Endocrine disruption caused by environmental chemicals: Effects on key enzymes of steroid hormone metabolism of humans and animals

Susan Lo-Ullmann

Endocrine disruption caused by environmental chemicals: Effects on key enzymes of steroid hormone metabolism of humans and animals

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 15 – Biowissenschaften
der Johann Wolfgang Goethe-Universität Frankfurt am Main

von
Susan Lo-Ullmann
aus Köln

Frankfurt 2007
(D30)

Die vorliegende Arbeit wurde in der Zeit von November 2001 bis März 2006 am Institut für Klinische Biochemie, Abteilung Endokrinologie, Universitätsklinkum Bonn angefertigt.

Vom Fachbereich Biowissenschaften der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan:	Prof. Dr. Rüdiger Wittig
Gutachter:	Prof. Dr. Jörg Oehlmann, Institut für Ökologie, Evolution und Diversität
Gutachter:	Prof. Dr. Dietrich Klingmüller, Institut für Klinische Biochemie, Universitätsklinikum Bonn

Datum der Disputation: 10. Oktober 2007

Meiner Familie

You can´t win.
You can´t even break even.
You can´t stay out of the game.

Rules of thermodynamic

Voet & Voet

Index

Abbreviations

AAC	–	Androgenic/antiandrogenic compounds
AB	–	Assay buffer
ABC	–	ATP-binding cassette
AC	–	Adenylate cyclase
A. demin	–	Aqua demineralized
AGS	–	Adrenogenital syndrome
AMH	–	Anti-Müllerian hormone
5α-3α-androstanediol	–	5α-androstane-3α,17β-diol
5α-3β-androstanediol	–	5α-androstane-3β,17β-diol
5α-androstanedione	–	5α-androstane-3,17-dione
5β-3α-androstanediol	–	5β-androstane-3α,17β-diol
5β-androstanedione	–	5β-androstane-3,17-dione
Androstenedione	–	Androst-4-ene-3,17-dione
Androsterone	–	5α-androstane-3α-ol-17-one
AR	–	Androgen receptor
Aromatase	–	Cytochrome P450 aromatase
ATCC	–	American Type Culture Collection
ATP	–	Adenosine triphosphate
BPH	–	Benign prostatic hyperplasia
BRGM	–	Bureau de Recherches Geologiques et Minieres
cAMP	–	Cyclic adenosine 3´,5´cyclic monophosphate
CAS	–	Chemical abstracts service
cDNA	–	Complementary deoxyribonucleic acid
$CHCl_3$	–	Chloroform
CH_2Cl_2	–	Dichlormethane
CYP	–	Cytochrome P450
DBT	–	Dibutyltin (IUPAC)
DBT-CL	–	Dibutyl-dichloro-stannane (IUPAC)
p,p´DDE	–	1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl] benzene (IUPAC)
DDT	–	1-chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl] benzene (IUPAC)
Dehydroepiandrosterone	–	Androst-5-ene-3β-ol-17-one
DEPC	–	Diethyl pyrocarbonate
DES	–	Diethylstilbestrol
dNTP	–	Deoxynucleotide triphosphate
DHEA	–	*q.v.* dehydroepiandrosterone
DHEA-SO_4	–	Dehydroepiandrosterone sulfate
5α-DHT, DHT	–	*q.v.* dihydrotestosterone
5β-DHT	–	5β-androstane-17β-ol-3-one
Dihydrotestosterone	–	5α-androstane-17β-ol-3-one
3α-Diol	–	*q.v.* 5α-3α-androstanediol
3β-Diol	–	5α-androstane-3β,17β-diol

Diuron	–	3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (IUPAC)
DMEM	–	Dulbecco´s modified Eagle media
DNA	–	Deoxyribonucleic acid
DPM	–	Disintegrations per minute
DTE	–	Dithioerythritol
E1	–	*q.v.* estrone
E2	–	*q.v.* estradiol
ED	–	Endocrine disrupter
EDTA	–	Ethylendiamin tetra-acetate
EDSP	–	Endocrine Disruptor Screening Programme
Enon	–	*q.v.* androstenedione
EPA	–	Environmental Protection Agency
ER	–	Estrogen receptor
Estrone	–	[10]-estratriene-3α-ol-17-one
Estradiol	–	[10]-estratriene-3α,17β-diol
EtOH	–	Ethyl alcohol
FBS	–	Fetal bovine serum
Fe	–	Female
Fenarimol	–	(2-chlorophenyl)-(4-chlorophenyl)-pyrimidin-5-yl-methanol (IUPAC)
Fig.	–	Figure
Finasteride	–	N-(1,1-Dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-en-17-carboxamid (IUPAC)
Flutamide	–	2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide (IUPAC)
FP	–	Forward primer
FSH	–	Follicle stimulating hormone
GDP	–	Guanosine diphosphate
GnRH	–	Gonadotropin-releasing hormone
GTP	–	Guanosine triphophate
HB	–	Homogenization buffer
HCL	–	Hydrochloride
HPG axis	–	Hypothalamic-pituitary-gonadal axis
HRE	–	Hormone resposive element
3α-HSD	–	3α-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase
3β-HSD	–	3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase
17β-HSD 1	–	17β-hydroxysteroid dehydrogenase type 1
17β-HSD 3	–	17β-hydroxysteroid dehydrogenase type 3
HSP	–	Heat shock protein
IC_{20}/IC_{50} value	–	Concentration causing 20% or 50% inhibition of enzyme activity
IGB-Berlin	–	Institut für Gewässerökologie und Binnenfischerei Berlin

IUPAC	–	International Union of Pure and Applied Chemistry
KT	–	11-ketotestosterone
Letrozole	–	4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl)methyl] benzonitrile (IUPAC)
LH	–	Luteinizing hormone
Linuron	–	3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (IUPAC)
LNCaP	–	Lymph node carcinoma of prostate
Ma	–	Male
MBT	–	Monobutyltin (IUPAC)
MBT-Cl	–	Butyl-trichloro-stannane (IUPAC)
MDR	–	Multidrug resistance
MeOH	–	Methyl alcohol
Methyltestosterone	–	17β-hydroxy-17α-methylandrost-4-en-3-one (IUPAC)
MgCl2	–	Magnesiumchloride hexahydrate
mRNA	–	Messenger ribonucleic acid
MT	–	*q.v.* methyltestosterone
N_2	–	Nitrogen
NAD(H)	–	Nicotinadenin nucleotide (reduced)
NADP(H)	–	Nicotinadenin nucleotide phosphate (reduced)
$NaHCO_3$	–	Sodium hydrogen carbonate
Na-pyruvate	–	Sodium pyruvate
O_2	–	Oxygen
17,20βP	–	17,20β-progesterone
P450arom	–	*q.v.* aromatase
PBS	–	Phosphate buffered solution
PCOS	–	Polycystic ovary syndrome
PKA	–	Protein kinase A
PPi	–	Pyrophosphate
Prochloraz	–	N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl] imidazole-1-carboxamide (IUPAC)
5α-Re	–	*q.v.* 5α-reductase
5β-Re	–	*q.v.* 5β-reductase
RF	–	Retardation factor
RNA	–	Ribonucleic acid
RPMI	–	Roswell Park Memorial Institute
RP	–	Reverse primer
rRNA	–	Ribosomal ribonucleic acid
RNA	–	Ribonucleic acid
RT-PCR	–	Reverse transcriptase polymerase chain reaction

SEM	–	Standard error of mean
SHBG	–	Sex hormone binding globulin
T	–	*q.v.* testosterone
TE	–	Tris EDTA
TLC	–	Thin layer chromatography
TRIZMA® Base	–	Tris[hydroxymethyl]aminomethane
TRIZMA® HCl	–	Tris[hydroxymethyl]aminomethane hydrochloride
TBT	–	Tributyltin (IUPAC)
TBT-CL	–	Tributyl-chloro-stannane (IUPAC)
TPT	–	Triphenyltin (IUPAC)
TPT-Cl	–	Chloro-triphenyl-stannane (IUPAC)
Tris	–	2-amino-2-hydroxymethyl-1,3-propanediol
TSH	–	Thyroid stimulating hormone
UBrun	–	Brunel University West London
Vinclozolin	–	3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-1,3-oxazolidine-2,4-dione (IUPAC)
V	–	Volume
WHI	–	World Health Institute

1 Zusammenfassung

Viele Umweltchemikalien stehen im Verdacht, das endokrine System von Menschen und Tieren zu schädigen. Diese endokrinen Disruptoren können auf verschiedene Weise wirken und möglicherweise zu einer Beeinträchtigung der Gesundheit führen. Die genauen Mechanismen sind noch unklar. Die Forschungsgruppe der Abteilung Endokrinologie, Uniklinkum Bonn, untersucht verdächtige Substanzen vorwiegend hinsichtlich deren direkter Wirkung auf den Androgen- und Östrogenstoffwechsel.

Die Experimente wurden innerhalb des von der Europäischen Union geförderten Projekts „COMPRENDO – Comparative Research on Endocrine Disrupters – Phylogenetic Approach and Common Principles focussing on Androgenic/Antiandrogenic Compounds“ durchgeführt. Die folgenden Substanzen, die unter anderem in der Landwirtschaft und Plastikindustrie verwendet werden, sind in der Vergangenheit als potentiell androgen und/oder antiandrogen aufgefallen und wurden daher vom Konsortium zu Beginn des Projekts ausgewählt: Dibutylzinn-Dichlorid (DBT-Cl), Diuron, Fenarimol, Linuron, Monobutylzinn-Trichlorid (MBT-Cl), p,p´DDE, Prochloraz, Tributylzinn-Chlorid (TBT-Cl), Triphenylzinn-Chlorid (TPT-Cl) und Vinclozolin. Als Positiv-Kontrollsubstanzen wurden in den verschiedenen Experimenten Enzyminhibitoren sowie Rezeptoragonisten und –antagonisten eingesetzt, die für den medizinischen Bereich entwickelt wurden: Finasterid als 5α-Reduktase Typ 2 Inhibitor (Proscar®, MSD Sharp & Dohme), Letrozol als Aromatase-Inhibitor (Femara®, Novartis Pharma), Flutamid als Androgenrezeptor-Antagonist (ohne Patentschutz) und Methyltestosteron (MT, ohne Patentschutz) als Androgenrezeptor-Agonist.

Der Schwerpunkt der vorliegenden Arbeit lag auf der Untersuchung der Wirkung der Testsubstanzen auf die Aktivität der Cytochrom P450 Aromatase (Aromatase, P450arom) und der 5α-Reduktase Typ 2. Beide Enzyme sind von essentieller Bedeutung im humanen Sexualsteroidhormon-Stoffwechsel.

Die Aromatase ist unerlässlich für die Synthese von Östron und Östradiol aus den Substraten Androstendion und Testosteron. Dieses Enzym findet man in fast allen Tierarten und beim Menschen, in den meisten Fällen ist die Aromatase in Gonaden und Gehirn exprimiert. Eine Inhibition der Aromatase könnte zu einer Verschiebung des Verhältnisses

zwischen Androgenen und Östrogenen zugunsten der Androgene und somit möglicherweise zu einer Vermännlichung des beeinträchtigten Organismus führen. In unseren Experimenten zur Untersuchung der Aromatase dienten Plazentagewebe und Zellen der Chorionkarzinom-Zellinie JEG-3 als Enzymquelle.

Die 5α-Reduktase (5α-Re) katalysiert im Sexualsteroidhormon-Metabolismus die Umwandlung von Testosteron in das zehnfach stärker an den Androgenrezeptor bindende Androgen Dihydrotestosteron. Es sind zwei Isoenzyme bekannt, von denen in der Prostata überwiegend 5α-Re Typ 2 vorkommt, während in Zellen der Prostatakarzinom-Zelllinie LNCaP 5α-Re Typ 1 und 2 vorhanden sind. Eine Hemmung der 5α-Reduktase-Aktivität könnte zu einer Veränderung des normalen Hormongleichgewichts führen zugunsten des schwächeren Androgens Testosteron. Diese Verschiebung könnte eine Verweiblichung des beeinträchtigten Organismus bewirken.

In den Enzymtests hemmte die Organozinnverbindung Tributylzinn die Aktivität der Aromatase sowohl in Plazentahomogenat als auch in JEG-3-Zellen im Mittel bei Konzentrationen von 12,2 µM bzw. 6,4 µM auf halbmaximale Geschwindigkeit (=IC_{50}-Wert). Auch die Aktivität der 5α-Re in Prostatahomogenat und LNCaP-Zellen wurde von TBT gehemmt, die IC_{50}-Werte waren im Durchschnitt 4,0 bzw. 2,7 µM.

Die zweite Organozinnverbindung Triphenylzinn wurde ebenfalls als Inhibitor der Aromatase und 5α-Re nachgewiesen. Die durchschnittlichen Konzentrationen, die zu einer halbmaximalen Enzymaktivität führten, waren bei der Untersuchung der Aromatase in Plazenta 6,2 µM und in JEG-3-Zellen 3,8 µM hoch, bei der Untersuchung der 5α-Re lagen die mittleren Werte bei 3,9 µM in Prostata und 4,2 µM in LNCaP-Zellen.

Das Imidazol Prochloraz hemmte im Mittel die P450arom-Aktivität auf halbmaximale Geschwindigkeit bereits bei niedrigen Konzentrationen von 0,06 µM in den Chorionkarzinomzellen und 0,024 µM im Plazentahomogenat. Die Aktivität der 5α-Reduktase wurde dagegen erst bei höheren Konzentrationen beeinflusst, die mittleren IC_{50}-Werte betrugen 12,4 µM in den Prostatahomogenat-Untersuchungen und 53,2 µM in den Tests mit LNCaP-Zellen.

Das Fungizid Fenarimol hemmte die Aromataseaktivität auf 50% bei durchschnittlichen Konzentrationen von 2,5 µM und 2,0 µM in Plazentahomogenat bzw. in JEG-3-Zellen. Die 5α-Re-Aktivität in Prostatahomogenat wurde erst bei einer mittleren

Konzentration von 24,1 µM halbmaximal gehemmt, die 5α-Re-Aktivität in LNCaP-Zellen wurde von keiner der hier verwendeten Fenarimol-Konzentrationen bis zu 50% gehemmt.

Im ersten Teil einer weiteren Studie wurde die inhibitorische Wirkung von TPT auf die Enzyme P450arom, 5α-Re Typ 2, 3β-HSD Typ 2, 17β-HSD Typ 1 und 17β-HSD Typ 3 untersucht. Im zweiten Teil wurde in den Enzym-Experimenten 100 µM TPT für eine komplette Inhibition eingesetzt und die Enzymaktivität in Abhängigkeit der Verwendung verschiedener Konzentrationen des starken Antioxidanz Dithioerythritol (DTE) untersucht. Als Enzymquelle diente in diesen Versuchen die Gewebe Plazenta (P450arom und 17β-HSD Typ 1), Prostata (5α-Re Typ 2), Nebenniere (3β-HSD Typ 2) und Testis (17β-HSD Typ 3). Um eine reelle Gefahr für den Menschen durch Organozinnverbindungen abzuschätzen, wurden außerdem Blutproben von acht gesunden Probanden auf Rückstände untersucht.

Es wurden signifikante Rückstände von Triphenylzinn (0,49-1,92 nmol/L) in den Blutproben festgestellt, während die Konzentrationen der anderen nachgeforschten Organozinnverbindungen nahe oder unter dem Detektionslimit lagen. Der Nachweis der hochgiftigen Substanz im Menschen ist bedenklich und untermauert die Dringlichkeit der Entwicklung und Etablierung von Methoden für die Risikoeinschätzung für Mensch und Tier.

Unsere Experimente dokumentieren eine konzentrationsabhängige, vollständige Inhibition der Aktivität aller getesteten Enzyme durch Triphenylzinn. Die IC_{50}-Werte waren im Durchschnitt bei 3β-HSD 4,0 µM, 17β-HSD Typ 3 4,2 µM, bei 17β-HSD Typ 1 10,5 µM, bei P450arom bei 1,5 µM und 5α-Re Typ 2 1,0 µM. Die Untersuchungen zeigten außerdem, dass das Antioxidanz DTE den schädigenden Effekt von TPT teilweise kompensieren und eine Enzymaktivität aufrechterhalten kann. Dieser schützende Effekt war unterschiedlich stark bei den getesteten Enzymen, bei 5α-Re konnten die eingesetzen Konzentrationen an DTE die supprimierten Aktivität durch TPT nicht wieder bis auf 50% anheben. Die maximale effektive Konzentration (=EC_{50}-Wert) des DTEs war bei den anderen Enzymen im Durchschnitt 0,9 mM bei 3β-HSD, 12,9 mM bei 17β-HSD Typ 3, 0,21 mM bei 17β-HSD Typ und 0,91 mM bei P450arom.

Die Fähigkeit von DTE antagonistisch auf die Inhibition der Enzymaktivität durch TPT zu wirken führt zu der Vermutung, dass der kritischen Angriffspunkt für TPT die

oxidierbaren Cysteinreste des Enzyms sind, die durch die Ausbildung von Disulfidbrücken entscheidend für die Tertiär- und Quartärstruktur sind.

Endokrine Disruptoren konnten sehr gut mit den Gewebetests nachgewiesen werden, in denen Plazenta-, Prostata-, Nebennieren- und Hodengewebe als Enzymquelle diente. Die Enzymaktivität in den Experimenten reagierte im Vergleich zu den Enzymaktivitäten in den Zelltests empfindlich auf die Testsubstanzen. Die Gewebetests waren außerdem einfacher und schneller zu handhaben. Dagegen spiegeln die Krebszell-Enzymtests auf Basis von JEG-3 Chorionkarzinomzellen und LNCaP Prostatakarzinomzellen zu einem gewissen Teil die Situation *in vivo* wider, da hier mit intakten Zellen gearbeitet wird.

Innerhalb des EU-Projekts COMPRENDO wurde ein Human-Biomonitoring zusammen mit unseren Partnern in Danzig durchgeführt. Dafür wurden Blut- und Urinproben von 60 Probanden in Polen und 15 Probanden in Deutschland gesammelt. Die Proben wurden auf Rückstände von p,p´DDE (S. Galassi, Universität Mailand), TBT, TPT (R. Jeannot, Bureau de Recherches Geologiques et Minieres, Orleans), Diuron, Fenarimol, Linuron und Vinclozolin (T. A. Albanis, Universität Ioannina) untersucht. In unserer Arbeitsgruppe wurden die Konzentrationen von Sexualhormonen überprüft. Es wurden außerdem Plazentaproben von 15 frisch entbundenen Müttern aus Polen und Deutschland gesammelt. Die Proben wurden auf Rückstände der oben genannten Chemikalien untersucht. Außerdem wurde die spezifische P450arom-Aktivität im Gewebe und der CYP19 mRNA-Gehalt gemessen.

Durch die Analyse auf Chemikalienrückstände wurden p´p-DDE Konzentrationen in Urin-, Blut- und Plazentaproben nachgewiesen. In einigen Urinproben der polnischen Probanden waren Konzentrationen an Monobultylzinn und Dibutylzinn vorhanden. Die gemessenen Sexualhormon-Konzentrationen im Blut korrelierten nicht mit den jeweiligen Chemikalienrückständen. im Blut oder Urin. Es konnte kein Zusammenhang zwischen den p´p-DDE Rückständen in den Plazenta-Proben und der jeweiligen Aromataseaktivität. Für die gemessenen Unterschiede zwischen den beiden Gruppen in Polen und Deutschland bezüglich der Sexualhormon-Konzentrationen, der spezifischen P450arom-Aktivität und des relativen CYP19-Gehalts sind daher andere Faktoren verantwortlich als die, die hier berücksichtigt wurden.

In einer weiterer Studie des Projekts COMPRENDO wurde die Wirkung von endokrinen Disruptoren auf die aquatischen Organismen *Xenopus laevis* (Krallenfrosch – W. Kloas, Institut für Gewässerökologie und Binnenfischerei, IGB-Berlin), *Rutilus rutilus* (Rotauge – W. Kloas, s.o.) und *Pimephales promelas* (Dickkopfelritze – S. Jobling, Universität Brunel) untersucht. Im Vorfeld wurde die Präsenz der oben genannten Enzyme des Steroidhormon-Metabolismus in den Organen Gehirn und Gonaden in männlichen und weiblichen Kontrolltieren überprüft. Im Gehirn von *Xenopus laevis* wurde zum ersten mal 5β-Reduktase-Aktivität nachgewiesen.

In den Expositionsversuchen am IGB-Berlin wurden das Wasser in den Aquarien mit 0,1 µM Letrozol, 0,1 µM Methyltestosteron, 0,1 µM Vinclozolin oder 0,01 µM TPT versetzt. Die Krallenfrösche und Rotaugen wurden 2 Wochen in einem der Becken gehalten. In den Experimenten der Universität Brunel waren die Dickkopfelritzen drei Wochen lang Wasser mit verschiedenen Konzentrationen Methyltestosteron, p,p´DDE und TPT ausgesetzt.

Die Gehirne und Gonaden der Versuchstiere wurden nach der Chemikalienexposition entnommen. Die Organe wurden entsprechend der Vorversuche zur qualitativen Analyse spezifisch auf veränderte Aktivitäten von Aromatase, 5α-Re, 17β-HSD oder 5β-Reduktase untersucht.

Bei *Rutilus rutilus* und *Xenopus laevis* führte die Exposition mit den Substanzen Methyltestosteron und Letrozol zu Veränderungen der Enzymaktivitäten, verglichen mit den Enzymaktivitäten der Kontrolltiere, deren Hälterungswasser mit Ethanol versetzt war. Im Unterschied zur starken inhibitorischen Wirkung von TPT in unseren Enzymtests fanden wir keine veränderten P450arom- oder 5α-Re-Aktivitäten in den untersuchten Organen, außer in den Ovarien von *Pimephales promelas*, die einer Konzentration von 320 ng TPT/L ausgesetzt waren. In dieser Gruppe war die mittlere P450arom-Aktivität erhöht, wahrscheinlich durch eine zu starke Hochregulierung des Enzyms, um die vorausgegangene Inhibition durch TPT zu kompensieren.

Mehrere der getesteten Chemikalien inhibieren die Aktivität eines oder mehrerer der getesteten Enzyme des Sexualsteroidhormon-Stoffwechsels bei Mensch und Tier und können daher von klinischer Relevanz sein. Die Ergebnisse der verschiedenen Experimente sind allerdings nicht immer übereinstimmend, daher lassen sich Schlussfolgerungen möglicher Gefahren für den Menschen und Tiere nur schwer ziehen. Für eine bessere

Risikoabschätzung werden Studien mit verschiedenen Kurz- und Langzeit-Testsystemen *in vitro* und *in vivo* benötigt.

2 Summary

Many environmental chemicals are suspected of disturbing the human and animal endocrine system. These so-called endocrine disruptors can operate in many ways. The interaction of endocrine disruptive effects that eventually endanger human health is still unclear. However, one of the basic mechanisms of endocrine disruption is the inhibition of key enzymes in the hormone metabolism.

In this study, we focused on the inhibitory potency of suspected endocrine disrupting compounds on aromatase (P450arom) and 5α-reductase (5α-Re) activities in human tissue and human cancer cells. Both enzymes are essential for the human sexl steroid hormone metabolism. We were able to demonstrate that the organotin compounds tributyltin (TBT) and triphenyltin (TPT) are potent unspecific inhibitors of P450arom and 5α-Re activity. Prochloraz and fenarimol inhibited P450arom activity at low concentrations (IC_{50}<2 μM), while 5α-Re activity was only impaired at higher concentrations (IC_{50}>10 μM). While the human tissue assay proved to be more practical and sensitive as a screening tool for putative endocrine disruptors, the cell assay reflected partly the situation *in vivo*.

In another experimental series, we investigated the inhibitory effect of TPT on P450arom, 5α-Re, 3β-HSD type 2, 17β-HSD type 1 and type 3 alone and in combination with the strong antioxidant dithioerythrithol (DTE). TPT inhibited unspecifically all enzymes that were tested. The experiments also showed that DTE is able to compensate the adverse effects of TPT, and that the effectiveness of the compensatory activity of DTE differs among the enzymes investigated. The suppressed 5α-Re activity could not be reactivated with DTE. Conceivably, cysteine residues that are responsible for the tertiary and quarternary structure of the enzyme are critical targets for TPT.

A human sampling study was undertaken with the COMPRENDO partner in Gdansk. 60 Polish and 15 German blood samples were investigated for chemical residues and sex hormone concentrations. In addition, 15 placenta samples from Poland and Germany, respectively, were tested for chemical residues, P450arom activities and CYP19 mRNA contents. The chemical analysis was performed by the COMPRENDO partners in Milan (p,p´DDE), Orleans (TBT and TPT) and Ioannina (diuron, fenarimol, linuron und vinclozolin). The results showed that individual sex hormone concentrations in blood were not correlated with chemical body burden. The detected differences in sex hormone concentrations, specific aromatase activity and relative CYP19 mRNA content of Polish

and German donors were presumably the result of other factors than the ones determined in this study.

Another task of the EU-project was the investigation of the effects of chemical exposure of the aquatic model organisms *Pimephales promelas, Rutilus rutilus* and *Xenopus laevis*. We investigated the specific P450arom and 5α-Re activities in brain and gonads of the animals. During the qualitative investigation of the androgen metabolism in *Xenopus laevis* brain, 5β-reductase activity was discovered for the first time. In contrast to the inhibitory potency of TPT discovered in our enzyme assays, TPT exposure of aquatic model organisms had no observed effect on enzyme activity in the organs investigated, except for P450arom activities in female gonads of *Pimephales promelas* at 320 ng TPT/L. In this group, mean P450arom activities were elevated, possibly as a result of an overshooting upregulation due to the inhibition of P450arom by TPT. The exposure of *Rutilus rutilus* and *Xenopus laevis* to the effector substances methyltestosterone and letrozole resulted in slightly different mean enzyme activities compared to the control group.

In conclusion, many of the tested pesticides are able to inhibit P450arom and 5α-Re, and thus might be of clinical relevance. However, results are not always coherent, and possible risks for human and wildlife health are therefore difficult to predict. Risk assessment will require large studies with an additional number of short and long term *in vitro* and *in vivo* assays. Any extrapolation to humans should be very meticulously performed.

3 General introduction

In the mid-nineties, scientific attention was drawn to the topic "Chemical compounds with endocrine effects". Since then, the causality between contamination with environmental chemicals and the occurrence of reproductive disorders in human and wildlife is being investigated. This topic is of such importance because we are facing a multitude of chemicals in our daily life. An endocrine disruptor (ED) is any "external substance that causes adverse health effects in an intact organism or its offspring as a result of a change in hormonal function" (EU-workshop in Weybridge, 1996). Endocrine disruption can occur in several elements of the endocrine system and through different mechanisms. Due to the complexity of the hormonal regulatory system, extrapolations of experiments to human health effects are difficult. However, the results of a number of *in vitro* and *in vivo* models have already contributed to understand the current risk of ED on humans and animals.

3.1 Environmental chemicals and endocrine disruption

A large number of pesticides and additives used in agriculture and industry has been reported to possess endocrine modulating activity (Bretveld et al. 2006; Gillesby and Zacharewski 1998; Kavlock and Ankley 1996). For most endocrine active compounds, their hormonal action is unintended. The substances acting as ED are chemically heterogeneous, and the observed phenomena are caused by different influences on sex hormone signaling pathways (Gillesby and Zacharewski 1998; Sonnenschein and Soto 1998). The effects can be direct or indirect, with agonistic or antagonistic action (Fig. 3–1, Bretveld et al. 2006; Degen et al. 1999). Direct impacts include the interaction with hormone receptors, the change of the density of hormone receptors in the cells of target tissues and the modulation of post-receptor signaling (Kelce et al. 1994; Duax et al. 1985). Indirect mechanisms affect steroid hormone transport proteins, steroid hormone synthesis, metabolism and secretion (Sonnenschein and Soto 1998). As hormones act at extremely low levels – *i.e.*, parts per trillion – exposures to minimal amounts of ED may be of concern, particularly during sensitive periods of fetal development.

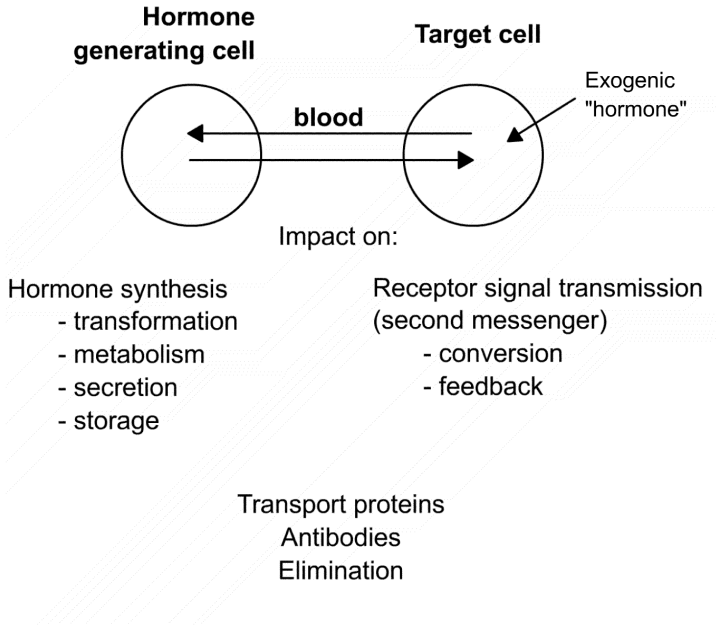


Fig. 3–1 Possible impact of endocrine disrupters on the endocrine system.

3.1.1 Effects of endocrine disruptors on wildlife animals

It is difficult to determine to what extent endocrine disrupters (ED) affect reproduction or population size of wildlife species. This is because many factors such as habitat restriction, human interference and changes to natural food supplies can play an important role on population size. However, in a number of cases there is data supporting the causal connection between biological disorganisation and the exposure to a specific chemical agent. Endocrine disruption has been postulated as the cause of harmful effects in various wildlife species, *e.g.*, (a) impaired reproductive functions of seals exposed to PCBs, (b) abnormal male reproductive organs with altered sex hormone levels due to exposure to metabolites of DDT, (c) hermaphroditism, vitellogenin in males and altered testes development in roach due to sewage effluents, (d) eggshell thinning with embryo mortality of water birds exposed to DDE and PCBs, and (e) imposex in marine neogastropods caused by water contamination with tributyltin, bisphenol A and octylphenol (Jobling and Tyler 2006; Oehlmann et al. 2000; Safe 2000; Schulte-Oehlmann et al. 2000; Vos et al. 2000; Tyler et al. 1998; Guillette and Guillette 1996).

3.1.2 Effects of endocrine disruptors on human health

In most countries, human population size and the average age, as an indicator for life standard, has increased during the recent decade, mainly due to advances in health care and hygiene. However, at least in some parts of the world, people have to face problems that are related to the chronic exposure to toxic or endocrine active chemicals, *e.g.*, reproduction disorders and increased incidence of certain cancers that are suggested to be triggered by chemical exposure (Solomon and Schettler 2000). Symptoms with suspected relation to endocrine disruption include:

a) temporal reduction in sperm counts and quality (Swan 2006b; Younglai et al. 1998),
b) altered fertility (Bretveld et al. 2006; Jarow 2003),
c) increased incidence of testicular, prostate and breast cancer (Liu et al. 1999; McKiernan et al. 1999; Levy et al. 1998; Haas and Sakr 1997; Bergstrom et al. 1996; Wang and Cao 1996; Wolff et al. 1996),
d) developmental abnormalities, like hypospadia and cryptorchidism, in reproductive organs (Damgaard et al. 2006; Swan 2006a; Skakkebaek 2004; Steinhardt 2004; Skakkebaek 2003; McLachlan et al. 2001; Paulozzi 1999; Guillette and Guillette 1996),
e) altered sex ratios (Allan et al. 1997),
f) effects on neurological and intellectual function in children due to prenatal chemical exposure (Jacobson and Jacobson 1997; Schaefer 1994).

In addition to accidental and unwanted chemical exposure, hormonally active compounds are in widespread use for beneficial medical health care with sometimes alarming side-effects. One of the most severe clinical cases was discovered in 1971, when clinicians traced back the occurrence of rare cervical and vaginal carcinoma in young women to maternal use of the synthetic estrogen diethylstilbestrol (DES) during pregnancy. Sons of DES-treated mothers were at risk of genital anomalies and abnormal spermatogenesis. This was the first example of transplacental carcinogenesis in humans, indicating that the fetus may suffer the greatest risk of adverse effects of endocrine disruption (Giusti et al. 1995; Herbst et al. 1971).

It is possible that any ED in the body, depending on a “critical window” of exposure, induces or promotes a developmental impairment of reproductive organs and brain. Data exploring children´s or fetuses´ exposure and sensitivity towards chemicals is still very limited. Children do have a higher metabolic rate compared to adults: they eat more food and breathe more air per pound of body weight. In addition, they come in much closer contact to their environment through crawling, touching and tasting. Thus, in comparison to adults children are disproportionately higher exposed to toxic chemicals in our environment (Landrigan 2001; Goldman and Koduru 2000; Chance and Harmsen 1998).

3.1.3 Models for the detection of endocrine disruption

In the last years, scientists have been increasingly trying to throw light on the mode of endocrine disrupting actions and hierarchy of observed effects. For example, ED exposure of pre-pubertal rats can influence endocrine processes following the sensitivity hierarchy: changes in anogenital distance > induction of hypospadias > induction of ectopic testes (Ashby 2000). As the underlying mechanisms of the observed effects until now remain hidden, results from *in vitro* experiments may fill these gaps.

The challenge is to find the connection between results from the test-tube and the laboratory animal in accordance to „in vitro simplicitas, in vivo veritas“. The Endocrine Disruptor Screening Program (EDSP), organized by the Environmental Protection Agency (EPA), has selected several assays and grouped them in two classes. Tier 1 screening assays identify the agents that may have hormone-related activity: *in vitro* (a) AR or ER binding and transcriptal activation (human or rat cells), (b) steroidogenesis assay (human testis); *in vivo* (c) three-day uterotrophic assay with subcutaneous administration (rat), (d) 5–7 day Hershberger assay (rat), (e) metamorphosis test (*Xenopus laevis*), (f) gonadal recrudescence (*i.e.*, *Pimephales promelas*), and (g) 20-day pubertal female assay with thyroid end points (rat). Tier 2 assays are selected assays with the capability to characterize adverse effects and dose response relationships for agents positive-tested in tier 1: mammalian reproductive, two-generation toxicity, reproduction and life-cycle test in (a) rat, (b) bird, (c) fish, and (d) mysid shrimp (webpage EPA).

