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ABSTRACT

Aromatase, a key steroidogenic enzyme that catalyses the conversion of androgens to estrogens, represent a target for endocrine disrupting chemicals. However, little is known about the effect of pollutants on aromatase enzymes in fish. In this study, we first optimised a rainbow trout (*Oncorhynchus mykiss*) microsomal aromatase assay to measure the effects of 43 substances belonging to diverse chemical classes (steroidal and non steroidal aromatase inhibitors, pesticides, heavy metals, organotin compounds, dioxins, polycyclic aromatic hydrocarbons) on brain and ovarian aromatase activities *in vitro*. Our results showed that 12 compounds were able to inhibit brain and ovarian aromatase activities in a dose-dependent manner with IC_{50} values ranging from the low nM to the high µM range depending on the substance: steroidal and non steroidal inhibitors of aromatase (4-hydroxyandrostenedione, androstatrienedione, aminogluthethimide), imidazole fungicides (clotrimazole, imazalil, prochloraz), triazole fungicides (difenoconazole, fenbuconazole, propiconazole, triadimenol), the pyrimidine fungicide fenarimol and methylmercury. Overall, this study demonstrates that rainbow trout brain and ovarian microsomal aromatase assay is suitable for evaluating potential aromatase inhibitors *in vitro* notably with respect to environmental screening. The results highlight that methylmercury and some pesticides that are currently used throughout the world, have the potential to interfere with the biosynthesis of endogenous estrogens in fish.

Key words: aromatase, brain, endocrine disrupting chemicals, ovary, rainbow trout, tritiated water assay.

INTRODUCTION

It has now been well established that many environmental pollutants are able to disturb the normal physiology and endocrinology of organisms. These substances, termed Endocrine Disrupting Chemicals (EDCs), have been defined as “exogenous substances that cause adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function” (OECD,1997). Exposure of fish to EDCs has been associated with reproductive adverse effects at both individual and population level in a variety of fish species (Jobling *et al.*, 2002; Brion *et al.*, 2004; Nash *et al.*, 2004; Mills and Chichester, 2005). These substances have multiple modes of action since they can potentially act on the synthesis, secretion, transport, action and elimination of endogenous hormones (Segner *et al.*, 2003). To date, research has focused mainly on compounds that interfere with sex steroids receptors, particularly the estrogen receptor. However, the endocrine system may also be disrupted by environmental substances through pathways and mechanisms other than those that are ER-mediated. The knowledge of critical molecular and biochemical targets of EDCs in fish is thus of a great interest.

The biosynthesis of steroid hormones represents a target for EDC action, particularly the steps catalysed by cytochrome P450-dependent enzymes (Monod *et al.*, 1993). In vertebrates, an essential sex-related enzyme is aromatase (P450aro). Aromatase is an enzymatic complex including a NADPH-dependent cytochrome P450 reductase and a cytochrome P450 aromatase which catalyzes the final, rate-limiting step in the conversion of androgens into estrogens (Simpson *et al.*, 1994). In fish, this enzyme has been shown to be mainly expressed in ovary and brain. In mammals except pig, only one gene encodes the aromatase while in several teleost fish such as zebrafish (*Danio rerio*), goldfish *(Carassius auratus*) or rainbow trout (*Oncorhynchus mykiss*), aromatase is encoded by two different genes : cyp19a (or cyp19A1) and cyp19b (cyp19A2) (Callard and Tchoudakova, 1997; Kishida and Callard, 2001; Dalla Valle *et al.*, 2002). These two distinct genes generate two structurally and functionally different aromatase proteins, CYP19A1 or P450 Aro A (AroA), and CYP19A2 or P450 Aro B (AroB) (Tchoudakova and Callard, 1998; Chiang *et al.*, 2001a; Blazquez and Piferrer, 2004). These genes have distinct expression patterns : the brain aromatase activity is mainly due to the expression of the cyp19b gene while the ovarian aromatase activity is mainly due to the expression of the cyp19a gene (Tchoudakova and Callard, 1998; Chiang *et al.*, 2001b; Forlano *et al.*, 2001; Kishida and Callard, 2001; Trant *et al.*, 2001; Dalla Valle *et al.*, 2002; Menuet *et al.*, 2005).

In teleost fish, brain aromatase activity is much higher than in mammals (Pasmanik and Callard, 1985; Pasmanik and Callard, 1988). This very high expression of brain aromatase could be linked to the capability of fish brain to grow during adulthood (Gelinas *et al.*, 1998; Forlano *et al.*, 2001). In the gonads, transcription of the aromatase gene has been proposed as a key step in the process of ovarian differentiation. For instance, in the rainbow trout (*Oncorhynchus mykiss*), inhibition of ovarian aromatase in undifferentiated female resulted in a complete masculinization of an all-female population (Guiguen *et al.*, 1999). Moreover, during the female reproductive cycle, ovarian secretion of 17β-estradiol controls the hepatic synthesis of vitellogenin, a phospho-lipoprotein corresponding to the major precursor of embryonic trophics reserves (Flouriot *et al.*, 1997).

Recent studies have reported alterations of brain and/or ovarian P450 aromatase activities in wild fish collected from contaminated sites (Noaksson *et al.*, 2001; Orlando *et al.*, 2002; Noaksson *et al.*, 2003; Lavado *et al.*, 2004) suggesting that fish populations are exposed to substances that perturb the biosynthesis of estrogens. However the nature (and the levels) of substances involved in these biological responses remains to be determined. In fish, a few studies have shown that environmental chemicals can interfere with aromatase activity. In these studies, the range of substances was limited to some pesticides (Monod *et al*., 1993, Noakson *et al*., 2003, Ankley *et al*., 2005) and polycyclic aromatic hydrocarbons (PAHs) (Monteiro *et al*., 2000). Thus, there is a need to provide a broader, more systematic knowledge on aromatase inhibiting potencies of environmental chemicals. Further, most of the available studies have analysed only gonadal aromatase and it remains to be determined whether the brain form is likewise affected.

Considering the critical role of aromatase in development and reproduction in fish as well as the large number of chemical substances that can potentially affect this enzyme, the aim of this study was to investigate whether several substances belonging to diverse chemical classes (pesticides, polycyclic aromatic hydrocarbon, heavy metals) affect brain and ovarian aromatase activities in rainbow trout (*Oncorhynchus mykiss*) *in vitro*. For this purpose, we first optimized a microsomal aromatase assay to measure the effect of xenobiotics on aromatase activity in fish.

MATERIALS AND METHODS

Reagents and chemicals

[1β-3H (N)]androst-4-ene-3,17-dione (specific activity 25.3 Ci / mmol) was purchased from Perkin Elmer (France). Glucose-6-phosphate dehydrogenase was obtained from Fluka (France). β-NADPH tetrasodium salt, β-NADP sodium salt, glucose-6-phosphate dipotassium salt were purchased from Sigma-Aldrich (France). Diuron, heavy metals (triphenylarsine, cadmium chloride, and methyl mercury), PAHs (Benzo-[a]-pyrene (B[a]P) and chrysene), pentachlorophenol, 4-hydroxyandrostenedione, aminoglutethimide and clotrimazole were purchased from Sigma-Aldrich (France). Aldrin, alpha-cypermethrin, amitrol, atrazine, benomyl, bupirimate, chlordane, difenoconazole, endosulfan, fenarimol, fenbuconazole, fipronil, heptachlor, imazalil, iprodion, isoproturon, mecoprop, methoxychlor, metolachlor, parathion-methyl, permethrin, prochloraz, propiconazole, simazine, triadimenol, trifluralin, and vinclozolin comes from Riedel-de-Haën (France). Tri-n-butyltin chloride (TBT) was obtained from Acros Organics (France). 2,3,7,8 TCDD was obtained from Promochem (France), lead acetate was obtained from Rectapur (France) and 1,4,6-androstatrien-3,17-dione was obtained from Steraloïds (USA). Azimsulfuron comes from Du Pont (France), and oxadiazon from Supelco (France). Reagents and chemicals were of the highest purity.

