Adrenocorticolysis induced by 3-MeSO₂-DDE

Mechanisms of action, kinetics and species differences

VERONICA LINDSTRÖM
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Abstract

The DDT metabolite 3-methylsulphonyl-DDE (3-MeSO₂-DDE) induces cell death specifically in the adrenal cortex of mice after a cytochrome P45011B1 (CYP11B1)-catalysed bioactivation. This substance is not only an environmental pollutant, but also a suggested lead compound for an improved chemotherapy of adrenocortical carcinoma (ACC). The aim of the thesis was to further investigate this compound in terms of kinetics, cell death mechanisms and species differences. The pharmacokinetics of 3-MeSO₂-DDE and the current drug for ACC, o,p'-DDD, was studied during 6 months following a single dose in minipigs. The elimination was slower for 3-MeSO₂-DDE than for o,p'-DDD, indicated by a lower clearance and longer t½ in plasma and subcutaneous fat. Both substances remained in fat tissue during the whole study period. Unlike o,p'-DDD, 3-MeSO₂-DDE was retained also in liver. The adequacy of the murine adrenocortical cell line Y-1 was evaluated for studies of adrenotoxic compounds. The Y-1 cells proved to be an appropriate test system for future mechanism studies, since CYP-catalysed irreversible binding, inhibited corticosterone production induced by 3-MeSO₂-DDE and o,p'-DDD were successfully demonstrated. Cell death of 3-MeSO₂-DDE in the mouse adrenal cortex was implied to be necrotic. Early apoptotic signalling (i.e. up-regulation of caspase-9) was observed, although it seemed to be interrupted by ATP-depletion and anti-apoptotic actions by heat shock protein 70, resulting in lack of activation of caspase-3. Using cultured adrenal tissue slices, two not previously studied species were examined ex vivo regarding adrenal binding of 3-MeSO₂-[¹⁴C]DDE. Binding was found in the hamster adrenal cortex and in assumed cortical cells in the medulla, while the guinea pig adrenal was devoid of binding. This emphasises the species specificity in bioactivation of 3-MeSO₂-DDE. The thesis forms a basis for further investigations in the human adrenocortical cell line H295R and provides new knowledge of importance for toxicological risk assessment of 3-MeSO₂-DDE.

Keywords: 3-methylsulphonyl-DDE, o, p'-DDD, CYP11B1, adrenal cortex, tissue-specific toxicity, bioactivation, kinetics, Y-1 cells

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LIST OF PAPERS

This thesis is based on the papers listed below, which will be referred to in the text by their roman numerals.


II Hermansson, V.*, Asp, V., Bergman, Å., Bergström, U. and Brandt I. Comparative CYP-dependent binding of the adrenocortical toxicants 3-methylsulphonyl-DDE and o,p’-DDD in Y-1 adrenal cells. Archives of Toxicology, 2007 (in press) Available at: http://www.springerlink.com/content/100462/

III Asp, V., Lindström, V., Bergström, U. and Brandt I. Cytotoxicity and decreased corticosterone production in adrenocortical Y-1 cells by o,p’-DDD and methylsulphonated derivatives of p,p’-DDE. Manuscript

IV Lindström, V., Asp, V., Brandt, I. and Bergström, U. Mode of cell death induced by 3-methylsulphonyl-DDE in the mouse adrenal cortex. Manuscript

V Lindström, V., Brandt, I. and Lindhe, Ö. Species differences in 3-methylsulphonyl-DDE bioactivation by adrenocortical tissue. Submitted manuscript

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*Veronica Lindström, former Hermansson.
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<th>Description</th>
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<tr>
<td>3-MeSO₂-DDE</td>
<td>2-(4-chloro-3-methylsulphonylphenyl)-2-(4-chlorophenyl)-1,1-dichloroethene</td>
</tr>
<tr>
<td>o,p’-DDD</td>
<td>2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane</td>
</tr>
<tr>
<td>p,p’-DDE</td>
<td>2,2-bis(4-chlorophenyl)-1,1-dichloroethene</td>
</tr>
<tr>
<td>11-DOC</td>
<td>11-deoxycorticosterone</td>
</tr>
<tr>
<td>ACC</td>
<td>adrenocortical carcinoma</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
</tr>
<tr>
<td>b.w.</td>
<td>body weight</td>
</tr>
<tr>
<td>C</td>
<td>corticosterone</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CL</td>
<td>clearance</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>F</td>
<td>bioavailability</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>λₜ</td>
<td>lambda z</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NPSH</td>
<td>nonprotein sulphydryls</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>t¹⁄₂</td>
<td>half-life</td>
</tr>
<tr>
<td>V₉</td>
<td>volume of distribution</td>
</tr>
</tbody>
</table>
INTRODUCTION