3.2 The Endocrine system

The endocrine system is found in all mammals, birds and fish. It regulates many functions of the body, including metabolism, growth and function of the reproductive system, and the development of the brain and nervous system. The endocrine system implies ductless glands located throughout the body that secrete chemical messengers (hormones) which are released into the bloodstream and circulate within the body, and receptors in various organs and tissues recognize and respond to them.

3.2.1 Sex hormones

In general, sex hormones are classified into male and female hormones. Androgen representatives are testosterone, androsterone and dihydrotestosterone (DHT), while the estrogen estradiol and the gestagen progesterone are the major female hormones. Sex hormones affect sexual differentiation and maturation, including the growth and function of the reproductive organs, the development of secondary sex characteristics, and the behavioral patterns.

3.2.2 Sexual differentiation

One of the central hormones in sexual differentiation is testosterone. The development of the male phenotype is induced by perinatal testosterone and anti-Müllerian hormone (AMH). Masculinization includes the development of the brain, the persistence of the Wolffian duct system, and the differentiation of male external genitalia (Simpson and Rebar 1995). In the target tissue or organ, testosterone is locally converted into the more potent androgen DHT. In females, it is largely the absence of testosterone and AMH that results in phenotypic and endocrinotypic female development, like the formation of Müllerian ducts and degeneration of the Wolffian ducts. The same tissue that forms the penis and scrotum in males forms the clitoris, labia, and vagina in females. At puberty, estrogens stimulate the development of female secondary sex characteristics, including enlargement of the breasts and onset of menstruation (Simpson and Rebar 1995).

3.2.3 Androgen and estrogen action

Androgens are primary responsible for the maturation of male sex organs, the stimulation of spermatogenesis and the formation of male accessory glands, the development of secondary sexual characteristics, and the growth of muscle mass.

Estrogens promote the proliferation of follicular thecal cells, endometrial stromal and epithelial cells. They are also responsible for the development of female secondary sex characteristics. The main production of estrogens occurs in the ovaries before menopause. During pregnancy, the formation of estrogens shifts into the placenta. Minor quantities are also produced in testis and adrenal glands (Andersen et al. 2002; Simpson et al. 1997). They are also synthesized extraglandulary in adipose tissue (Deslypere et al. 1985). After the menopause, the lack of estrogens results in several more or less severe symptoms, which often can be restored by the intake of synthetic hormones. Yet, hormone replacement therapy has become disreputable because of the World Health Institute (WHI) study in 2000 (Prelevic et al. 2005; Henderson and Feigelson 2000).

One of the major actions of androgens and estrogens is to initiate the production of proteins. Since androgens and estrogens can pass through the phospholipid membranes of the cell, androgen and estrogen receptors (AR and ER) can be located directly in the nucleus. Androgen and estrogens trespass into the nucleus and bind to the AR and ER, respectively, which causes the separation of an associated heat shock protein (HSP). This allows the formation of hormone receptor dimers, which can bind to the hormone response element (HRE) to promote transcription of the associated genes.

Until now, there is only one active form of the androgen receptor known, while two different estrogen receptor subtypes (α and β) have been detected (Mosselman et al. 1996). They form mixed dimers in the presence of ligands: ERα (αα), ERβ (ββ) and ERαβ (αβ) (Li et al. 2004). The ERα is found in the endometrium, breast cancer cells, ovarian stroma cells and the hypothalamus (Yaghmaie et al. 2005), while the ERβ has been documented in kidneys, brain, bones, heart, lungs, intestinal mucosa, prostate, and endothelial cells (Babiker et al. 2002). More recently, receptors have been shown to have actions that are independent of their interaction with DNA. Some AR and ER associate with the cell surface membrane or are found in the cytoplasm. Hormone binding can cause rapid changes in cell functions independent of changes in gene transcription, such as changes in the ion transport (Kampa and Castanas 2006; Wehling et al. 2006; Zivadinovic and Watson 2005; Heinlein and Chang 2002).

3.2.4 Steroidogenesis

All steroid hormones derive from cholesterol and contain the same cyclopentano-phenanthrene ring. The various enzymes involved in the synthesis of steroid hormones are members of the cytochrome P450 class. The nomenclature of the enzymes indicates the site of hydroxylation.

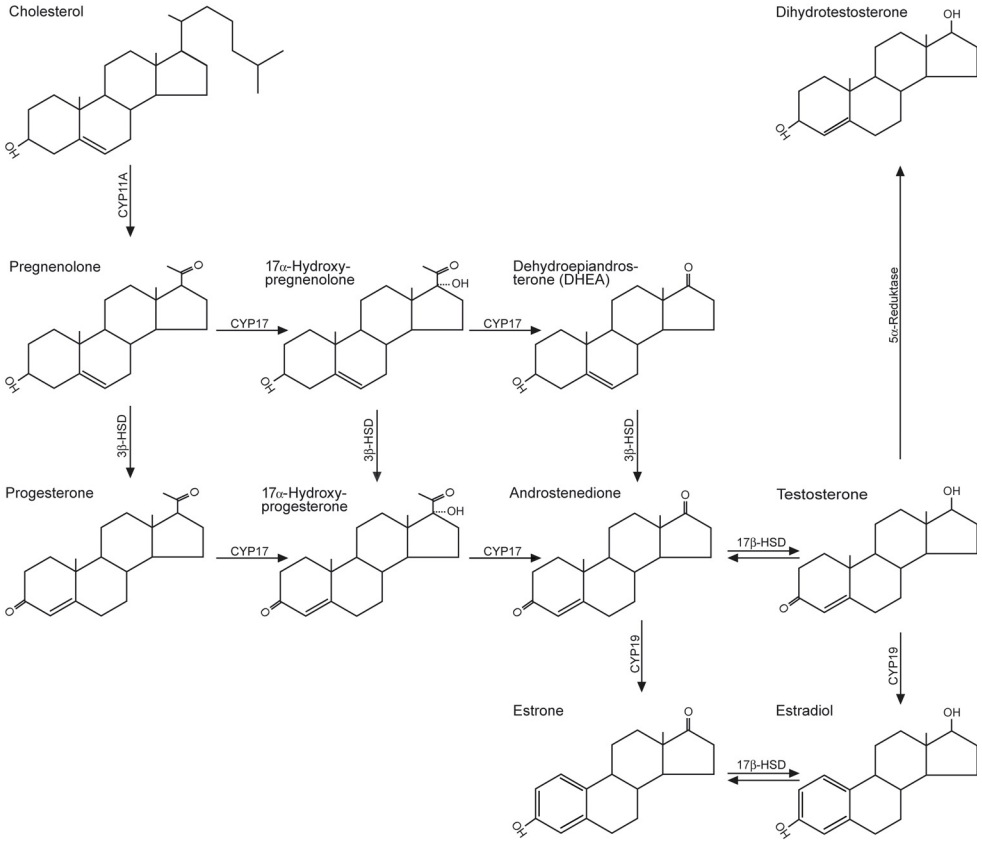


Fig. 3–2 Main steroid hormone pathway in gonads (Sanderson and van den Berg 2003). Enzymes involved are CYP11A (P450scc-desmolase), CYP17 (17β-hydroxylase and 17,20 desmolase), 3β-HSD (3β-dehydrogenase $\Delta^{4,6}$-isomerase), 17β-HSD and CYP19 (P450 arom).

The cascade of conversions of C_{27} cholesterol to the 18-, 19-, and 21-carbon sex steroid hormones (Fig. 3–2) is introduced by the cleavage of a 6-carbon residue from cholesterol to produce pregnenolone, catalyzed by the side chain cleaving enzyme P450scc-desmolase (CYP11A). Pregnenolone (C_{21}) is converted into 17α-OH-pregnenolone by the addition of a hydroxygroup by CYP17. The same CYP17 catalyzes the cleavage of the C17 side chain

of 17α-OH-pregnenolone. The resulting steroid DHEA is one of the direct precursors for androgens. In a similar pathway, the production of androstenedione is carried out via progesterone after 3β-hydroxylation of pregnenolone by 3β-HSD. Androstenedione, like DHEA, is a direct precursor for androgen and estrogen metabolism.

3.2.5 Key enzymes of the human androgen and estrogen pathway

3β-hydroxysteroid dehydrogenase

Two different isoforms of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD type 1 and 2, EC 1.1.1.145) catalyze the transformation of Δ^5–3β-hydroxysteroids into their corresponding ketosteroids (Simard et al. 1996). 3β-HSD is membrane-bound and the reaction uses NAD as proton acceptor (Labrie et al. 1994).

There are several important 3β-hydrogenization steps in steroid metabolism. The one that we focus on is the conversion from DHEA into androstenedione as a precursor for androgen and estrogen metabolism. 3β-HSD type 1 is predominantly expressed in placenta, breast and skin, while adrenal cortex, testis and ovaries are the primary sites for 3β-HSD type 2 activity (Labrie et al. 1994). Ancillary sites of action include the skin, liver, adipose tissue, breast, lung, endometrium, prostate, brain and epididymis (Labrie et al. 1994; Milewich et al. 1991). The widespread distribution of 3β-HSD suggests the importance of intracrine formation of sex steroids in peripheral target tissues (Luu The et al. 1989).

17β-hydroxysteroid dehydrogenase

The key step in the formation and re-inactivation of all estrogens and androgens is catalyzed by members of the 17β-hydroxysteroid dehydrogenases (17β-HSDs) family. The reaction is a reversible oxidoreduction at position C17. To date, ten different human 17β-HSDs have been cloned, sequenced and characterized (Yang et al. 2005). 17β-HSDs are omnipresent in the body, *i.e.,* they were found in all 25 tissues of a rhesus monkey (Martel et al. 1994). The 17β-HSDs provide the cells with the intracellular concentration of each sex steroid according to local needs (Labrie et al. 2000). In the present study, we have focused on 17β-HSD type 1 and 3. Both types are important in the androgen and estrogen metabolism.

17β-HSD Type 1 (17β-HSD1, EC 1.1.1.62) is a cytosolic protein that catalyzes predominantly the conversion of the low active estrogen E1 into highly active E2, using

NAD(P)H as cofactor (Green et al. 1999). 17β-HSD1 ensures a high level of estradiol formation, especially in ovary, placenta and breast (Labrie et al. 2000). Its molecular structure and corresponding gene was the first to be elucidated of the 17β-HSD family (Peltoketo et al. 1999).

17β-HSD Type 3 (17β-HSD3, EC 1.1.1.63) is a microsomal isozyme which uses NADP(H) as a cofactor and is predominantly expressed in the testes. The preferred substrate is androstenedione, which is being transformed into testosterone. 17β-HSD Type 3 deficiency causes male pseudohermaphroditism (Geissler et al. 1994).

Cytochrome P450 aromatase

Cytochrome P450 aromatase (EC 1.14.14.1, P450arom) is essential for the estrogen biosynthesis from C19 precursor androgens (Simpson et al. 1997). The membrane-bound P450arom enzyme system was first discovered in placental tissue and consists of the cytochrome P450arom and NADPH-cytochrom P450 reductase. Cytochrome P450arom is responsible for the introduction of the conjugated π-electron system into ring A of the steroid, as a result of the cleavage of the C19-methyl group (Fig. 3–3). NADPH-Cytochrom P450 reductase allocates the required reducing equivalents. The enzyme reaction of 1 mol substrate requires 3 mol NADPH and 3 mol O_2, while 1 mol H_2O and 1 mol formic acid (HCOOH) are released.

The P450arom gene CYP19 is localized on chromosome 15q21.1. In total, 10 exons (70 kb) encode the protein that consists of 503 amino acids (Corbin et al. 1988). Different tissue specific promoters were identified, thereof the most important ones in extraglandular tissue (P1.3 and P1.4), in placental tissue (P1.1), in ovary and breast cancer (PII).

The enzyme P450arom is not only found in humans, but has been identified in several other species. P450arom is an old enzyme from the evolutional perspective, and can therefore be found widespread in various species from mollusks to mammals. In most species, P450arom is expressed in gonads and brain (Steckelbroeck et al. 1999b; Simpson et al. 1997). In teleost fish, P450arom activity in the brain is comparable higher than in mammalian brains (Gonzalez and Piferrer 2002). The reason for this is still unclear. P450arom in the placenta can only be found in females of primates, horses, cattle, and pigs.

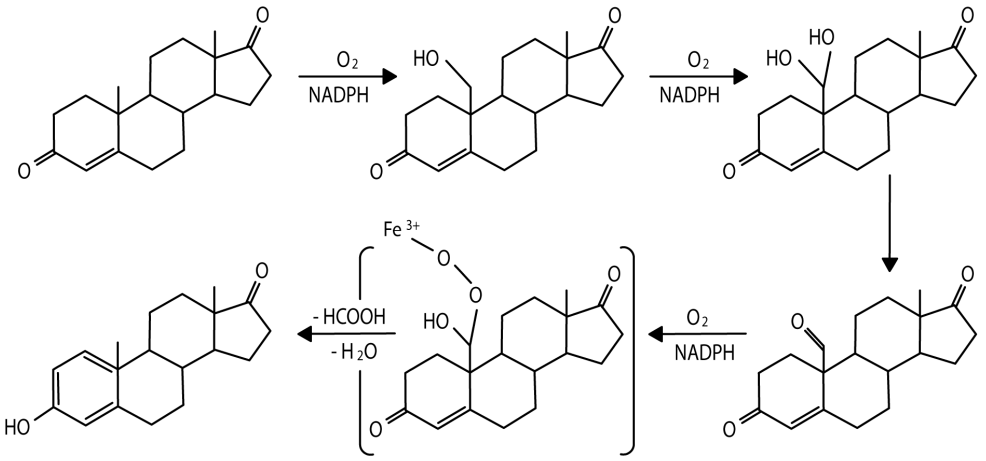


Fig. 3–3 P450arom catalyzed transformation of androstenedione into estradiol.

According to its key function, changes in P450arom content or catalytic activity have direct impact on the estrogen level. The importance is underlined by the fact that world-wide only eight female and two male patients are known with primary P450arom deficiency. Typical symptoms are pseudohermaphroditism, primary amenorrhoea, missing breast development, hypogonadism, and polycystic ovaries (Ito et al. 1993). Due to the missing closure of epiphyses, affected men are comparably tall, infertile and suffer from osteoporosis (Carani et al. 1997).

5α-reductase

The microsomal enzyme 3-oxo-5α-steroid 4-dehydrogenase, synonym of 5α-reductase (5α-Re, E.C. 1.3.99.5), is responsible for the irreversible conversion of testosterone (T) into the approximately 10 times more potent androgen dihydrotestosterone (DHT) (Ntais et al. 2003; Rizner et al. 2003; Wilson et al. 1993). DHT is indispensable for the normal virilization and function of the male external genitals and prostate (Wilson 1996; Harris et al. 1992). Two isoforms of the catalyzing enzyme have been identified in humans and animals so far (Wilson et al. 1993). Both isozymes are transiently expressed in newborn skin and scalp. Isozyme 1 is permanently expressed in skin from the time of puberty. Type 2 isozyme is predominant in fetal genital skin, male accessory sex organs and in prostate, including benign prostatic hyperplasia and prostate adenocarcinoma tissues (Ntais et al.

2003; Thigpen et al. 1993). High levels of endogenous androgens have long been considered as risk factors for prostate cancer, therefore it has been postulated that certain 5α-Re polymorphisms or the damage of androgen inactivation pathways support the pathogenesis of prostate cancer (Ntais et al. 2003). The expression pattern of 5α-Re isozymes in prostate cancer tissues and most prostatic tumor cells are still under investigation (Ntais et al. 2003; Zhu et al. 1998). In LNCaP cells, both isozymes of 5α-Re are present and functionally important (Zhu et al. 1998).

3.3 Outline of this study

A number of pesticides act as androgenic and/or anti-androgenic compounds (AAC). As a part of the EU-project COMPREDO, the suspected AACs dibutyltin-dichloride (DBT-Cl), diuron, fenarimol, linuron, monobutyltin-dichloride (MBT-Cl), p,p´DDE, prochloraz, tributyltin-dichloride (TBT-Cl), triphenyltin-dichloride (TPT-Cl), vinclozolin, as well as the pharmaceuticals finasteride, flutamide, letrozole (Letro), and methyltestosterone (MT) were selected for detailed investigation (Schulte-Oehlmann et al. 2006). The consortium expected to find endocrine disrupters causing multiple positive responses in the test-systems among these substances.

This study intended to clarify the following questions:

1. Do the selected AAC affect the activity of human P450arom and 5α-reductase?
2. Which human cancer cell lines can be used for the screening of the disruptive potential of a test substance on P450 arom and 5α-reductase activity?
3. How does the endocrine disruptor triphenyltin (TPT) inhibit enzyme activity? Does the use of the strong antioxidant dithioerythritol (DTE) influence the inhibitory potency of TPT?
4. Which enzyme activities are detectable in brain and gonadal tissue of the aquatic model animals *Pimephales promelas*, *Rutilus rutilus* and *Xenopus laevis*? Does the exposure of these aquatic animals to suspected and known AAC result in altered enzyme activities in their brains and/or gonads?
5. Is there any relationship between chemical body burden (organotin, p,p´DDE, linuron, diuron, vinclozolin, and fenarimol) and sex hormone concentration in donor blood, placental aromatase activity or amount of placental CYP19 mRNA?

The thesis is structured as follows:

Chapter 4 gives information on general materials and methods used in most of the experiments of the studies.

Chapter 5 describes the examination of inhibitory effects on human P450arom activity in placenta tissue and chorion carcinoma cells (JEG-3) caused by the test compounds. In a human sample study, 60 Polish and 15 German donors were interviewed and sampled for blood and urine. The samples were examined for pesticidal body burden, measured by COMPRENDO partners in Ioannina, Milan and Orleans, while the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), androstenedione (Enon), testosterone (T), DHT, and estradiol (E2) in blood serum were determined in Bonn. In addition, 16 German and 14 Polish mothers provided placenta samples for the investigation of residual chemicals, as well as for the analysis of specific P450arom activity and CYP19 mRNA expression.

Chapter 6 focuses on the examination of the test substances regarding their effect on human 5α-reductase activity in prostate tissue and prostate carcinoma cells (LNCaP). Possible risks for human reproductive and sexual development caused by altered 5α-Re activity are discussed, and the suitability of the tissue und the cancer cell model is reviewed.

TPT has been long known to cause masculinization of female animals (imposex) in some meso- and neogastropods via endocrine disruption (Schulte-Oehlmann et al. 2000). Therefore, a closer investigation of the effects of TPT on several enzymes of human sex steroid metabolism was carried out as laid out in **chapter 7**. The target enzymes were (a) 3β-HSD in adrenal gland, (b) 17β-HSD in testis, (c) 5α-reductase in prostate, and (d) P450arom and (e) 17β-HSD in placenta. In another experimental series, the use of the strong antioxidant dithioerythritol (DTE) during the enzyme assay was used to clarify whether TPT is an endocrine disruptor as defined above.

In **chapter 8**, animal exposure experiments were used to learn more about the influence of suspected endocrine disrupters on the hormone system of selected model animals. The experiments with *Xenopus laevis*, *Rutilus rutilus* (IGB-Berlin) and *Pimephales promelas* (UBrun) were initiated with sexually mature adults that were exposed to the chemicals of interest for 21 days. A number of endpoints related specifically to

endocrine function were assessed by the respective COMPRENDO partner, including alterations in secondary sexual characteristics, gonadal condition and sperm mobility. In this study, the exposed animals were investigated for changes in enzyme activities through various tissue samples.

4 General materials

4.1 Laboratory equipment

Listing 4–1 Laboratory equipment

Device	Manufacturer
ABI Prism® 7700	Applied Biosystems, Foster City, USA
Autoclave type 23	Melag, Berlin, Germany
Centrifuges:	
Minifuge Sigma 113	B. Braun, Melsungen, Germany
Centrifuge Z 382 K	Hermle, Wehingen, Germany
Centrifuge PKR	Beckman Coulter, Krefeld, Germany
Cooling facilities:	
Refrigerator (no name)	GFL, Burgwedel, Germany
Deep freezer GFL 6343-6345	GFL, Burgwedel, Germany
DNA Thermo Cycler Cetus	PerkinElmer, Rodgau-Jügesheim, Germany
Fluorometer Polarstar Galaxy	BMG Labtech, Offenburg, Germany
Homogenizer Teflon-Glas Potter S	B. Braun, Melsungen, Germany
Ice machine	Ziegra, Isernhagen, Germany
Incubator	Heraeus, Hanau, Germany
IMMULITE® 2000 Analyzer	DPC-Biermann, Bad Nauheim, Germany
Liquid scintillation counter 1409	Wallac, Turku, Finland
Luminometer Centro LB 960	Berthold Technologies, Bad Wildbad, Germany
Magnetic stirrer Mag-O	Gerhardt, Königswinter, Germany
Microscope Axiovert S100	Zeiss, Jena, Germany
pH-meter Portatest 655	Knick, Berlin, Germany
Photometer:	
Ultrospec 2000	Pharmacia Biotech, Uppsala, Sweden
UV-Vis 550 S	PerkinElmer, Rodgau-Jügesheim, Germany
Pipettes:	
10 µL, 100 µL, 1000 µL	Eppendorf, Hamburg, Germany
Multipette	Eppendorf, Hamburg, Germany
TLC analyzer Tracemaster 20	Berthold Technologies, Bad Wildbad, Germany
TLC chamber	Desaga, Wiesloch, Germany
Safety work bench LaminAir® TL 2448	Kendro, Langenselbold, Germany

Sonic device Labsonic 2000	B. Braun, Melsungen, Germany
Vibrofix VF 1 Electronic	IKA-Labortechnik, Staufen, Germany
Water quench GFL 1083	GFL, Burgwedel, Germany
Weighing machine:	
Scale 1264 MP	Sartorius, Göttingen, Germany
Acute scale Sartorius	Sartorius, Göttingen, Germany

4.2 Consumables

Listing 4–2 Consumables

Material	Manufacturer
Blue Max™ Falcon® (15 mL, 50 mL)	Becton, Dickinson and Company, Plymouth, United Kingdom
Cell culture plates (6-, 12-, 96-well)	Greiner Bio-one, Frickenhausen, Germany
Centrifuge tubes	Greiner Bio-one, Frickenhausen, Germany
Combitips	Eppendorf, Hamburg, Germany
Cryo tubes	Greiner Bio-one, Frickenhausen, Germany
Cuvettes, expendable (10x4x45 mm)	Sarstedt, Nümbrecht, Germany
Gloves, Safeskin® powder free, expendable	Kimberly-Clark, Forchheim, Germany
Leukosilk®	BSN Medical, Hamburg, Germany
Membrane filters (0.2 mm)	Schleicher & Schüll, Dassel, Germany
Micropipettes, expendable (10 µL)	Brand, Wertheim, Germany
Petri dishes, Falcon®	Becton, Dickinson and Company, Plymouth, United Kingdom
Pipettes, expendable, sterile	Greiner Bio-one, Frickenhausen, Germany
Pipette tips	Greiner Bio-one, Frickenhausen, Germany
QIAshredder	Qiagen, Hilden, Germany
Scalpels, expendable	B. Braun, Melsungen, Germany
Silica TLC foils, Polygram® (0.2 mm)	Macherey-Nagel, Düren, Germany
Silica thin layer plates	Merck, Darmstadt, Germany
Silica thin layer plates, channeled	Whatman, Maidstone, United Kingdom
Scintillation vials (20 mL)	Greiner Bio-one, Frickenhausen, Germany
Vakutainer® reaction tubes	Becton, Dickinson and Company, Plymouth, United Kingdom
Weighing dishes, Rotilabo®, expendable	Carl Roth GmbH, Karlsruhe, Germany

4.3 Chemicals

4.3.1 Radioactive steroids

Listing 4–3 Radioactive steroids

Steroids	Specific radioactivity
[1β-^{3}H(N)]-androst-4-ene-3,17-dione	25.9 Ci/mmol
[4-^{14}C]-[10]-estratriene-3β-ol-17-one	51.3 mCi/mmol
[4-^{14}C]-androst-5-ene-3β-ol-17-one	53.8 mCi/mmol

All radioactive steroids were bought from NEN Perkin-Elmer™ (Rodgau, Germany). Purification of radioactive substrates was performed according to Steckelbroeck (1999a; 1999b). In brief, 2 µL of the purchased radioactive labeled steroids were analyzed for their purity grade via thin layer chromatography (TLC). If substrate radioactivity was below 95%, the whole substrate was applied onto a TLC glass plate that was cleaned beforehand during an overnight flow in CH_2Cl_2/MeOH (180 mL + 20 mL) as mobile phase. The radio labeled substrate was separated by TLC in CH_2Cl_2/acetone (92.5 mL + 7.5 mL) under optimal conditions, and the plate was scanned for radioactivity in a plate scanner Tracemaster 20. When the radioactivity of the substrate was identified, the according silica gel area was scraped from the plate and eluated with 2 mL EtOH, MeOH, and $CHCl_3$, respectively. The eluate was dried under N_2 and redissolved in approximately 10 mL oxygen-free EtOH. Disintegrations per minute (dpm) were measured in an aliquote of the stock solution to determine the final concentration.

4.3.2 Reference steroids

Unlabeled 5α-androstane-3,17-dione (5α-androstanedione), androst-4-ene-3,17-dione (androstenedione), 5α-androstane-17β-ol-3-one (5α-DHT), 5α-androstane-3β-ol-17-one (androsterone), androst-4-ene-17β-ol-3-one (testosterone), 5α-androstane-3β,17β-diol (3β-diol), [10]-estratriene-3β-ol-17-one (E1), [10]-estratriene-3β,17β-diol (E2), androst-5-ene-3β-ol-17-one (DHEA), and androst-5-ene-3β,17β-diol were purchased from Sigma-Aldrich® (Seelze, Germany). The 5β-reduced steroids 5β-androstane-3,17-dione (5β-androstanedione), and 5β-androstane-17β-ol-3-one (5β-DHT) were kindly provided by Prof. Siekmann (Dept. of Clinical Biochemistry, University Bonn, Germany). Steroid

solutions of approximately 10 mg/mL solvent were prepared in EtOH and $CHCl_3$ at varying ratios according to each reference steroids´ solubility.

4.3.3 Test compounds

The test compounds were purchased by the coordinator in Frankfurt and distributed to the different COMPRENDO partners in order to ensure equal quality in the divers experiments. The substances are listed in Table 4–1.

Table 4–1 Part A Basic data of the test compounds.

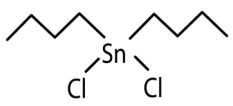
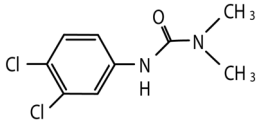
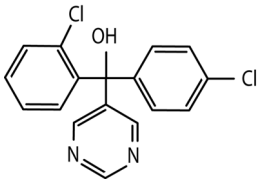
	DBT-Cl	**Diuron**	**Fenarimol**
Manufacturer	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany
Cas number	683-18-1	330-54-1	60168-88-9
Chemical name (IUPAC)	Dibutyl-dichloro-stannane	3-(3,4-dichlorophenyl)-1,1-dimethyl-urea	(2-chlorophenyl)-(4-chlorophenyl)-pyrimidin-5-yl-methanol
Group	Organotin	Phenyl urea	Pyrimidine
Molecular structure			
Application	Urethane and esterification catalyst in the plastic industry, heat stabilizer	All-out herbicide for maintenance of road and path network	Fungicide used on many crops, lawn care
Mode of action	Inhibition of alpha-keto oxidase activity	Inhibition of photosynthesis (PS II)	Inhibitor of C14-demethylase in sterol biosynthesis
Suspected damage	Toxic to aquatic organisms, irritant to skin, immunosuppressive	Toxic to aquatic organisms, teratogenic	Estrogenic, adverse effective on the fertility of male mice and rats, toxic to gastro-intestine and liver
References	Boyer 1989; IPCS	IPCS	Vinggaard et al. 2005; IPCS

Table 4–1 Part B Basic data of the test compounds.

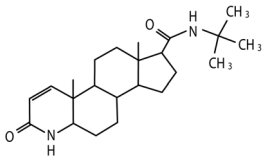
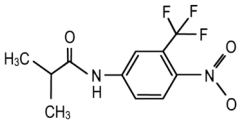
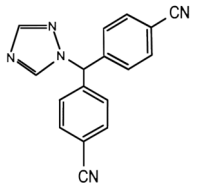
	Finasteride	**Flutamide**	**Letrozole**
Manufacturer	MSD Sharp & Dohme, Haar, Germany	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany
Cas number	98319-26-7	13311-84-7	112809-51-5
Chemical name (IUPAC)	N-(1,1-Dimethylethyl)- 3-oxo-(5α,17β)-4-azaandrost-1-en-17-carboxamid	2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide	4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl)methyl]benzonitrile
Group	Nonsteroidal antiandrogen	-	Nonsteroidal P450arom inhibitor
Molecular structure			
Application	BPH, alopecia, prostate cancer	Medical antiandrogen used in prostate cancer and PCOS therapy	Breast cancer and prostate cancer therapy
Mode of action	Inhibition of 5α-Re, predominately isozyme 2	Androgen antagonist	Binding to Hem
Suspected damage	-	Toxic to development, neurotoxic	-
References	IPCS	Diamanti-Kandarakis et al. 1998; IPCS	IPCS

Table 4–1 Part C Basic data of the test compounds.

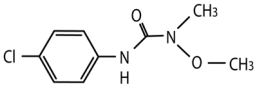
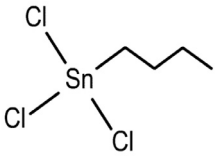
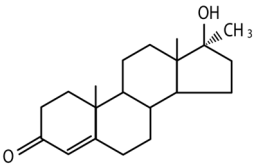
	Linuron	**MBT-Cl**	**Methyltestosteron**
Manufacturer	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany
Cas number	1746-81-2	1118-46-3	58-18-4
Chemical name (IUPAC)	3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea	Butyl-trichloro-stannane	17ß-hydroxy-17α-methylandrost-4-en-3-one
Group	Phenyl urea	Organotin	Synthetic androgen
Molecular structure			
Application	All-out herbicide for road and path network	Intermediate for PVC stabilizers, heat stabilizer, glass coatings	Anabolic, Klinefelter syndrome therapeutic agent
Mode of action	Inhibition of photosynthesis, suspected AR antagonist	-	AR agonist
Suspected damage	Androgenic, very toxic to aquatic organisms	-	Chronic use can cause menstrual irregularities and virilization in women
References	Lambright et al. 2000; Gray et al. 1999b	Boyer 1989; IPCS	IPCS

Table 4–1 Part D Basic data of the test compounds.

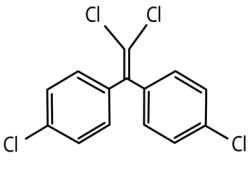
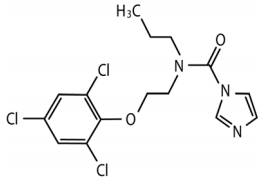
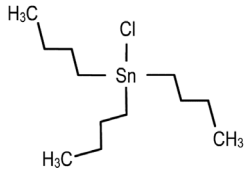
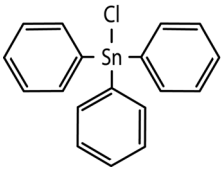
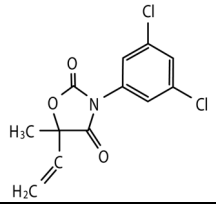
	p,p´DDE	**Prochloraz**	**TBT-Cl**
Manufacturer	Sigma-Aldrich, Seelze, Germany	Sigma-Aldrich, Seelze, Germany	Merck, Darmstadt, Germany
Cas number	72-55-9	67747-09-5	1461-22-9
Chemical name (IUPAC)	1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)-ethenyl]benzene	N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl] imidazole-1-carboxamide	Tributyl-chloro-stannane
Group	Organochlorine	Imidazole	Organotin
Molecular structure			
Application	Pesticide	Agricultural fungicide, cytostatica and P450arom inhibitor in medicine	Antifouling, broadband biocide
Mode of action	-	Blocks active center of P450 enzymes	Endocrine disruption
Suspected damage	Androgenic, carcino-genic, endocrine disrupt-tive, gastro-intestinal toxic, neurotoxic	-	Endocrine disruptive, immunotoxic, irritant
References	Kelce et al. 1995; IPCS; PAN	Vinggaard et al. 2002; IPCS; PAN	Grote et al. 2004; Boyer 1989; IPCS

Table 4–1 Part E Basic data of the test compounds.

	TPT-Cl	**Vinclozolin**
Manufacturer	Merck, Darmstadt, Germany	Sigma-Aldrich, Seelze, Germany
Cas number	639-58-7	50471-44-8
Chemical name (IUPAC)	Chloro-triphenyl-stannane	3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-1,3-oxazolidine-2,4-dione
Group	Organotin	Dicarboximide
Molecular structure		
Application	Antifouling, broadband biocide, agricultural fungicide (potato, sugar beet)	Fungicide for uses on fruits and vegetables
Mode of action	Endocrine disruption	NADH cytochrome C reductase in lipid peroxidation, metabolites are suspected to act as AR antagonists
Suspected damage	Developmental and reproductive toxic, immunotoxic, irritant	Endocrine disruptive, teratogenic
References	Grote et al. 2004; Boyer 1989; IPCS; PAN	Gray et al. 1999a; Kelce et al. 1994; IPCS; PAN

4.4 Solvents and basic ingredients

Listing 4–4 Solvents, reagents and kits

Solvent, reagent or kit	Manufacturer
Acetic acid	Merck-Schuchardt, Hohenbrunn, Germany
Albumine standard (2 mg/mL)	Sigma-Aldrich, Seelze, Germany
Acetone	Merck, Darmstadt, Germany
Benzol, p.A.	Merck, Darmstadt, Germany
Acetic acid, 100 % p.A.	Merck, Darmstadt, Germany
Chloroform ($CHCl_3$), lichrosolv®	Merck, Darmstadt, Germany
Cyclohexan, lichrosolv®	Merck, Darmstadt, Germany
Citric acid monohydrate	Sigma-Aldrich, Seelze, Germany
Dextran T 70	Sigma-Aldrich, Seelze, Germany
Diethyl pyrocarbonate	Sigma-Aldrich, Seelze, Germany
Dichlormethan (CH_2Cl_2), lichrosolv®	Merck, Darmstadt, Germany
Diethylether, p.A.	Merck, Darmstadt, Germany
DMEM, liquid media, phenolred-free	Biochrom, Berlin, Germany
DTE	Sigma-Aldrich, Seelze, Germany
EDTA	Sigma-Aldrich, Seelze, Germany
Ethyl alcohol, p.A.	Merck, Darmstadt, Germany
Ethyl acetate, lichrosolv®	Merck, Darmstadt, Germany
Fetal bovine serum (FBS)	Biochrom, Berlin, Germany
Fetal bovine serum (FBS), charcoal stripped	Biochrom, Berlin, Germany
Folin & Ciocalteus phenol reagent	Sigma-Aldrich, Seelze, Germany
Gentamycine sulfate	Biochrom, Berlin, Germany
Liquid scintillation cocktail Ultima Gold™	Perkin-Elmer, Rodgau, Germany
4-methoxybenzaldehyd, p.A	Sigma-Aldrich, Seelze, Germany
Methyl alcohol, lichrosolv®	Merck, Darmstadt, Germany
$MgCl_2$	Merck, Darmstadt, Germany
NAD	Hoffman-La Roche, Grenzach-Wyhlen, Germany
NADPH	Hoffman-La Roche, Grenzach-Wyhlen, Germany
Norit, charcoal	Sigma-Aldrich, Seelze, Germany

RPMI, dry media, phenolred-free	Biochrom, Berlin, Germany
Sodium bismutate	Sigma-Aldrich, Seelze, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium hydroxide solution (1N)	KMF, Lohmar, Germany
Sodium pyruvate (Na-pyruvate)	Merck, Darmstadt, Germany
Sulphuric acid, p.A.	Merck, Darmstadt, Germany
Toluene, p.A.	Merck, Darmstadt, Germany
TRIZMA® Base	Sigma-Aldrich, Seelze, Germany
TRIZMA® HCl	Sigma-Aldrich, Seelze, Germany
Ultima Gold®, scintillation cocktail	Perkin-Elmer, Rodgau, Germany

4.5 Recipes of buffers, reagents and media

Assay buffer (AB)

For the preparation of the acidic part of the solution, 3.362 g (0.16 M) of citric acid monohydrate and 0.203 g (0.01 M) $MgCl_2$ were dissolved in 100 mL A. demin. The basic solution consisted of 1.938 g (0.16 M) TRIZMA® HCl, and 0.203 g $MgCl_2$ dissolved in 100 mL A. demin. The basic solution was adjusted to pH 5.5 and 7.4 with the acidic solution, respectively.