Origin of fish, fish maintenance and dissections

Female rainbow trout (*Oncorhynchus mykiss*) were obtained from two experimental fish farms (INRA, Gournay-sur-Aronde, France and INRA, Jouy-en-Josas, France). The fish were maintained in the laboratory under a natural photoperiod in 500 litres tanks supplied with dechlorinated tap water (temperature 15.5 +/- 1.3°C, pH 8.06 +/- 0.18, dissolved oxygen 8.80 +/- 0.18 mg/l, conductivity 667.0 +/- 6.5 µS/cm), and were fed twice a week with granules NEO prima 5 (Le gouessant aquaculture, France). Fish were killed by cranial blow, then measured and weighed. The brain and the ovaries were removed and the gonads weighed to determine the gonado-somatic index (GSI). After removal, tissues were rinsed in ice cold KCl (0.15 M). In order to obtain sufficient material for preparation of brain and gonad microsomes, brains or ovaries of fish were pooled according to their GSI and different batches of brain and ovarian microsomes were constituted such as follows : for each tissue, 4 batches of microsomes were obtained from females having GSI < 1% ("low GSI", N = 12 fish /

batch), 2 other batches were obtained from females having GSI comprised between 8 % and 13 % ("medium GSI", N = 13 fish / batch) and 2 batches were obtained from females having GSI > 13 % ("high GSI", N = 17 fish / batch).

Preparation of brain and ovarian microsomes

Pooled brain or gonad tissues were homogenized with a Teflon potter homogenizer in a 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM PMSF, 1 mM EDTA and 20 % glycerol (v/v) in a ratio of 1:2 (w:v). After centrifugation of the homogenates (10,000 *g*, 20 min, 4°C), the supernatants were collected and centrifuged at 100,000 *g* (90 min, 4°C). The microsomal pellet was then resuspended in the same buffer as used for the homogenisation (100 µl / fish) and the total amount of microsomal protein determined (Bradford, 1976) using BSA (Bovine Serum Albumin, Sigma-Aldrich, France) as standard. Microsomes were then aliquoted and stored at –80°C until used.

Measurement of brain and ovarian aromatase activities

Aromatase activity was determined by the tritiated water assay which measures the release of tritiated water during the conversion of [1β-3H (N)]androst-4-ene-3,17-dione to estrone (Thompson and Siiteri, 1974). Optimal concentrations of brain and gonads microsomal proteins were determined as well as concentration of substrate, the duration and the temperature at which the enzymatic reaction occurred. The resulting assay protocol is described below. For the aromatase assay, 200 µg of brain or ovarian microsomal proteins were added to a potassium phosphate buffer (50 mM) containing a NADPH-generating system and consisting of 20 µM β-NADPH, 1 mM β-NADP, 10 mM glucose-6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase. To assess the effect of test compounds on aromatase activity, substances were dissolved in either dimethylsulfoxide (DMSO) or ethanol Solvent concentration did not exceed 0.2 % of the final reaction mixture, i.e. 500 µl. Test compounds were incubated with microsomes during 1 hour at 27°C. The rationale for choosing 1 hour of incubation was to increase sensitivity of the aromatase assay. Indeed in a preliminary experiment, we showed that brain and ovarian IC_{50} (defined as the concentration of chemical required for 50% inhibition of aromatase activity) calculated for 4-hydroxyandrostenedione were respectively 1.8 and 4.5 times lower for one hour of incubation compared to ten minutes (data not shown). After one hour at 27°C, the reaction was started by addition of 75 nM of [1β-3H (N)]androst-4-ene-3,17-dione. Appropriate controls without substrate (tissue control), cofactors, and substitution of microsomal proteins for BSA, and addition of the specific aromatase inhibitor 4-hydroxyandrostenedione at a concentration of 0.5 µM were used. After 30 min at 27°C, the reaction was stopped by the addition of 1 ml of chloroform. After extensive vortexing for 30 sec, the tubes were centrifuged at 3000 *g* (10 min, 4°C). The aqueous layer was removed and extracted again with 1 ml of chloroform. The aqueous layer was mixed with charcoal (5 %, w/v) to eliminate remaining organic compounds, vortexed for 30 sec and centrifuged at 4000 *g* (20 min, 4°C). Then two aliquots of the supernatant (2×150 µl) were mixed with 750 µl of scintillation liquid (OptiPhase 'Hi safe' 3, Perkin Elmer, France) in two different wells of a 24-well plate (Flexibles plates 24-w, Perkin Elmer, France) and counted for 2 min in a Liquid Scintillation Counter (Microbeta, Perkin Elmer, France). As a first screening step all xenobiotics were tested at 10 µM. For IC_{50} determination, all compounds were tested at concentrations between 10 nM and 100 µM except aminoglutethimide for which one concentration was added (250 µM) and androstatrienedione and 4-hydroxyandrostenedione which were tested between 0.01 nM and 1 µM.

Data analysis and statistics

The enzyme kinetic parameter (Vmax and Km) were calculated from Lineweaver-Burk inverse plots. The aromatase inhibition potency of the tested substances was expressed as IC_{50} calculated using the Regtox macro for Microsoft Excel freely available at http://eric.vindimian.9online.fr/download.html REGTOX_EV7.0.5.xls (Vindimian *et al*., 1983). Data were also expressed by the relative potency of aromatase inhibition (RPAI) calculated as the ratio of IC_{50} value of 4-hydroxyandrostenedione to test compound. Experimental data were expressed as mean ± standard deviation (N = 3 independent experiments performed in ducplicate). The SPSS™ software version 10.1 for Windows (SPSS, USA) was used for statistical analysis.

RESULTS

Establishment and optimisation of the microsomal aromatase assay

Subcellular localisation of the aromatase activity and specificity of the aromatase assay

Aromatase activity was measured in the microsomal and cytosolic fraction of brain and gonads. In both brain and ovaries, the highest aromatase activity was located in the microsomal fraction (> 90 % in the microsomes and < 10 % in the cytosol, data not shown), which is in agreement with the subcellular localisation of the aromatase enzyme, i.e. the endoplasmic reticulum (Simpson *et al*., 1994). In controls (without substrate or cofactor, substitution of microsomes for BSA or with 4 hydroxyandrostenedione), no aromatase activity was found (data not shown). All together, these experiments showed that the method is specific of the aromatization reaction.

Effect of amount of microsomal protein and time of incubation on microsomal aromatase activity

In the tritiated water assay, quantification of aromatase activity depends on degree of linearity of the reaction with respect to time and amount of total protein. At all concentrations of microsomal proteins tested (0.1, 0.2 and 0.5 mg), aromatase activities in brain and ovaries were linear for up to 30 minutes (Fig. 1). Accordingly, brain and ovarian aromatase activities measured after 30 min were proportional to the amount of microsomal proteins in the assay (data not shown). Based on these results, 200 µg of microsomal proteins and 30 min of incubation time were chosen as standard procedure for the subsequent measurements on brain and ovarian aromatase activities.

Effect of substrate concentration on brain and ovarian microsomal aromatase activities : determination of affinity for androstenedione (Km) and maximum reaction rates (V_{max})

In order to ensure saturating concentration of substrate available for the enzyme, the effect of substrate concentration on brain and ovarian aromatase activities was assessed. In brain and ovarian microsomes, aromatase activity was maximal at 75 nM of radiolabelled androstenedione and above (Fig 2A, B). This concentration was chosen to ensure that the substrate was not limiting.

By using the established aromatase assay, the rainbow trout aromatase affinity for androstenedione (Km) and its maximum reaction rate (V_{max}) were determined in both brain and ovarian microsomes (Fig 2A, B). The Km values for androstenedione in brain and ovary were 9.9 +/- 2.6 nM and 7.48 +/- 1.14 respectively without significant difference. In contrast, V_{max} were much higher in brain (V_{max} = 453.7 +/- 24.5) than in ovary (51.0 +/- 1.5 fmol/mg/min) (Fig 2A, B).