The adrenal gland

The adrenal comprise two separate endocrine organs; the cortex and the medulla. The adrenal cortex produces steroidal hormones, while the medulla produces mainly catecholamines (adrenaline, noradrenaline and dopamine). The cortex is divided into three functional and histological zones; an outer zona glomerulosa, a central zona fasciculata and an inner zona reticularis. Zona glomerulosa produces mineralocorticoids (aldosterone), while zona fasciculata and reticularis produce mainly glucocorticoids (cortisol/corticosterone), but also androgens like dehydroepiandrosterone (DHEA). Mineralocorticoids are essential for the regulation of sodium and potassium homeostasis and are secreted after release of angiotensin II from the kidney. Glucocorticoid synthesis is regulated via the hypothalamic-pituitary-adrenal (HPA) axis. Different kinds of physiological stress cause release of the corticotrophin-releasing hormone (CRH) and vasopressin from the hypothalamus, stimulating the pituitary to release the adrenocorticotrophic hormone (ACTH) which reaches the adrenal via the blood stream. ACTH increases the production of glucocorticoids, which in turn can inhibit both CRH and ACTH release by a negative feed-back mechanism. All corticosteroids are derived from cholesterol, which is taken up from plasma lipoproteins (Gwynne et al. 1976) or synthesised in situ from acetate in the adrenal cortex. Cholesterol is also stored in intracellular lipid droplets in the adrenal cortex, mainly in zona fasciculata (Kraemer 2007). Upon ACTH stimulation, cholesterol is transported to the mitochondria by the steroidogenic acute regulatory protein (StAR) (Clark et al. 1994). Cholesterol is cleaved by a side-chain-cleaving enzyme (CYP11A1, classically named CYP450scc) into pregnenolone. Then, depending on which zone the cholesterol has entered, it comes across various steroidogenic CYP enzymes (Figure 1), which transform pregnenolone into functional corticosteroids. Long-term, ACTH, via cyclic adenosine monophosphate (cAMP), increases transcription of genes encoding steroid hydroxylases such as CYP11A1 and CYP11B1 (John et al. 1986; Waterman and Bischof 1996). The conversion of cholesterol to pregnenolone as well as the final step in corticosterone,
cortisol and aldosterone synthesis occur in the mitochondria, since all CYP11 enzymes are located to the mitochondrial inner membrane. All other enzymatic reactions in the adrenocortical steroidogenesis (shown in Figure 1) take place in the endoplasmic reticulum (ER).

![Steroid synthesis diagram](image)

**Figure 1.** Schematic illustration of the steroid synthesis in the adrenal cortex. 3β-HSD = 3β-hydroxysteroid dehydrogenase, 17-KSR = 17-ketosteroid reductase.

The adrenal as a target organ for toxicity

Toxic insult to the adrenal may have serious consequences for the steroid hormone synthesis. Because of the essential functions of the adrenocortical hormones (Rosol et al. 2001), this could cause homeostatic imbalance. The adrenal is considered a sensitive organ for toxicity and many xenobiotics accumulate in this organ. This is due to several factors such as the high blood supply and high lipid content of the adrenal. Since cholesterol for steroidogenesis is taken up from plasma lipoproteins there are mechanisms for lipoprotein uptake, which may also promote uptake of lipophilic compounds (Colby 1996). The high content of CYP enzymes gives a high capacity of the organ to bioactivate certain xenobiotics into reactive compounds. During adrenal steroid synthesis, reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide are formed. Normally, ROS are handled by antioxidant systems such as superoxide dismutase (SOD), catalase, α-tocopherol and glutathione (GSH) (Hornsby and Crivello 1983b). However, at toxic doses, xenobiotics may overwhelm the antioxidant system by enhancing ROS formation through uncoupling of CYP or through redox cycling. The adrenocorticolytic polycyclic aromatic hydrocarbon (PAH) DMBA causes necrosis to the rat adrenal cortex through a proposed genera-
tion of ROS via activation by CYPs (Hallberg 1990). ROS can cause oxidative damage to DNA, proteins and also initiate lipid peroxidation which can result in CYP inactivation (Hornsby and Crivello 1983a). The adrenal cortex, containing storages of lipids, is sensitive to damage by lipid peroxidation. Oxidative damage to lipids can result in break down of biological membranes and finally end with cell death (Colby 1996). Carbon tetrachloride, for instance, causes necrosis to the adrenal cortex after initiation of lipid peroxidation. However, the initiation requires a CYP-catalysed bioactivation (Colby 1981; Colby et al. 1994). Reactive intermediates formed through interaction with adrenal enzymes may also bind covalently to proteins or DNA and cause downstream toxic effects.