DMEM

Phenolred-free DMEM, containing 4.5 g/L glucose and Na-pyruvate, was supplemented with 0.04 g/L gentamycine sulfate, 10 % heat-inactivated FBS and 2 mM glutamine.

Heat inactivated FBS

FBS was inactivated during a 30 min incubation at 56°C in a water bath.

Homogenization buffer

Homogenization buffer (HB) consisted of 10 mM TRIZMA® HCL and 1 mM EDTA, dissolved in A. demin and adjusted to pH 7.4.

Norit/Dextran solution

5% (w/w) activated charcoal and 0.5% (w/w) dextran were shaken in A. demin for at least 2 hours.

RPMI-1640

10.42 g phenolred-free RPMI-1640 dry media, 0.11 g Na-pyruvate (=1 mM), 0.04 g gentamycine sulfate and 2 g $NaHCO_3$ were dissolved in 200 mL A. demin. The pH-value was adjusted to 7.4 and 100 mL heat-inactivated FBS was added. The media was filled with A. demin up to 1 liter. The media was sterile filtered through a 0.2 µM pore membrane filter, filled into 100 mL autoclaved bottles and stored at 2–8°C.

Spraying reagent

100 mL pure acetic acid were mixed with 2 mL sulphuric acid and 1 mL 4-methoxybenz-aldehyd.

5 Effects of various pesticides on human aromatase, and the investigation of residual chemicals in relation to aromatase activity, CYP19 mRNA expression in placenta and sex hormone concentrations in blood samples of donors

Parts of this chapter are published in:

Alléra A, Lo S, King I, Steglich F, Klingmüller D.

Impact of androgenic/antiandrogenic compounds (AAC) on human sex steroid metabolizing key enzymes. Toxicology. 2004, December 1;205(1-2):75-85.

5.1 Abstract

Human populations are exposed to different kinds and amounts of pesticides, industrial pollutants and/or other synthetic compounds. Work-related exposure is mainly suspected in the agricultural sector and the dockyards, lifestyle-related exposure may happen through the regular consumption of sea-food. Apart from the toxic side-symptoms at high concentrations, several pesticides are known or suspected to interfere with the endocrine system at lower concentrations, and thus are labeled as Endocrine Disruptors (ED). The interference with the endocrine system might impair the development and maintenance of the male and female reproductive system. The present study was carried out to determine whether 10 suspected androgenic or antiandrogenic ED (DBT, diuron, fenarimol, linuron, MBT, p,p´DDE, prochloraz, TBT, TPT and vinclozolin) affect P450arom activity in placenta and JEG-3 chorion carcinoma cells.

To investigate endocrine effects in relation to pesticide body burden, we determined the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), androstenedione (Enon), testosterone (T), DHT and estradiol (E2) in the serum of 59 male and 16 female donors from two different European regions. In parallel, urine and serum samples of the donors were analyzed for chemical residues by the COMPRENDO partners in Milan (p,p´DDE), Ioannina (diuron, fenarimol, linuron and vinclozolin), and France (TBT and TPT).

16 German and 14 Polish mothers provided placenta samples for the investigation of residual chemicals, as well as for the analysis of specific P450arom activity and CYP19 mRNA expression. The present study and subsequent investigations was intended to clarify impairment of human health due to chemical exposure.

5.2 Introduction

Endocrine modulating activity has been reported for many chemicals, including naturally occurring substances (*e.g.*, coumestrol and genistein), pharmaceuticals (*e.g.*, tamoxifen, diethylstilbestrol and ethynylestradiol), pesticides (*e.g.*, dieldrin, toxaphene, endosulfan, DDT and vinclozolin), industrial chemicals (*e.g.*, bisphenol A, PCBs and phthalate plasticizers), and heavy metals (IOPC 2002; McLachlan et al. 2001; Solomon and Schettler 2000).

The structural diversity of these so-called endocrine disruptors (ED) suggests that effects are caused through a number of different pathways (Gillesby and Zacharewski 1998). These interactions include indirect mechanisms like the alteration of the synthesis, the release, the transport, the metabolism or the clearance of hormones, and direct mechanisms like the interaction with hormone receptors (Sonnenschein and Soto 1998). Endocrine effects will potentially occur at lower exposure levels than most other toxic effects, due to the fact that the endocrine systems usually work with extremely low hormone concentrations (Kelce et al. 1994; Duax et al. 1985). Since fetal development and sexual differentiation is under direct control and regulation of gonadal steroid hormones, it is during this period that the effects of EDs may be particularly severe (Chance and Harmsen 1998).

Aromatase is an enzyme which catalyzes the transformation of androgens into estrogens (Simpson et al. 1997). It therefore plays a major role in the maintenance of certain processes within the body and in normal fetal development. Cases of genetic aromatase deficiency are rare, probably due to the indispensability of this enzyme, and thus high lethality of fetuses with such deficiencies. A rare disorder in this context is the placental aromatase deficiency, with disturbed production and transfer of estrogen from the placenta to the baby in the expectant mother (Holt et al. 2005; Shozu et al. 1991). The symptom is a temporary masculine appearance of mother and daughter (karyotype XX). Furthermore, estrogen has a protective effect on bone via multiple mechanisms (Shapiro 2005). In estrogen deficiency states due to natural menopause, chemotherapy-induced ovarian failure, treatment with gonadotropin-releasing hormone (GnRH) agonists or treatment with aromatase inhibitors, bone resorption predominates and bone loss follows.

Aromatase disturbance caused by chemical body burden could be the reason for increased incidences of human reproductive disorders like polycystic ovary syndrome (PCOS) and carcinomas of hormone sensitive tissues (*e.g.,* breast and testis). This trend can

only partly be attributed to improved detection methods and/or recently identified genetic factors (Kortenkamp 2006; Ntais et al. 2003; Skakkebaek et al. 2001; Hsing et al. 2000; Kelce et al. 1998; Colborn et al. 1993; Sharpe and Skakkebaek 1993).

Some epidemiologic studies have stated associations between exposure to specific pesticides or industrial chemicals and steroid hormone levels in serum (Akingbemi and Hardy 2001; Steenland et al. 1997). For the investigation of endocrine effects in relation to human chemical contaminants, blood and urine samples were collected in Germany and Poland (Prof. Dr. J. Falandysz, University of Gdansk), from 16 female and 59 male donors classified into five subgroups: (a) German control group, (b) Polish control group, (c) Polish fish consumers (diet-related ED exposure), (d) Polish farmers (work-related ED exposure) and (e) Polish dockers (work-related ED exposure). We measured the concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone sulfate (DHEA-SO_4), androstenedione (Enon), testosterone (T), dihydrotestosterone (DHT), and estradiol (E2) in all serum samples.

LH and FSH are glycoproteins secreted from the b-cells of the adenopituitary, controlled by hypothalamic GnRH. LH stimulates androgen production in the Leydig cells in men, while FSH promotes the development of the gonadal tissue and spermatogenesis. LH and FSH levels help to differentiate hypogonadism (hyper- or hypogonadotropic) (Sina et al. 1975).

DHEA circulating in the blood originates almost entirely from the adrenals. It is only weak androgenic, but is the precursor for Enon and T (Vermeulen 1983). Thus, it can indirectly cause hirsutism or virilization (Vermeulen 1983). DHEA-SO_4 has a half-life of nearly a full day, is stable in within-day release and unlike T, DHEA-SO_4 does not circulate bound to a carrier protein. It is therefore an appropriate indicator for hyperandrogenism in hirsutism and polycystic ovary syndrome (Buvat et al. 1983).

Androstenedione is the direct precursor of T and can be used, in combination with other androgens, as an indicator for an impaired androgen balance (Ilondo et al. 1982). Enon levels underlie a circadian release, with slightly higher concentrations in the morning compared to the late afternoon. Women suffering from hirsutism or polycystic ovaries show abnormally high Enon levels in serum, resulting from increased androgen production in the ovaries. In postmenopausal women with osteoporosis, levels are significantly reduced, while increased values indicate congenital adrenal hyperplasia and Cushing syndrome. Enon concentrations in females depend on the estrous cycle.

Testosterone in men is responsible for the development of male secondary characteristics, the accessory sexual organs, the prostate and the seminal vesicles. Most of body hair follicles respond to T and DHT. Hypogonadismus, cryptorchism, Klinefelter syndrome, adrenogenital syndrome (AGS), sub function of the pituitary and cirrhosis of the liver can be related to low T levels in men, while increased levels can result from T producing tumors, high sportive activity or androgen resistance (Dejager et al. 2002; Doldi et al. 1996; Odell and Swerdloff 1976). T in women mostly results from peripheral conversion of Enon, produced in the adrenal and the ovaries. Women with polycystic ovaries, adrenal tumors or hyperplasia show increased T levels.

Dihydrotestosterone (DHT) is the strongest human androgen. In males, approx. 70% of DHT derives from peripheral conversion of T. Lower DHT levels are found in men with Klinefelter´s syndrome, damage of the seminiferous tubules, patients with anorchia, and in elderly men.

E2 is produced in minor concentrations in the testis, and is being directly synthesized from Enon or T in the periphery, like adipose tissues, brain, skin and mamma tissue (Oettel 2003). In women, E2 is mainly produced in the ovaries. E2 concentrations vary within the estrous cycle.

In parallel to steroid hormone measurements, urine and serum samples of the donors were analyzed for residues of TBT and TPT (Dr. R. Jeannot, Dr. T. Dagnac, Bureau de Recherches Geologiques et Minieres, Orleans, France), diuron, fenarimol, linuron and vinclozolin (Prof. Dr. T. A. Albanis, Dr. V. Sakkas, University of Ioannina, Greece) and p,p´DDE (Prof. Dr. S. Galassi, University of Milan, Italy). In addition to body fluid sampling, 16 German and 14 Polish mothers provided placenta samples to measure specific P450arom activity and CYP19 expression in relation to residual chemicals.

There is an increasing number of studies showing the outstanding role of P450arom, identifying this enzyme as the key switch controlling androgen vs. estrogen production, due to its position at the end of the gonadal steroidogenic pathway (Jeyasuria and Place 1998). P450arom catalyzes the transformation of androstenedione (Enon) and testosterone (T) to estrone (E1) and estradiol (E2), respectively. Thus, we investigated the direct impact of the selected suspicious and control substances (Table 5–1) on aromatase (P450arom) activity in human placenta tissue and chorion carcinoma cells from the celline JEG-3.

5.3 Material and Methods

5.3.1 Chemicals

Enzyme assay

EDTA, TRIZMA™ base, TRIZMA™ HCl and citric acid were used for the preparation of the required buffers. [1β-^{3}H]-androstenedione (25.9 Ci/mmol) served as substrate in the P450arom assay, NADPH was used as cofactor. The test compounds are listed in Listing 5–1. Further information concerning the material is given in the general section, chapter 4.

Listing 5–1 Test substances that were investigated concerning their effect on P450arom activity in placenta tissue and JEG-3 chorion carcinoma cells.

Substance	Cas no.	Application / source
DBT	683-18-1	Esterification catalyst
Diuron	330-54-1	Herbicide
Fenarimol	060168-88-9	Fungicide
Flutamide	13311-84-7	Pharmaceutical, non-steroidal antiandrogen
Letrozole	112809-51-5	Non-steroidal P450arom inhibitor
Linuron	330-55-2	Herbicide
MBT	1118-46-3	PVC stabilizer
MT	58-18-4	Pharmaceutical, synthetic androgen
p,p´DDE	72-55-9	Most prevalent metabolite of DDT
Prochloraz	67747-09-5	Fungicide
TBT	56-35-9	Broad-spectrum biocide
TPT	668-34-8	Fungicide, molluscicide
Vinclozolin	50471-44-8	Fungicide

Testosterone, androstenedione, estradiol, DHEA-SO_4, and DHT levels

All materials necessary for the *in vitro* diagnostic of T, Enon, E2 and DHEA-SO_4 in serum were purchased from DPC-Biermann (Bad Nauheim, Germany). Components supplied with the diagnostic kits were the Bead Pack, containing the pre-coated beads with the different antibodies, the reagent and the adjustors. Multi-diluent, chemiluminescent substrate, probe wash, probe cleaning kit, disposable reaction tubes and tube caps were purchased separately

from DPC-Biermann (Bad Nauheim, Germany). All materials necessary for the quantitative determination of DHT were purchased from IBL (Hamburg, Germany).

Quantification of CYP19 mRNA in placenta samples

Diethyl pyrocarbonate (DEPC) and β-mercaptoethyl alcohol were purchased from Boehringer (Ingelheim, Germany). The RNeasy® Mini Kit (mini spin columns, RLT lysis buffer, RW1 washing buffer, RPE washing buffer and RNase-free tubes), the QIAshredder^{TM} spin columns, proteinkinase K, RNase free DNase set, dNTP Mix and the QuantiTect SYBR® Green PCR kit were obtained from Qiagen (Hilden, Germany). The SuperScriptTM II reverse transcriptase, random primers, dNTP mix and the RiboGreen® RNA Quantitation kit – including RiboGreen® RNA quantitation reagent, 20xTE buffer (200 mM TrisHCl, 20 mM, EDTA, pH 7.5) and ribosomal RNA standard (16S and 23S rRNA of E. coli) – were purchased from Invitrogen (Karlsruhe, Germany). RNasin was obtained from Promega (Mannheim, Germany).

5.3.2 Source and preparation of human placenta

Placental tissue was removed after caesarian section and stored on ice. It was fragmented, cut into smaller pieces, washed with KCL and frozen in liquid nitrogen. The tissue samples were stored at −80° C until further processing. The study was approved by the local ethics committee and informed consent from all tissue donors or their family members was obtained.

Tissue homogenization was carried out as described previously (Steckelbroeck et al. 1999a). In brief, 20 mg placental tissue (wet weight) was homogenized in HB and sonificated. Homogenates were spinned down and supernatants were stored as cell-free fractions at −80°C. Samples of the tissue preparation were collected for the protein determination according to Lowry (1951). Measurements were conducted in the linear range of protein content *versus* enzyme activity.

5.3.3 Measurement of aromatase activity in placenta tissue

Measurement of P450arom activity based on the proportional release of $^{3}H_2O$ and estrone from [1β-^{3}H]-Enon (Steckelbroeck et al. 1999a). For the investigation of chemical effects on P450arom activities, incubations were performed in duplicate, at pH 7.4. The results are

the means of three determinations. Placenta homogenates were 1/6 v/v diluted with HB. Test compound dilutions were prepared in EtOH and added to the tissue preparations to obtain final concentrations of 0.001 µM to 100 µM. [1β-^{3}H]-Enon diluted in assay buffer and NADPH dissolved in HB at a final concentration of 0.05 µM and 3 mM, were added to the tissue preparations, respectively. The aqueous phases of the incubations were transferred into reaction tubes and stripped from the remaining steroids twice with $CHCl_3$ and once with dextran-coated charcoal solution. Radioactivity in a sample of the aqueous phase was counted as automatically quench-corrected disintegrations per minute (dpm) with a Wallac 1409 liquid scintillation counter.

5.3.4 Chorion carcinoma cell line JEG-3

The chorion carcinoma celline JEG-3 was obtained from DSMZ (Braunschweig, Germany). Cells were cultured in phenolred-free DMEM, which was supplemented with 0.04 g/L gentamycine sulfate, 10% heat-inactivated FBS and 2 mM glutamine before use. Cells were seeded in 75 cm^2 cell culture flasks and incubated at 95% humidity, 5% CO_2 and 37°C. When confluency was 75%, cells were subcultured or used in the enzyme assays. Medium was changed every 2–3 days.

5.3.5 Measurement of aromatase activity in JEG-3

Approximately 100,000 JEG-3 cells were seeded into each well of 12-well plates and incubated until 75% confluency. Two wells with cell-monolayers were carefully washed with phosphate buffered solution (PBS) and lysed in 0.05 N NaOH for protein determination according to Lowry (1951). Culture media in the other wells was exchanged with incubation media, which contained 10% charcoal-stripped FBS and [1β-^{3}H]-Enon at a final concentration of 0.5 µM. A volume of 2.5 µL ethanolic test compound solution per mL medium was added to the wells, with final concentrations of 0.01-100 µM. Cell incubations with 2.5 µL EtOH/mL medium served as reference aromatase activities, while incubations with medium and EtOH, but without cells resulted in blank values. Cells were incubated in a humidified atmosphere, 5% CO_2 and 37°C. Reactions were stopped after 1 h by chilling on ice. Medium of the wells was transferred into test tubes and stripped from remaining steroids as described previously (Steckelbroeck et al. 1999a). Values reported are the means of three determinations.

5.3.6 Urine, blood and placenta of Polish and German donors

All Polish donors were inhabitants of the Gdansk region. 15 dockers (male n=15, age 48±4 years), 15 farmers (male n=13, female n=2; age 42±11 years), 15 people with a fish preference in their diet (male n=11, female n=4; age 47±15 years) and 15 people (male n=10, female n=5; age 38±14 years) that did not belong to any of these groups. Blood and urine samples of 15 Germans (male n=10, female n=5) were taken, the average age of the donors was 40±15 years. Placenta tissue and urine samples of 14 Polish mothers (28±5 years) and 16 German mothers (31±6 years) were taken.

5.3.7 CYP19 mRNA quantification in placenta samples

1 mL diethyl pyrocarbonate (DEPC) stock solution was added to 1 L A. demin, mixed until bubble-free and used for the decontamination of all equipment used for RNA-extraction. 60 mg frozen tissue of each placenta sample was homogenized with a rotor-stator homogenizer in 1200 µL RLT®-lysis buffer, supplemented with β-mercaptoethyl alcohol in an autoclaved glass mortar with a tightly fitting glass pestle.

Total RNA was extracted according to the "RNeasy Mini Protocol for the Isolation of Total RNA from Animal Tissues". In brief, EtOH was added to the tissue lysate, protein and DNA content were removed within two different digestion-centrifugation steps. Purified total RNA was eluted in 30 µL RNase-free water. RNA concentrations were measured with a RiboGreen® quantification kit, using a polar star galaxy fluorometer as detection system. Samples were verified for purity by photometric A260/A280 ratio analysis.

RNA samples were reverse transcribed into cDNA, using SuperScript™ II reverse transcriptase, 50 ng random primers, dNTP mix (10 mM) and RNasin ribonuclease inhibitor (40 U). 20 ng cDNA each of the different placenta samples and the calibrator (CYP19 mRNA from JEG-3 cells) were used as template for the detection of CYP19 target gene and 18S mRNA reference ("housekeeping") gene.

Real-time quantification was performed according to the SYBR® Green protocol on an ABI PRISM® 7700 sequence detection system. 300 nM each of the following primers were used:

CYP19 sequences:

Primer forward	5´- TTG GAA GGA TGC ACA GAC TCG - 3´
Primer reverse	5´- GGC GAT GTA CTT TCC TGC ACA - 3´

18S sequences:

Primer forward	5´- ACG GCT ACC ACA TCC AAG GA - 3´
Primer reverse	5´- AAG GAT TTA AAG TGG ACT CAT TCC A - 3´

The thermo profile for the real-time PCR is given in Table 5–1. Relative quantification of CYP19 mRNA amount was accomplished by comparative $-2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and given as arbitrary units relative to the calibrator.

Table 5–1 Thermo profile of the real-time PCR performed for the quantification of CYP19 mRNA.

PCR:	**Time**	**Temperature**
Initial activation step	15 min	50°C
Three-step-cycling:		
Denaturation	15 s	94°C
Annealing	30 s	58°C
Extension	30 s	72°C
Number of cycles: 40		

5.3.8 Sex hormone analysis of donor blood

LH, FSH, DHEA-SO_4, T, Enon, and E2 levels were measured in vitro with an IMMULITE® 2000 Analyzer according to the principle of sandwich (LH and FSH) and competitive (DHEA-SO_4, T, Enon and E2) chemiluminescence immunoassay, following the manufacturers guideline (DPC-Biermann, Bad Nauheim, Germany). In brief, serum samples were controlled for complete clot formation and spinned down. One of the beads coated with the respective rabbit anti-bodies was dispensed into each cuvette. In the competitive immunoassay, the samples and the alkaline phosphatase conjugated to T, Enon, E2 and DHEA-SO_4 in a human protein-based matrix were added to the beads, respectively.

In the sandwich immunoassay, the samples and the alkaline phosphatase conjugated to anti-LH and anti-FSH were added to the beads. The mixtures were incubated for 30 min (LH, FSH and DHEA-SO_4) and 60 min (T, E2 and Enon) at 37°C. All unbound compounds

were removed by several washing steps. The appropriate luminogenic substrate was added. After 5 min, the luminescent signal was detected with the IMMULITE®, generated by the decay of the instable product built from the reaction of the substrate and alkaline phosphatase bound to the bead. The luminescence signal is direct proportional to the amount of bound alkaline phosphatase, and anti-proportional to the amount of DHEA-SO_4, T, Enon and E2 and direct proportional to the amount of LH and FSH in the serum samples.

DHT levels were measured by enzyme immunoassay, following the manufacturer´s manual (IBL, Hamburg, Germany). The principle of the competitive binding scenario is that the unlabeled antigen present in standards, control and patient samples compete with the enzyme-labeled antigen (DHT-conjugate) for a limited number of antibody binding sites on the microwell plate. In brief, 50 µL of each calibrator, control and specimen sample was pipetted into the wells of the microwell strips that were supplied with the kit. 100 µL of the conjugate working solution was added into each well and the mixture was incubated for 1 hour at room temperature. The wells were washed with diluted wash buffer and dried. Enzyme substrate was pipetted into each well and the mixture was incubated for 10–15 minutes at room temperature. 50 µL of stopping solution was added into each well. After 20 min, the absorbance at 450 nm was measured on a microtiter plate reader.

5.3.9 Data analysis

Enzyme assays

Product formation given in dpm was calculated into pmol/h/mg protein enzyme activity when protein content was determined. Results were analyzed with the non-linear curve-fitting software FigSys (Biosoft, Cambridge, UK). The software was also used to estimate the concentration of the test compound that led to 20% and 50% inhibition of enzyme activity.

Quantification of mRNA and sex hormone analysis

The software GraphPad Prism (GraphPad Software, San Diego, USA) was used to visualize the data, presented as mean values with ±SEM. Column analysis was used to determine if data sets were normally distributed (Kolmogorov-Smirnov test). Variances were tested for homogeneity. Comparison between two groups was performed with the two-tailed t-test for unpaired samples (normal distribution, variances homogeneous), the Mann-Whitney test (Gaussian distribution not assumed, variances homogeneous) or the t-test with Welch´s

correction (normal distribution, equal variances not assumed). In case of more than two groups to compare, differences were verified by either one-way analyses of variance (ANOVA), followed by a post-hoc Tukey´s multiple comparison test (normal distribution, variances homogeneous) or the Kruskal-Wallis test connected with a post-hoc Dunn´s multiple comparison test (Gaussian distribution and/or equal variances not assumed).

5.4 Results

5.4.1 Impact of test compounds on human P450arom activity in placenta tissue and JEG-3 cells

Each test compound was applied in the P450arom enzyme assays at 4–8 different concentrations between 0.001 and 100 µM. Values from the samples incubated with 3 µL EtOH represented 100% enzyme activity and served as reference. Specific P450arom activity in JEG-3 cells was 20 pmol product/h/mg protein. Data from JEG-3 and placenta enzyme experiments was plotted and curve-fitted, *e.g.*, prochloraz (Fig. 5–1A) and fenarimol (Fig. 5–1B). Resulting IC_{20} and IC_{50} values are given in Table 5–2 in descending order of P450arom inhibiting magnitude.

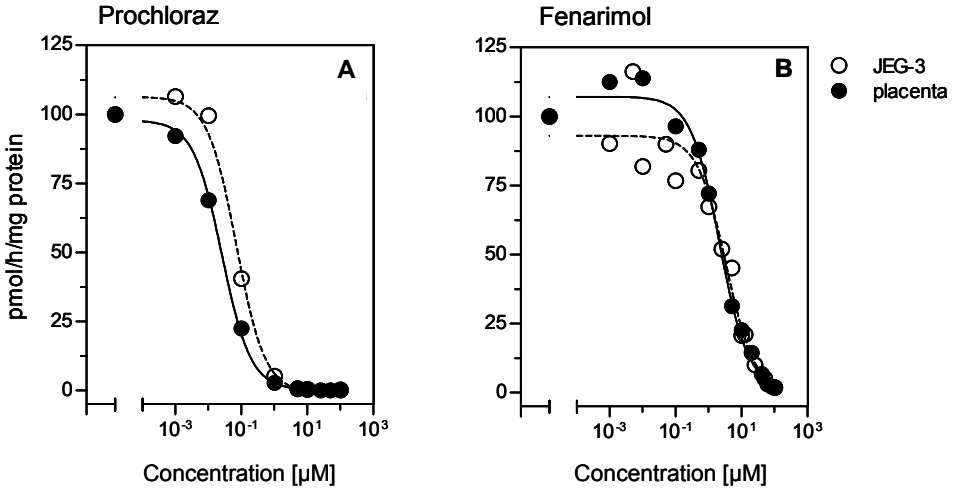


Fig. 5–1 Effect of prochloraz (A) and fenarimol (B) on aromatase (P450arom) activity in JEG-3 cells and human placenta tissue, given as % activity compared to EtOH controls (100 %).

Table 5–2 Inhibitory effects expressed as IC_{20} and IC_{50} values of test compounds (DBT, diuron, fenarimol, linuron, MBT, p,p´DDE, TPT, TBT and vinclozolin) and control substances (letrozole, prochloraz, MT, flutamide and finasteride) on P450arom activity in placenta tissue homogenate and JEG-3 cells.

P450arom activity	JEG-3		Placenta	
	IC_{20} [µM]	IC_{50} [µM]	IC_{20} [µM]	IC_{50} [µM]
Letrozole	0.0001	0.0005	0.0001	0.0005
Prochloraz	0.02	0.06	0.005	0.024
Fenarimol	0.11	1.99	0.67	2.50
MT	0.31	2.76	0.80	5.50
TPT	0.47	3.81	1.50	6.20
TBT	0.58	12.2	3.10	6.40
Flutamide	-	-	5.20	27.2
DBT	-	-	25.2	73.0
Finasteride	-	-	29.7	77.3
Diuron	-	-	-	-
Linuron	-	-	-	-
MBT	-	-	-	-
p,p´DDE	-	-	-	-

5.4.2 Aromatase activity and CYP19 mRNA quantification in placenta samples

Chemical analysis performed by the COMPRENDO partners in Milan (Prof. Dr. S. Galassi) revealed p,p´DDE contamination in all placenta samples (Fig. 5–2C), but individual values did not correlate with aromatase activity or with CYP19 mRNA expression. Comparison between the German and Polish group showed a significantly lower mean aromatase activity (Fig. 5–2A) and CYP19 mRNA expression (Fig. 5–2B) in German mothers than in Polish mothers, while there was a tendency (not significant) for higher p,p´DDE residues in German placenta samples (Fig. 5–2C).

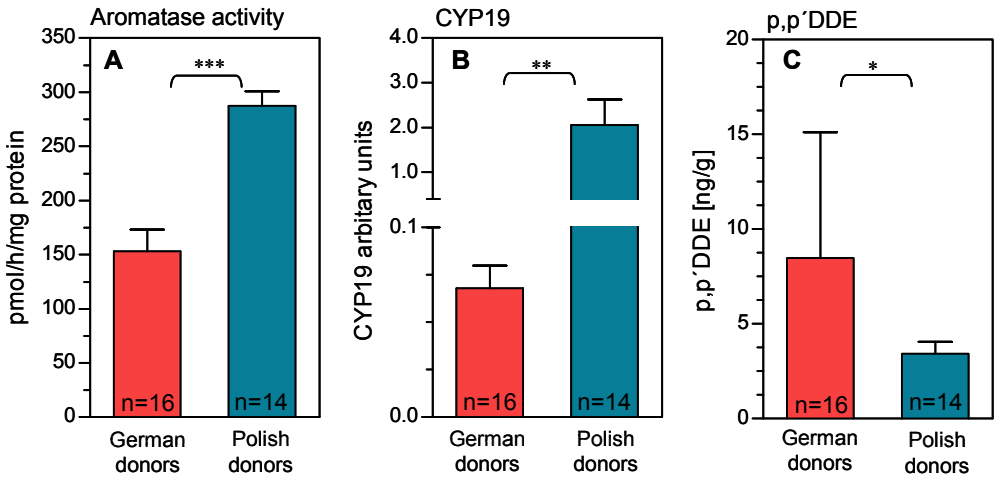


Fig. 5–2 Mean values and SEM of (A) aromatase activity, (B) CYP19 mRNA and (C) p,p´DDE concentrations in placenta samples of German and Polish donors. Mean values of aromatase activity and CYP19 mRNA expression were significantly lower in German donors, while p,p´DDE analysis revealed slightly higher contamination than in Polish donors.
Statistical analysis was performed as follows: (A) t-test with Welch´s correction, (B) t-test with Welch´s correction and (C) Mann-Whitney test. **** = $p<0.01$, ** = $p<0.05$, * = $p<0.1$*

5.4.3 Chemical and sex hormone analysis of male donor blood and urine

Chemical analysis performed by the COMPRENDO partners in Milan and Orleans revealed p,p´DDE contamination in serum of all male donors (Fig. 5–3A), MBT residues in seven urine samples of Polish dockers (n=7, mean of 107 ng/L, data not shown) and two urine samples of fish consumers (n=2, mean of 39.5 ng/L, data not shown), and DBT residues in urine of three dockers (n=3, mean of 59 ng/L, data not shown).

Chemical residues in urine or serum did not correlate with any of the sex hormone level determined in serum. Group analysis of serum sex hormone concentration resulted in significantly lower mean E2 levels (Fig. 5–3B) and slightly higher FSH levels (not significant) in German men compared to Polish men of all groups.

5.4.4 Chemical and sex hormone analysis of female donor blood and urine

Chemical analysis performed by the COMPRENDO partners in Milan revealed p,p´DDE contamination in serum of all female donors. There was no correlation to individual sex hormone concentrations, and no significant differences between grouped Polish and German donors could be found (Fig. 5–4C). DBT was detected in one of the urine samples

of a Polish women with fish diet by the COMPRENDO partners in Orleans (n=1, DBT concentration 87 ng/L, data not shown). Mean concentrations of Enon (Fig. 5–4A) and T (Fig. 5–4B) in serum of Polish women were significantly higher ($p<0.05$) than those of German women.