Effect of incubation temperature of microsomes on aromatase activity

Variations of the incubation temperature resulted in significant changes in aromatase activity (Fig. 3A, B). In brain and in ovarian microsomes, aromatase activity was maximal at 18°C and 27°C respectively. At 37°C, aromatase activities in both brain and gonad were completely inhibited. In order to verify that temperature did not influence the effects of xenobiotics in the aromatase assay, microsomes were exposed to known aromatase inhibitors and incubated at 18 or 27°C. As shown by the Figure 4, the calculated brain and ovarian IC_{50} for 4-hydroxyandrostenedione and prochloraz were the same at both temperatures. Based on this data, the incubation temperature used for the microsomal assay was set at 27°C.

Brain and ovarian aromatase activities measured in the different batches of microsomes

Whatever the batch of microsomes, brain aromatase activity was systematically higher than ovarian activity (Table I). Moreover, brain and ovarian aromatase activities were significantly different among batches : in microsomes isolated from the “high GSI” group, brain aromatase activity was significantly higher than in microsomes isolated from fish of the “low” and “medium” GSI groups. In contrast, no aromatase activity was measured in ovarian microsomes isolated from females of the “high GSI” group. From a practical point of view, the effect of environmental pollutants on brain and ovarian aromatase activities was tested on microsomes of the “medium GSI group”, where both brain and ovarian aromatase activities were high.

Effect of environmental pollutants on brain and ovarian aromatase activities

Forty three environmental chemicals from different chemical classes were tested for their ability to interfere with brain and ovarian aromatase activities. As a first screening step, experiments were conducted by testing all the compounds at 10µM except for few of them which where tested at lower concentrations due to solubility limitations. The results from these experiments are summarized in Table II. Twelve out of 43 substances were potent inhibitors of aromatase activity *in vitro* at 10 µM both in the brain and ovaries: androstatrienedione, 4-hydroxyandrostenedione, aminoglutethimide, clotrimazole, fenarimol, difenoconazole, fenbuconazole, imazalil, prochloraz, propiconazole, triadimenol, and methylmercury (Table II). None of the other tested chemicals exhibited any significant effect on aromatase activity at the concentrations tested. For the active substances, dose-response experiments were then conducted to determine their IC_{50} values. The results are summarised in Table III. Steroidal compounds (4-hydroxyandrostenedione and androstatrienedione) were found to be the most potent aromatase inhibitors with IC_{50} values in the low nM range. The non steroidal compound aminoglutethimide was found to be the least potent aromatase inhibitor among all the inhibiting substances tested in this study with a relative potency of aromatase inhibition (RPAI) for brain and ovary equal to 4.6 10^{-5} and 4.1 10^{-6} respectively compared to 4-hydroxyandrostenedione. In contrast, the imidazole fungicide clotrimazole inhibited the brain and ovarian aromatase activities with a potency of inhibition in brain close to that of 4-hydroxyandrostenedione ($RPAI_{brain}$ = 0.8 and $RPAI_{ovary}$ = 0.009). With the exception of methylmercury, all the aromatase inhibiting xenobiotics were fungicides belonging to the triazole, imidazole and pyrimidine families and were characterized by IC_{50} values ranging from the low to the high µM range (Table III). Linear regression analysis showed that there exists a significant and positive correlation between the inhibitory action of a substance on the ovarian aromatase and on the brain aromatase ($R^2 = 0.85$; Pearson's test, $p< 0.01$, Fig. 5). Interestingly, despite this overall correlation, tissue specific effects were highlighted for certain chemicals. For the four fungicides (clotrimazole, imazalil, prochloraz and propiconazole), no significant differences of their brain and ovarian IC_{50} values were noted. In contrast, 4 out of 12 compounds (4-hydroxyandrostenedione, androstatrienedione, difenoconazole, and methylmercury) showed ovarian IC_{50} values significantly lower than those measured in the brain (student's t test, $p<0.05$). IC_{50} of aminoglutethimide and fenbuconazole were also lower than brain IC_{50} but the difference was not statistically significant (Student's –t test, p = 0.06 and 0.07 respectively). Finally, triadimenol had stronger inhibitory effect on the brain than on the ovarian aromatase activity (Student's t test, $p<0.05$). Fenarimol had the same pattern of inhibitory effect as triadimenol but the difference was not statistically significant.

DISCUSSION

This paper describes the potential endocrine disrupting activity of xenobiotics by assessing their capacities to inhibit *in vitro* brain and ovarian aromatase activities of rainbow trout. We first optimized the tritiated water assay, characterized aromatase activity in the two target tissues and determined the kinetic parameters of the aromatase in brain and ovarian microsomes. Then we showed that several environmental substances are able to inhibit both brain and ovarian aromatase activities in a dose-dependent manner, indicating that they can potentially interfere with biosynthesis of endogenous estrogens and alter the androgen : estrogen ratio. Among them, methylmercury and the triazole fungicide fenbuconazole were newly identified as *in vitro* inhibitors of aromatase activity in a vertebrate model.

Rainbow trout brain and ovarian microsomal aromatase assay

The tritiated water assay to measure aromatase activity has been already applied to several fish species, including goldfish (*Carassius auratus*) (Pasmanik and Callard, 1985), medaka (*Oryzias latipes*) (Melo and Ramsdell, 2001), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) (Noaksson *et al.*, 2001), sea bass (*Dicentrarchus labrax*) (Gonzalez and Piferrer, 2002; Gonzalez and Piferrer, 2003) and rainbow trout (*Oncorhynchus mykiss*) (Monod *et al.*, 1993; Shilling *et al.*, 1999). We aimed to optimize the assay in such a way that it fits to both ovarian and brain aromatase activities, and to determine whether brain and ovarian aromatase differ in their key kinetic parameters, Vmax and Km. Amount of protein, length of incubation time, and substrate concentration were found to influence aromatase activity, a finding which is in accordance with the observations of Gonzalez and Piferrer (2002) on aromatase activity in sea bass. Also temperature influenced aromatase activity. The temperature effect on aromatase activity can be associated with decreasing cyp19 mRNA expression in tilapia with increasing water temperature (D'Cotta *et al.*, 2001; Tsai *et al.*, 2003). Aromatase activity of rainbow trout showed lower temperature maxima than aromatase activity of sea bass (30°C and 40°C for brain and ovary, respectively, in sea bass (Gonzalez and Piferrer, 2002)), a difference that might be related to the fact that rainbow trout is a cold water teleost fish species while sea bass live at higher temperatures.

Comparison of the catalytic properties

To our knowledge, this study is the first to report Vmax and Km values for both the brain and ovarian P450 aromatase in rainbow trout. The Vmax value of the brain aromatase is nine-fold higher than that of ovarian aromatase (453.72 ± 24.50 and 51.04 ± 1.52 fmol/mg/min respectively). These results are in accordance with the ten-fold higher Vmax of goldfish brain aromatase isoform compared to ovary isoform (Zhao *et al.*, 2001) and are in line with the four-fold higher Vmax of sea bass brain aromatase compared to ovary (Gonzalez and Piferrer, 2002). Moreover, the Vmax calculated for the ovaries is similar to the Vmax reported by Shilling *et al.* (1999) for rainbow trout (71.1 fmol/mg/min). While brain exhibited higher catalytic activity compared to the ovary, brain and ovarian Km values were not significantly different, demonstrating similar and very high affinities of both aromatases for androstenedione. Our data are well in accordance with Km values reported in literature for other teleost fish species : 5 nM in goldfish brain homogenates (Pasmanik and Callard, 1988), 8.2 nM in goldfish brain microsomes (Zhao *et al.*, 2001), 4.1 and 3.4 nM in brain and ovarian sea bass microsomes respectively (Gonzalez and Piferrer, 2002). However, it should be noted that our ovarian Km value is in disagreement with that reported by Shilling *et al.* (1999) for rainbow trout who found a 40-fold higher value.