Adrenocortical carcinoma and o,p′-DDD

Adrenocortical carcinoma (ACC) is a rare tumour with an incidence of 1-2 per million/year (Grondal et al. 1990; Gatta et al. 2006). The tumour is often discovered at an advanced clinical stage, resulting in a poor prognosis and short survival after diagnosis (Gatta et al. 2006). In about 60% of the cases, tumours are functional, i.e. steroid hormone overproducing (Wooten and King 1993; Haak et al. 1994; Tauchmanova et al. 2004). Among functional tumours, overproduction of glucocorticoids, resulting in Cushing’s syndrome, is most frequently found (Haak et al. 1994; Tauchmanova et al. 2004), but overproduction of androgens (androgenital syndrome) and aldosterone (Conns syndrome) occurs as well. Besides cortisol hypersecretion, patients with Cushing’s syndrome show some typical features, such as muscle weakness, hypertension and obesity (Lewis et al. 1999). For diagnosis of ACC, hormone screening tests, imaging with computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET) are techniques used (Allolio et al. 2004).

The primary and most effective treatment of ACC is surgical removal of the tumour (Jarolim 2003). Cytotoxic drugs are used for inoperable tumours, when complete removal of tumour tissue is impossible and as an adjuvant after surgery. o,p′-DDD (mitotane), the main drug of choice for treatment of ACC, causes cell death in the adrenal cortex following a CYP-catalysed hydroxylation of the side-chain β-carbon and a subsequent spontaneous dehydrochlorination. A resulting reactive acyl chloride, binds covalently to primarily mitochondrial proteins (Martz and Straw 1980; Cai et al. 1995a). Steroidogenesis is also affected since o,p′-DDD inhibits the activity of CYP11B1 and CYP11A1 (Hart and Straw 1971; Hart et al. 1971). Oxidative stress through the production of ROS may contribute to the cytotoxicity (Schteingart 2000). o,p′-DDD is usually administered orally as 500 mg tablets. The dose has to be adjusted to the patient depending on tolerability. The daily dose during long-term treatments is normally 1-3 g, but can vary up to
10-12 g (EMEA 2005). Reports and evaluations of the medical treatment with \( o,p' \)-DDD show that there are severe side effects, such as gastrointestinal irritation and CNS toxicity (80% and 40% of patients respectively) (EMEA 2005). Symptoms of neurological toxicity include confusion, vertigo, depression and tremor (Hutter and Kayhoe 1966; Van Slooten et al. 1984; Bollen and Lanser 1992; Haak et al. 1994; Gicquel et al. 2001; EMEA 2005). The therapeutic efficacy of \( o,p' \)-DDD seems to be inadequate in a majority of patients and there are also problems with poor compliance. A retrospective study of published reports on ACC patients treated between 1972 and 1992 concluded that only 35% of tumours responded to treatment with \( o,p' \)-DDD (Wooten and King 1993). Protocols combining \( o,p' \)-DDD with other cytostatics like cisplatin, doxorubicin, etoposide have been introduced, with variable results (Berruti et al. 1998; Bonacci et al. 1998; Khan et al. 2000). \( o,p' \)-DDD seems to be a P-glycoprotein antagonist \textit{in vitro}, and has been used also in order to inhibit the drug efflux of for example doxorubicin, vincristine and etoposide in ACC cells (Khan et al. 2000; Abraham et al. 2002). Since ACC is a rare disease, little research has been focused on developing new therapeutic alternatives.

3-MeSO\(_2\)-DDE

Two methyl sulphones are formed from \( p,p' \)-DDE: 3-MeSO\(_2\)-DDE and 2-MeSO\(_2\)-DDE (Weistrand and Noren 1997). These compounds were classically found in adipose tissue (Jensen and Jansson 1976), but 3-MeSO\(_2\)-DDE is also present in human milk as well as in liver and blood serum in humans, polar bears and seals (Letcher et al. 1995; Weistrand and Noren 1997; Noren and Meironyte 2000; Chu et al. 2003; Larsson et al. 2004; Verreault et al. 2005; Jorundsdottir et al. 2006). 3-MeSO\(_2\)-DDE is a lipophilic compound, which can be transferred via mothers milk and decrease plasma corticosterone levels in suckling mice pups (Jönsson et al. 1992; Jönsson 1994). 3-MeSO\(_2\)-DDE is also transferred over the placenta and taken up in the adrenal cortex of mouse foetuses (Jönsson et al. 1995).