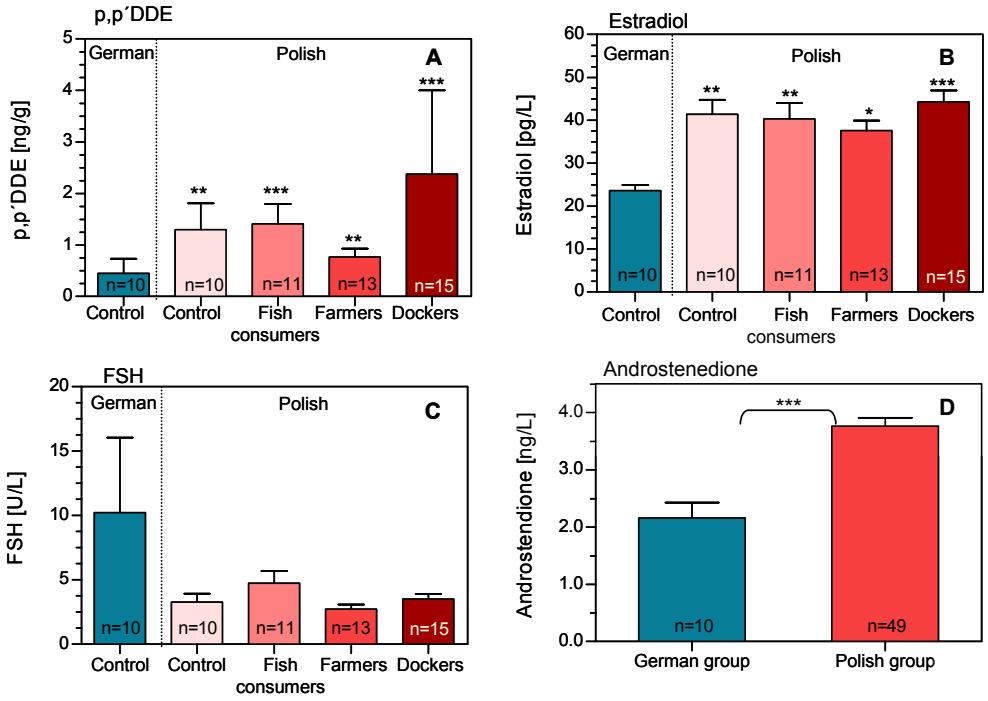


Fig. 5–3 Mean concentrations and SEM of (A) p,p´DDE, (B) estradiol, (C) FSH and (D) androstendione in serum of male donors. Mean of p,p´DDE, E2 and androstendione values in serum of Polish men were significantly higher than in serum of German men. Variation of FSH values in German donor serum was very high (C), but the mean value was higher than in the Polish subgroups (not significant).
Statistical analysis was performed as follows: (A) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test, (B) ANOVA with post-hoc Tukey´s multiple comparison test, (C) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test and (D) t-test for unpaired samples.
**** = $p<0.01$, ** = $p<0.05$, * = $p<0.1$*

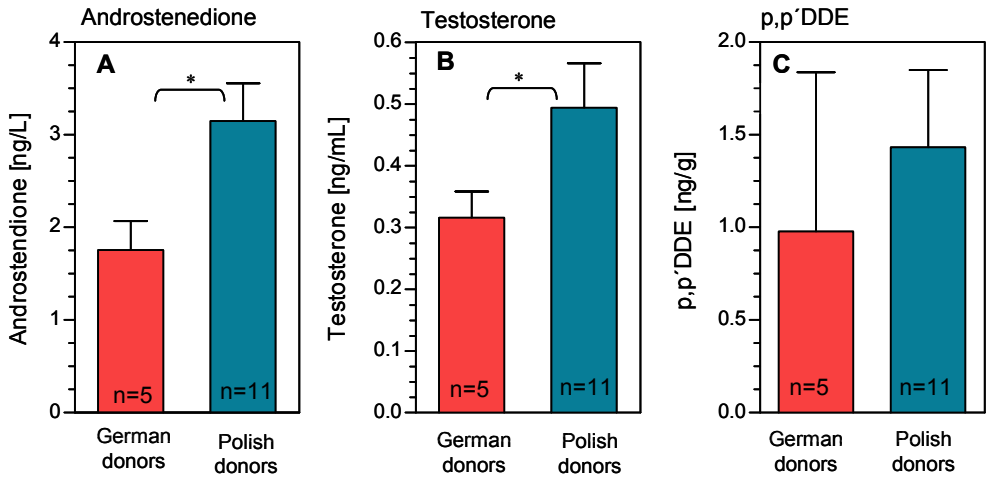


Fig. 5–4 Mean concentrations and SEM of (A) androstenedione, (B) testosterone, and (C) p,p´DDE concentrations in serum samples of German and Polish female donors. Mean Enon and T values of Polish donors were significantly higher compared to mean values resulting from German serum samples.
Statistical analysis was performed as follows:(A) t-test for unpaired samples, (B) t-test with Welch´s correction and (C) t-test for unpaired samples. **** = $p<0.01$, ** = $p<0.05$, * = $p<0.1$*

5.5 Discussion

In the present study, our main concern was to investigate the changes in human P450arom activity, the enzyme that catalyzes the formation of estrogens, caused by ED exposure or body contamination. Prochloraz, MT, fenarimol and the triorganotin compounds TBT and TPT significantly inhibited P450arom both in placental microsomes and in JEG-3 cells. In body, the reduced activity of this enzyme could result in a change of the natural balance between androgens and estrogens in favor of the androgens.

The importance of estrogens in women, as well as in men, has become more and more evident in recent years. The effects of estrogens are mediated via the estrogen receptors ER-α and ER-β. Estrogen receptors are expressed not only in reproductive tissues, but they are also found in myocardial, endothelial and vascular smooth muscle cells, liver, breast, brain and bone (Chlebowski 2005). The classical signaling pathway for estrogen action is the ligand-dependent receptor activation pathway, in which activated estrogen receptors are transcription factors that alter gene expression and increase protein synthesis (Voet and Voet 2004). In addition, the non-nuclear estrogen-signaling pathway through cell-membrane estrogen receptors supports the rather slow classical pathway with the feasibility

of rapid responses to estrogen (Toran-Allerand 2004; Pietras and Szego 1999). Thus, decreased levels of estrogens could result in multiple slow and fast reaction body disorders.

However, MT, TPT, TBT and prochloraz likewise impair 5α-Re 2 activity, responsible for the transformation of T into the much stronger androgen DHT, in the prostate and in LNCaP prostate carcinoma cells (chapter 4). The first consequence of the inhibition of both aromatase and 5α-Re 2 could be a drastic increase of circulating T concentrations, due to the missing "flush" via aromatization and 5α-reduction. The synthesis of T is probably warranted by the multiple 17β-HSDs isozymes present in the body (Yang et al. 2005). The excessive exposure to androgens and/or reduced estrogens may cause clinical problems, such as ovarian dysfunction, osteoporosis and hirsutism (Saitoh et al. 2001).

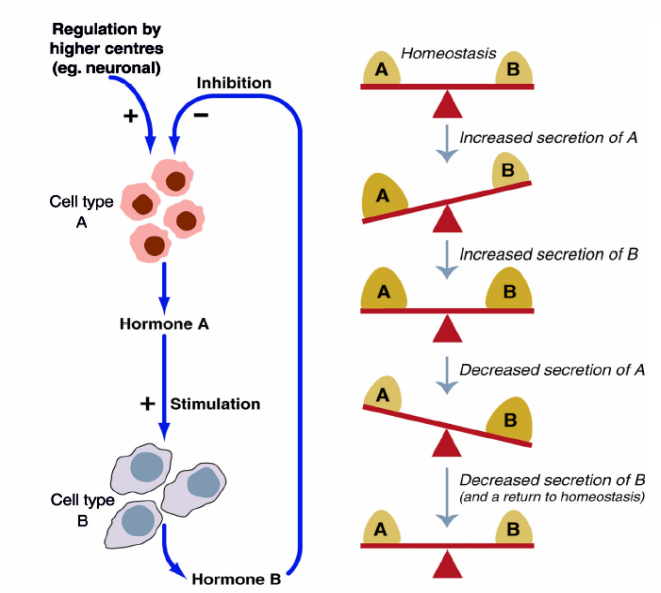


Fig. 5–5 Diagram of the feedback principle to maintain homeostasis in an endocrine system, with A representing a cell type that secretes hormone A and regulates the production of hormone B by cell type B. Hormone B exerts a negative feedback on the secretion of hormone A (IOPC 2002).

In the long run, reduced levels of estrogens might also induce an upregulation of P450arom to compensate the missing steroid hormones, causing occasional overshoots (Fig. 5–4) (Nakanishi et al. 2002). Thus, the prediction of the outcome of an ED with P450arom inhibitory power is extremely difficult.

The medication of several strong P450arom inhibitors, like letrozole and MT, against estrogen-dependent mammacarcinoma is promising (Andersen et al. 2002; Sanderson et al. 2002; Kelloff et al. 1998), however, the presence of accumulated endocrine acting body

contaminants, *e.g.* prochloraz or fenarimol, need to be considered due to possible synergistic effects. Suppression of local estrogen production sites with tissue-selective drugs is highly recommended. Several publications report that third-generation aromatase inhibitors like anastrozole and letrozole are showing greater efficacy in the treatment of ER-positive tumors than tamoxifen (Jakesz et al. 2005; Goss 2003). Thus, all ED with indirect interaction with the hormone system, like the ones we found in the present study, might be more effective and dangerous than those with direct impact on the hormone receptor.

In 2000, a study of the German Federal Institute for Consumer Protection (BgVV 1995) drew attention to the potential risk of organotin residues in food and consumables. According to the investigation, fish products are the key source for TPT, with concentrations of 10–30 µg/kg wet weight. Fish products like tuna and swordfish are also contaminated with p,p´DDE, showing mean liver concentrations of 81–135 ng/g wet weight (Kannan et al. 2002).

The chemical analysis by the COMPRENDO partners in France and Italy proved p,p´DDE contamination in urine, blood and placenta samples of several subjects. Fish consumers were not exceptionally high burdened, but significantly higher blood concentrations of p,p´DDE were found in Polish men, taking all subgroups together, compared to German men. This tendency of higher concentrations in Polish donors was not found in samples of female placenta and serum samples. The higher exposure of Polish men compared to the control group in Germany was also reflected by MBT and DBT residues found in Polish urine samples. However, individual sex hormone concentrations in blood were not correlated with p,p´DDE, MBT or DBT body burden. Previous investigations of sex hormone concentrations in relation to p,p´DDE contamination revealed no correlations (Cocco et al. 2004; Hagmar et al. 2001; Persky et al. 2001), except for a study carried out in North Carolina among African-American farmers, in which elevated serum levels of p,p´DDE were positively correlated with decreased total testosterone concentrations (Martin et al. 2002).

To the author´s knowledge, another study about the relation between organotin contamination and serum sex hormone concentrations in blood has not been performed so far. The significant differences in aromatase activities and CYP19 mRNA expression (placenta samples), E2 and Enon levels (male serum), Enon and T levels (female serum) probably are the result of other factors than determined in this study, *e.g.*, genetic factors,

body fat, alcohol consumption and/or chemicals other than the ones determined (Onland-Moret et al. 2005; Allen et al. 2003).

At present, clear evidence for the impairment of human health by ED in the environment is still missing. However, there is alarming data about significant levels of ED detected in human body fluids and tissues, and some of these contaminants are associated with developmental and reproductive anomalies in laboratory species (Akingbemi and Hardy 2001). Low-dose chronic or multiple chemical exposure with impact on P450arom might effect our progeny or cause ailments with as yet unknown etiologies, like Alzheimer´s disease, autism or cerebral palsy (Goldman and Koduru 2000).

6 Effects of Various Pesticides on Human 5α-Reductase Activity in Prostate and LNCaP Cells

This chapter is published as:

Lo S, King I, Allera A, Klingmüller D.

Effects of various pesticides on human 5alpha-reductase activity in prostate and LNCaP cells. Toxicology In Vitro. 2007, April; 21(3):502-508.

6.1 Abstract

Certain pesticides are able to disturb the sex hormone system and to act as antiandrogens. While the different underlying mechanisms remain unclear, inhibition of 5α-reductase, the enzyme which is indispensable for the synthesis of DHT and thus normal masculinization, appears to be one of the sensitive targets for endocrine disruption. We therefore tested several endocrine disrupters with antiandrogenic *in vivo* effects *in vitro* for their influence on 5α-reductase activity in two different test systems: (a) an enzyme assay with human Lymph Node Carcinoma of Prostate (LNCaP) cells and (b) an enzyme assay with human prostate tissue homogenate. The selected pesticides and industrial compounds were monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), triphenyltin (TPT), diuron, fenarimol, linuron, p,p´DDE, prochloraz and vinclozolin. The synthetic androgen methyltestosterone (MT) and the synthetic antiandrogen flutamide, as well as the 5α-reductase type 2 inhibitor finasteride served as control compounds.

The effect of the organotin compounds DBT, TBT and TPT on enzyme activity was approximately the same in both test systems, with IC_{50} values ranging between 2.7 and 11.2 μM. While in prostate tissue, methyltestosterone and prochloraz proved to be stronger inhibitors (IC_{50} values of 1.9 and 12.4 μM) than in LNCaP cells (IC_{50} values of 13.2 and 53.2 μM). The inhibitory impact of finasteride was approximately 130 times stronger in prostate tissue than in LNCaP cells. Fenarimol, flutamide, linuron and p,p´DDE inhibited 5α-reductase activity only at very high concentrations ($IC_{50} \geq 24$ μM) in prostate homogenates, and not at all in LNCaP cells. On average, the IC_{20} values were 3.5 times lower than the IC_{50} values. Diuron, MBT and vinclozolin exerted no effect in either of the test systems. The finding of pesticides acting as 5α-reductase inhibitors might be of clinical relevance. As a screening tool for putative ED, the tissue assay is the more practical and sensitive method. However, the human cancer cell assay can, to some extent, reflect

particular cell processes since the living cell is able to compensate moderate toxicological effects of the ED on cell viability, and possibly also their impact on 5α-reductase activity.

6.2 Introduction

Androgens are responsible for the normal masculinization of the body, including the brain, the formation of male genitalia and male behavior. 5α-reductase (5α-Re) is one of the key enzymes in human androgen metabolism. It is responsible for the irreversible conversion of testosterone into 5α-dihydrotestosterone (5α-DHT), the most potent human AR agonist (Rizner et al. 2003). During sex differentiation, 5α-DHT is indispensable for the development of male external genitals, the prostate, and the maturation of facial and body hair (Wilson 1996; Harris et al. 1992).

In general, 5α-DHT is locally converted from circulating testosterone in the tissue or organ, mainly the prostate, which requires 5α-DHT for its development, growth, differentiation, and function. The two isoforms of 5α-Re in humans and animals (Wilson et al. 1993) are encoded by different genes, and differ in their biochemical properties and cellular localization (Negri-Cesi and Motta 1994). Both isozymes are transiently expressed in skin and scalp of newborns. 5α-Re type 1 remains to be expressed in skin, liver and brain from the time of puberty onwards (Steckelbroeck et al. 2001; Stoffel-Wagner et al. 1998; Luu-The et al. 1994), and regulates the proper balance between androgens and estrogens (Mahendroo et al. 1999; 1997). 5α-Re type 2 is predominantly present in fetal genital skin, male accessory sex organs and the prostate (Ntais et al. 2003; 2001; Thigpen et al. 1993).

Mechanisms that result in impaired 5α-DHT concentrations include 1) mutated expression of 5α-reductase, 2) modified 5α-reductase activity, 3) changed concentrations of precursor hormones, and 4) disordered inactivation of 5α-DHT due to, *e.g.,* misregulation of 3-ketosteroid reductases. Substantially elevated DHT levels in relation to testosterone are considered to be risk factors for benign prostatic hyperplasia (BPH) and prostate cancer (Ntais et al. 2003), while decreased DHT/testosterone ratios during sex differentiation are supposed to result in the so-called testicular dysgenesis syndrome (TDS) (Skakkebaek et al. 2006; Fisher 2004; Silver and Russell 1999; Nordenskjold et al. 1998; Paulozzi et al. 1997). TDS is a disease pattern with the symptoms of poor semen quality, testis cancer, undescended testis, and hypospadias. Experimental and epidemiological studies suggest that TDS is a result of disruption of embryonal programming and gonadal

development during fetal life (Fisher 2004; Safe 2000; Vos et al. 2000; Sonnenschein and Soto 1998; Davidson and Yager 1997; Paulozzi et al. 1997; Toppari et al. 1996). Mutations of the 5α-Re type 2 gene lead to pseudohermaphroditism of varying degrees (Bahceci et al. 2005; Hackel et al. 2005; Migeon and Wisniewski 2003), while, to the best of the author´s knowledge, a disease pattern resulting from 5α-Re type 1 deficiency has not been reported yet.

A large number of chemicals used in agriculture and industry, including the selected test compounds monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), triphenyltin (TPT), diuron, fenarimol, linuron, p,p´DDE, prochloraz, and vinclozolin, have been reported to possess antiandrogenic potency. We investigated in this study, if these antiandrogenic effects are the result of 5α-Re inhibition.

Triorganotin compounds like TBT and TPT are assumed to interfere with androgen metabolism due to their potential to induce virilization of females (imposex) in marine snails, possibly caused by an impaired balance of sex hormones in favor of androgens (Oehlmann et al. 2000; Morcillo and Porte 1999; Oehlmann et al. 1996). In addition, reports on rat exposure showed smaller litter size (Ema et al. 1997; Harazono et al. 1996) and dose-dependent reduction of thymus weight (Snoeij et al. 1985). Administration of DBT resulted in increased incidence of rat fetuses with malformation, *e.g.,* exencephaly and cleft jaw (Ema et al. 1995). The organochlorine p,p´DDE, a persistent metabolite of the pesticide DDT, induced abnormalities in sex development of male rats, probably by binding to the androgen receptor (Gray et al. 1999b; Kelce et al. 1995). Vinclozolin and linuron administration to pregnant rats resulted in incomplete development of the reproductive tract in male offspring, *e.g.,* female-like anogenital distance, cleft phallus with hypospadia, small or even absent sex accessory glands, indicating antiandrogenic potency of these ED (Lambright et al. 2000; Gray et al. 1999a; Gray et al. 1999b; Monosson et al. 1999). As shown by the Hershberger assay, oral administration of the azolfungicides fenarimol and prochloraz considerably reduced the weight of ventral prostate, seminal vesicles and bulbourethral glands of castrated and testosterone-treated male rats (Vinggaard et al. 2005; Vinggaard et al. 2002). Both chemicals inhibit P450arom activity as demonstrated in human placenta tissue and human JEG-3 cells (Andersen et al. 2002).

The exact mechanisms of endocrine modulation of these chemicals are still unclear, but the key enzyme 5α-Re is certainly one of the sensitive targets. We therefore tested the

selected endocrine disrupters for their effect on 5α-Re activity in two different test systems, which were (a) an enzyme assay with human Lymph Node Carcinoma of Prostate (LNCaP) cells and (b) an enzyme assay with human prostate tissue homogenate.

6.3 Materials and methods

6.3.1 Chemicals

Enzyme assay

EDTA, TRIZMA™ base, TRIZMA™ HCl and citric acid were used for the preparation of the required buffers. [1β-^{3}H]-androstenedione (25.9 Ci/mmol) served as substrate in the 5α-Re assay, NADPH was used as cofactor. The test compounds are listed in Listing 6–1. Further information concerning the materials is given in the general section, chapter 4.

6.3.2 Source and preparation of prostate tissue

Macroscopically normal prostate tissue samples were obtained from patients undergoing prostate ectomy. Prostate samples were immediately frozen in liquid N_2 and stored at −80°C. The study was approved by the local ethics committee and informed consent was obtained from all tissue donors or their family.

Prostate samples were treated as previously described (Steckelbroeck et al. 2001). Tissues were weighed, transferred into a glass mortar filled with HB and homogenized with a tightly fitting glass pestle. Samples were transferred into plastic tubes and pulse ultrasonificated at 50 Watt. The homogenates were spinned down for 15 min at 4°C and 600 g. The supernatant, containing the microsomal fraction, was divided into aliquots and stored in liquid N_2. Protein concentrations were determined according to Lowry (1951).

Listing 6–1 Test substances that were investigated concerning their effect on 5α-reductase activity in prostate tissue and LNCaP prostate carcinoma cells.

Substance	Cas no.	Application / source
DBT	683-18-1	Esterification catalyst
Diuron	330-54-1	Herbicide
Fenarimol	060168-88-9	Fungicide
Finasteride	98319-26-7	Pharmaceutical, 5α-Re inhibitor
Flutamide	13311-84-7	Pharmaceutical, non-steroidal antiandrogen
Linuron	330-55-2	Herbicide
MBT	1118-46-3	PVC stabilizer
MT	58-18-4	Pharmaceutical, synthetic androgen
p,p´DDE	72-55-9	Most prevalent metabolite of DDT
Prochloraz	67747-09-5	Fungicide
TBT	56-35-9	Fungicide, molluscicide
TPT	668-34-8	Fungicide, molluscicide
Vinclozolin	50471-44-8	Fungicide

6.3.3 Enzyme assays in prostate tissue

Enzyme assays in prostate homogenate were performed as described (Steckelbroeck et al. 2001). In brief, prostate homogenates were diluted in homogenization buffer (HB) so that in a 50 µL aliquot, approximately 10% of substrate was converted during a 30 min incubation period. Control incubations without tissue were performed with 50 µL HB. 3 µL each of the test compound dilution was added to the tissue-containing aliquot. The mixture was constantly shaken at 37°C. Preincubation was stopped by chilling, and 100 µL of the [^{3}H]-androstenedione-solution (0.1 µM in assay buffer) was added to the mixture. The enzyme reaction was started by adding 50 µL HB containing 3 mM NADPH. The tubes were capped, vortexed, shaken as described above for 30 min and reactions were stopped by chilling on ice.

6.3.4 LNCaP cells in culture

Cell culture media components, unless otherwise specified, were purchased from Biochrom (Berlin, Germany). RPMI 1640 dry medium, supplemented with 2 mM L-glutamine and 11.11 mM D-glucose, was dissolved according to the manufacturer´s instruction. 40 µg/mL gentamycine sulfate and 2 g/L sodium bicarbonate were added. The medium was supplemented with 10% (v/v) FBS (full medium) or 10% (v/v) charcoal-stripped FBS (experimental medium). LNCaP (ATCC, Manassas, VA) cells were routinely grown in full RPMI 1640 in an atmosphere of 5% CO_2 under saturating humidity at 37°C.

6.3.5 Enzyme assays in LNCaP cells

LNCaP enzyme assays were performed in 12-well plates at 75% cell confluence with minor modifications as described (Negri-Cesi and Motta 1994). Cell medium was replaced by 300 µL medium with or without (control) the compound of interest. The maximum concentration used was below the toxic level of the respective test compound (Allera et al. 2004). In order to get blank values, control incubations (without cells) were performed in the same manner as cell samples. The enzyme assay was started by adding 300 µL medium containing 0.5 µM [^{3}H]-androstenedione as substrate and stopped after 60 min by chilling on ice. Samples of the incubation medium were transferred to glass tubes. Aliquots of cells were washed with PBS and lysed in 0.05 N NaOH for protein determination.

6.3.6 Isolation and quantification of the enzyme products

The enzyme products resulting from catalytic activity of different enzymes in prostate homogenate or in LNCaP cells are shown in Fig. 6–1. The products were extracted from the tissue homogenate or cell incubation medium with 1 mL $CHCl_3$/MeOH (2/1, v/v) and 1 mL ethylacetate, respectively. Samples of the organic phases were evaporated to dryness under N_2, redissolved in a mixture of 35 µL $CHCl_3$ and 15 µL EtOH containing 25 µg each of the unlabelled reference steroid, and applied to silica thin layers (Merck, Darmstadt, Germany). Steroids were separated by TLC in dichloromethane-acetone (92.5/7.5, v/v) as mobile phase and stained with a mixture of acetic acid, H_2SO_4 and 4-methoxybenzaldehyde (100/2/1, v/v/v), followed by heating at 130°C. Spots were cut out and transferred into counting vials containing 15 mL of liquid scintillation cocktail. Radioactivity was counted as automatically quench-corrected dpm in a Wallac 1409 liquid scintillation analyzer.

The ratio of the product to total radioactivity recovered from a single TLC lane was calculated and respective blank values were subtracted. Specific 5α-Re activity was expressed as pmol / mg protein / h. Product formation in the absence of the compound of interest was defined as 100% enzyme activity.

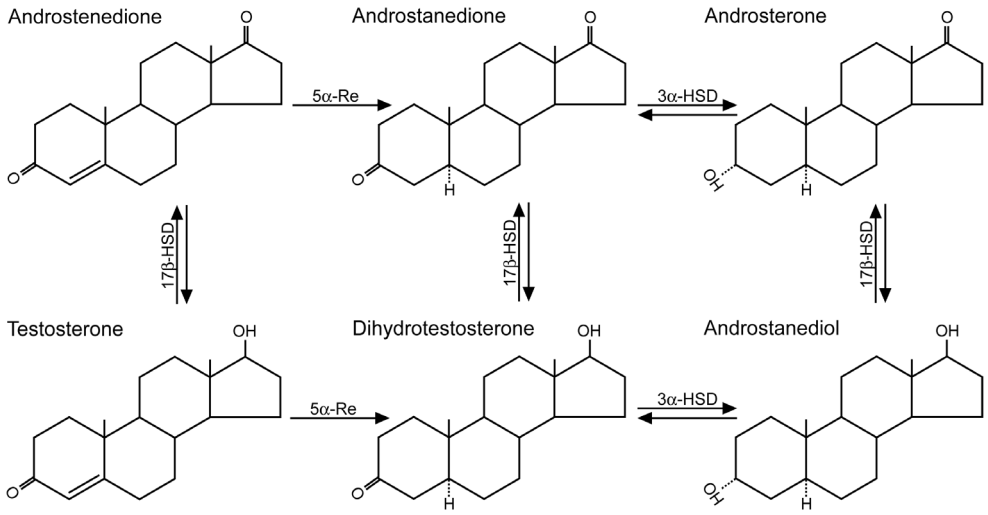


Fig. 6–1 Major androgen metabolism in human prostate and LNCaP cells. The enzymes 3α-hydroxysteroid dehydrogenase (3α-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and 5α-reductase (5α-Re) catalyze the oxidoreduction at position 3 and 17, and the reduction at position 5 of the steroid, respectively.

6.3.7 Data analysis

Analysis of enzyme kinetics was carried out using a computer assisted non-linear curve-fitting method (FigSys, Biosoft, UK). The Quick Fit option of the software automatically calculated the IC_{20} and IC_{50} values, representing the concentrations of the respective compound causing 20% and 50% inhibition of enzyme activity.

6.4 Results

6.4.1 Effect of cell culture medium supplemented with normal and charcoal-stripped FBS on 5α-reductase in LNCaP cells

To exclusively investigate the effect of the compound of interest, the culture medium had to be free of the endogenous steroids usually present in FBS. Thus, the use of a medium supplemented with charcoal-stripped FBS was considered. However, preliminary investigation first has to clarify if the lack of essential nutrients in charcoal-stripped FBS influences 5α-Re activity. The continuous product formation of 17β-HSD is important, because this enzyme just like 5α-Re catalyzes the substrate androstenedione. For that reason, we investigated the time-course of enzyme activity in LNCaP cells.

5α-Re activity rose in the same, time dependent manner in both media (Fig. 6–2), as well as 17β-HSD. The use of stripped FBS thus meets the requirements stated above.

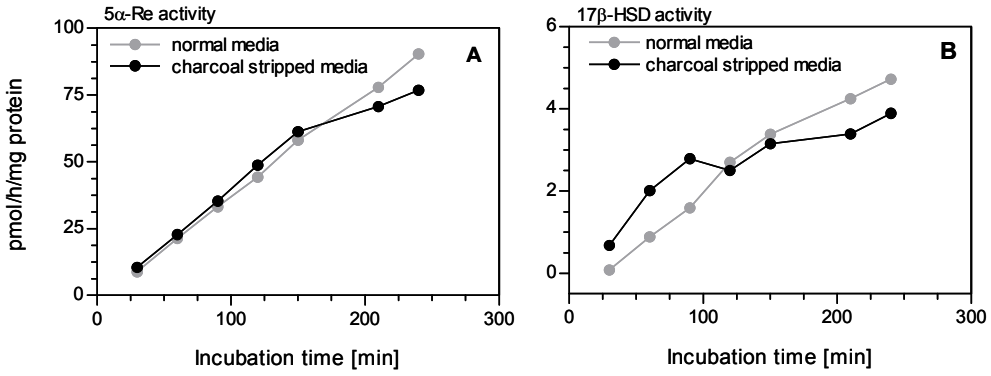


Fig. 6–2 (A) 5α-reductase activity and (B) 17β-HSD activity in LNCaP cells incubated in medium supplemented with 10% normal FBS or 10% charcoal-stripped FBS versus incubation time.

6.4.2 Impact of the pesticides on human 5α-reductase activity in prostate tissue homogenate and LNCaP cells

Specific 5α-Re activity was approximately 50 and 65 pmol/mg protein/h in the tissue and the cell assay. The concentrations of the compounds causing a 20% and 50% enzyme inhibition (IC_{20} and IC_{50} values) are given in Table 6–1. None of the compounds revealed the inhibitory power of the specific 5α-Re inhibitor finasteride, with IC_{50} values of 1 nM (IC_{20} value=0.23 nM) in tissue homogenate and 130 nM (IC_{20} value=30 nM) in LNCaP.

Table 6–1 Inhibitory effects (expressed as IC_{20} and IC_{50} values) of test compounds (DBT, diuron, fenarimol, linuron, MBT, p,p´DDE, TPT, TBT, vinclozolin) and control substances (finasteride, flutamide, MT) on 5α-reductase activity in prostate tissue homogenate and LNCaP cells.

5α-reductase activity	IC_{20} [µM]		IC_{50} [µM]	
	Prostate	LNCaP	Prostate	LNCaP
Finasteride	0.00023	0.03	0.001	0.13
MT	0.4	4.4	1.9	13.2
TPT	1.1	1.9	3.9	4.2
TBT	1.3	0.5	4.0	2.7
DBT	3.0	2.4	6.8	11.2
Prochloraz	4.6	14.4	12.4	53.2
Fenarimol	11.8	-	24.1	-
Flutamide	13.8	-	48.4	-
p,p´DDE	19.1	-	62.9	
Linuron	28.1	-	86.0	-
Diuron	-	-	-	-
MBT	-	-	-	-
Vinclozolin	-	-	-	-

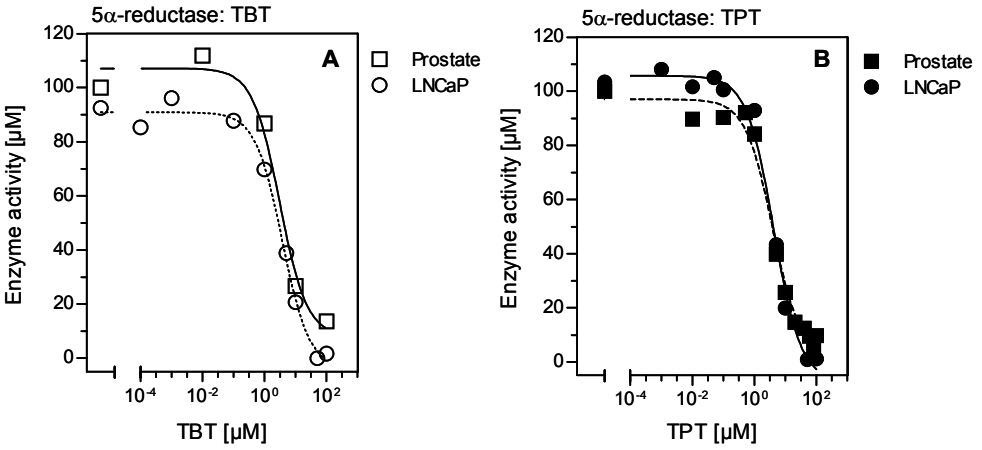


Fig. 6–3 Effect of (A) TBT and (B) TPT on 5α-reductase (5α-Re) activity, given as % activity compared to EtOH controls (100%), in human prostate homogenate and LNCaP cells.

TBT and TPT were the next strongest 5α-Re inhibitors (Fig. 6–3), followed by DBT and prochloraz. Fenarimol, flutamide, p,p´DDE, and linuron inhibited 5α-Re in prostate tissue only at very high concentrations, and no effect was observed in LNCaP cells. MBT, vinclozolin and diuron did not affect 5α-Re enzyme activity, neither in LNCaP cells nor in prostate tissue.

6.5 Discussion

Our study focused on the impact of the selected chemicals on 5α-Re activity, the enzyme which is indispensable for the formation of 5α-DHT. The two test systems that were used based on prostate tissue homogenate ("tissue assay"), exhibiting high 5α-Re 2 activity, and on LNCaP cells ("cell assay"), a human prostate carcinoma cell line known for high expression of both 5α-Re isoenzymes (Zhu et al. 2003).

It could be demonstrated that particularly the organotin compounds TBT, TPT, and DBT must be considered as hazardous environmental substances (Appel 2004). They inhibited human 5α-Re at low micromolar concentrations in both test systems. In addition, TBT and TPT have also been reported to act as inhibitors of other key enzymes of human sex steroid metabolism in various tissues, *e.g.,* P450arom in placenta, 3β-HSD in adrenal, 5α-Re 1 in brain, 17β-HSD type 1 in placenta tissues, and 17β-HSD type 3 in testis (Allera et al. 2004; Lo et al. 2003; McVey and Cooke 2003; Cooke 2002; Doering et al. 2002; Heidrich et al. 2001). The effective enzyme inhibiting concentrations of TBT and TPT were higher than those found in human blood or tissue so far (Lo et al. 2003; Wong et al. 1995), however, one cannot exclude a local bioaccumulation up to the harmful limit in, *e.g.*, sex hormone responsive tissues. Also, the "effective" *in vivo* enzyme inhibiting concentration remains unclear and therefore usually is set as 50% inhibition of normal enzyme activity (IC_{50} value). On average, chemical concentrations leading to a 20% lower DHT conversion were 3.5 times lower than the corresponding IC_{50} values, and might already have great impact on the endocrine system.

With the exception of TBT, the tissue assay proved to be more sensitive to 5α-Re affecting chemicals than the cell assay. The finding that the inhibitory effect of MT, prochloraz and, especially, finasteride on 5α-Re activity was considerably lower in LNCaP cells than in prostate tissue is possibly due to cellular detoxification and/or transport of the chemicals out of the cell by members of the MDR (multidrug resistant) or ABC (ATP-

binding cassette) exporter protein superfamilies often found in, *e.g.,* multidrug resistant cancers (Luckie 2003; van Brussel and Mickisch 2003; Larriba et al. 2001). In the tissue assay, p,p´DDE, fenarimol, flutamide, and linuron displayed very weak 5α-Re inhibition, while diuron, MBT, and vinclozolin did not affect enzyme activity. Therefore, it seems to be unlikely that the antiandrogenic effects observed *in vivo* (Lambright et al. 2000; Gray et al. 1999a; Gray et al. 1999b) are caused by impaired 5α-Re activity. It has been suggested that metabolites of the administered compound, *e.g.,* vinclozolin, might be the reason of antiandrogenic effects in rats (Zacharewski 1998; Kelce et al. 1994).

The advantages of the tissue assay are that it is easy to use and that it provides a ready-to-use enzyme source, while the cell assay can supply information on the cellular response to the chemical, and on the putative impact on the gene expression of sex hormone metabolizing key enzyme(s) (Nakanishi et al. 2002). However, when using the cell assay, it should be take into account that, *e.g.*, organotin compounds are highly cytotoxic, especially at incubation periods above 24 h (Allera et al. 2004). Furthermore, it must be emphasized that in addition to 5α-Re, other steroid-metabolizing enzymes, such as 17β-HSD, are present in prostate tissue and LNCaP cells, which could be affected by the discussed ED. Human risk assessment of ED will require a further number of short and long term *in vitro* and *in vivo* assays. Any extrapolation from our observations to humans should be very meticulously performed.

In conclusion, many of the tested pesticides are able to inhibit the 5α-Re activity, which may be of clinical relevance. The prostate tissue assay as a screening tool for putative ED is the more practical and sensitive method, while the cell assay partly reflects the situation *in vivo*.

7 Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism

This chapter is published as:

Lo S, Allera A, Albers P, Heimbrecht J, Jantzen E, Klingmüller D, Steckelbroeck S. Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism. Journal of Steroid Biochemistry and Molecular Biology. 2003, April; 84(5):569-576.