Effect of test chemicals on rainbow trout brain and ovarian aromatase activities

Aromatase activity inhibition by known aromatase inhibitors

In this study three aromatase inhibitors were used, namely two steroidal inhibitors, 4-hydroxyandrostenedione and androstatrienedione, and a non steroidal inhibitor, aminoglutethimide. Steroidal inhibitors are steroid analogues of androstenedione. They bind irreversibly to the active site of the enzyme while non steroidal inhibitor act reversibly on P450 aromatase by interacting with the heme prosthetic group of the enzyme (Brodie *et al.*, 1986; Yue and Brodie, 1997). The capacities of these pharmaceuticals to inhibit aromatase have been extensively studied in mammals notably within the context of estrogen-dependant cancer therapy (see Geisler and Lonning, 2005 for a review). By using different *in vitro* human systems (i.e., placental microsomes, human adrenocortical cells H295R, human choriocarcinoma-derived JEG3 cells), the human IC_{50} values reported for these molecules ranged from 0.0015 µM to 1.5 µM for 4-hydroxyandrostenedione and from 0.0146 µM to 55 µM for aminoglutethimide (see Table III). Our results clearly show that these compounds act as aromatase inhibitors in rainbow trout as well. That rainbow trout aromatase activity appears to be more susceptible than that of some mammalian species may reflect the high affinity of these compounds for fish aromatase (Zhao *et al.*, 2001). The inhibitory potency of the non steroidal aromatase inhibitor, aminoglutethimide, on brain and gonad aromatase activities of trout was much less expressed than that of the steroidal substances. This gives support to previous findings on goldfish and rainbow trout aromatase (Shilling *et al.*, 1999; Zhao *et al.*, 2001). By calculating the RPAI of aminoglutethimide for human aromatase based on IC_{50} data reported in different studies (Yue and Brodie, 1997; Ohno *et al.*, 2004), we found that aminoglutethimide was 10 to 1200-fold less effective in inhibiting rainbow trout than human aromatase. Similarly, letrozole, exhibited a 1000-fold less inhibitory potency in rainbow trout than in human (Shilling *et al.*, 1999). A recent study reported 53 % overall identity of the deduced amino-acid sequence of brain trout aromatase with that of human (Dalla Valle *et al.*, 2002). This may imply that there exist differences of tertiary structure between human and trout P450 aromatase that could explain differences in efficacy and mechanism of inhibition (Pelissero *et al.*, 1996; Shilling *et al.*, 1999). The data of the present study show that there exist differences in the inhibitory potency of substances for trout and mammalian aromatase. Additionally, differences are evident between brain and gonad P450 aromatase. This underlines that although some knowledge is available for the inhibitory action of chemicals on aromatase activity in mammals, this can not be linearly extrapolated to teleostean aromatase activity.

We found that the efficacy of inhibition of ovarian aromatase by aminoglutethimide and the two steroidal inhibitors were 5 and 60 fold higher respectively comparatively to brain. In the study of Zhao *et al.* (2001) it has been demonstrated that steroidal inhibitor have higher affinity for ovarian aromatase isozyme than brain aromatase in goldfish by determining the Ki. Given that rainbow trout aromatase activities in brain and ovary are supported by two structurally different proteins, i.e. P450AroA in ovary and P450AroB in brain (Dalla Valle *et al.*, 2002), it is possible that the differences in the amino-acid sequences of rainbow trout aromatase isozymes account for distinct response to aromatase inhibitors (IC_{50} values) as previously suggested for goldfish aromatases (Zhao *et al.*, 2001).

Aromatase Inhibition by environmental substances

Among the environmental substances tested in this study, nine inhibited brain and ovarian aromatase activities in a dose-dependent manner among which were seven triazole and imidazole fungicides. Due to their well described capacity to inhibit sterol 14-α-

demethylase which catalyses the synthesis of ergosterol, an essential membrane component in yeast and fungi, azole fungicides are widely used as antimycotic agents in agriculture and in human and veterinary therapies (Zarn *et al.*, 2003). Various imidazole-like compounds have been shown to inhibit aromatase in human placenta microsomes, and in different cell lines expressing aromatase (Mason *et al.*, 1987; Ayub and Levell, 1990; Vinggaard *et al.*, 2000; Andersen *et al.*, 2002; Sanderson *et al.*, 2002; Ohno *et al.*, 2004). In fish, data are more scarce. Previous studies reported that some imidazole fungicides (clotrimazole, prochloraz, imazalil) are able to inhibit aromatase activity in fish (Monod *et al.*, 1993; Noaksson *et al.*, 2003; Ankley *et al.*, 2005). Our results confirm the capability of these imidazole fungicides to inhibit ovarian and also brain aromatase activities and extend the findings to other imidazole-like compounds. We found that clotrimazole was the most potent aromatase inhibitor of *in vitro* aromatase activity, both in brain and ovary, among all the tested imidazole-like compounds. This is similar to the results of Monod *et al.* (1993) who found that this substance was a much more potent ovarian aromatase inhibitor than prochloraz and imazalil (see Table III). In a cyprinid fish species, the roach (*Rutilus rutilus*), clotrimazole has also been shown to inhibit *in vitro* brain aromatase activity with an IC_{50} of 0.9 µM (Noaksson *et al.*, 2003). Brain and ovarian IC_{50} values obtained in the present study for clotrimazole were lower than those observed previously (Monod *et al.*, 1993; Noaksson *et al.*, 2003) which is probably due to the use of optimized assay conditions and the resulting enhanced sensitivity of the assay. Besides, species difference cannot be ruled out since the highest IC_{50} value for clotrimazole was reported for the roach. This hypothesis is further supported by the very low degree of inhibition of aromatase by fenarimol in the fathead minnow compared to rainbow trout (Ankley *et a*l., 2005). Nevertheless, IC_{50} values for prochloraz reported for the fathead minnow by Ankley *et al*. (2005) are similar to those reported in trout (Monod *et al.*, 1993). Whether there exists species difference in sensitivity of aromatase remains to be determined.

Among the imidazole-like compounds tested in this study, only benomyl and amitrol had no effect on aromatase activity. The absence of an inhibitory effect of these two molecules is likely due to their chemical structures. Although relationship between the structure and the biological activity as inhibitors of aromatase is not simple (Sanderson *et al.*, 2002), it has been shown that both molecules containing an imidazole ring fused to a benzene ring (e.g. benomyl) and molecules containing an imidazole ring without aromatic ring on the N-1 substituent (e.g. amitrol) are very weak inhibitors of human aromatase (Ayub and Levell, 1988). Therefore, our results strongly suggest that this type of structure activity described for human aromatase is also true for rainbow trout brain and ovarian aromatase.

Among the other substances tested, only methylmercury inhibited brain and ovarian aromatase activities in a dose-dependent manner. The ability of this substance to inhibit vertebrate aromatase has not been described before and the mechanism of inhibition remains to be determined. Molecules such as TBT, lead, TCDD, B[a]P, chrysene, atrazine, simazine, or vinclozoline are known to perturb aromatase expression *in vitro* in different cell systems (Letcher *et al.*, 1999; Monteiro *et al.*, 2000; Saitoh *et al.*, 2001; Sanderson *et al.*, 2001; Sanderson *et al.*, 2002; Taupeau *et al.*, 2003) but failed to produce any effect in our cell-free system. These differences rely most probably on the mechanism of action of these substances which are able of up- or down regulating the aromatase gene transcription (Saitoh *et al.*, 2001; Sanderson *et al.*, 2001; Sanderson *et al.*, 2002; Taupeau *et al.*, 2003).

The *in vitro* microsomal aromatase assay is limited to measuring the inhibitory effects of the test compound on aromatase enzymatic activity through direct interaction with the enzymatic complex but does not provide any information on the effect of the test agent on the gene / protein machinery. Nonetheless, the aromatase assay can be used for ecotoxicological screening in order to identifying and characterising the mode of action and endocrine effects of environmental contaminants on a key steroidogenic enzyme. Since brain and ovarian Log IC_{50} values were significantly and positively correlated (Fig 5), environmental contaminants can be tested either on brain or ovarian microsomes. However, brain microsomes can be recommended since brain aromatase activities can be measured whatever the maturity of fish.

