most likely due to the higher polarity of amino groups. In consequence, this would suggest that the binding of an amine-substituted aromatic ring could act similarly to a phenolic ring. This appears to be the first time that binding activity for molecules having an amine- rather than a hydroxyl-substituted aromatic ring has been reported. Possibly corroborative findings were reported by Anstead et al. (1997) who demonstrated that nitro-reduction of 2-nitrophenol to 2-aminophenol increased the binding affinity to the estrogen receptor in MCF-7 cells by a factor 12, although generally the binding affinity was extremely low when compared to E_2.

Although competitive binding of nitromusk metabolites to the ER of rainbow trout and xenopus was demonstrated in vitro with this study, the question remains as to the relevance of these findings for the in vivo and specifically the environmental situation. The comparison of the IC_{50} values with those of other known putative estrogenic pollutants in the environment, e.g. bisphe-

nol A, alkylphenol-polyethoxylates (nonylphenol), DDT metabolites, methoxychlor, kepone, metals, etc. shows that the concentrations necessary for 50% inhibition of E_2 binding to the respective ERs are also in the μM to mM range (Thomas and Smith, 1993; Shelby et al., 1996; Vonier et al., 1996; Nagel et al., 1997; Nimrod and Benson, 1997), while the endocrine modulating activity of environmentally relevant concentrations of the latter compounds in fish and amphibians remain to be confirmed. Similarly, no in vivo studies have yet been carried out with rainbow trout and/or xenopus to determine the endocrine modulating activity of nitromusk parent compounds or their metabolites. On the other hand, environmental concentration levels of both, nitromusk parent compound and metabolites, have been analyzed (Gatermann et al., 1998). The comparison of the IC_{50} values determined in the study presented here with the latter data would seem to allow assessment of the potential risk associated with the exposure of fish and amphibians to nitromusk metabolites. Indeed, the highest environmental concentrations of 2X, 4X and 2K, namely 10, 34 and 250 ng/l, respectively, were detected in the effluents of a sewage treatment plant (Gatermann et al., 1998). Dilution of effluent waters in the receiving surface waters, e.g. river Elbe, results in concentrations ranging between 1 and 9 ng/l. Consequently, fish and amphibians would be chronically exposed to concentrations 10 000- and 1 000 000-fold lower, respectively, than those shown to inhibit E_2 binding to the respective ER by 50%. However, as these organisms are chronically exposed to the metabolites that are lipophilic and hence bioaccumulative, and given that 4X has been observed at higher levels than the parent compound in fish (Rimkus et al., 1998, 1999), additional studies including in vivo experiments, should be considered to elucidate the risks posed by environmental nitromusk contamination.

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