7.1 Abstract

Organotins are known to induce imposex (=pseudohermaphroditism) in marine neogastropods and are suggested to act as specific endocrine disruptors, inhibiting the enzyme-mediated conversion of steroid hormones. Therefore, we investigated the *in vitro* effects of triphenyltin (TPT) on human 5α-reductase type 2 (5α-Re 2), cytochrome P450 aromatase (P450arom), 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD 3), 3β-HSD type 2, and 17β-HSD type 1 activity. First, the present study demonstrates that significant amounts of TPT occurred in the blood of eight human volunteers (0.17–0.67 μg organotin cation/L *i.e.* 0.49–1.92 nmol cation/L). Second, TPT showed variable inhibitory effects on all the enzymes investigated. The mean IC_{50} values were 0.95 μM for 5α-Re 2 (mean of n=4 experiments), 1.5 μM for P450arom (n=5), 4.0 μM for 3β-HSD 2 (n=1), 4.2 μM for 17β-HSD 3 (n=3) and 10.5 μM for 17β-HSD 1 (n=3). To exclude the possibility that the impacts of TPT were mediated by oxidizing essential thiol residues of the enzymes, the putative compensatory effects of the reducing agent dithioerythritol (DTE) were investigated. Co-incubation with DTE (n=3) resulted in dose-response prevention of the inhibitory effects of 100 μM deleterious TPT concentrations on 17β-HSD 3 (EC_{50} value of 12.9 mM; mean of n=3 experiments), 3β-HSD 2 (0.90 mM; n=3), P450arom (0.91 mM; n=3), and 17β-HSD 1 (0.21 mM; n=3) activity. With these enzymes, the use of 10 mM DTE resulted in an at least 80% antagonistic effect, whereas the effect of TPT on 5α-Re 2 was not compensated.

In conclusion, the present study shows that TPT acts as an unspecific, but significant inhibitor of human sex steroid hormone metabolism and suggests that the inhibitory effects are mediated by the interaction of TPT with critical cysteine residues of the enzymes.

7.2 Introduction

Organotin compounds are widely used as unselective biocides for pest control, with an estimated annual production of approximately 50,000 tons. Derivatives of dialkyltin compounds such as dibutyltin (DBT), diphenyltin (DPT), and dioctyltin (DOT) are used in industry as stabilizers in polyvinylchloride (PVC) and as catalysts in various products, whereas trialkyltins, including tributyltin (TBT) and triphenyltin (TPT), are used in agriculture as fungicides and pesticides (Takahashi et al. 1999; Fent 1996; Boyer 1989; Molin and Wahlberg 1975). TBT and TPT additives in coatings for marine vessels prevent sessile animals, which need to adhere to a substrate during their life cycle, to settle down. Organotins are ubiquitous environmental pollutants especially relevant for water ecosystems (Duncan 1980). Photochemical and biochemical influences lead to successive degradation of organotins, and TPT disintegrates into diphenyltin, monophenyltin and inorganic tin (Duncan 1980). However, organotins stored in sediments are stable up to several years. This is a potential threat to aquatic life as a consequence of natural resuspension and particulate consumption by benthic organisms which live in seabeds and riverbeds (Sarradin et al. 1995; Fent et al. 1991). TPT compounds are rather selective in their action against fungal species, demonstrating a low risk for fungal resistance, a low volatility and a relatively rapid disintegration to "non-toxic" compounds by sunlight. Consequently, they were utilized for pest and fungal plant pathogen control (Duncan 1980). Since August 2002, the use of TPT acetate and TPT hydroxide has been banned within the European Union.

The extensive use of organotins as biocides leads to an ongoing contamination of aquatic and terrestic environment. In the aquatic environment, a strong food chain accumulation of organotins has been noticed (Stab et al. 1996). Examination of marine vertebrates showed considerable concentrations in liver and kidney, as well as in hair, nails and feathers (Bhosle et al. 2004; Coelho et al. 2002; Kannan and Falandysz 1997). The main sources of organotin intake for humans are seafood contaminated by the exposure to antifouling agents (Takahashi et al. 1999) and drinking water contaminated by the leaching from PVC water pipes (Sadiki and Williams 1999). Organotins also effectively penetrate through the skin (Cooke et al. 2004; Adeeko et al. 2003; Hasan et al. 1984). Reasonable concentrations have been detected in higher species, including mammals, in liver, kidney, brain and blood samples (Kannan et al. 1999; Kannan et al. 1996; Fait et al. 1994; Fent et al. 1991).

Both TBT and TPT are reported to affect the immune system, the nervous system and the hormone system as well as embryogenesis (Ema et al. 1997; Cima et al. 1996; Snoeij et al. 1985). TBT is known to act as an endocrine disruptor causing imposex in various female prosobranch snails. Investigations have shown that incorporated TBT induces an increased testosterone/estradiol ratio in snails suggesting that TBT inhibits cytochrome P450 aromatase (P450arom) (Bettin et al. 1996). Previously, TBT and TPT were shown to act as inhibitors of human P450arom activity (Heidrich et al. 2001; Saitoh et al. 2001). As a consequence, most developed nations have imposed a ban on TBT-based antifouling paints for vessels under 25 m in length since the late 1980s (Batley et al. 1992). Other triorganic tin compounds, such as TPT, have recently been found to likewise cause imposex (Horiguchi et al. 1998). Hence, TPT should similarly be considered as an endocrine disruptor. The release of environmental chemicals, such as pesticides, detergents, and plasticizers, is suggested to play a role in the observed increased incidence of male reproductive disorders.

The enzymes examined in the present study maintain the proper balance of androgens and estrogens in the human body. 3β-HSD converts Δ5-3β-hydroxysteroids into the corresponding Δ4-3-ketosteroids (Labrie et al. 1994). 17β-HSD 3 catalyzes the testicular conversion of the weak androgen Δ4-androstenedione into the strong androgen testosterone. Testosterone is the most abundant androgen in the male sex steroid hormone system. Dihydrotestosterone is synthesized from testosterone via 5α-Re 2 activity. It represents the most potent androgen naturally occurring and is indispensable for the normal virilization of the male external genitalia and prostate (1996; Wilson et al. 1993). P450arom is responsible for the conversion of C19 androgens into the corresponding C18 estrogens in a variety of tissues, including the ovary, testis, placenta, brain, and adipose tissue (Steckelbroeck et al. 1999a; Morishima et al. 1995). 17β-HSD 1 predominantly catalyzes the conversion of the weak estrogen estrone into the strong estrogen estradiol (Peltoketo et al. 1999).

Organotins possess both lipophilic and ionic properties. The former encourages their accumulation in lipids and their membrane toxicity, while the latter enables their binding to macromolecules (Kannan and Falandysz 1997; Gray et al. 1987). The biochemical effects of organotins on human sex steroid hormone metabolism remain to be elucidated. Therefore, we have studied the inhibitory effects of TPT on the *in vitro* activity of the key enzymes of human sex steroid hormone metabolism using human tissue samples. In several

experiments, it was demonstrated that sulfhydryl compounds antagonize the harmful effects of organotins (Costa 1985; Van der Bend et al. 1985; Byington et al. 1974; Byington 1971). It is suggested that organotins interact with any thiol residues accessible, thus, the sulfhydryl antagonist prevents modification of the tertiary structure of the proteins. Therefore, we also investigated the *in vitro* effects of the reducing agent dithioerythritol (DTE) on TPT inhibited enzyme activities. To elucidate the potential risk of TPT-evoked endocrine disruption in humans, the content of a variety of organotin compounds was determined in blood samples of eight healthy adult human volunteers.

7.3 Materials and Methods

7.3.1 Chemicals

Steroids and other chemicals

25.9 Ci/mmol [1β-^{3}H]-androst-4-ene-3,17-dione (androstenedione or Enon), 51.3 mCi/mmol [4-^{14}C]-[10]-estratriene-3α-ol-17-one (estrone), and 53.8 mCi/mmol [4-^{14}C]-androst-5-ene-3β-ol-17-one (dehydroepiandrosterone or DHEA) were obtained from New England Nuclear Co. (Dreieich, Germany) and purified by thin layer chromatography (TLC) prior to use. Non-radioactive reference steroids (5α-androstane-3,17-dione (androstanedione), androstenedione, 5α-androstane-17β-ol-3-one (dihydrotestosterone or DHT), 5α-androstane-3α-ol-17-one (androsterone), androst-4-ene-17β-ol-3-one (testosterone) and 5α-androstane-3α,17β-diol (3α-androstanediol or 3α-Diol), estrone, [10]-estratriene-3α,17β-diol (estradiol), DHEA, androst-5-ene-3β,17β-diol (androstenediol)), EDTA, Folin & Ciocalteu´s phenol reagent, TRIZMA^{TM} (a,a,a-tris-(hydroxymethyl)-methylamin), TRIZMA^{TM}-HCl, citric acid, sodium potassium tartrat, activated charcoal (Niorit A), and dithioerythritol were purchased from Sigma^{TM} Chemical Company (Deisenhofen, Germany). Atamestane was provided by Schering (Berlin, Germany). Finasteride was purchased from MSD Sharp & Dohme (Haar, Germany). Dextran T-710 was obtained from Pharmacia Biotech (Uppsala, Sweden). The liquid scintillation cocktail, Ultima Gold^{TM}, was obtained from Packard-Instrument, B.V., Chemical Operations (Groningen, Netherlands). NADPH was purchased from Roche (Mannheim, Germany).

Triphenyltin chloride and all other chemicals were purchased from Merck A.G. (Darmstadt, Germany). All chemicals were purchased at the highest grade commercially available.

Buffers

Homogenization buffer (HB) contained 10 mmol/L TRIZMA^{TM}-HCl and 1 mM EDTA at pH 7.4. Assay buffer consisted of 160 mmol/L TRIZMA^{TM}-citrate and 10 mM $MgCl_2$ and was adjusted to the indicated pH values.

7.3.2 Source and preparation of tissues

Human term placenta (cytosolic 17β-HSD 1 activity; microsomal P450arom activity) was obtained following caesarean section. Macroscopically normal testicular tissue (microsomal 17β-HSD 3 activity) was obtained from patients with testicular germ cell tumor undergoing orchiectomy. Macroscopically normal prostate tissue (microsomal 5α-Re 2 activity) was obtained from patients with bladder cancer undergoing cystectomy and prostatectomy. Macroscopically normal adrenal tissue (microsomal 3β-HSD 2 activity) was obtained from patients with kidney cancer undergoing nephrectomy. All utilized human tissue samples were immediately frozen in liquid nitrogen after removal and stored at –80°C until further processing. The study was approved by the local ethics committee and informed consent from all tissue donors or their family members had been obtained.

All steps of tissue preparation were carried out at 4°C. According to the enzyme content, 25 to 200 mg tissue wet weight were homogenized and centrifuged as described previously (Steckelbroeck et al. 2001; 1999a; 1999b). The cell-free supernatants were used as microsomal preparations for the investigation of P450arom, 3β-HSD 2, 17β-HSD 3, and 5α-Re 2 activity. Aliquots were stored in liquid nitrogen until utilization in the experiments.

To prepare a cytosolic fraction for the 17β-HSD 1 assay the cell-free placental homogenate was further centrifuged at 100,000 g for 60 min. The obtained soluble supernatant was stored in liquid nitrogen until utilization in the experiments. Aliquots of all tissue preparations were removed for protein determination (Lowry et al. 1951).

7.3.3 Incubation procedures

The *in vitro* activities of P450arom, 3β-HSD 2, 5α-Re 2, 17β-HSD 1, and 17β-HSD 3 were determined with some modifications according to methods described previously (Steckelbroeck et al. 2001; 1999a; 1999b). Briefly, solutions of the substrates were prepared in assay buffer. The measurement of enzyme activity was conducted with 1 µM final concentration of [4-^{14}C]-DHEA (3β-HSD 2), with 0.5 µM final concentration of [4-^{14}C]-estrone (17β-HSD 1) and 0.1 µM final concentration of [1β-^{3}H]-androstenedione (17β-HSD 3, 5α-Re 2, and P450arom). Stock solutions of the test compounds were prepared in EtOH. The assay buffer was adjusted to pH 7.5 for the measurement of the activity of P450arom, 3β-HSD 2, 17β-HSD 1, and 17β-HSD 3 and to pH 5.5 for the measurement of 5α-Re 2 activity.

The reaction mixture contained 50 µL assay buffer with 0 to 10 mM DTE, 50 µL of the tissue preparation and 3 µL EtOH containing the test compound at the indicated concentrations. "Blank" reactions were incubated tissue-less. Reference enzyme activities were determined with incubations of tissue homogenate and 3 µL EtOH. To achieve equilibrium of the substances in the tissue preparation, the preliminary reaction mixture was preincubated for 5 min with constant shaking at 37°C. Preincubation was stopped by chilling. Then, 50 µL assay buffer containing the substrate was added to the reaction mixture. The reactions were started by the addition of another 50 µL HB containing 3 mM final concentration of the required cofactor (NAD for measurement of 3β-HSD 2 activity and NADPH for measurement of P450arom, 17β-HSD 1, 17β-HSD 3, and 5α-Re 2 activity). All incubations were performed in duplicate. Reaction tubes were capped, vortexed and incubated for 30 min with constant shaking at 37°C. Reactions were stopped by chilling.

7.3.4 Product isolation assay

Measurement of 3β-HSD 2, 5α-Re 2, 17β-HSD 1, and 17β-HSD 3 *in vitro* activity based on product isolation post incubation according to methods described previously (Steckelbroeck et al. 2001; 1999a; 1999b). Briefly, steroids were extracted with a mixture of MeOH/$CHCl_3$ (1/2, v/v) from the incubation mixtures, 100 µL of the tritium containing organic phase for the determination of 3β-HSD 2, 5α-Re 2, and 17β-HSD 3 activity, and 300 µL of the ^{14}C containing organic phase for the determination of 17β-HSD 1 were

evaporated to dryness and redissolved in a mixture of 35 µL $CHCl_3$ and 15 µL EtOH containing 25 µg each of non-radioactive reference steroids.

Metabolites were separated by TLC. The silica sheets were dried and stained by spraying with a mixture of acetic acid/ H_2SO_4/4-methoxybenzaldehyde (100/2/1, v/v/v) and charred at 135°C. Within each lane, the zones corresponding to the stained reference steroids were cut out and transferred into counting vials containing 15 mL liquid scintillation cocktail. Radioactivity was counted as automatically quench-corrected dpm with a Wallac 1409 liquid scintillation counter.

The relative amount of each corresponding radioactive steroid was calculated, in percentage, with the total radioactivity recovered from a single TLC lane set as 100%. Blank values were subtracted from tissue metabolism rates. Enzyme activity was assessed by quantifying the formation of radioactive labeled products.

7.3.5 Tritiated water-release assay

Measurement of P450arom *in vitro* activity was based on the proportional release of 1β-^{3}H from [1β-^{3}H]-androstenedione into ^3H_2O during the P450arom catalyzed reaction (Steckelbroeck et al. 1999a). In brief, organic compounds were extracted from incubation mixtures by adding ice-cold $CHCl_3$. 350 µL of the aqueous phase was stripped from remaining steroids with 5% dextran-coated charcoal and 1.5 mL $CHCl_3$. 250 µL of the highly purified aqueous phase containing ^3H_2O was quantified as quench-corrected dpm by counting for 15 min using a Wallac 1409 liquid scintillation counter. The amount of ^3H_2O was corrected for dilution and the blank values were subtracted.

7.3.6 Determination of different organotin compounds in human blood

Determination of the concentration of monobutyltin, dibutyltin, tributyltin, tetrabutyltin, monooctyltin, dioctyltin, and triphenyltin in the blood of eight human volunteers (Table 7–1) was conducted by GALAB (Geesthacht, Germany) according to a "one unique extraction-derivatization step" method described previously (Kuballa and Wilken 1995). Blood was sampled in organotin-free glassware. The organotin compounds were extracted with tetra-methyl-ammonium-hydroxide and MeOH, alkylated with sodium tetraethylborate and transferred by extraction with hexane into the organic phase. An aliquot of the hexane layer containing the tetrasubstituted organotincompounds was separated using capillary gas-chromatography (Perkin-Elmer GC 8400, Überlingen, Germany), and detecting and

quantifying the organotins via atomic emission spectrometry (GC-AED from Hewlett Packard, Agilent). According to GALAB, the recovery of the procedure was 75–100%. The chromatographic data were processed with a Perkin-Elmer Nelson 2600 software package. A column from ICT (Frankfurt, Germany) was used (DB 1701, length 30 m, id 0.32 mm, 0.24 µm film thickness). The temperature program of the GC was 80–280°C at 30°C/min. The flow rate of the helium carrier gas was 1.8 mL/min at 80°C. For atomization, a heated quartz furnace was used, with furnace gases hydrogen (120 mL/min) and air (35 mL/min). The temperature of the transfer line was constant at 250°C; the pre-furnace temperature was 280°C and the atomization temperature 700°C. The 286.3 nm tin line was generated by an electrode-less discharge lamp operated at 7 W.

7.3.7 Data analysis

Dose-response analyses were performed with a computer-assisted non-linear curve-fitting method using the linear dose versus effect model (FigP 2.7, Biosoft, Cambridge, UK). To calculate inhibitor concentration resulting in 50% inhibition (IC_{50} value) or the concentration of enhancer provoking a response halfway between baseline and maximum (EC_{50} value) the QuickFit option of the software was used.

7.4 Results

7.4.1 Determination of different organotin species in human blood

The blood of eight healthy human volunteers was analyzed for the presence of different organotin compounds. As shown in Table 7–1, TPT is the major organotin compound found in human blood (0.17–0.67 µg organotin cation/L *i.e.* 0.49–1.92 nmol cation/L). Furthermore, we were able to demonstrate the presence of minor concentrations of TBT in human blood, whereas the concentrations of monobutyltin, dibutyltin, tetrabutyltin, monooctyltin as well as dioctyltin were below the detection limit of 0.02 µg organotin cation/L.

Table 7–1 The serum of eight human volunteers was analyzed. The gender, age, profession, and body mass index (BMI) of the subjects are listed. Organotin cations were determined by GC-MIP-AED. The content is expressed as µg organotin cation/L. Mean of TPT blood concentration was 0.38 µg/L (n=4 females) and 0.19 µg/L (n=4 males), mean of TBT was 0.0275 µg/L TBT (females) and 0.005 µg/L TBT (males).

Gender	Female	Female	Male	Male	Male	Female	Female	Male
Age in years	18	50	54	53	54	31	27	41
Profession	student	teacher	civil engineer	physician	reporter	physician	student	camera-man
BMI	25	24	27	22	25	21	20	24
Monobutyltin	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Dibutyltin	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Tributyltin	0.04	0.02	<0.02	0.02	<0.02	0.05	<0.02	<0.02
Tetrabutyltin	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Monooctyltin	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Dioctyltin	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Triphenyltin	0.32	0.67	0.17	0.32	0.23	0.35	0.18	0.23

7.4.2 Dose-response analyses of the inhibitory effects of TPT on the key enzymes of human sex steroid hormone metabolism

The putative inhibitory effects of TPT on the activity of key enzymes of human sex steroid hormone metabolism were investigated. For this purpose, incubations with TPT at various concentrations were carried out. Product formation in the absence of the inhibitor represented 100% enzyme activity. TPT demonstrated dose-response inhibitory effects on all the enzymes investigated. In Fig. 7–2A, the inhibition of 17β-HSD 1 activity is shown as an example. The average IC_{50} values for the different enzymes, calculated according to data analysis, are given in Table 7–2.

The investigation of the inhibition of 17β-HSD 1 activity by TPT revealed an IC_{50} value of 10.5 µM (n=3 experiments; Fig. 7–1A). As shown in Table 7–2, TPT showed similar dose-responsive inhibitory effects on all the enzymes investigated with the lowest IC_{50} value of 0.95 µM for 5α-Re 2 (Fig. 7–2A).

Table 7–2 Activity of the enzymes investigated corresponding to 100% non-inhibited enzyme activity in the absence or the presence of DTE. Moreover, the detected mean IC_{50} values of TPT and mean EC_{50} values of DTE are listed. **ND=not detectable*

	3β-HSD 2	17β-HSD 3	17β-HSD 1	P450arom	5α-Re 2
Activity / nmol/h/mg protein	2.9–29.0	0.013–0.020	3.4–11.5	0.20–0.32	0.03–0.13
Activity with 10mM DTE / nmol/h/mg protein	3.5–34.4	0.045–0.28	2.327–21.435	0.29–0.42	0.013–0.034
IC_{50} (TPT) / μM	4.0 (n=1)	4.2 (n=3)	10.5 (n=3)	1.5 (n=5)	0.95 (n=4)
EC_{50} (DTE) / mM	0.90 (n=3)	12.9 (n=3)	0.21 (n=3)	0.91 (n=3)	ND* (n=3)

7.4.3 Compensatory effects of DTE on the TPT inhibited enzyme activity

Further experiments were conducted to examine the putative compensatory effects of DTE on the inhibitory effects of TPT. For this purpose, the reaction mixtures contained 100 μM TPT final concentration and varying DTE concentrations from 0–10 mM. Product formation in the absence of TPT and the presence of 10 mM DTE final concentration represented 100% enzyme activity. DTE concentrations above 10 mM resulted in decreasing enzyme activities (data not shown). All experiments were conducted thrice.

With the exception of 5α-Re 2, DTE demonstrated a strong dose-responsive compensatory effect on the TPT-inhibited activity of the enzymes under debate (Fig. 7–1 to 7–5, Table 7–2). 17β-HSD 1 showed the lowest EC_{50} value (0.21 mM) observed (Fig. 7–4B). The EC_{50} values of the other enzymes investigated are listed in Table 2. DTE resulted in an approximately 80% antagonistic effect with 17β-HSD 1, P450arom, 3β-HSD 2, and 17β-HSD 3. In contrast to the other enzymes, DTE resulted in only very weak compensatory effects on TPT inhibited 5α Re 2 activity (Fig. 7–2B). An EC_{50} value for this enzyme was not detectable.

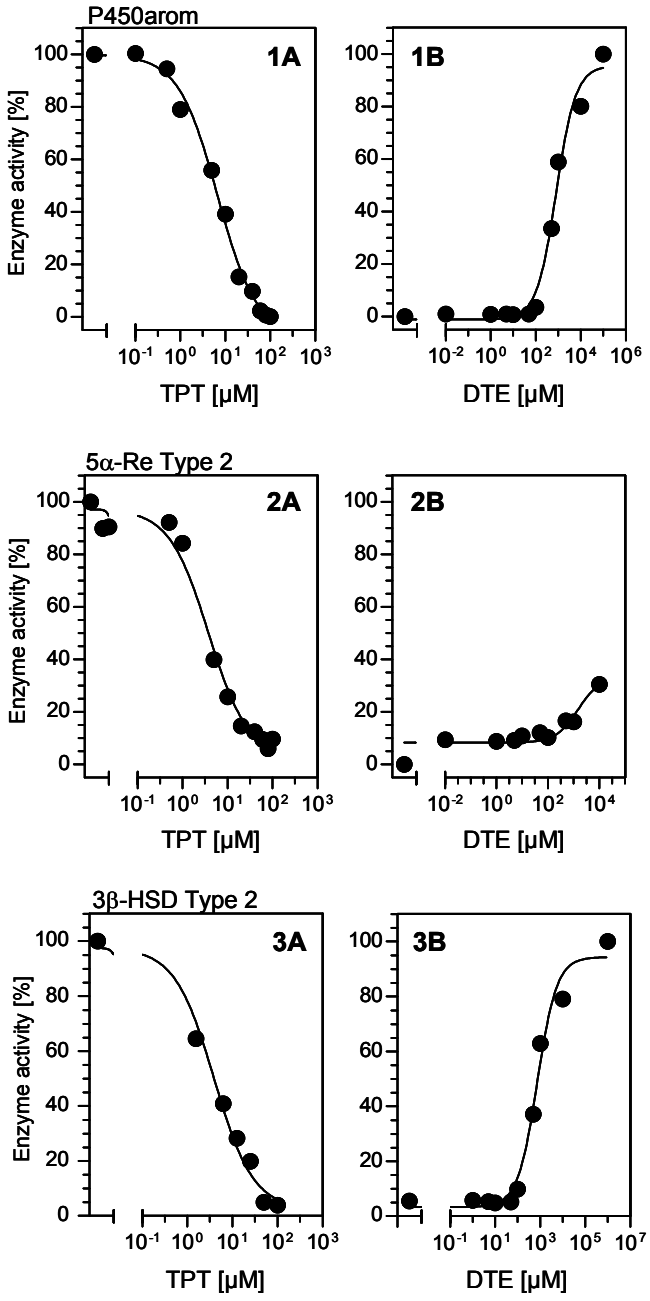
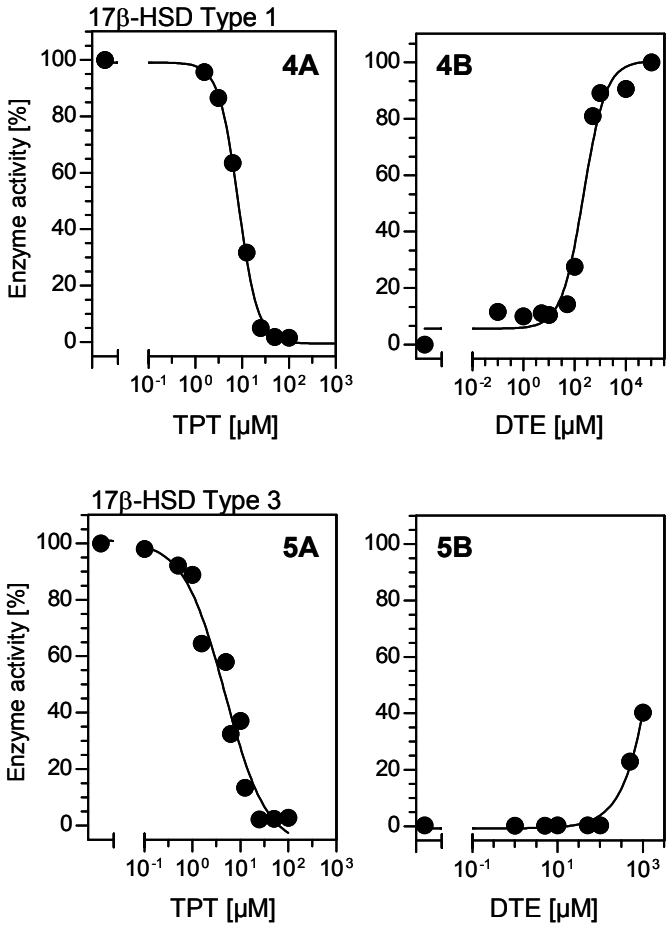


Fig. 7-1 to 7-5 The inhibitory effect of increasing TPT concentrations on human P450arom (Fig. 7-1), 5α-Re type 2 (Fig. 7-2), 3β-HSD type 2 (Fig. 7-3), 17β-HSD type 1 (Fig. 7-4) and 17β-HSD type 3 (Fig. 7-5). Activities were investigated using microsomal placenta (Fig. 7-1), prostate (Fig. 7-2), adrenal (Fig. 7-3), testis (Fig. 7-5) and cytosolic placenta (Fig. 7-4) preparations with 0.05 µM (Fig. 7-1) and 0.1 µM androstenedione (Fig. 7-2, 7-5), 1 µM DHEA (Fig. 7-3) and 1 µM estrone (Fig. 7-4), respecively. As cofactor, 3 mM NADPH was used in enzyme assay described in Fig. 7-1, 7-2, 7-4, and 7-5, and 3 mM NAD was used in Fig. 7-3, at pH value of 7.5 (Fig. 7-1, 7-3, 7-4, 7-5) and 5.5 (Fig. 7-2). The results represent mean values of n=3 assays performed in duplicate. The compensatory effect of increasing DTE concentration were determined at 100 µM deleterious TPT concentrations.



7.5 Discussion

In the present study, investigations of organotin body burden demonstrated the presence of significant amounts of TPT in human blood, while the other organotin compounds were close to the detection limit (Table 7–1). Women showed a slightly higher average blood concentration of TPT and TBT, which possibly reflects the higher percentages of body fat in women (Blaak 2001) and the accumulation of tinorganic compounds in lipids (Kannan and Falandysz 1997).

Men get in touch with organotins probably via consumption of contaminated food. Marine fishery products contain up to 455 ng TBT/g wet weight fish muscle (Kannan and Falandysz 1997), whereas agriculture products are more likely to be contaminated with TPT due to its use as pesticide (Duncan 1980). Different diets would be expected to result

in different organotin loads in human tissues and blood. Two studies verify hepatic TBT deposition (Takahashi et al. 1999; Kannan and Falandysz 1997). Japanese people, who consume a considerably higher dietary intake of fish, showed significantly higher liver TBT loads than Polish people. The chemical analysis performed by the COMPRENDO partner in France (BRGM) revealed MBT residues in urine samples of seven Polish dockers and two fish consumers, and DBT contamination in urine of two dockers and one fish consumer (chapter 5), confirming the body burden with organotin compounds.

Moreover, our experiments clearly document a dose-dependent complete inhibition of all the steroidogenic enzymes investigated. These results indicate that TPT is an unspecific inhibitor of human sex steroid hormone metabolism. Several mechanisms could account for the loss of the enzymes activity caused by TPT: (a) binding of TPT to cell membrane components, indirectly leading to inhibition of the catalytic activity of the enzymes (Gray et al. 1987), (b) inhibition of components of the electron transport chain affecting the availability of the coenzymes necessary for full enzyme activity, and (c) binding of the organotin to the proteins itself, resulting in direct destruction of the catalytic activity of the enzymes. It is very unlikely that binding of TPT to cell membrane components is the cause of the inhibitory potency of TPT, since TPT affects not only microsomal enzymes, but also the activity of the cytosolic 17β-HSD 1. The specific inhibition of components of the electron transport chain can also be excluded, since TPT inhibits both NADPH- and NAD-dependent enzyme activities. Previously, it was suggested that TPT binds to specific amino acids, such as cysteine and histidine, leading to an impeded enzyme activity (Fent et al. 1991). Thiol compounds abolish TBT mediated haemolysis (Gray et al. 1987) and the protective effect of the thiol compounds were attributed to a chemical interaction of the tinorganic Lewis acid, with the thiol Lewis base indicating a putative reaction of organotins with cysteine residues of proteins (Gray et al. 1987; Byington et al. 1974; Byington 1971).

Treatment with DTE partly resulted in a substantial compensation of the adverse effects of TPT. The EC_{50} values of DTE varied in a wide range, indicating that the effectiveness of the compensatory activity of DTE differs among the enzymes investigated. DTE resulted in an approximately 80% antagonistic effect with 17β-HSD 1, P450arom, 3β-HSD 2, and 17β-HSD 3. Interestingly, DTE demonstrated hardly any compensatory effect on TPT inhibited 5α-Re (Fig. 7–2B). Equally, this enzyme showed the highest sensibility (lowest IC_{50} value) towards TPT inhibition (Fig. 7–2A). Accordingly, 17β-HSD 1 shows

the highest IC_{50} value for TPT at the lowest EC_{50} value for DTE. The inhibitory power of most of the other ED screened in this study decreased in the presence of DTE, regardless of the mechanism of inhibition. Thus, methodological details might be the cause for different ED outcomes for the same chemical in different laboratories.

In order to identify a relation between the aminoacic sequence of the enzyme and its interaction with the inhibitor or the antagonist, the ratio between the number of amino acids and the number of cysteine residues of the enzyme was calculated. Noteworthy is that 5α-Re 2 features the highest frequency of cysteine residues (3.41 cysteine residues per 100 amino acids), whereas the other enzymes demonstrate reasonably lower cysteine contents (2.26 cysteine residues per 100 amino acids in 17β-HSD 3, 1.83 in 17β-HSD 1, 1.39 in P450arom and 1.35 in 3β-HSD 2). The present data gives reason to believe that critical (in terms of accessibility) cysteine residues are responsible for the inhibitory effects of TPT. Consequently, one might suggest that 5α-Re 2 is characterized by the occurrence of a relatively high number of critical cysteine residues compared to the other enzymes. In proteins, the proper pairing of cysteine residues and maintenance of disulfide bonds is essential for normal structure and activity. In eukaryotic cells, the naturally occurring thiol compound glutathione prevents the formation of disulfide bonds in the cytosol and catalyzes their formation in the endoplasmic reticulum. The inhibitory *in vitro* effects of TPT could possibly be attenuated by glutathione in vivo. On the other hand, an adverse effect of TPT loads on glutathione functions might also be suggested, since a distinct proper ratio of the reduced and oxidized form of the thiol compound is required in the cytosol and the endoplasmic reticulum, respectively (Lodish et al. 1995).

Endocrine disrupting or interfering effects of organotins observed in non-mammalian species can only cautiously be extrapolated to humans, but effects targeting the endocrine system will potentially occur at lower exposure levels than most other toxic effects. The *in vitro* effects of TPT on the key enzymes of human sex steroid hormone metabolism might therefore indicate possible risks for the endocrine system *in vivo*. Hormonal imbalance, caused by endocrine modulators, has been associated with negative outcomes such as cancer in hormone sensitive tissues, declining reproductive health including semen quality, congenital anomalies, and even brain diseases (Jacobsen et al. 2006; Jorgensen et al. 2006; Kortenkamp 2006; Skakkebaek et al. 2006; Jouannet et al. 2001; Henderson and Feigelson 2000; Juberg 2000; Birge 1997; McEwen 1997). The IC_{50} values of TPT detected are

relatively high (0.95–10.5 µM), compared to the average TPT content found in human blood samples (0.49–1.92 nM organotin cation). Yet, it was shown that the lipophilic substances TPT and TBT are accumulated at high concentrations in specific organs and tissues, such as liver, fat and brain tissue (Harino et al. 2000). Interestingly, fat tissue expresses reasonable P450arom activity and is the main source for estrogens in postmenopausal women (Deslypere et al. 1985). Moreover, steroid hormone imbalance is observed in patients with liver diseases, indicating the importance of steroid hormone metabolism in this organ (Yoshitsugu and Ihori 1997; Kley and Kruskemper 1978). A study on the neurotoxicity of organotins proved that TPT crosses the blood-brain barrier and causes deficits in the learning ability in rats (Lehotzky et al. 1982). Noteworthy, steroid hormones play a crucial role for the functions of the central nervous system (McEwen et al. 1991), and a complex system of enzymes catalyzing the metabolism of steroid hormones exists in the brain (Mellon and Griffin 2002b, 2002a). Altogether, even relatively low TPT loads might affect the endocrine system due to putative enrichment of the compound in fat and membrane-rich brain tissue.

In conclusion, the present study demonstrates a sigificant TPT blood load in humans and indicates that TPT acts as an unspecific inhibitor of the key enzymes of human sex steroid hormone metabolism. Moreover, the experiments show that DTE is able to compensate the adverse effects of TPT and that the effectiveness of the compensatory activity of DTE differs among the enzymes investigated. Conceivably, critical cysteine residues are responsible for the inhibitory effects of TPT. Explicit studies concerning human load of organotin compounds are still lacking. Consequently, we emphasize the importance of further studies to evaluate patho-physiological effects of organotin compounds on the human sex steroid hormone metabolism.