Several of the aromatase inhibiting substances found in our study are known to be present in the aquatic environment and to accumulate in fish. Methylmercury is a well-known contaminant of the aquatic food web that may adversely affect reproduction of wild population of fish (Drevnick and SandHeinrich, 2003). In a recent survey of pharmaceuticals in aquatic environment, it has been shown that the fungicide clotrimazole was present at low concentrations (up to 33 ng/L) in some UK estuaries and rivers (Thomas and Hilton, 2004; Roberts and Thomas, 2006). In surface water of French rivers, difenoconazole, fenbuconazole, iprodion, prochloraz and propiconazole have been measured at mean concentrations in the low ng/L range with maximal concentration of 1.75 µg/L for propiconazole (IFEN, 2001). As a consequence, all these substances could potentially represent a risk for fish populations and may be involved in altered aromatase activity that have been observed in wild fish population inhabiting contaminated water (Noaksson *et al*., 2001; Orlando *et al*., 2002; Noaksson *et al*., 2003; Lavado *et al*., 2004; Noaksson *et al*., 2004; Noaksson *et al*., 2005; Martin-Skilton *et al*., 2006). However, few studies have been conducted to assess the *in vivo* effect of these compounds on brain and ovarian aromatase activities in fish and it would be advisable to determine to which extent the inhibitory potency of xenobiotics on *in vitro* aromatase activity can be extrapolated to the *in vivo* situation. Our results concerning the inhibitory effect of methylmercury on brain and ovarian aromatase activities are consistent with the decreasing circulating concentrations of estradiol measured in female fathead minnow fed with methylmercury (Drevnick and Sandheinrich, 2003). Similarly, prochloraz in the fathead minnow caused a suite of *in vivo* responses consistent with inhibition of steroidogenesis, i.e. decrease of estradiol and vitellogenin synthesis in female (Ankley *et al.*, 2005). However, the same authors showed that the endocrine effects of fenarimol were more ambiguous with notably an increased concentration of circulating estradiol in female. Shilling *et al.* (1999) failed to demonstrate any effect on aromatase and vitellogenin synthesis in juvenile female rainbow trout exposed to clotrimazole. All these data suggest that extrapolation of *in vitro* study of xenobiotics interference with aromatase enzyme (and more generally steroidogenic enzyme) to the *in vivo* situation is not an easy task. It should be related to the *in vivo* pharmaco-toxicokinetic and the multiple mode of action of these molecules on the endocrine system as well as the complexity of the synthesis pathway (Andersen *et al.*, 2002; Sanderson and Van den Berg, 2003; Ankley *et al.*, 2005). Taken together, further *in vivo* studies are needed.

CONCLUSION

The optimized aromatase activity assay with rainbow trout microsomes developed in this study is a suitable test for screening for aromatase inhibiting activities of environmental contaminants. The assay can distinguish inhibitory effects in the brain and the ovary, and we show for the first time that the two tissues differ in their sensitivity to chemical inhibition. While ovary exhibited a higher sensitivity to steroidal inhibitors as compared to brain, the sensitivity of the two aromatases to environmental substances was broadly similar indicating that brain and ovarian aromatase are relevant biochemical target of EDCs. Our study also indicates that methylmercury and some pesticides (clotrimazole, imazalil, prochloraz, fenbuconazole, propiconazole, difenoconazole, triadimenol, and fenarimol), that are currently used throughout the world, have the potential to interfere with the biosynthesis of endogenous estrogens. Based on these results, it can be suggested that these compounds will affect the functioning of the HPG axis and in turn sexual development and reproduction of fish. However, further *in vivo* studies are needed to support these hypothesis.

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Table I : Aromatase activity (AA) measured in the different batches of microsomes isolated from brain or ovary of females rainbow trout having different GSI. The number of batch / group of GSI is detailed in the Materials and Methods. *n* = number of independent measures performed on different batches of brain or ovarian microsomes. Different letters indicate statistically different values (Kruskall-Wallis test, and Mann-Whitney U test, $p < 0.05$). BDL = Below Detection Limit.

GSI	AA in brain microsomes (fmol/mg/min)	AA in ovarian microsomes (fmol/mg/min)
Low GSI	153.9 ± 59.3 [a,d]	43.8 ± 6.0 [c]
< 1 %	*n = 16*	*n = 27*
Medium GSI	146.8 ± 5.1 [a]	107.7 ± 27.6 [d]
8 % - 13 %	*n = 13*	*n = 11*
High GSI	428.9 ± 46.5 [b]	BDL
> 13 %	*n = 15*	*n= 3*

Table II : Effect of environmental pollutants on aromatase activity (AA) in microsomes prepared from brain and ovary of rainbow trout. All chemicals were tested at 10 µM except 4OHA (4-hydroxyandrostenedione), ATD (androstatrienedione), aldrin, heptachlor, and TCDD. The other concentrations are indicated in the table. Values are means of triplicates ± standard deviation. * indicate a significant difference compared to control (Student's t test, $p < 0.05$).

CHEMICALS FAMILY	CHEMICALS	% OF CONTROL AA (BRAIN)	% OF CONTROL AA (OVARY)
	Control	100.0 ± 8.4	100 ± 10.3
STEROIDAL INHIBITOR	4OHA (1µM)	2.7 ± 0.4 *	3.1 ± 3.1 *
	ATD (1µM)	3.9 ± 0.9 *	2.1 ± 0.2 *
NON STEROIDAL INHIBITOR	Aminoglutethimide	84.7 ± 2.8 *	70.7 ± 1.3 *
HEAVY METALS	Cadmium chloride	106.1 ± 8.6	98.6 ± 10.3
	Lead acetate	101.8 ± 7.8	99.2 ± 12.0
	Methyl mercury	75.9 ± 2.1 *	15.7 ± 26.7 *
	Triphanylarsine	107.8 ± 3.7	86.4 ± 1.5
	H3AsO4	100.1 ± 2.6	100.0 ± 4.4
PAH	Benzo[a]Pyrene	98.3 ± 2.0	102.9 ± 0.9
	Chrysene	99.7 ± 1.1	105.2 ± 4.7
INSECTICIDES			
organochlorine	Aldrin (100nM)	92.5 ± 2.2	89.9 ± 4.7
	Chlordane	105.5 ± 10.5	110.0 ± 9.5
	Endosulfan	111.7 ± 0.3	107.6 ± 0.4
	Heptachlor (100nM)	89.7 ± 8.8	89.7 ± 11.7
	Methoxychlor	113.5 ± 2.3	84.5 ± 1.6
pyrazole	Fipronil	107.2 ± 1.4	101.7 ± 19.4
pyrethroids	Alpha-cypermethrin	83.7 ± 5.9	100.2 ± 5.0
	Permethrin	87.3 ± 8.4	94.1 ± 4.9
FUNGICIDES			
benzimidazol	Benomyl	116.7 ± 0.4	102.3 ± 0.6
triazole	Difenoconazole	81.7 ± 2.7 *	75.9 ± 2.9 *
	Fenbuconazole	7.1 ± 0.9 *	6.7 ± 3.2 *
	Propiconazole	10.1 ± 0.6 *	5.9 ± 0.5 *
	Triadimenol	53.2 ± 1.5 *	69.8 ± 0.7 *
imidazole	Clotrimazole	3.6 ± 0.1 *	0.0 ± 0.7 *
	Imazalil	3.8 ± 0.5 *	2.6 ± 0.2 *
	Prochloraz	18.0 ± 0.1 *	10.8 ± 0.4 *
dicarboximide	Iprodion	115.1 ± 13.4	102.3 ± 4.9
	Vinclozoline	100.5 ± 14.7	111.1 ± 11.7
pyrimidine	Bupirimate	102.2 ± 8.9	92.2 ± 5.3
	Fenarimol	46.4 ± 1.1 *	56.9 ± 0.8 *
HERBICIDES			
	Oxadiazon	87.5 ± 5.1	81.4 ± 5.7
chloroacetanilide	Metolachlor	106.0 ± 4.5	109.6 ± 1.1
chlorotriazine	Atrazine	114.7 ± 4.2	104.7 ± 3.2
	Simazine	98.0 ± 14.1	108.7 ± 8.8
dinitroaniline	Trifluralin	103.9 ± 12.6	102.1 ± 4.7
phenoxypropionic	Mecoprop	105.8 ± 3.3	99.7 ± 5.4
phenylurea	Diuron	97.7 ± 3.9	85.2 ± 4.5
	Isoproturon	93.9 ± 8.1	89.9 ± 1.9
pyrimidinylsulfonylurea	Azimsulfuron	112.9 ± 3.7	95.3 ± 18.7
triazole	Amitrol	101.0 ± 0.9	93.8 ± 5.2
OTHERS	Pentachlorophenol	109.8 ± 9.6	98.3 ± 1.6
	TCDD (10-9 M)	112.0 ± 2.6	101.3 ± 3.1
	TCDD (10-8 M)	116.7 ± 6.0	89.6 ± 2.6
	Tributyltin chloride	91.4 ± 1.9	86.6 ± 0.1

Table III : Comparative inhibition of aromatase activities in brain and ovarian microsomes in rainbow trout. The relative inhibition potency was calculated in comparison with 4-hydroxyandrostenedione. Dose-response experiments were conducted three times for each compound tested. Published IC50 values of the studied pesticides for placental assay, cells based assays and rainbow trout ovarian assay are also reported.