8 Aquatic model organisms for the investigation of endocrine disruption

8.1 Abstract

There is still uncertainty of how to assess new chemicals with putative endocrine disrupting potency. One of the most difficult tasks is the extrapolation across species from laboratory animals to endangered wildlife species, or even to humans. In the present study, the aquatic vertebrates *Xenopus laevis*, *Rutilus rutilus,* and *Pimephales promelas* were chosen as animal models. First, *Xenopus laevis* of different life stages and sexes were extensively investigated for androgen metabolizing enzyme distribution in various tissue samples. To the author´s best knowledge, 5β-reductase (5β-Re) was for the first time discovered in brain tissue of both sexes. Aromatase (P450arom) and 5α-reductase (5α-Re) activity in gonadal and brain tissue samples of all the selected aquatic animals were analyzed for their suitability as endpoints in an endocrine disruption *in vivo* model. For this purpose, animals were exposed to methyltestosterone (MT), TPT, letrozole (Letro), and vinclozolin (Vin), and subsequently investigated for changes in target enzyme activities.

In female *Xenopus laevis*, exposure to 0.1 µM MT significantly ($p<0.001$) elevated specific 5α-Re activity in brain, and slightly lowered P450arom activity in gonads. Vin exposure significantly ($p<0.05$) elevated 5α-Re activity in female brain.

In *Rutilus rutilus*, exposure to 0.1 µM MT significantly ($p<0.001$) elevated 17β-HSD activity in female brain and elevated ($p<0.01$) P450arom activity in male brain. Exposure to 0.1 µM Vin elevated ($p<0.01$) 17β-HSD activity in female brain.

In TPT-exposed *Pimephales promelas* (320 ng/L), a significant increase of P450arom activity in female gonads was detected. Exposure to 30 ng/L MT significantly ($p<0.01$) elevated P450arom activity in female gonads, while 10 ng/L MT lowered ($p<0.01$) P450arom activity in female brain. P450arom activity in male brain (300 ng/L MT) was slightly elevated.

In conclusion, androgen metabolism of *Xenopus laevis* proved to be sex and age dependent, and androgen inactivation seems to be catalyzed by 5β-Re. For the assessment of putative endocrine disrupting chemicals, the selected enzymes P450arom, 5α-Re, 17β-Re and 5β-Re proved less suitable as distinctive endpoints, because of high variability between individuals, low responsiveness to chemical exposure and inconsistent reaction, even to the control substances MT and Letro.

8.2 Introduction

Environmental chemicals can cause ecosystemic damage, inter alia by perturbing the endocrine system of wildlife species. One of the best documented examples deals with the application of TBT: the successful use of TBT-antifouling paints against disturbing organisms on ship hulls in the 1970s, the collapse of the shellfish industry in Arcachon due to reproductive failure and severe shell malformation in the 1980s, the symptoms of imposex in approximately 150 different marine mollusk species, the investigation of TBTs toxicology and, at last, the partial prohibition of the application of TBT paints on vessels under 25 m in length (Santillo et al. 2001; Schulte-Oehlmann et al. 2000; Yamabe et al. 2000). This ban improved the situation within marinas and sheltered harbors, and some regional recovery of affected mollusk populations has been recorded since (Santillo et al. 2001; Matthiessen et al. 1995). One of the reasons for the comparatively fast prohibition of TBT as an antifoulant was the most commonly associated adverse effect: the virilization of female marine gastropods, called imposex (Smith 1981). As it was possible to reproduce this highly sensitive, chemical-specific phenomenon under laboratory conditions, and thus prove the direct causal relationship, the need for TBT regulation and control was readily accepted. The effects on wildlife and human population of most other environmental chemicals are much more circumstantial, and resulting problems seem to be less pressing. Thus, the supply of suitable and sensitive bioassays for chemical assessment is of environmental, political and economical importance.

A lot of time and effort has been put into the search and validation of model animals for *in vivo* studies – suitable in terms of application, evaluation, and extrapolation on reproductive processes in wild life and human health. Particularly nonmammalian vertebrate species have been taken into consideration, as HPG axes are surprisingly similar to mammals in their operation, the pattern of feedback mechanisms, and the hormones involved (Fig. 8–1). In amphibian and teleost species, hypothalamic GnRH(s), analog to those found in mammals, trigger the release of GTH-I (≅ FSH) and GTH-II (≅ LH) from the pituitary (Sower et al. 2004; Sherwood et al. 1994). GTH-I mainly regulates gonadal growth and gamete formation, while GTH-II is responsible for gamete release (Nagahama 1994). As in mammals, the major androgens are testosterone and DHT. Most of the teleost species use 11-ketotestosterone as signaling hormone for masculinization, additionally or as an equivalent to DHT (Borg 1994).

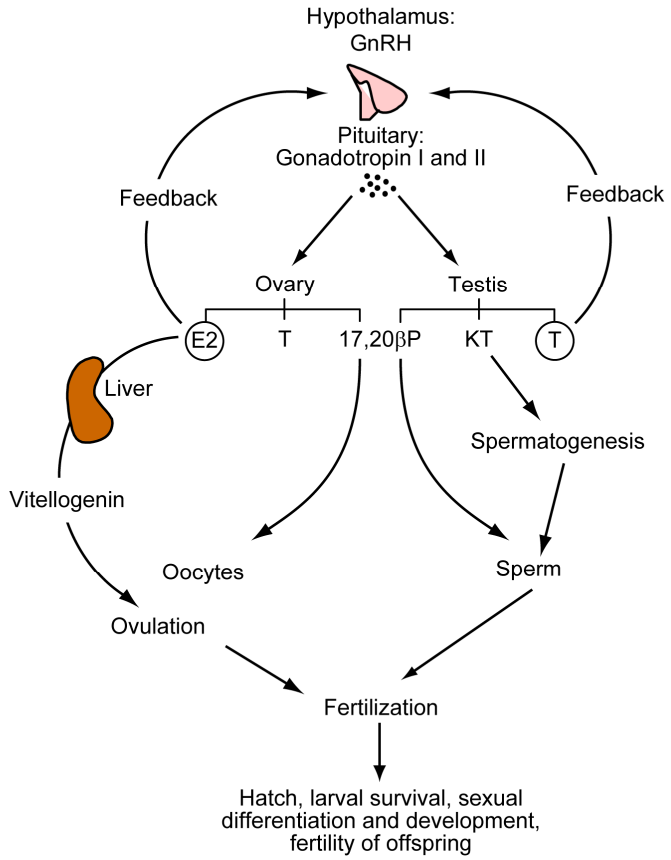


Fig. 8–1 HPG axis in male and female teleost fish (Kime 1998)
E2=estradiol, T=testosterone, 17,20βP=17,20β-progesterone, KT=11-ketotestosterone

In this context, two different types of androgen receptors have been discovered in teleosts (Sperry and Thomas 1999a, 1999b). In female teleost, oocyte maturation and ovulation are regulated by E2, vitellogenin and the progesterone-like molecule 17α,20β-P (Nagahama 1994). It is noteworthy that female amphibians and teleosts exhibit high levels of androgens as well as estrogens during the reproductive phase. Another remarkable point is that teleost fish express high levels of P450arom in brain, 100–1000 times higher than in mammals (Callard et al. 1990). The reason for this is yet unclear, but adult fish brains retain a remarkable potential for neurogenesis, continue to grow throughout life and can even regenerate after damage (Kishida and Callard 2001).

In the present study, one anuran and two teleost species have been selected for the investigation of putative endocrine disruptors. The chemical exposures of *Xenopus laevis* and *Rutilus rutilus* were conducted by partner IGB-Berlin (Dr. I. Lutz and C. van Ballegooy, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin), while the chemical exposures of *Pimephales promelas* were conducted by partner UBrun (D. Hala, University of Brunel, UK).

Xenopus laevis is a member of the family Pipidae, all of whose members are wholly aquatic (Fig. 8–2). The mating season lasts for 10 months in wildlife and extends to perennial mating under laboratory conditions. *Xenopus laevis* was longtime used as a bioassay to determine human pregnancy, as female animals respond with spontaneous egg deposition when exposed to human chorionic gonadotropin (HCG) present in urine of pregnant women.

Xenopus laevis has also been extensively employed in experiments on sex reversal. Larvae raised in water containing estradiol can display reversion of genetic males to fully functional females, if the exposure occurs at a critical period of development. In contrast, exposure of females to exogenous androgens does not appear to interfere with normal gonadal development (Guillette et al., 1995). The high sensitivity to environmental chemicals and its aquatic way of living predestine *Xenopus laevis* as a suitable candidate for aquatic studies. *Xenopus laevis* has already successfully been used to screen estrogen receptor binding capacity of endocrine disruptors (Huang et al. 2005; Kloas et al. 1999; Lutz and Kloas 1999).

Fig. 8–2 Photography of Xenopus laevis.
Wikipedia, GNU Free Documentation licence

Rutilus rutilus (Cyprinid) is common to lowland rivers throughout Europe (Fig. 8–3). In wildlife, these fish form shoals and spawn in early spring. Although they are considered as comparably resistant against poor water quality, incidences of intersex have been reported, with specimen that develop both male and female reproductive ducts and impaired hormone levels (Arukwe 2001; Ebrahimi et al. 1995; Jobling et al. 1995).

Fig. 8–3 Photography of Rutilus rutilus (Cyprinid)
Wikipedia, GNU Free Documentation licence

Pimephales promelas (Cyprinid) is a fast growing, easy to handle species (Fig. 8–4). Sex can be determined via body coloring, shape of the head and size of adipose tissue near the back fin. Being a standard test fish of the US Environmental Protection Agency (EPA), the development and growth of *Pimephales promelas* under chemical exposure is well described and can serve as reference. *Pimephales promelas* spawns continuously. Animals are rather small (<7.5 cm), therefore the size of liver, gonads and brain samples is limited.

Fig. 8–4 Photography of Pimephales promelas (Cyprinid)
Wikipedia, GNU Free Documentation licence

A number of endpoints related specifically to endocrine function of the test animals was evaluated in Berlin and Brunel, including alterations in secondary sexual characteristics, gonadal condition, and concentrations of sex steroids. In the present study, we investigated the effects of chemical short-time exposure on P450arom and 5α-Re activity in gonadal and brain tissue of adult aquatic vertebrates. In addition to the enzymes in focus, 5β-Re and 17β-HSD activities were measured if applicable.

8.3 Methods

8.3.1 Source of tissues

The exposure experiments with *Xenopus laevis* and *Rutilus rutilus* were designed and conducted by Prof. Dr. W. Kloas, Dr. I. Lutz and Christoph van Ballegooy (IGB-Berlin). In brief, *Xenopus laevis* male and female animals were kept in basins, and husbandry water of each basin was supplemented with 0.1 µM letrozole (Letro), 0.1 µM methyltestosterone (MT), 0.01 µM TPT, and 0.1 µM vinclozolin (Vin), respectively. Each exposure was performed in duplicate. After a 14 day exposure, animals were sacrificed, and gonads and brains were removed. The organs were snap-frozen in N_2 and stored at –20°C until delivery to Bonn.

For the investigation of androgen metabolism in different life stages of *Xenopus laevis*, brain and gonad samples of adult, juvenile and larval animals were collected from IGB-Berlin.

The exposure study with *Pimephales promelas* was designed and performed by Dr. S. Jobling and David Hala (UBrun). The pair-breeding assay was a 42-day study with the reproductive performance of paired fish being monitored and recorded over a three week pre-exposure period followed by a three week exposure period to various concentrations of MT, p,p´DDE and TPT. After exposure, animals were sacrificed, and gonads and brains were removed. The organs were snap-frozen in N_2 and stored at –20°C until delivered to the university hospital in Bonn.

8.3.2 Tissue preparation

Animal tissue homogenates were prepared according to modified methods described previously (Steckelbroeck et al. 1999a). In brief, each tissue sample deriving from the exposure experiment was homogenized in homogenization buffer (HB) and sonificated. For the investigation of androgen metabolism in different life stages of *Xenopus laevis*, tissue samples were pooled. Homogenates were spinned down and supernatants were stored as cell-free fractions at –80°C. Samples of the tissue preparation were collected for the protein determination according to Lowry (1951). Measurements were conducted in the linear range of protein content *versus* enzyme activity.

Table 8–1 Tissue samples of the laboratory animals Xenopus laevis, Rutilus rutilus, and Pimephales promelas with scheduled enzyme activity tests. Pimephales promelas male brain samples deriving from TPT exposure experiment were too small for androgen metabolism analysis. Testicular samples from p,p´DDE experiment were also very small, but it was possible to pool them for the analysis.
0=not tested, X=tested

Species	Sex	Organ	P450arom	17β-HSD	5α-Re	5β-Re
Xenopus laevis	female	ovary	X	0	0	0
		brain	X	X	X	X
	male	testis	0	X	X	X
		brain	0	X	X	X
Rutilus rutilus	female	ovary	X	0	0	0
		brain	X	0	0	0
	male	testis	0	X	X	X
		brain	X	X	X	X
Pimephales promelas	female	ovary	X	0	0	0
		brain	X	0	0	0
	male	testis	0	X	X	X
		brain	X	X	X	X

8.3.3 Enzyme activity in different tissues of the model animals

The tritium-labeled water release assay was used to measure P450arom activity in the tissue samples mentioned above (Table 8–1), according to methods described previously (chapter 3). 17β-HSD and 5α-Re activity were measured in the tissue samples (Table 8–1) according to the product isolation assay established by Steckelbroeck (2001; 1999b). The effects of tissue concentration, incubation temperature and pH on the different enzyme activities were analyzed, and quantification of enzyme activities of exposed animals was performed under these optimized conditions (data not shown).

8.3.4 Validation of 5β-reductase activity assay in brain of *Xenopus laevis*

5β-Re activity was discovered in female and male brain tissue of *Xenopus laevis*. We conducted a number of methodological experiments to establish the optimal incubation conditions of the new assay, using preparations of female brain tissue (50 mg fresh tissue per mL HB, age of approximately 5 years) in a procedure similar to that described for 5α-Re activity. This included the analysis of the effects of substrate concentration (0.05–5 µM, 24°C, pH 7.4), incubation temperature (22–37°C, 0.1 µM, pH 7.4), and pH (5.5–7.5, 0.1 µM, 34°C) *versus* enzyme activity. Products were analyzed by one-dimensional digital autoradiography, using an automated TLC-linear analyzer.

8.3.5 Data analysis

Analysis of enzyme kinetics was carried out using a computer assisted non-linear curve-fitting method (FigSys, Biosoft, United Kingdom). GraphPad Prism (GraphPad Software, San Diego, USA) was used to visualize and evaluate the enzyme activity results of exposed aquatic animals. GraphPad Prism was also used for the statistical analysis. Kolmogorov-Smirnov´s one-sample test was used to determine if data sets were normally distributed. Variances were tested for homogeneity. Differences between groups were verified by either one-way analyses of variance (ANOVA), followed by a post-hoc Tukey´s multiple comparison test in case of normal distribution and homogeneous variances, or the Kruskal-Wallis test connected with a post-hoc Dunn´s multiple comparison test in case of lack of normal distribution and/or inhomogeneous variances.

8.4 Results

8.4.1 Enzyme activities in brain and gonad samples of *Xenopus laevis*

Tissue samples and specific enzyme activities determined from control animals (n=6) are listed in Table 8–2.

Table 8–2 Mean specific P450arom, 5α-Re, 5β-Re, and 17β-HSD activities in brain, testis, and ovary samples of Xenopus laevis control animals (n=6). u.d.l.=under detection limit

Xenopus laevis	Male		Female	
Enzyme activity in pmol/h/mg protein	Brain	Testis	Brain	Ovary
P450arom	u.d.l.	u.d.l.	0.5 ± 0.09	0.46 ± 0.16
5α-Re	0.89 ± 0.4	57.3 ± 5.08	2.38 ± 0.42	not tested
5β-Re	6.32 ± 0.7	u.d.l.	23.5 ± 2.96	not tested
17β-HSD	9.34 ± 2.1	813 ± 57.3	16.5 ± 2.68	not tested

The analysis of the exposure experiment resulted in no significantly different mean P450arom activities, except for a significantly higher mean value ($p<0.001$) in ovaries of animals exposed to Letro compared to the mean value deriving from MT exposed animals (Fig. 8–5). We found significantly higher 5α-Re activities compared to control animals in female brains of MT exposed ($p<0.001$) and Vin exposed ($p<0.05$) animals (Fig. 8–6). 5α-Re activity was very low in male brain samples and variability of the control animals was very high. However, there was a tendency to lower 5α-Re activities in Letro and MT exposed animals. Analysis of male gonads resulted in approximately the same 5α-Re activities of individuals from all exposure groups. The mean 17β-HSD activities in brain and testis tissue of exposed animals were not significantly different from the control value, but results from Letro exposed animals were significantly lower compared to MT exposed animals ($p<0.05$, Fig. 8–7). In addition, a tendency for elevated 17β-HSD activities in male brain of TPT and Vin exposed animals, and slightly decreased activities in testis of Letro and MT exposed animals were detected. 5β-reductase (5β-Re) activity was 4 times higher in female brain (specific activity: 23.49±2.96 pmol/h/mg protein, n=6, Fig. 8–8) than in male brain (specific activity: 6.32±0.7 pmol/h/mg protein, n=6). We found no significant differences of 5β-Re activity between the exposure groups (Fig 8–8).

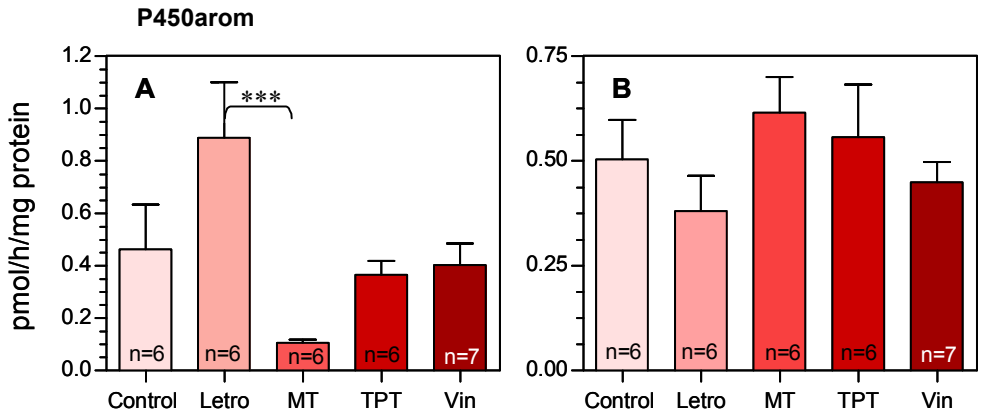


Fig. 8–5 Mean specific P450arom activity with SEM in (A) ovarial and (B) brain tissues samples (n=6–7) of female Xenopus laevis exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. P450arom activities detected in tissues of exposed animals were not significantly different from the control animals, except for ovarial P450arom activity of the Letro group compared to the MT group. Statistical analysis was performed as follows: (A) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test and (B) ANOVA with post-hoc Tukey´s multiple comparison test.
**** = p<0.01, ** = p<0.05, * = p<0.1*

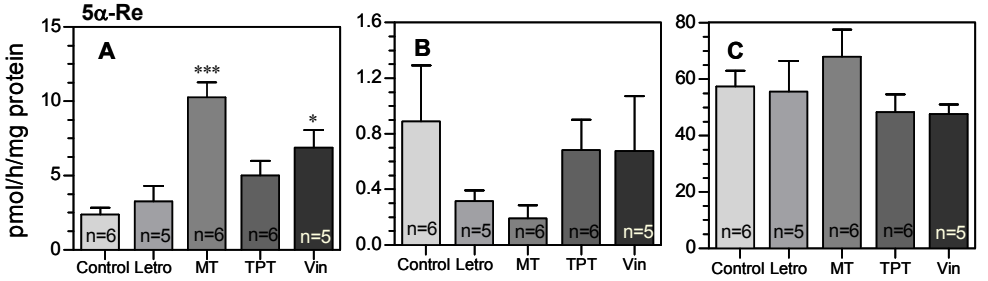


Fig. 8–6 Mean specific 5α-Re activity with SEM in (A) female brain, (B) male brain, and (C) testicular samples (n=5–7) of Xenopus laevis exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. 5α-Re activity detected in female brain samples of MT and Vin exposed animals was significantly higher in the control group, while there were no significant differences in the enzyme activities detected in male tissue samples.
Statistical analysis was performed as follows: (A) ANOVA with post-hoc Tukey´s multiple comparison test, (B) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test and (C) ANOVA with post-hoc Tukey´s multiple comparison test. **** = p<0.01, ** = p<0.05, * = p<0.1*

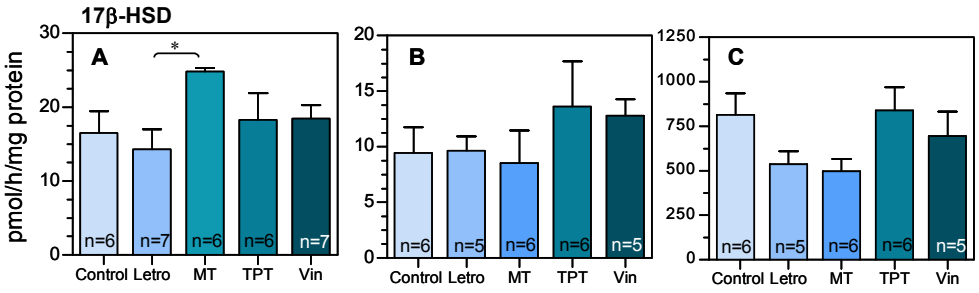


Fig. 8–7 Mean specific 17β-HSD activity with SEM in (A) female brain, (B) male brain, and (C) male gonad samples (n=5–7) of Xenopus laevis exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. 17β-HSD activity detected in exposure groups was not significantly different from the control group, except for 17β-HSD activity in female brain of the Letro group compared to the MT group.
Statistical analysis was performed as follows: (A) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test and (B,C) ANOVA with post-hoc Tukey´s multiple comparison test
**** = p<0.01, ** = p<0.05, * = p<0.1*

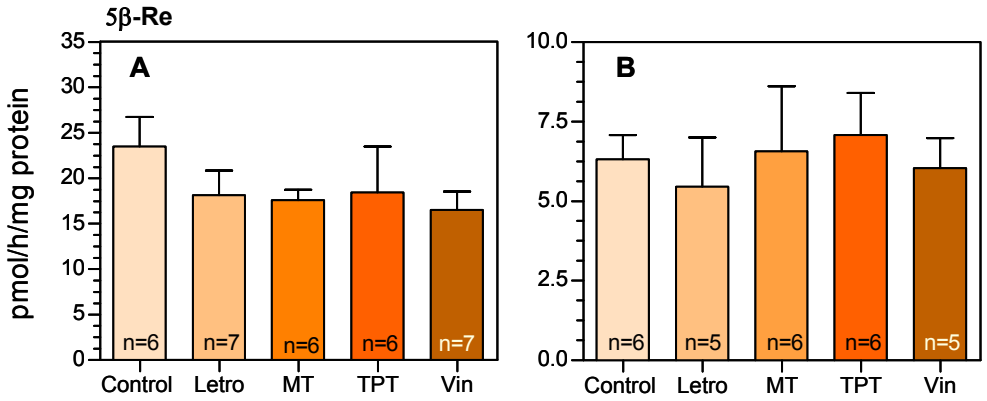


Fig. 8–8 Mean specific 5β-Re activity with SEM in (A) female brain and (B) male brain tissues samples (n=5–7) of Xenopus laevis exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. 5β-Re activity detected in exposure groups was not significantly different from the control group.
Statistical analysis was performed with the ANOVA following the post-hoc Tukey´s multiple comparison test.
**** = p<0.01, ** = p<0.05, * = p<0.1*

8.4.2 Assay validation of 5β-reductase activity in *Xenopus laevis*

Androgen metabolizing enzymes in the brain were identified after incubation of a tissue preparation of one of the female control animals. The one-dimensional TLC β-Scan shows the different peaks of the radioactive products, and the stained TLC glass plate (Fig. 8–9) shows the corresponding reference steroids as stained dots.

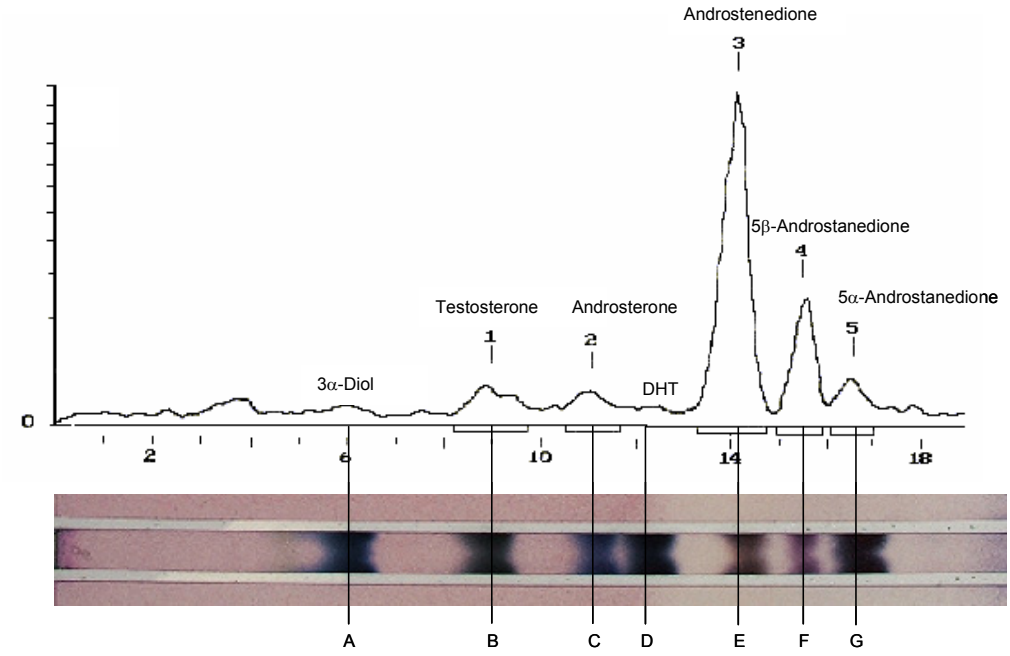


Fig. 8–9 β-Scan of the TLC-separated androstenedione metabolites in female brain of Xenopus laevis after enzyme in vitro assay. The TLC was stained for the identification of the different unlabelled steroids that were added to the incubation extracts. After staining, the coloured areas of the reference steroids together with the corresponding RF-values were used to identify the β-signals of the products, which were testosterone (peak 1, stain B), androsterone (peak 2, stain C), 5β-androstandione (peak 4, stain F) and 5α-androstandione (peak 5, stain G). 3α-Diol (stain A) and DHT (stain D) could not be found after the incubation.

The different radioactive signals in the autoradiogram were 1=testosterone, 2=androsterone, 3=androstenedione, 4=5β-androstanedione, and 5=5α-androstanedione, proving the presence of 17β-HSD, 5α-Re and 5β-Re in *Xenopus laevis* brain. The calculated intra-assay coefficient of variation was approximately 5.3% (n=32). Variations in any of the three variables substrate concentration (Fig. 8–10A), incubation temperature (Fig. 8–10B), and pH (Fig. 8–10C) resulted in minor changes in 5β-Re activity.

According to the results shown in Fig. 8–10, 5β-Re activity measurements should be carried out at pH 6.5, a substrate concentration of 0.5 µM androstenedione, and 34°C incubation temperature.

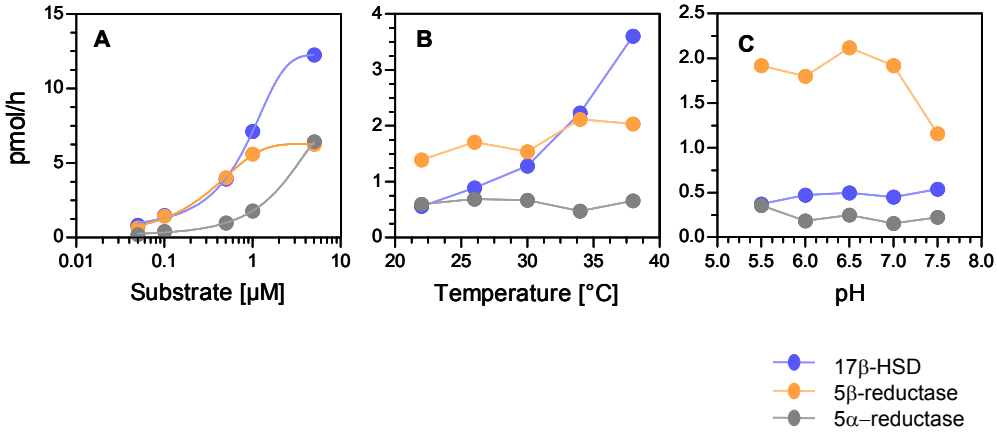


Fig. 8–10 Effects of (A) substrate concentration, (B) incubation temperature, and (C) pH-value on 5β-Re activity in brain tissue of Xenopus laevis. Approximately 50 mg fresh tissue per mL HB was used for the enzyme assays.

8.4.3 Enzyme activities in brain and gonad samples of *Rutilus rutilus*

Tissue samples and specific enzyme activities determined from control animals are listed in Table 8–3. In a preliminary test, P450arom activity in fish brain rose with higher incubation temperature. Ovarial P450arom activity was very low and did not change within the temperature range tested (22°C, 27°C, 32°C, and 37°C, Fig. 8–11). Testicular homogenates showed no P450arom activity (data not shown).

Table 8–3 Mean specific P450arom, 5α-Re, and 17β-HSD activities in brain, testis, and ovary samples of Rutilus rutilus control animals (female n=9, male n=21).
n.t.=not tested, u.d.l.=under detection limit

Rutilus rutilus	Male		Female	
pmol/h/mg protein	Brain	Testis	Brain	Ovary
P450arom	4.28 ± 0.3	n.t.	3.55 ± 0.4	u.d.l.
5α-Re	88.7 ± 3.8	73.4 ± 5.6	n.t.	n.t.
17β-HSD	57.1 ± 5.3	24.8 ± 2.3	n.t.	n.t.

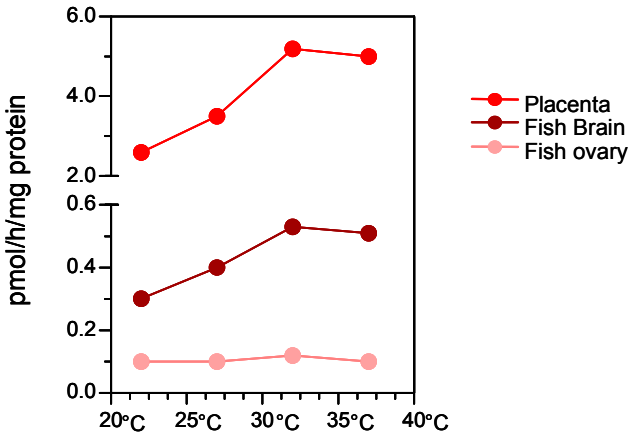


Fig. 8–11 Effect of temperature on P450arom activity in human placenta, female Rutilus rutilus brain and ovary. Enzyme assays were performed with a substrate concentration of 0.05 µM androstenedione, 3 mM NADPH as cofactor and an incubation time of 30 min.

Ovarial P450arom activity of *Rutilus rutilus* exposed to different test chemicals was under the detection limit of our methods (data not shown).

Mean P450arom activities in brain tissue samples of exposed female animals did not show any significant differences (Fig. 8–12A). Letro and MT exposed male animals showed significantly decreased ($p<0.001$) and increased ($p<0.01$) mean activities, respectively (Fig. 8–12B). The only significant difference in 5α-Re activity between groups was the higher mean value in testis of TPT exposed animals compared to Letro exposed animals (Fig. 8–13). 17β-HSD activities detected in testicular samples were not significantly different from one another, but enzyme activity in male brain samples of MT ($p<0.001$) and Vin ($p<0.01$) exposed animals were both significantly higher compared to control animals (Fig 8–14).