Chemical name	Brain IC50 (µM)	Ovarian IC50 (µM)	Ratio Brain/Ovarian IC50	RPAI brain	RPAI ovary	IC50 in placental assay (µM)	IC50 in cells based assays (µM)	IC50 in rainbow trout ovarian assay (µM)
4-OH androstenedione	0.009 ± 0.001	0.00015 ± 0.00003	60.0	1	1	0.04 [a]- 1.4 [b]	0.0011 [c] - 0.08 [d]	#1.5 [e]
Androstatrienedione	0.015 ± 0.003	0.00025 ± 0.00018	60.0	0.6	0.6	-	-	##0.5 [f]
Aminoglutethimide	197 ± 54	36.8 ± 12.0	5.4	4.6E-05	4.1E-06	1 [g]-130 [f]	2.25 [c] -15.8 [d]	39 [f] , Ki = 2.4 [h]
Clotrimazole	0.011 ± 0.004	0.016 ± 0.001	0.7	0.8	0.009	0.43 [i] - 1.8 [j]		0.05 [k] , 0.9 * [l]
Imazalil	0.43 ± 0.03	0.32 ± 0.21	1.3	2.1E-02	4.7E-04	0.04 [m] - 0.15 [j]	0.0044 [c]-0.1 [n]	5 [k]
Prochloraz	1.3 ± 0.4	1.0 ± 0.5	1.3	6.9E-03	1.5E-04	0.34 [m] - 0.70 [j]	0.1 [n]	5 [k], (11-7.2)**[o]
Fenbuconazole	1.3 ± 0.4	0.21 ± 0.05	6.2	6.9E-03	7.1E-04	-	-	-
Propiconazole	0.9 ± 0.3	0.9 ± 0.6	1.0	1.0E-02	1.7E-04	6.5 [m]	0.96 [c] - 5 [n]	-
Difenoconazole	70 ± 7	29 ± 6	2.4	1.3E-04	5.2E-06	-	4 [n]	-
Triadimenol	11 ± 0.7	26 ± 2	0.4	8.2E-04	5.8E-06	21 [m]	12.6 [p]	-
Fenarimol	6 ± 1	18 ± 6	0.3	1.5E-03	8.3E-06	4.1*** [q] - 10 [m]	2 [m, c]	-
Methylmercury	11 ± 1	0.78 ± 0.07	14.1	8.2E-04	1.9E-04	-	-	-

RPAI: relative potency of aromatase inhibition, # = 80% of inhibition, ## = 100% of inhibition, * = roach brain microsomes, ** = S9 fraction from brain fathead minnow, *** = rat ovarian microsomes (a) France et al., 1987; (b) Geelen et al, 1991; (c) Ohno et al., 2004; (d) Yue and Brodie, 1997; (e) Shilling et al., 1999; (f) Pelissero et al., 1996; (g) Le Bail et al., 2000; (h) Zhao et al., 2001; (i) Ayub and Levell, 1990; (j) Mason et al;1987; (k) Monod et al., 1993; (l) Noakson et al., 2003; (m) Vingaard et al., 2000, (n) Sanderson et al., 2002, (o) Ankley et al., 2005, (p) Trosken et al. (2004), (q) Hirsh et al (1987).

FIGURE LEGENDS

Fig. 1. Effect of the amount of microsomal proteins and incubation time on the aromatase activities (AA) in rainbow trout ovaries (A) and brain (B). In all experiments, the concentration of substrate was 75 nM and the reaction was carried out at a temperature of 27°C. Each value represents the mean of two independent experiments ± SD.

Fig. 2. Saturation analysis of aromatase activity (AA) in : (A) ovarian microsomes, and (B) brain microsomes of rainbow trout. Brain and ovaries were incubated 30 min at 27°C with increasing concentrations of 1-β[3H]-androstenedione from 2 to 600 nM. Inserts : Lineweaver-Burk plots to determine the Vmax and the Km.

Fig. 3. Effect of temperature on the rainbow trout aromatase activities (AA) in brain (A) and ovary (B).The concentration of substrate was 75 nM and the amount of protein 200 µg. AA were expressed as percent of maximal AA measured for each tissue.

Fig. 4. Effect of temperature on brain and ovarian IC_{50} values for two aromatase inhibiting substances (prochloraz and 4-hydroxyandrostenedione (4-OHA)). IC_{50} values calculated in ovary (A) and brain (B) for each substance at 18°C and 27°C were not significantly different (Student's t test, $p > 0.05$).

Fig. 5. Linear regression analysis between brain and ovarian LOG (IC_{50}) values. Brain and ovarian IC_{50} data were positively and significantly correlated (Pearson's test, $p<0.01$).

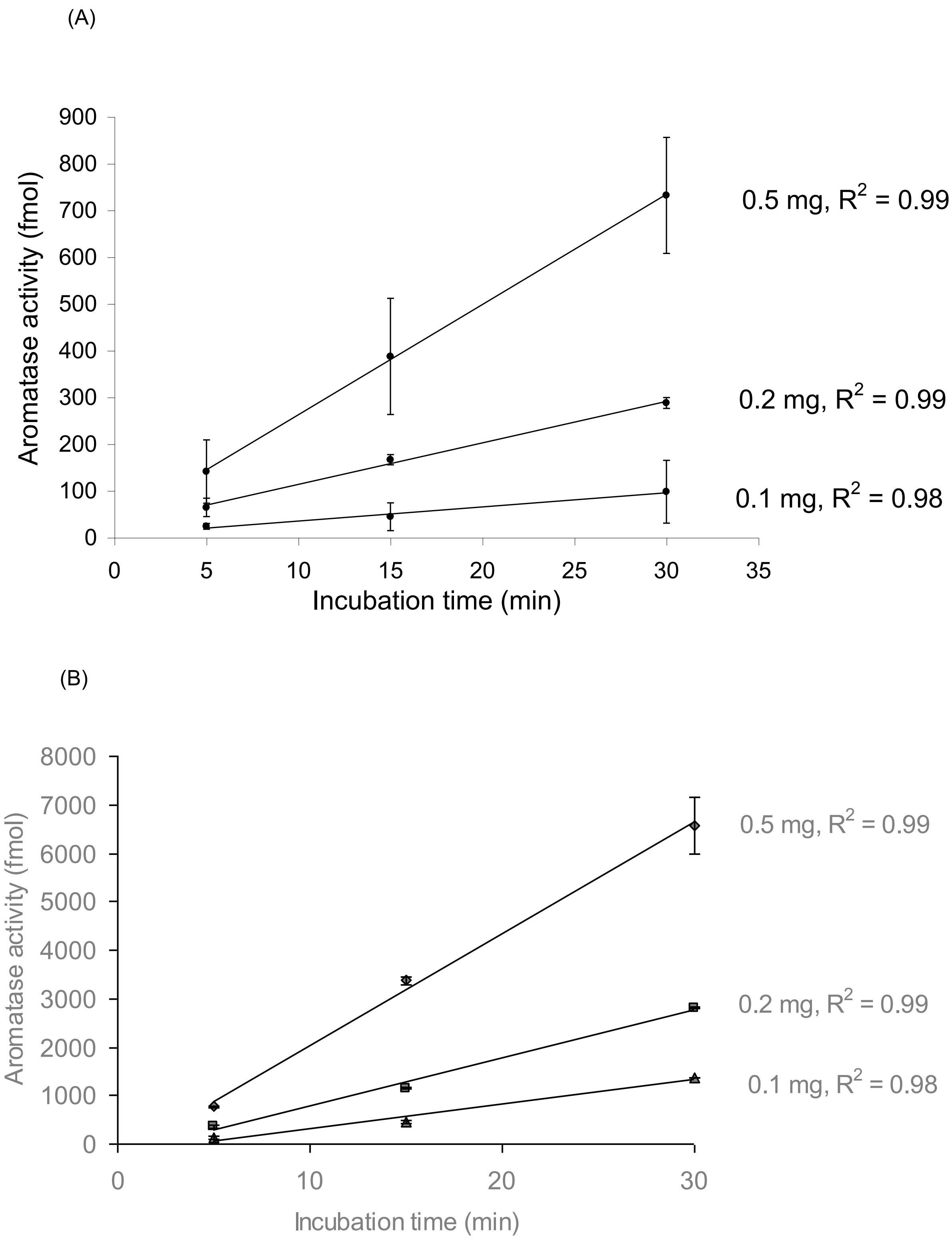
(A)
900
800
700
600
500
400
300
200
100
0
Aromatase activity (fmol)
0
5
10
15
20
25
30
35
Incubation time (min)
0.5 mg, R^2 = 0.99
0.2 mg, R^2 = 0.99
0.1 mg, R^2 = 0.98
(B)
8000
7000
6000
5000
4000
3000
2000
1000
0
Aromatase activity (fmol)
0
10
20
30
Incubation time (min)
0.5 mg, R^2 = 0.99
0.2 mg, R^2 = 0.99
0.1 mg, R^2 = 0.98

Fig. 1.

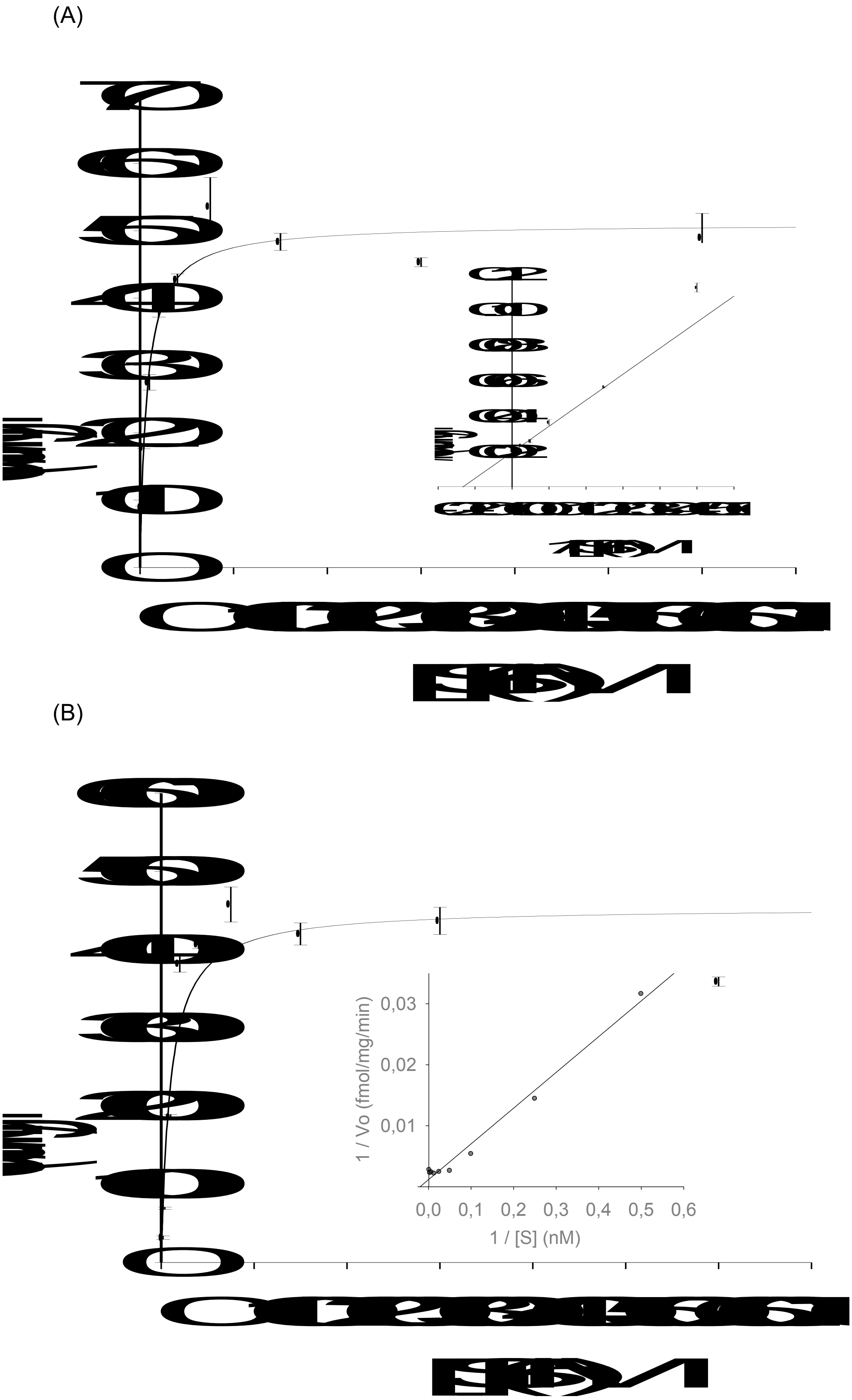

Fig. 2.

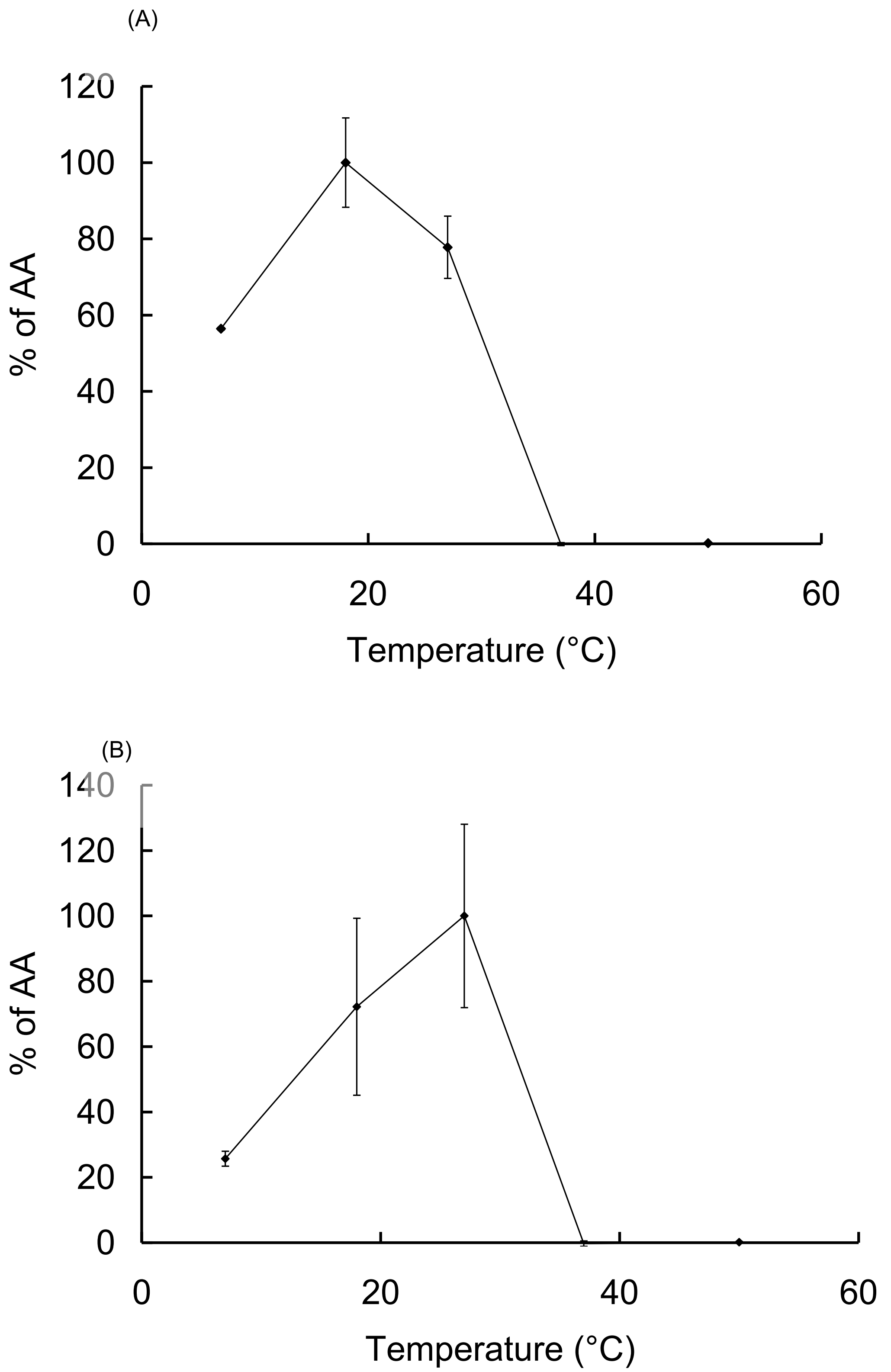
(A)
% of AA
120
100
80
60
40
20
0
0
20
40
60
Temperature (°C)
(B)
% of AA
140
120
100
80
60
40
20
0
0
20
40
60
Temperature (°C)