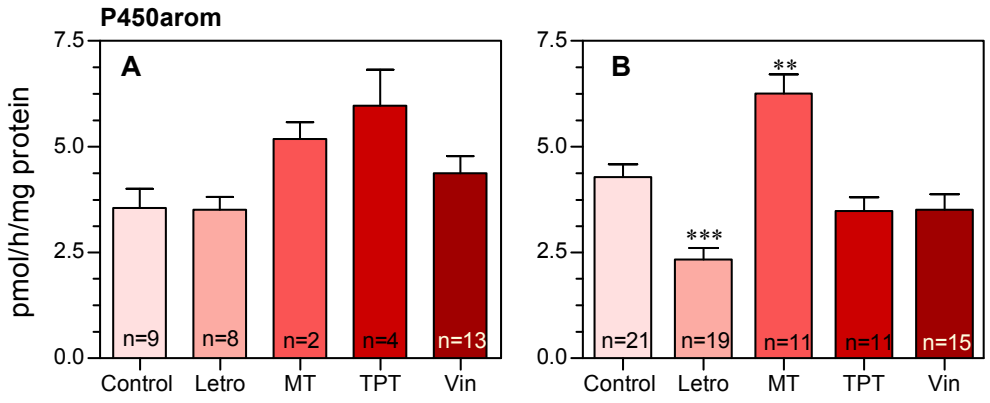


Fig. 8–12 Mean specific P450arom activity with SEM in (A) female and (B) male brain tissue samples (n=2–15) of Rutilus rutilus exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. P450arom activity detected in exposure groups was not significantly different in female tissues. Letro and MT exposed male animals had significantly lower (p<0.001) and higher (p<0.01) mean P450arom activities compared to control animals, respectively.
Statistical analysis was performed as follows: (A) Kruskal-Wallis with post-hoc Dunn´s multiple comparison test and (B) ANOVA with post-hoc Tukey´s multiple comparison test.
**** = p<0.01, ** = p<0.05, * = p<0.1*

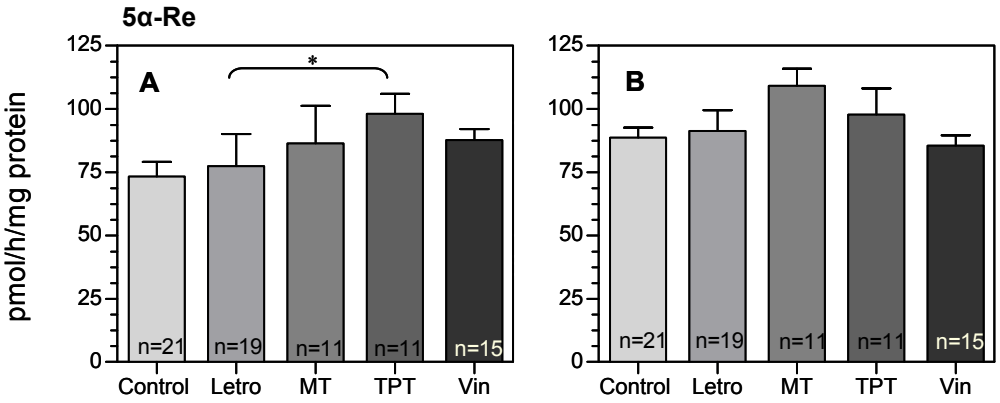


Fig. 8–13 Mean specific 5α-Re activity with SEM in male (A) gonad and (B) brain tissue samples (n=11–21) of Rutilus rutilus exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. 5α-Re activity detected in exposure groups was not significantly different from the control group, but values were significantly higher in TPT exposed animals than in Letro exposed animals.
Statistical analysis was performed with the Kruskal-Wallis test, following the Dunn´s multiple comparison test. **** = p<0.01, ** = p<0.05, * = p<0.1*

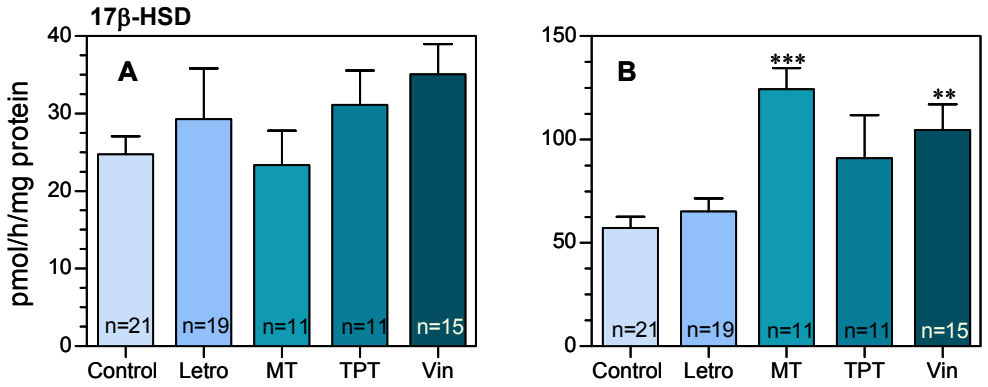


Fig. 8–14 Mean 17β-HSD activities with SEM in male (A) gonad and (B) brain tissue samples (n=11–21) of Rutilus rutilus exposed for 2 weeks to 0.1 µM Letro, MT, Vin, and 0.01 µM TPT. Mean 17β-HSD activities detected in testicular samples were not significantly different. 17β-HSD activity detected in male brain samples of MT ($p<0.001$) and Vin ($p<0.01$) exposed animals was significantly higher compared to control animals.
Statistical analysis was performed with the Kruskal-Wallis test, following the Dunn´s multiple comparison test. *** = $p<0.01$, ** = $p<0.05$, * = $p<0.1$*

8.4.4 Enzyme activities in brain and gonad samples of *Pimephales promelas*

Due to methodological problems during the exposure phase at the University of Brunel, *e.g.*, high mortality, white spot disease, and small tissue sizes, the results of the exposure experiments with *Pimephales promelas* are of limited relevance. Female animals were analyzed for P450arom activity in brain (n=8) and ovary (n=9), with mean activities of 9.19±0.8 and 0.16±0.02 pmol/h/mg protein, respectively. We found significantly elevated mean P450arom activities in ovaries from 320 ng TPT/L and 30 ng MT/L exposed animals (Fig. 8–15). The other analysis revealed no differences between the groups (data not shown). Mean specific activities in male brain tissue samples of control animals (n=10) were 7.29±2.31 pmol/h/mg protein (P450arom), 412±30 pmol/h/mg protein (17β-HSD), and 80.2±9.76 pmol/h/mg protein (5α-Re). Testicular mean specific enzyme activities was 42.4 pmol/h/mg protein (17β-HSD) and 15.0 pmol/h/mg protein (5α-Re), determined from pooled tissue samples.

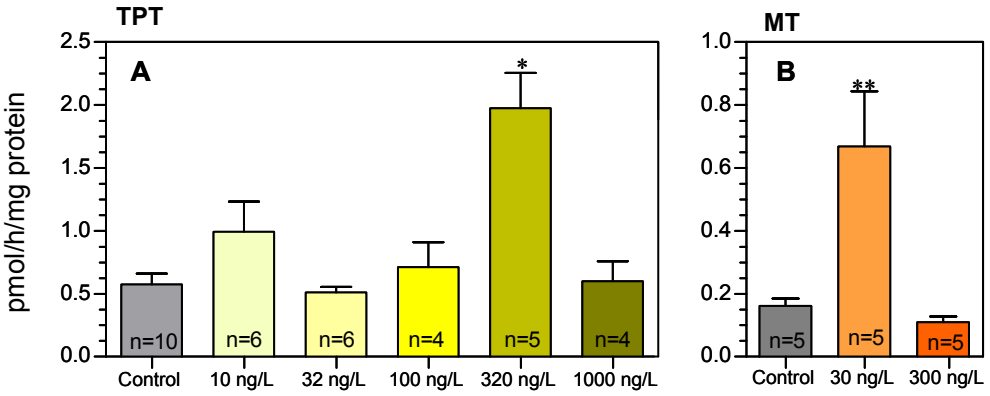
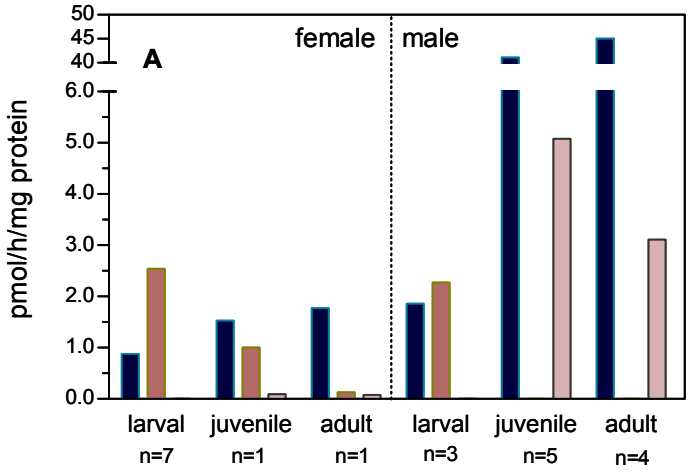


Fig. 8–15 Mean specific P450arom activity with SEM in ovary tissue samples (n=4–10) of Pimephales promelas exposed for three weeks to various concentrations of (A) TPT and (B) MT. The animals exposed to 320 ng/L TPT and 30 ng/L MT showed significantly higher P450arom activities than control animals.
Statistical analysis was performed with the Kruskal-Wallis test, following the Dunn´s multiple comparison test. **** = p<0.01, ** = p<0.05, * = p<0.1*

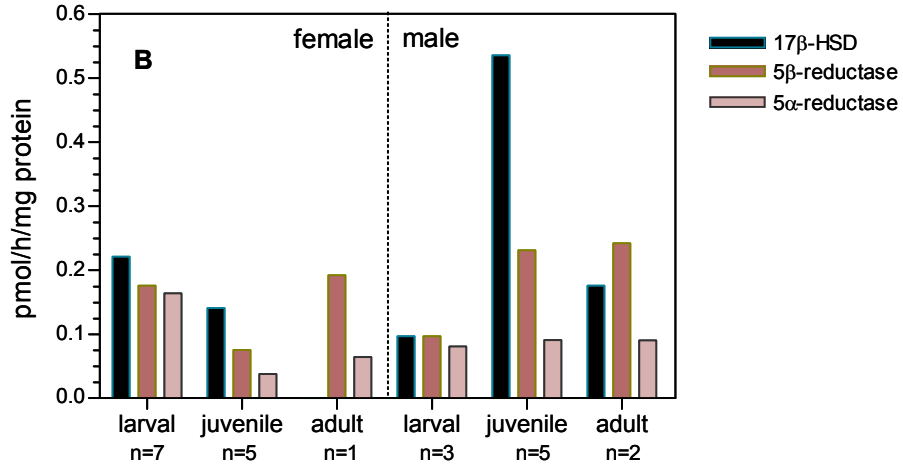
8.4.5 Identification of androgen metabolism at different life stages of *Xenopus laevis*

The enzyme activities detected in the different tissue homogenates are visualized in Fig. 8–16. In all female gonad samples, androgen metabolizing enzyme activity was higher compared to brain samples. In ovaries, 5β-Re continuously decreased with age, while it remained more or less stable at different life stages in brain tissue. In brain tissue samples, 5α-Re activity decreased, and 17β-HSD activity even completely disappeared with age.

In male testis, androgen metabolizing enzyme activity was much higher compared to brain tissue (Fig. 8–16). In testis, 17β-HSD and 5α-Re activities highly increased with age, while 5β-Re activity disappeared.



Gonad	Females			Males		
pmol/h/mg protein	larval	juvenile	adult	larval	juvenile	adult
17β-HSD	0.88	1.53	1.77	1.86	41.13	45.06
5β-reductase	2.54	1.00	0.13	2.27	0.00	0.00
5α-reductase	0.00	0.09	0.08	0.00	5.08	3.11



Brain	Females			Males		
pmol/h/mg protein	larval	juvenile	adult	larval	juvenile	adult
17β-HSD	0.22	0.14	0.00	0.10	0.54	0.18
5β-reductase	0.18	0.08	0.19	0.10	0.23	0.24
5α-reductase	0.16	0.04	0.06	0.08	0.09	0.09

Fig. 8–16 Mean specific enzyme activities in gonadal (A) and brain (B) tissue samples of Xenopus laevis at different life stages (adult=5–6 years, juvenile=3 years+10 months, larval=1 year+4 months).

8.5 Discussion

Some of the problems found in fish, including decreased fecundity, genital abnormalities, altered behavior patterns, and response to stress and disease, are now observed in human populations (Colborn 2004). Thus, it is possible that aquatic vertebrates give an early warning of endocrine effects that later become apparent in other wildlife species, and ultimately in humans. In comparison to terrestrial vertebrates, they are more exposed to ED because of the high interchange with the aquatic environment through body surface and/or the gills. Several fish species respond to hormone active chemicals with elevated plasma vitellogenin levels or even the generation of intersex gonads (Kirby et al. 2004; Kleinkauf et al. 2004; Cho et al. 2003; De Metrio et al. 2003). Amphibians likewise react very sensitive to endocrine disruption, and this may contribute to their global decline (Carey and Bryant 1995). Severe ED treatment during the critical time of sex differentiation can result in sex reversal (Kloas 2002; Kloas et al. 1999). The selected model organisms *Xenopus laevis*, *Rutilus rutilus,* and *Pimephales promelas* have already proved as very suitable model organisms.

It has been proposed that P450arom is a critical enzyme for ovarian differentiation in fish and amphibians (Baroiller and D'Cotta 2001; Melo and Ramsdell 2001). As expected, higher P450arom activities were detected in female gonad samples compared to male ones. For unknown reasons, no P450arom activity could be detected in ovarian tissue samples deriving from exposure experiments of *Rutilus rutilus*. High P450arom activity was present in teleost brain of both sexes, its function remaining speculative (Forlano et al. 2001). P450arom activity rose linear with temperature (24–37°C) in all tissues investigated, regardless of the much lower husbandry temperatures of the animals (Gonzalez and Piferrer 2002). As fish and amphibian are poikilothermic, the elevated P450arom activities due to higher ambient temperatures might contribute to sex determination during the critical, thermosensitive periods of embryonic development. In *Xenopus laevis*, it has been reported that low temperatures (16°C) yield more males, and high temperatures (26°C) more females in laboratory animals (Kobel 1996). The specific P450arom activities in ovarian and brain tissue samples from exposure experiments presented here were obtained with incubation temperatures at 37°C, because tissue size was very small and we needed highest attainable values.

In mammalians, 5β-Re is involved in cholesterol and bile acid metabolism, in addition to the clearance of cortisol in the liver (Westerbacka et al. 2003; Charbonneau and

The 2001). 5α- and 5β-Re work in concert with 3α-HSDs to convert 5α/5β-dihydrosteroids into 5α/5β-tetrahydrosteroids (Penning et al. 2000; 1986). Brain 5β-Re, which converts testosterone into 5β-DHT, is unique to birds (Hutchison and Steimer 1981). With highest levels in nonbreeding individuals and seasons, 5β-Re seems to function as an inactivation shunt for androgens (Balthazart 1989; Massa et al. 1979). To the best of the author´s knowledge, 5β-Re activity in amphibian brain has not been reported to date. In the present study, 5β-Re was present in brain of both sexes of *Xenopus laevis* at all three life stages investigated. In addition, it was found in all female gonads, but solely in juvenile testis, supporting the assumption that 5β-Re is responsible for androgen inactivation.

It could be demonstrated in chapter 3-5 that in particular the organotin compounds TBT and TPT inhibit phase I enzyme (P450arom and 5α-Re) *in vitro* activities. In contrast to these findings, we did not detect any significant differences of enzyme activities in tissues samples of TPT exposed *Xenopus laevis* or *Rutilus rutilus*, with the exception of elevated P450arom activities in female gonads of *Pimephales promelas* (320 ng/L TPT). It is possible that the organotin compounds are metabolized into less hazardous metabolites like MBT, an organotin compound which proved to be ineffective in the *in vitro* enzyme tests.

The anti-androgenic potency of p,p´DDE and vinclozolin has been demonstrated with androgen receptor reporter gene assays, rat *in vitro* experiments and in the adult male guppy (Xu et al. 2006; Makita et al. 2003; Baatrup and Junge 2001; Sunami et al. 2000; Kelce et al. 1995). In the exposure experiments, p,p´DDE did not alter the selected enzyme activities in *Pimephales promelas*. Vin exposure resulted in some changes of 5α-Re and 17β-HSD in *Xenopus laevis* and *Rutilus rutilus* (Table 8–4). Obviously, there is no uniform pattern concerning the effects on enzyme activities.

However, *Rutilus rutilus* and *Xenopus laevis* exposed to the control substances MT and Letro resulted in, though not significant, but at least slightly different mean enzyme activities compared to the control group. According to the findings that some of the test compounds with inhibitory potency caused an increased enzyme gene expression in human cell lines (Nakanishi et al. 2002), probably as a compensatory process in the negative feedback mechanism of the endocrine control loop, the strong P450arom inhibitor Letro should have caused an upregulation of P450arom enzyme activity in the ovary and the brain. This is only the case in ovarial tissue samples of *Xenopus laevis*. On the contrary,

Letro exposure caused elevated P450arom activities in brain tissue of male *Rutilus rutilus*. Another finding is that brain and gonadal enzyme activity changes are equally frequent. P450arom activity as an endpoint seems to be a little more sensitive to chemical exposure than 17β-HSD, 5α-Re, and 5β-Re.

Table 8–4 Changes in enzyme activities in different tissue samples of exposed model animals. (+/–)=tendency of elevated/decreased mean enzyme activity, +/++/+++=significantly (p<0.05/p<0.01/p<0.001) elevated mean enzyme activity, –/– –/– – – =significantly (p<0.05/ p<0.01/ p<0.001) decreased mean enzyme activity, 0=no significant change in mean enzyme activity

Xenopus laevis		**Letro**	**MT**	**TPT**	**Vin**
P450arom	Ovary	(+)	(–)	0	0
	Female brain	0	0	0	0
5α-Re	Testis	0	0	0	0
	Female brain	0	+++	0	+
	Male brain	(–)	(–)	0	0
17β-HSD	Testis	0	0	0	0
	Female brain	0	(+)	0	0
	Male brain	0	0	0	0
5α-Re	Female brain	0	0	0	0
	Male brain	0	0	0	0
Rutilus rutilus		**Letro**	**MT**	**TPT**	**Vin**
P450arom	Female brain	0	(+)	(+)	0
	Male brain	– – –	++	0	0
5α-Re	Testis	0	0	(+)	0
	Male brain	0	0	0	0
17β-HSD	Testis	0	0	0	(+)
	Male brain	0	+++	0	++
Pimephales promelas			**MT**	**TPT**	**p,p´DDE**
P450arom	Ovary		++ (30 ng/L)	+ (320 ng/L)	0
	Testis		0	0	0
	Female brain		0	0	0
5α-Re	Male brain		0	0	0
	Testis		0	0	0
17β-HSD	Male brain		0	0	0
	Testis		0	0	0

In conclusion, the present study demonstrated sex and age dependent differences in androgen metabolism in *Xenopus laevis*. For the assessment of putative endocrine disrupting chemicals, the selected enzyme activities (P450arom, 5α-Re, 17β-Re, and 5β-Re) proved less suitable as distinctive endpoints because of high variability between individuals, inconsistent effects of the control substances MT and Letro, and low sensitivity. The *in vivo* outcome is probable influenced by various factors like absorption, metabolism, excretion rates, bioaccumulation potential, and pharmacokinetics. In addition to these endpoint-specific reasons, the general setup was inapplicable due to high mortality (*Pimephales promelas*) and difficulty in sex determination (*Rutilus rutilus*), resulting in disproportionate numbers of female and male exposed animals. In addition, tissue size was sometimes insufficient for the realization of the enzyme assay.

Although the use and scope of the models to detect endocrine effects in this study are limited, it is clear that such models are the only source to provide relevant data for risk assessment. These promising models need further development before they can be considered as the sole basis for regulatory action. As it is very unlikely that a putative endocrine disruptor exclusively targets a specific metabolic function or hormone-dependent signaling step, several endpoints like organ weight and serum concentration of sex steroid hormones together with enzyme activity may provide a clearer picture of endocrine potency of the test compound. Nevertheless, enzyme determination after chemical exposure appears of potential value and should be subject to further development and validation.

9 References

Adeeko A, Li D, Forsyth DS, Casey V, Cooke GM, Barthelemy J, Cyr DG, Trasler JM, Robaire B and Hales BF (2003) Effects of in utero tributyltin chloride exposure in the rat on pregnancy outcome. Toxicol Sci 74: 407-415.

Akingbemi BT and Hardy MP (2001) Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. Ann Med 33: 391-403.

Allan BB, Brant R, Seidel JE and Jarrell JF (1997) Declining sex ratios in Canada. Cmaj 156: 37-41.

Allen NE, Reichardt JK, Nguyen H and Key TJ (2003) Association between two polymorphisms in the SRD5A2 gene and serum androgen concentrations in British men. Cancer Epidemiol Biomarkers Prev 12: 578-581.

Allera A, Lo S, King I, Steglich F and Klingmuller D (2004) Impact of androgenic/antiandrogenic compounds (AAC) on human sex steroid metabolizing key enzymes. Toxicology 205: 75-85.

Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM and Bonefeld-Jorgensen EC (2002) Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol Appl Pharmacol 179: 1-12.

Appel KE (2004) Organotin compounds: toxicokinetic aspects. Drug Metab Rev 36: 763-786.

Arukwe A (2001) Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. Mar Pollut Bull 42: 643-655.

Ashby J (2000) Getting the problem of endocrine disruption into focus: the need for a pause for thought. Apmis 108: 805-813.

Baatrup E and Junge M (2001) Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy Poecilia reticulata. Environ Health Perspect 109: 1063-1070.

Babiker FA, De Windt LJ, van Eickels M, Grohe C, Meyer R and Doevendans PA (2002) Estrogenic hormone action in the heart: regulatory network and function. Cardiovasc Res 53: 709-719.

Bahceci M, Ersay AR, Tuzcu A, Hiort O, Richter-Unruh A and Gokalp D (2005) A novel missense mutation of 5-alpha reductase type 2 gene (SRD5A2) leads to severe male pseudohermaphroditism in a Turkish family. Urology 66: 407-410.

Balthazart J (1989) Correlation between the sexually dimorphic aromatase of the preoptic area and sexual behavior in quail: effects of neonatal manipulations of the hormonal milieu. Arch Int Physiol Biochim 97: 465-481.

Baroiller JF and D'Cotta H (2001) Environment and sex determination in farmed fish. Comp Biochem Physiol C Toxicol Pharmacol 130: 399-409.

Batley GE, Scammell MS and Brockbank CI (1992) The impact of the banning of tributyltin-based antifouling paints on the Sydney rock oyster, Saccostrea commercialis. Sci Total Environ 122: 301-314.

Bergstrom R, Adami HO, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Akre O and Hakulinen T (1996) Increase in testicular cancer incidence in six European countries: a birth cohort phenomenon. J Natl Cancer Inst 88: 727-733.

Bettin C, Oehlmann J and Stroben E (1996) TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgol Mar Res 50: 299-317.

BgVV (1995) Planung toxikologischer Prüfungen zur Bewertung von Chemikalien im Rahmen des Chemikaliengesetzes. *In* D. Kayser and R. Roll [eds.].

Bhosle NB, Garg A, Jadhav S, Harjee R, Sawant SS, Venkat K and Anil AC (2004) Butyltins in water, biofilm, animals and sediments of the west coast of India. Chemosphere 57: 897-907.

Birge SJ (1997) The role of estrogen in the treatment of Alzheimer's disease. Neurology 48: S36-41.

Blaak E (2001) Gender differences in fat metabolism. Curr Opin Clin Nutr Metab Care 4: 499-502.

Borg B (1994) Androgens in teleost fishes. Comp Biochem Physiol 109C: 219-245.

Boyer IJ (1989) Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55: 253-298.

Bretveld RR, Thomas CC, Scheepers PP, Zielhuis GG and Roeleveld NN (2006) Pesticide exposure: the hormonal function of the female reproductive system disrupted? Reprod Biol Endocrinol 4: 30.

Buvat J, Dewailly D, Marcolin G, Buvat-Herbaut M, Racadot A and Fossati P (1983) Investigative strategy of hyperandrogenism in women. Horm Res 18: 106-116.

Byington KH (1971) Effects of triphenyltin compounds on the adenosine triphosphatase activity of beef heart submitochondrial particles. Biochem Biophys Res Commun 42: 16-22.

Byington KH, Yeh RY and Forte LR (1974) The hemolytic activity of some trialkyltin and triphenyltin compounds. Toxicol Appl Pharmacol 27: 230-240.

Callard G, Schlinger B, Pasmanik M and Corina K (1990) Aromatization and estrogen action in brain. Prog Clin Biol Res 342: 105-111.

Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS and Simpson ER (1997) Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337: 91-95.

Carey C and Bryant CJ (1995) Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ Health Perspect 103 Suppl 4: 13-17.

Chance GW and Harmsen E (1998) Children are different: environmental contaminants and children's health. Can J Public Health 89 Suppl 1: S9-13, S10-15.

Charbonneau A and The VL (2001) Genomic organization of a human 5beta-reductase and its pseudogene and substrate selectivity of the expressed enzyme. Biochim Biophys Acta 1517: 228-235.

Chlebowski RT (2005) Bone health in women with early-stage breast cancer. Clin Breast Cancer 5 Suppl: S35-40.

Cho SM, Kurihara R, Strussmann CA, Uozumi M, Yamakawa H and Yamasaki T (2003) Histological abnormalities in the gonads of konoshiro gizzard shad (Konosirus punctatus) from coastal areas of Japan. Environ Sci 10: 25-36.

Cima F, Ballarin L, Bressa G, Martinucci G and Burighel P (1996) Toxicity of organotin compounds on embryos of a marine invertebrate (Styela plicata; tunicata). Ecotoxicol Environ Saf 35: 174-182.

Cocco P, Loviselli A, Fadda D, Ibba A, Melis M, Oppo A, Serra S, Taberlet A, Tocco MG and Flore C (2004) Serum sex hormones in men occupationally exposed to dichloro-diphenyl-trichloro ethane (DDT) as young adults. J Endocrinol 182: 391-397.

Coelho MR, Bebianno MJ and Langston WJ (2002) Routes of TBT uptake in the clam Ruditapes decussatus. II. Food as vector of TBT uptake. Mar Environ Res 54: 193-207.

Colborn T (2004) Neurodevelopment and endocrine disruption. Environ Health Perspect 112: 944-949.

Colborn T, vom Saal FS and Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101: 378-384.

Cooke GM (2002) Effect of organotins on human aromatase activity in vitro. Toxicol Lett 126: 121-130.

Cooke GM, Tryphonas H, Pulido O, Caldwell D, Bondy GS and Forsyth D (2004) Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part 1: Toxicology, histopathology and clinical chemistry. Food Chem Toxicol 42: 211-220.

Corbin CJ, Graham-Lorence S, McPhaul M, Mason JI, Mendelson CR and Simpson ER (1988) Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc Natl Acad Sci U S A 85: 8948-8952.

Costa LG (1985) Inhibition of gamma-[3H]aminobutyric acid uptake by organotin compounds in vitro. Toxicol Appl Pharmacol 79: 471-479.

Damgaard IN, Skakkebaek NE, Toppari J, Virtanen HE, Shen H, Schramm KW, Petersen JH, Jensen TK and Main KM (2006) Persistent pesticides in human breast milk and cryptorchidism. Environ Health Perspect 114: 1133-1138.

Davidson NE and Yager JD (1997) Pesticides and breast cancer: fact or fad? J Natl Cancer Inst 89: 1743-1744.

De Metrio G, Corriero A, Desantis S, Zubani D, Cirillo F, Deflorio M, Bridges CR, Eicker J, de la Serna JM, Megalofonou P and Kime DE (2003) Evidence of a high percentage of intersex in the Mediterranean swordfish (Xiphias gladius L.). Mar Pollut Bull 46: 358-361.

Degen GH, Foth H, Kahl R, Kappus H, Neumann HG, Oesch F and Schulte-Hermann R (1999) VCI position paper on "endocrine active substances", p. 30-36. DGPT-Forum.

Dejager S, Bry-Gauillard H, Bruckert E, Eymard B, Salachas F, LeGuern E, Tardieu S, Chadarevian R, Giral P and Turpin G (2002) A comprehensive endocrine description of Kennedy's disease revealing androgen insensitivity linked to CAG repeat length. J Clin Endocrinol Metab 87: 3893-3901.

Deslypere JP, Verdonck L and Vermeulen A (1985) Fat tissue: a steroid reservoir and site of steroid metabolism. J Clin Endocrinol Metab 61: 564-570.

Diamanti-Kandarakis E, Mitrakou A, Raptis S, Tolis G and Duleba AJ (1998) The effect of a pure antiandrogen receptor blocker, flutamide, on the lipid profile in the polycystic ovary syndrome. J Clin Endocrinol Metab 83: 2699-2705.

Doering DD, Steckelbroeck S, Doering T and Klingmuller D (2002) Effects of butyltins on human 5alpha-reductase type 1 and type 2 activity. Steroids 67: 859-867.

Doldi N, Bassan M, Belvisi L, Messa A and Ferrari A (1996) Expression of 17 alpha-hydroxylase and aromatase in the syndrome of androgen resistance: a case report. Gynecol Endocrinol 10: 281-284.

Duax WL, Griffin JF, Weeks CM and Korach KS (1985) Molecular conformation, receptor binding, and hormone action of natural and synthetic estrogens and antiestrogens. Environ Health Perspect 61: 111-121.

Duncan J (1980) The toxicology of molluscicides. The organotins. Pharmacol Ther 10: 407-429.

Ebrahimi M, Singh PB and Kime DE (1995) Biosynthesis of 17,20 alpha-dihydroxy-4-pregnen-3-one, 17,20 beta-dihydroxy-4-pregnen-3-one, and 11-ketotestosterone by testicular fragments and sperm of the roach, Rutilus rutilus. Gen Comp Endocrinol 100: 375-384.

Ema M, Kurosaka R, Amano H and Ogawa Y (1995) Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats. J Appl Toxicol 15: 297-302.

Ema M, Miyawaki E, Harazono A and Ogawa Y (1997) Effects of triphenyltin chloride on implantation and pregnancy in rats. Reprod Toxicol 11: 201-206.

EPA http://www.epa.gov/ord/htm/researchstrategies.htm.

Fait A, Ferioli A and Barbieri F (1994) Organotin compounds. Toxicology 91: 77-82.

Fent K (1996) Ecotoxicology of organotin compounds. Crit Rev Toxicol 26: 1-117.

Fent K, Lovas R and Hunn J (1991) Bioaccumulation, elimination and metabolism of triphenyltin chloride by early life stages of minnows Phoxinus phoxinus. Naturwissenschaften 78: 125-127.

Fisher JS (2004) Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction 127: 305-315.

Forlano PM, Deitcher DL, Myers DA and Bass AH (2001) Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J Neurosci 21: 8943-8955.

Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW and Andersson S (1994) Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3. Nat Genet 7: 34-39.

Gillesby BE and Zacharewski TR (1998) Exoestrogens: Mechanisms of action and strategies for identification and assessment. Environmental Toxicology and Chemistry 17: 3-14.

Giusti RM, Iwamoto K and Hatch EE (1995) Diethylstilbestrol revisited: a review of the long-term health effects. Ann Intern Med 122: 778-788.

Goldman LR and Koduru S (2000) Chemicals in the environment and developmental toxicity to children: a public health and policy perspective. Environ Health Perspect 108 Suppl 3: 443-448.

Gonzalez A and Piferrer F (2002) Characterization of aromatase activity in the sea bass: effects of temperature and different catalytic properties of brain and ovarian homogenates and microsomes. J Exp Zool 293: 500-510.

Goss PE (2003) Emerging role of aromatase inhibitors in the adjuvant setting. Am J Clin Oncol 26: S27-33.

Gray BH, Porvaznik M, Flemming C and Lee LH (1987) tri-n-Butyltin: a membrane toxicant. Toxicology 47: 35-54.

Gray LE, Jr., Ostby J, Monosson E and Kelce WR (1999a) Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. Toxicol Ind Health 15: 48-64.

Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL and Ostby J (1999b) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 15: 94-118.

Green VL, Speirs V, Landolt AM, Foy PM and Atkin SL (1999) 17Beta-hydroxysteroid dehydrogenase type 1, 2, 3, and 4 expression and enzyme activity in human anterior pituitary adenomas. J Clin Endocrinol Metab 84: 1340-1345.

Grote K, Stahlschmidt B, Talsness CE, Gericke C, Appel KE and Chahoud I (2004) Effects of organotin compounds on pubertal male rats. Toxicology 202: 145-158.

Guillette LJ, Jr. and Guillette EA (1996) Environmental contaminants and reproductive abnormalities in wildlife: implications for public health? Toxicol Ind Health 12: 537-550.

Haas GP and Sakr WA (1997) Epidemiology of prostate cancer. CA Cancer J Clin 47: 273-287.

Hackel C, Oliveira LE, Ferraz LF, Tonini MM, Silva DN, Toralles MB, Stuchi-Perez EG and Guerra-Junior G (2005) New mutations, hotspots, and founder effects in Brazilian patients with steroid 5alpha-reductase deficiency type 2. J Mol Med 83: 569-576.

Hagmar L, Bjork J, Sjodin A, Bergman A and Erfurth EM (2001) Plasma levels of persistent organohalogens and hormone levels in adult male humans. Arch Environ Health 56: 138-143.

Harazono A, Ema M and Ogawa Y (1996) Pre-implantation embryonic loss induced by tributyltin chloride in rats. Toxicol Lett 89: 185-190.

Harino H, Fukushima M and Kawai S (2000) Accumulation of butyltin and phenyltin compounds in various fish species. Arch Environ Contam Toxicol 39: 13-19.

Harris G, Azzolina B, Baginsky W, Cimis G, Rasmusson GH, Tolman RL, Raetz CR and Ellsworth K (1992) Identification and selective inhibition of an isozyme of steroid 5 alpha-reductase in human scalp. Proc Natl Acad Sci U S A 89: 10787-10791.

Hasan M, Haider SS and Bajpai VK (1984) The biochemical and ultrastructural studies on the effects of trimethyltin on regional brain lipid profiles and lipid peroxidation. Ind Health 22: 107-116.

Heidrich DD, Steckelbroeck S and Klingmuller D (2001) Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids 66: 763-769.

Heinlein CA and Chang C (2002) The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 16: 2181-2187.

Henderson BE and Feigelson HS (2000) Hormonal carcinogenesis. Carcinogenesis 21: 427-433.

Herbst AL, Ulfelder H and Poskanzer DC (1971) Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284: 878-881.

Holt HB, Medbak S, Kirk D, Guirgis R, Hughes I, Cummings MH and Meeking DR (2005) Recurrent severe hyperandrogenism during pregnancy: a case report. Journal of clinical pathology 58: 439-442.

Horiguchi T, Hyeon-Seo C, Shiraishi H, Shibata Y, Soma M, Morita M and Shimizu M (1998) Field studies on imposex and organotin accumulation in the rock shell, Thais clavigera, from the Seto Inland Sea and the Sanriku region, Japan. Sci Total Environ 214: 65-70.

Hsing AW, Tsao L and Devesa SS (2000) International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 85: 60-67.

Huang YW, Matthews JB, Fertuck KC and Zacharewski TR (2005) Use of Xenopus laevis as a model for investigating in vitro and in vivo endocrine disruption in amphibians. Environ Toxicol Chem 24: 2002-2009.

Hutchison JB and Steimer T (1981) Brain 5beta-reductase: a correlate of behavioral sensitivity to androgen. Science 213: 244-246.

Ilondo MM, Vanderschueren-Lodeweyckx M, Vlietinck R, Pizarro M, Malvaux P, Eggermont E and Eeckels R (1982) Plasma androgens in children and adolescents. Part II. A longitudinal study in patients with hypopituitarism. Horm Res 16: 78-95.

IOPC (2002) Global assessment of the state-of-the-science of endocrine disruptors. *In* T. Damstra, S. Barlow, A. Bergman, R. J. Kavlock and G. v. d. Kraak [eds.]. WHO.

IPCS Chemical Safety Information from Intergovernmental Organizations http://www.inchem.org/.

Ito Y, Fisher CR, Conte FA, Grumbach MM and Simpson ER (1993) Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. Proc Natl Acad Sci U S A 90: 11673-11677.

Jacobsen R, Moller H, Thoresen SO, Pukkala E, Kjaer SK and Johansen C (2006) Trends in testicular cancer incidence in the Nordic countries, focusing on the recent decrease in Denmark. Int J Androl 29: 199-204.

Jacobson JL and Jacobson SW (1997) Evidence for PCBs as neurodevelopmental toxicants in humans. Neurotoxicology 18: 415-424.

Jakesz R, Jonat W, Gnant M, Mittlboeck M, Greil R, Tausch C, Hilfrich J, Kwasny W, Menzel C, Samonigg H, Seifert M, Gademann G, Kaufmann M and Wolfgang J (2005) Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. Lancet 366: 455-462.

Jarow JP (2003) Endocrine causes of male infertility. Urol Clin North Am 30: 83-90.

Jeyasuria P and Place AR (1998) Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase (CYP19). J Exp Zool 281: 428-449.

Jobling S, Reynolds T, White R, Parker MG and Sumpter JP (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect 103: 582-587.

Jobling S and Tyler CR (2006) Introduction: The ecological relevance of chemically induced endocrine disruption in wildlife. Environ Health Perspect 114 Suppl 1: 7-8.