Fig. 3.

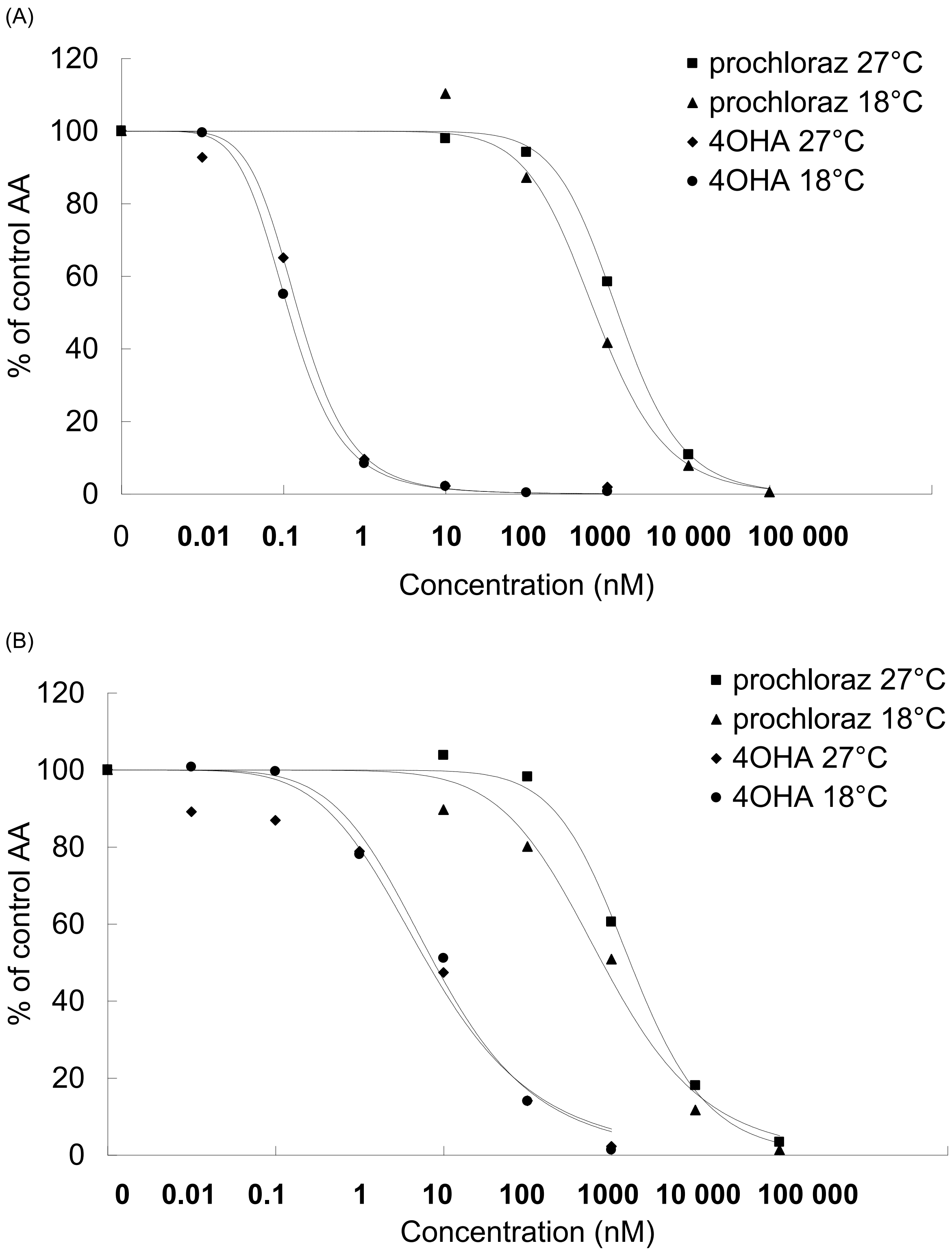
(A)
120
100
80
60
40
20
0
% of control AA
0
0.01
0.1
1
10
100
1000
10 000
100 000
Concentration (nM)
prochloraz 27°C
prochloraz 18°C
4OHA 27°C
4OHA 18°C
(B)
120
100
80
60
40
20
0
% of control AA
0
0.01
0.1
1
10
100
1000
10 000
100 000
Concentration (nM)
prochloraz 27°C
prochloraz 18°C
4OHA 27°C
4OHA 18°C

Fig. 4.

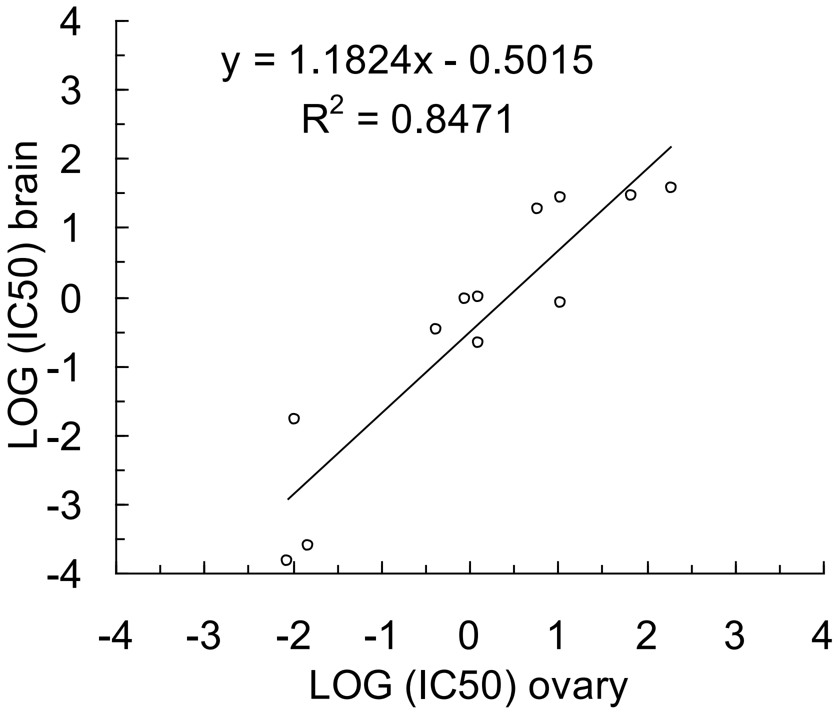
y = 1.1824x - 0.5015
R2 = 0.8471
4
3
2
1
0
-1
-2
-3
-4
LOG (IC50) brain
-4
-3
-2
-1
0
1
2
3
4
LOG (IC50) ovary

Fig. 5.