Jorgensen N, Asklund C, Carlsen E and Skakkebaek NE (2006) Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. Int J Androl 29: 54-61; discussion 105-108.

Jouannet P, Wang C, Eustache F, Kold-Jensen T and Auger J (2001) Semen quality and male reproductive health: the controversy about human sperm concentration decline. Apmis 109: 333-344.

Juberg DR (2000) An evaluation of endocrine modulators: implications for human health. Ecotoxicol Environ Saf 45: 93-105.

Kampa M and Castanas E (2006) Membrane steroid receptor signaling in normal and neoplastic cells. Mol Cell Endocrinol 246: 76-82.

Kannan K, Corsolini S, Focardi S, Tanabe S and Tatsukawa R (1996) Accumulation pattern of butyltin compounds in dolphin, tuna, and shark collected from Italian coastal waters. Arch Environ Contam Toxicol 31: 19-23.

Kannan K, Corsolini S, Imagawa T, Focardi S and Giesy JP (2002) Polychlorinated-naphthalenes, -biphenyls, -dibenzo-p-dioxins, -dibenzofurans and p,p'-DDE in bluefin tuna, swordfish, cormorants and barn swallows from Italy. Ambio 31: 207-211.

Kannan K and Falandysz J (1997) Butyltin residues in sediment, fish, fish-eating bird, harbour porpoise and human tissues from the polish coast of the baltic sea. Marine Pollution Bulletin 34: 203-207.

Kannan K, Senthilkumar K and Giesy JP (1999) Occurrence of butyltin compounds in human blood. Environmental Science and technology 33: 1776-1779.

Kavlock RJ and Ankley GT (1996) A perspective on the risk assessment process for endocrine-disruptive effects on wildlife and human health. Risk Anal 16: 731-739.

Kelce WR, Gray LE and Wilson EM (1998) Antiandrogens as environmental endocrine disruptors. Reprod Fertil Dev 10: 105-111.

Kelce WR, Monosson E, Gamcsik MP, Laws SC and Gray LE, Jr. (1994) Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicol Appl Pharmacol 126: 276-285.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA and Wilson EM (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375: 581-585.

Kelloff GJ, Lubet RA, Lieberman R, Eisenhauer K, Steele VE, Crowell JA, Hawk ET, Boone CW and Sigman CC (1998) Aromatase inhibitors as potential cancer chemopreventives. Cancer Epidemiol Biomarkers Prev 7: 65-78.

Kime D [ed.] (1998) Endocrine disruption in fish. Kluwer Academic Publishers, Boston-Dordrecht-London.

Kirby MF, Allen YT, Dyer RA, Feist SW, Katsiadaki I, Matthiessen P, Scott AP, Smith A, Stentiford GD, Thain JE, Thomas KV, Tolhurst L and Waldock MJ (2004) Surveys of plasma vitellogenin and intersex in male flounder (Platichthys flesus) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: temporal trends, 1996 to 2001. Environ Toxicol Chem 23: 748-758.

Kishida M and Callard GV (2001) Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142: 740-750.

Kleinkauf A, Scott AP, Stewart C, Simpson MG and Leah RT (2004) Abnormally elevated VTG concentrations in flounder (Platichthys flesus) from the Mersey estuary (UK)--a continuing problem. Ecotoxicol Environ Saf 58: 356-364.

Kley HK and Kruskemper HL (1978) [Liver and steroid hormones. III. Changes in steroid synthesis, concentration and metabolism in liver diseases]. Med Klin 73: 1155-1162.

Kloas W (2002) Amphibians as a model for the study of endocrine disruptors. Int Rev Cytol 216: 1-57.

Kloas W, Lutz I and Einspanier R (1999) Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. Sci Total Environ 225: 59-68.

Kobel (1996) Allopolyploid speciation., p. 391-401. *In* T. RC and K. HR [eds.], The Biology of Xenopus. Clarendon Press, Oxford.

Kortenkamp A (2006) Breast cancer, oestrogens and environmental pollutants: a re-evaluation from a mixture perspective. Int J Androl 29: 193-198.

Kuballa J and Wilken R (1995) Speciation and genotoxicity of butyltin compounds. Analyst 120: 667-673.

Labrie F, Luu-The V, Lin SX, Simard J, Labrie C, El-Alfy M, Pelletier G and Belanger A (2000) Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. J Mol Endocrinol 25: 1-16.

Labrie F, Simard J, Luu-The V, Pelletier G, Belghmi K and Belanger A (1994) Structure, regulation and role of 3 beta-hydroxysteroid dehydrogenase, 17 beta-hydroxysteroid dehydrogenase and aromatase enzymes in the formation of sex steroids in classical and peripheral intracrine tissues. Baillieres Clin Endocrinol Metab 8: 451-474.

Lambright C, Ostby J, Bobseine K, Wilson V, Hotchkiss AK, Mann PC and Gray LE, Jr. (2000) Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. Toxicol Sci 56: 389-399.

Landrigan PJ (2001) Children's environmental health. Lessons from the past and prospects for the future. Pediatr Clin North Am 48: 1319-1330.

Larriba S, Bassas L, Egozcue S, Gimenez J, Ramos MD, Briceno O, Estivill X and Casals T (2001) Adenosine triphosphate-binding cassette superfamily transporter gene expression in severe male infertility. Biol Reprod 65: 394-400.

Lehotzky K, Szeberenyi MJ, Horkay F and Kiss A (1982) The neurotoxicity of organotin: behavioural changes in rats. Acta Biol 33: 15-22.

Levy IG, Iscoe NA and Klotz LH (1998) Prostate cancer: 1. The descriptive epidemiology in Canada. Cmaj 159: 509-513.

Li X, Huang J, Yi P, Bambara RA, Hilf R and Muyan M (2004) Single-chain estrogen receptors (ERs) reveal that the ERalpha/beta heterodimer emulates functions of the ERalpha dimer in genomic estrogen signaling pathways. Mol Cell Biol 24: 7681-7694.

Liu S, Wen SW, Mao Y, Mery L and Rouleau J (1999) Birth cohort effects underlying the increasing testicular cancer incidence in Canada. Can J Public Health 90: 176-180.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif 25: 402-408.

Lo S, Allera A, Albers P, Heimbrecht J, Jantzen E, Klingmuller D and Steckelbroeck S (2003) Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism. J Steroid Biochem Mol Biol 84: 569-576.

Lodish HF, Hilton DJ, Klingmuller U, Watowich SS and Wu H (1995) The erythropoietin receptor: biogenesis, dimerization, and intracellular signal transduction. Cold Spring Harb Symp Quant Biol 60: 93-104.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Luckie DBJHW, Marija Krha, Mauri E. Krouse (2003) CFTR and MDR: ABC Transporters with Homologous Structure but Divergent Function. Current Genomics 4: 109-121.

Lutz I and Kloas W (1999) Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. Sci Total Environ 225: 49-57.

Luu-The V, Sugimoto Y, Puy L, Labrie Y, Lopez Solache I, Singh M and Labrie F (1994) Characterization, expression, and immunohistochemical localization of 5 alpha-reductase in human skin. J Invest Dermatol 102: 221-226.

Luu The V, Lachance Y, Labrie C, Leblanc G, Thomas JL, Strickler RC and Labrie F (1989) Full length cDNA structure and deduced amino acid sequence of human 3 beta-hydroxy-5-ene steroid dehydrogenase. Mol Endocrinol 3: 1310-1312.

Mahendroo MS, Cala KM, Landrum DP and Russell DW (1997) Fetal death in mice lacking 5alpha-reductase type 1 caused by estrogen excess. Mol Endocrinol 11: 917-927.

Mahendroo MS, Porter A, Russell DW and Word RA (1999) The parturition defect in steroid 5alpha-reductase type 1 knockout mice is due to impaired cervical ripening. Mol Endocrinol 13: 981-992.

Makita Y, Matsuura T, Ogata R, Romero Y, Omura M, Tanaka A, Hirata M and Inoue N (2003) Systemic effects of orally administered p, p'-DDE on immature male Wistar rats during pubertal period. J Occup Health 45: 223-227.

Martel C, Melner MH, Gagne D, Simard J and Labrie F (1994) Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey. Mol Cell Endocrinol 104: 103-111.

Martin SA, Jr., Harlow SD, Sowers MF, Longnecker MP, Garabrant D, Shore DL and Sandler DP (2002) DDT metabolite and androgens in African-American farmers. Epidemiology 13: 454-458.

Massa R, Davies DT, Bottoni L and Martini L (1979) Photoperiodic control of testosterone metabolism in the central and peripheral structures of avian species. J Steroid Biochem 11: 937-944.

Matthiessen P, Waldock R, Thain JE, Waite ME and Scrope-Howe S (1995) Changes in periwinkle (Littorina littorea) populations following the ban on TBT-based antifoulings on small boats in the United Kingdom. Ecotoxicol Environ Saf 30: 180-194.

McEwen BS (1997) Hormones as regulators of brain development: life-long effects related to health and disease. Acta Paediatr Suppl 422: 41-44.

McEwen BS, Coirini H, Danielsson A, Frankfurt M, Gould E, Mendelson S, Schumacher M, Segarra A and Woolley C (1991) Steroid and thyroid hormones modulate a changing brain. J Steroid Biochem Mol Biol 40: 1-14.

McKiernan JM, Goluboff ET, Liberson GL, Golden R and Fisch H (1999) Rising risk of testicular cancer by birth cohort in the United States from 1973 to 1995. J Urol 162: 361-363.

McLachlan JA, Newbold RR, Burow ME and Li SF (2001) From malformations to molecular mechanisms in the male: three decades of research on endocrine disrupters. Apmis 109: 263-272.

McVey MJ and Cooke GM (2003) Inhibition of rat testis microsomal 3beta-hydroxysteroid dehydrogenase activity by tributyltin. J Steroid Biochem Mol Biol 86: 99-105.

Mellon SH and Griffin LD (2002a) Neurosteroids: biochemistry and clinical significance. Trends Endocrinol Metab 13: 35-43.

Mellon SH and Griffin LD (2002b) Synthesis, regulation, and function of neurosteroids. Endocr Res 28: 463.

Melo AC and Ramsdell JS (2001) Sexual dimorphism of brain aromatase activity in medaka: induction of a female phenotype by estradiol. Environ Health Perspect 109: 257-264.

Migeon CJ and Wisniewski AB (2003) Human sex differentiation and its abnormalities. Best Pract Res Clin Obstet Gynaecol 17: 1-18.

Milewich L, Shaw CE, Doody KM, Rainey WE, Mason JI and Carr BR (1991) 3 beta-Hydroxysteroid dehydrogenase activity in glandular and extraglandular human fetal tissues. J Clin Endocrinol Metab 73: 1134-1140.

Molin L and Wahlberg JE (1975) Toxic skin reactions caused by tributyltin oxide (tbto) in socks. Berufsdermatosen 23: 138-142.

Monosson E, Kelce WR, Lambright C, Ostby J and Gray LE, Jr. (1999) Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. Toxicol Ind Health 15: 65-79.

Morcillo Y and Porte C (1999) Evidence of endocrine disruption in the imposex-affected gastropod Bolinus brandaris. Environ Res 81: 349-354.

Morishima A, Grumbach MM, Simpson ER, Fisher C and Qin K (1995) Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80: 3689-3698.

Mosselman S, Polman J and Dijkema R (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392: 49-53.

Nagahama Y (1994) Endocrine regulation of gametogenesis in fish. The International journal of developmental biology 38: 217-229.

Nakanishi T, Kohroki J, Suzuki S, Ishizaki J, Hiromori Y, Takasuga S, Itoh N, Watanabe Y, Utoguchi N and Tanaka K (2002) Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. J Clin Endocrinol Metab 87: 2830-2837.

Negri-Cesi P and Motta M (1994) Androgen metabolism in the human prostatic cancer cell line LNCaP. J Steroid Biochem Mol Biol 51: 89-96.

Nordenskjold A, Magnus O, Aagenaes O and Knudtzon J (1998) Homozygous mutation (A228T) in the 5alpha-reductase type 2 gene in a boy with 5alpha-reductase deficiency: genotype-phenotype correlations. Am J Med Genet 80: 269-272.

Ntais C, Polycarpou A and Ioannidis JP (2003) SRD5A2 gene polymorphisms and the risk of prostate cancer: a meta-analysis. Cancer Epidemiol Biomarkers Prev 12: 618-624.

Odell WD and Swerdloff RS (1976) Male hypogonadism. West J Med 124: 446-475.

Oehlmann J, Markert B, Stroben E, Schulte-Oehlmann U, Bauer B and Fioroni P (1996) Tributyltin biomonitoring using prosobranchs as sentinel organisms. Anal Bioanal Chem 354: 540-545.

Oehlmann J, Schulte-Oehlmann U, Tillmann M and Markert B (2000) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9: 383-397.

Oettel M (2003) Testosterone metabolism, dose-response relationships and receptor polymorphisms: selected pharmacological/toxicological considerations on benefits versus risks of testosterone therapy in men. Aging Male 6: 230-256.

Onland-Moret NC, Peeters PH, van der Schouw YT, Grobbee DE and van Gils CH (2005) Alcohol and endogenous sex steroid levels in postmenopausal women: a cross-sectional study. J Clin Endocrinol Metab 90: 1414-1419.

PAN Pesticide Action Network UK http://www.pan-uk.org/About/index.htm.

Paulozzi LJ (1999) International trends in rates of hypospadias and cryptorchidism. Environ Health Perspect 107: 297-302.

Paulozzi LJ, Erickson JD and Jackson RJ (1997) Hypospadias trends in two US surveillance systems. Pediatrics 100: 831-834.

Peltoketo H, Luu-The V, Simard J and Adamski J (1999) 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. J Mol Endocrinol 23: 1-11.

Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N and Ratnam K (2000) Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochem J 351: 67-77.

Penning TM, Smithgall TE, Askonas LJ and Sharp RB (1986) Rat liver 3 alpha-hydroxysteroid dehydrogenase. Steroids 47: 221-247.

Persky V, Turyk M, Anderson HA, Hanrahan LP, Falk C, Steenport DN, Chatterton R, Jr. and Freels S (2001) The effects of PCB exposure and fish consumption on endogenous hormones. Environ Health Perspect 109: 1275-1283.

Pietras RJ and Szego CM (1999) Cell membrane estrogen receptors resurface. Nat Med 5: 1330.

Prelevic GM, Kocjan T and Markou A (2005) Hormone replacement therapy in postmenopausal women. Minerva Endocrinol 30: 27-36.

Rizner TL, Lin HK, Peehl DM, Steckelbroeck S, Bauman DR and Penning TM (2003) Human type 3 3alpha-hydroxysteroid dehydrogenase (aldo-keto reductase 1C2) and androgen metabolism in prostate cells. Endocrinology 144: 2922-2932.

Sadiki AI and Williams DT (1999) A study on organotin levels in Canadian drinking water distributed through PVC pipes. Chemosphere 38: 1541-1548.

Safe SH (2000) Endocrine disruptors and human health--is there a problem? An update. Environ Health Perspect 108: 487-493.

Saitoh M, Yanase T, Morinaga H, Tanabe M, Mu YM, Nishi Y, Nomura M, Okabe T, Goto K, Takayanagi R and Nawata H (2001) Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochem Biophys Res Commun 289: 198-204.

Sanderson JT, Boerma J, Lansbergen GW and van den Berg M (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicol Appl Pharmacol 182: 44-54.

Sanderson JT and van den Berg M (2003) Interactions of xenobiotics with the steroid hormone biosynthesis pathway. Pure Appl Chem 75: 1957-1971.

Santillo D, Johnston P and Langston WJ (2001) Chapter 13: Tributyltin (TBT) antifoulants: a tale of ships, snails and imposex., p. 135-148. *In* P. Harremoës et al. [eds.], Late Lessons from Early Warnings: the Precautionary Principle 1896-2000. Office for Official Publications of the European Communities, European Environment Agency Environmental Issue Report.

Sarradin PM, Lapaquellerie Y, Astruc A, Latouche C and Astruc M (1995) Long term behaviour and degradation kinetics of tributyltin in a marina sediment. Sci Total Environ 170: 59-70.

Schaefer M (1994) Children and toxic substances: confronting a major public health challenge. Environ Health Perspect 102 Suppl 2: 155-156.

Schulte-Oehlmann U, Albanis T, Allera A, Bachmann J, Berntsson P, Beresford N, Carnevali DC, Ciceri F, Dagnac T, Falandysz J, Galassi S, Hala D, Janer G, Jeannot R, Jobling S, King I, Klingmuller D, Kloas W, Kusk KO, Levada R, Lo S, Lutz I, Oehlmann J, Oredsson S, Porte C, Rand-Weaver M, Sakkas V, Sugni M, Tyler C, van Aerle R, van Ballegoy C and Wollenberger L (2006) COMPRENDO: Focus and approach. Environ Health Perspect 114 Suppl 1: 98-100.

Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J, Watermann B and Scherf S (2000) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyltin as a xeno-androgen. Ecotoxicology 9: 399-412.

Shapiro S (2005) Oral oestrogen-progestagen contraceptives, menopausal treatment, and cancer. The lancet oncology 6: 736; author reply 737.

Sharpe RM and Skakkebaek NE (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341: 1392-1395.

Sherwood NM, Parker DB, McRory JE and Lescheid DW (1994) Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone., p. 3-66. *In* N. M. Sherwood and C. L. Hew [eds.], Molecular Endocrinology of Fish. Fish Physiology. Academic Press Inc.

Shozu M, Akasofu K, Harada T and Kubota Y (1991) A new cause of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab 72: 560-566.

Silver RI and Russell DW (1999) 5alpha-reductase type 2 mutations are present in some boys with isolated hypospadias. J Urol 162: 1142-1145.

Simard J, Durocher F, Mebarki F, Turgeon C, Sanchez R, Labrie Y, Couet J, Trudel C, Rheaume E, Morel Y, Luu-The V and Labrie F (1996) Molecular biology and genetics of the 3 beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. J Endocrinol 150 Suppl: S189-207.

Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE, Hinshelwood MM, Graham-Lorence S, Sun T, Fisher CR, Qin K and Mendelson CR (1997) Aromatase expression in health and disease. Recent Prog Horm Res 52: 185-213; discussion 213-184.

Simpson JL and Rebar RW (1995) Normal and Abnormal sexual differentiation and development., p. 788-850. *In* K. L. Becker [ed.], Principles and practice of endocrinology and metabolism. Lippincott.

Sina D, Schuhmann R, Abraham R, Taubert HD and Dericks-Tan JS (1975) Increased serum FSH levels correlated with low and high sperm counts in male infertile patients. Andrologia 7: 31-37.

Skakkebaek NE (2003) Testicular dysgenesis syndrome. Horm Res 60 Suppl 3: 49.

Skakkebaek NE (2004) Testicular dysgenesis syndrome: new epidemiological evidence. Int J Androl 27: 189-191.

Skakkebaek NE, Jorgensen N, Main KM, Rajpert-De Meyts E, Leffers H, Andersson AM, Juul A, Carlsen E, Mortensen GK, Jensen TK and Toppari J (2006) Is human fecundity declining? Int J Androl 29: 2-11.

Skakkebaek NE, Rajpert-De Meyts E and Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16: 972-978.

Smith BS (1981) Male characteristics on female mud snails caused by antifouling bottom paints. J Appl Toxicol 1: 22-25.

Snoeij NJ, van Iersel AA, Penninks AH and Seinen W (1985) Toxicity of triorganotin compounds: comparative in vivo studies with a series of trialkyltin compounds and triphenyltin chloride in male rats. Toxicol Appl Pharmacol 81: 274-286.

Solomon GM and Schettler T (2000) Environment and health: 6. Endocrine disruption and potential human health implications. Cmaj 163: 1471-1476.

Sonnenschein C and Soto AM (1998) An updated review of environmental estrogen and androgen mimics and antagonists. J Steroid Biochem Mol Biol 65: 143-150.

Sower SA, Nucci NV and Silver MR (2004) Family of Gonadotropin- Releasing Hormone, p. 3043 pgs. *In* L. Martini [ed.], Encyclopedia of Endocrine Diseases. Elsevier Academic Press.

Sperry TS and Thomas P (1999a) Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 140: 1602-1611.

Sperry TS and Thomas P (1999b) Identification of two nuclear androgen receptors in kelp bass (Paralabrax clathratus) and their binding affinities for xenobiotics: comparison with Atlantic croaker (Micropogonias undulatus) androgen receptors. Biol Reprod 61: 1152-1161.

Stab JA, Traas TP, Stroomberg G, van Kesteren J, Leonards P, van Hattum B, Brinkman UA and Cofino WP (1996) Determination of organotin compounds in the foodweb of a shallow freshwater lake in the Netherlands. Arch Environ Contam Toxicol 31: 319-328.

Steckelbroeck S, Heidrich DD, Stoffel-Wagner B, Hans VH, Schramm J, Bidlingmaier F and Klingmuller D (1999a) Characterization of aromatase cytochrome P450 activity in the human temporal lobe. J Clin Endocrinol Metab 84: 2795-2801.

Steckelbroeck S, Stoffel-Wagner B, Reichelt R, Schramm J, Bidlingmaier F, Siekmann L and Klingmuller D (1999b) Characterization of 17beta-hydroxysteroid dehydrogenase activity in brain tissue: testosterone formation in the human temporal lobe. J Neuroendocrinol 11: 457-464.

Steckelbroeck S, Watzka M, Reichelt R, Hans VH, Stoffel-Wagner B, Heidrich DD, Schramm J, Bidlingmaier F and Klingmuller D (2001) Characterization of the 5alpha-reductase-3alpha-hydroxysteroid dehydrogenase complex in the human brain. J Clin Endocrinol Metab 86: 1324-1331.

Steenland K, Cedillo L, Tucker J, Hines C, Sorensen K, Deddens J and Cruz V (1997) Thyroid hormones and cytogenetic outcomes in backpack sprayers using ethylenebis(dithiocarbamate) (EBDC) fungicides in Mexico. Environ Health Perspect 105: 1126-1130.

Steinhardt GF (2004) Endocrine disruption and hypospadias. Adv Exp Med Biol 545: 203-215.

Stoffel-Wagner B, Watzka M, Steckelbroeck S, Wickert L, Schramm J, Romalo G, Klingmuller D and Schweikert HU (1998) Expression of 5alpha-reductase in the human temporal lobe of children and adults. J Clin Endocrinol Metab 83: 3636-3642.

Sunami O, Kunimatsu T, Yamada T, Yabushita S, Sukata T, Miyata K, Kamita Y, Okuno Y, Seki T, Nakatsuka I and Matsuo M (2000) Evaluation of a 5-day Hershberger assay using young mature male rats: methyltestosterone and p,p'-DDE, but not fenitrothion, exhibited androgenic or antiandrogenic activity in vivo. J Toxicol Sci 25: 403-415.

Swan SH (2006a) Prenatal phthalate exposure and anogenital distance in male infants. Environ Health Perspect 114: A88-89.

Swan SH (2006b) Semen quality in fertile US men in relation to geographical area and pesticide exposure. Int J Androl 29: 62-68.

Takahashi S, Mukai H, Tanabe S, Sakayama K, Miyazaki T and Masuno H (1999) Butyltin residues in livers of humans and wild terrestrial mammals and in plastic products. Environ Pollut 106: 213-218.

Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD and Russell DW (1993) Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. J Clin Invest 92: 903-910.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jr., Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J and Skakkebaek NE (1996) Male reproductive health and environmental xenoestrogens. Environ Health Perspect 104 Suppl 4: 741-803.

Toran-Allerand CD (2004) Estrogen and the brain: beyond ER-alpha and ER-beta. Exp Gerontol 39: 1579-1586.

Tyler CR, Jobling S and Sumpter JP (1998) Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28: 319-361.

van Brussel JP and Mickisch GH (2003) Multidrug resistance in prostate cancer. Onkologie 26: 175-181.

Van der Bend RL, Duetz W, Colen AM, Van Dam K and Berden JA (1985) Differential effects of triphenyltin and 8-azido-ATP on the ATP synthesis, ATP-Pi exchange, and ATP hydrolysis in liposomes containing ATP synthase and bacteriorhodopsin. Arch Biochem Biophys 241: 461-471.

Vermeulen A (1983) Androgen secretion after age 50 in both sexes. Horm Res 18: 37-42.

Vinggaard AM, Jacobsen H, Metzdorff SB, Andersen HR and Nellemann C (2005) Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. Toxicology 207: 21-34.

Vinggaard AM, Nellemann C, Dalgaard M, Jorgensen EB and Andersen HR (2002) Antiandrogenic effects in vitro and in vivo of the fungicide prochloraz. Toxicol Sci 69: 344-353.

Voet D and Voet J (2004) Biochemistry. John Wiley & Sons Inc. 2nd edition, ISBN 0-471-58651-X.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I and Vethaak AD (2000) Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol 30: 71-133.

Wang PP and Cao Y (1996) Incidence trends of female breast cancer in Saskatchewan, 1932-1990. Breast Cancer Res Treat 37: 197-207.

Wehling M, Schultz A and Losel R (2006) Nongenomic actions of estrogens: exciting opportunities for pharmacology. Maturitas 54: 321-326.

Westerbacka J, Yki-Jarvinen H, Vehkavaara S, Hakkinen AM, Andrew R, Wake DJ, Seckl JR and Walker BR (2003) Body fat distribution and cortisol metabolism in healthy men: enhanced 5beta-reductase and lower cortisol/cortisone metabolite ratios in men with fatty liver. J Clin Endocrinol Metab 88: 4924-4931.

Wilson JD (1996) Role of dihydrotestosterone in androgen action. Prostate Suppl 6: 88-92.

Wilson JD, Griffin JE and Russell DW (1993) Steroid 5 alpha-reductase 2 deficiency. Endocr Rev 14: 577-593.

Wolff MS, Collman GW, Barrett JC and Huff J (1996) Breast cancer and environmental risk factors: epidemiological and experimental findings. Annu Rev Pharmacol Toxicol 36: 573-596.

Wong C, Kelce WR, Sar M and Wilson EM (1995) Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J Biol Chem 270: 19998-20003.

Xu LC, Sun H, Chen JF, Bian Q, Song L and Wang XR (2006) Androgen receptor activities of p,p'-DDE, fenvalerate and phoxim detected by androgen receptor reporter gene assay. Toxicol Lett 160: 151-157.

Yaghmaie F, Saeed O, Garan SA, Freitag W, Timiras PS and Sternberg H (2005) Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice. Neuro Endocrinol Lett 26: 197-203.

Yamabe Y, Hoshino A, Imura N, Suzuki T and Himeno S (2000) Enhancement of androgen-dependent transcription and cell proliferation by tributyltin and triphenyltin in human prostate cancer cells. Toxicol Appl Pharmacol 169: 177-184.

Yang SY, He XY and Schulz H (2005) Multiple functions of type 10 17beta-hydroxysteroid dehydrogenase. Trends Endocrinol Metab 16: 167-175.

Yoshitsugu M and Ihori M (1997) [Endocrine disturbances in liver cirrhosis--focused on sex hormones]. Nippon Rinsho 55: 3002-3006.

Younglai EV, Collins JA and Foster WG (1998) Canadian semen quality: an analysis of sperm density among eleven academic fertility centers. Fertil Steril 70: 76-80.

Zacharewski T (1998) Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays. Environ Health Perspect 106 Suppl 2: 577-582.

Zhu YS, Cai LQ, You X, Cordero JJ, Huang Y and Imperato-McGinley J (2003) Androgen-induced prostate-specific antigen gene expression is mediated via dihydrotestosterone in LNCaP cells. J Androl 24: 681-687.

Zhu YS, Katz MD and Imperato-McGinley J (1998) Natural potent androgens: lessons from human genetic models. Baillieres Clin Endocrinol Metab 12: 83-113.

Zivadinovic D and Watson CS (2005) Membrane estrogen receptor-alpha levels predict estrogen-induced ERK1/2 activation in MCF-7 cells. Breast Cancer Res 7: R130-144.

10 Publications presented in this thesis

1) Lo S, King I, Allera A, Klingmuller D. Effects of various pesticides on human 5α-reductase activity in prostate and LNCaP cells. Toxicology In Vitro. 2007, April, 21 (3): 502-508.

   The author of this thesis conducted all experiments of publication 1) and wrote the manuscript. The coauthors contributed to the reviewing process of the manuscript.

2) Allera A, Lo S, King I, Steglich F, Klingmüller D. Impact of androgenic / antiandrogenic compounds (AAC) on human sex steroid metabolizing key enzymes. Toxicology. 2004, December 1;205(1-2):75-85.

   The author of this thesis performed the testing of AAC on P450arom activities in placenta tissue and JEG-3 cells, and wrote those parts in publication 2) concerning these experiments. The coauthors of publication 2) performed the experiments concerning sulfatase and sulfotransferase activities. The first author of the manuscript combined all different parts to a coherent publication, while the last author contributed to the reviewing process of the manuscript.

3) Lo S, Allera A, Albers P, Heimbrecht J, Jantzen E, Klingmuller D, Steckelbroeck S (2003) Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism. Journal of Steroid Biochemistry and Molecular Biology. 2003, Apr;84(5):569-76.

   The authour of this thesis performed all experiments of publication 3) and wrote the majority of the manuscript. The coauthors contributed to the reviewing process of the manuscript.

Acknowledgements

This dissertation and the research involved were made possible thanks to the invaluable and unfailing support of many people.

My sincere gratitude goes to my supervisor Prof. Dr. Dietrich Klingmüller who gave me the opportunity to join the Department of Endocrinology at the Institute of Clinical Biochemistry of the University Hospital in Bonn. I am thankful for his guidance through the graduation, and for his constant willingness to help and support me.
I am deeply grateful to Prof. Dr. Jörg Oehlmann, the biological referee of my thesis, for his contributions and support.
During the first year, my advisor PD Stephan Steckelbroeck has helped me enormously along the way by introducing me into the research field of endocrinology, and by passing his inexhaustible ideas and energy to me.

I extend my gratitude to the team of the Endocrinology Clinic Niclaas Bliesener, Ingrid Hufschmidt, Ulla Krey and Uta Wolber who over the years have helped me in many ways. Thank you for the funny lunch breaks! I am especially thankful for the excellent assistance of Petra Wegener-Toper. Also, I appreciate the support of Helmut Lemoch, who taught me the basic methods of cell culture. PD Dr. Michael Ludwig has helped me enormously with all molecular biological questions.

I am grateful to the EU for funding my work as part of the COMPRENDO-project. My research was shaped immensely through the fruitful communications and collaboration at COMPRENDO and I bow before the coordinator Dr. Ulrike Schulte-Oehlmann.

It is impossible to credit all the help and kindness of my friends. The warmest thanks go to Astrid, Claudia, Corry, Elmar, Katrin, Marco, Nicole, Norbert and William. Special thanks to the members of the vocal group “Chili Chicks“ Anne, Astrid, Birgit, Catherine, Elsgin, Martha, Marielle, Myrna, Prasanna, Susanne, and the Emsland pony riders Beate, Detlef, Heike, Kerstin, Jasmin and Wolfgang for all the fun we had! Particular thanks to my former flat-mates in Bremen (Marc, Lucie, Sandra and Philipp, adopted flat-mate) and in Cologne (Birgit, Grazyna, Luciano, Nadine and Uwe).

My deepest gratitude and appreciation go to my beloved parents and my dear sister Vivien who proof-read this thesis.

All my love goes to Philip who has been at my side through all the time of becoming a PhD.

Curriculum vitae

Susan Lo-Ullmann
Mauritiussteinweg 30
50676 Cologne

Date of Birth: July 2, 1972
Place of Birth: Cologne, Germany

University Education

October 2001 – April 2006 University Hospital Bonn

Scientific employee at the Department of Endocrinology, Institute of Clinical Biochemistry, University Hospital Bonn. The graduation about the "Endocrine disruption caused by environmental chemicals: Effects on key enzymes of steroid hormone metabolism of humans and animals" was supervised by Prof. Dr. D. Klingmüller of the University Hospital Bonn and Prof. Dr. J. Oehlmann of the Johann Wolfgang Goethe-University Frankfurt. Scientific associate of the EU-project COMPRENDO – Comparative research on Endocrine Disrupters" (http://www.comprendo-project.org).

Academic teachers:

Prof. Dr. T.A. Albanis
Prof. Dr. J. Falandysz
Prof. Dr. Dietrich Klingmüller
PD Dr. Michael Ludwig
Prof. Dr. S. Oredsson
Dr. U. Schulte-Oehlmann
Prof. Dr. C. Tyler
Prof. Dr. D. Candia Carnevali
Prof. Dr. S. Galassi
Prof. Dr. W. Kloas
Prof. Dr. Oehlmann
Dr. C. Porte
PD Dr. Stephan Steckelbroeck
Dr. Matthias Watzka

October 1995 – April 1999 University in Bremen

Biological studies with specialization in marine biology, graduation at the Alfred-Wegener-Institut in Bremerhaven in April 1999. The title of the diploma thesis was "Diatomeenblüte in der Nordsee im Frühjahr 1998: Sukzession der Arten in verschiedenen Wassermassen" and was supervised by Prof. Dr. Victor Smetacek and Prof. Dr. Ulrich Bathmann.

Academic teachers:

PD Dr. Rainer Amon
Prof. Dr. Ulrich Bathmann
Prof. Dr. Hermann Cordes
Prof. Dr. Wolfgang Heyser
Prof. Dr. Gunter-Otto Kirst
Prof. Dr. Hans-Otto Pörtner
Prof. Dr. Victor Smetacek

October 1992 – June 1995 **Philipps-University Marburg**

Biological studies at the beginning of winter semester 1992/93, passing of the intermediate diploma in October 1994.

Academic teachers:

Prof. Dr. Walter Arnold
Prof. Dr. Gerhard Heldmaier
Prof. Dr. Klaus Kalmring
Prof. Dr. Christoph Kirchner
Prof. Dr. Harald Plachter
Prof. Dr. Renate Renkawitz-Pohl
Prof. Dr. Karl-August Seitz
Prof. Dr. Günter Throm

School Education

August 1991 – October 1992 **Fu Jen and National Normal University, Taipei, Taiwan**

Chinese studies at the Mandarin Training Center

August 1988– June 1991 **Friedrich-Dessauer-Gymnasium in Höchst**

Senior high school: Three years
Final examination: University-entrance diploma

August 1982– June 1988 **Eichendorffschule Kelkheim**

Junior high school: Six years
Final examination: Secondary school level certificate

August 1978– June 1982 **Grundschule Liederbach**

Elementary school: Four years

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorgelegte Dissertation

„Endocrine disruption caused by environmental chemicals: Effects on key enzymes of steroid hormone metabolism of humans and animals“

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe. Ich erkläre weiterhin, dass Entlehnungen aus Schriften, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.
Ich habe bisher an keiner anderen Universität ein Gesuch um Zulassung zur Promotion eingereicht oder die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Köln, den.....

Susan Lo-Ullmann