3-MeSO\(_2\)-DDE causes severe toxicity in the adrenal cortex of mice (Figure 2), resulting in extensive cell death specifically in the glucocorticoid-producing \textit{zona fasciculata} (Lund et al. 1988). Autoradiography revealed a highly specific irreversible metabolite binding to the adrenal cortex, which could not be demonstrated for the analogous substance 3,3’-(bis)MeSO\(_2\)-DDE (Lund et al. 1988). A high irreversible binding of 3-MeSO\(_2\)-DDE has also been demonstrated in mouse adrenal homogenates (300g supernatants) and this binding was decreased by addition of glutathione and the CYP-inhibitors metyrapone or carbon monoxide to the incubation medium (Lund et al. 1988). In a following study in mice, the mitochondria were proposed as the primary target for 3-MeSO\(_2\)-DDE induced toxicity in the adrenal cortex.
It was shown that mitochondrial damage arises after 6 h following a single i.p. dose as low as 3 mg/kg body weight (b.w.) (Jönsson et al. 1991). In addition, irreversible binding to adrenal homogenates was higher in the mitochondrial than in the microsomal fractions and the binding could be inhibited by metyrapone and the corticosterone precursor 11-deoxycorticosterone (11-DOC) (Jönsson et al. 1991). This was the first time CYP11B1 was proposed to contribute to the specific binding and toxicity. It was later confirmed that CYP11B1 indeed bioactivates 3-MeSO2-DDE into a reactive intermediate, which can bind covalently to adrenocortical proteins (Lund and Lund 1995). The structure of the putative reactive intermediate and the target proteins of the covalent binding are, however, still unknown.

![Figure 2](image)

\textit{Figure 2.} Histological sections of the adrenal cortex 24 hours after a single injection of (A) vechicle (corn oil) or (B) 50 mg/kg 3-MeSO2-DDE in mice.

The adrenal toxicity of 3-MeSO2-DDE has been investigated using several methods. Initially, investigations were performed \textit{in vivo} in mice, and \textit{in vitro}, using adrenal homogenates both from mice (Lund et al. 1988; Jönsson et al. 1991) and humans (Jönsson and Lund 1994). Later, the effects on hormone production were investigated in the murine adrenocortical cell line Y-1 (Johansson et al. 1998) and the human adrenocortical cell line H295R (Johansson et al. 2002). Studies of binding and toxicity \textit{ex vivo} were enabled by development of a test system based on precision-cut adrenal tissue slices from humans and other species (Lindhe et al. 2001; Lindhe et al. 2002). Using this system, adrenal slices of a standardised thickness are kept in culture with maintained cell viability and continuous hormone production up to 48 h. The CYP-dependent irreversible binding, mitochondrial damage and corticosterone inhibition by 3-MeSO2-DDE in the mouse adrenal \textit{zona fasciculata} have been reproduced in mouse adrenal slices (Lindhe et al. 2001). Furthermore, reduced CYP11B1 activity and binding of 3-MeSO2-DDE in \textit{zona fasciculata} and \textit{reticularis} have subsequently been demonstrated also in human adrenal tissue slices \textit{ex vivo} (Lindhe et al. 2002).
The human adrenal cortex and 3-MeSO₂-DDE

The effects of 3-MeSO₂-DDE in the human adrenal cortex in vivo is crucial since 3-MeSO₂-DDE is a suggested lead compound for an improved treatment of ACC and Cushing’s syndrome and as an imaging agent for diagnosis of adrenocortical disorders using PET (Lindhe et al. 2002). In addition, since 3-MeSO₂-DDE is a metabolite of the persistent environmental pollutant p,p’-DDE and is ubiquitously present in human tissues, it is relevant to gather information for human toxicological risk assessment. For obvious reasons, studies on the human adrenal gland can not be performed in vivo. Human adrenal tissue for experiments in vitro and ex vivo is difficult to obtain. To predict the sensitivity of the human adrenal cortex by extrapolating data from other species is difficult, since major species differences have been shown regarding irreversible binding and toxicity of 3-MeSO₂-DDE. Binding and toxicity of 3-MeSO₂-DDE in glucocorticoid-producing cells of chicken and polar cod have been reported (Jönsson et al. 1994, Lindhe et al. unpublished), while adrenal tissue homogenates of mink and otter are devoid of binding (Jönssson et al. 1993; Lindhe et al. unpublished). The project strategy used to elucidate the sensitivity of the human adrenal cortex to 3-MeSO₂-DDE-induced toxicity is illustrated in Figure 3.

Figure 3. Overview of strategy to approach the question whether 3-MeSO₂-DDE is an adrenal cortex toxicant in humans. By collecting data from mice using different techniques the correlation between in vitro/ex vivo and in vivo data can be assessed.
As shown in Figure 3, data from studies on mouse adrenal cortex is used for an improved understanding of how well *in vitro/ex vivo* data correlates with *in vivo* results. A plausible estimation of the corresponding *in vitro/in vivo* correlation in humans could hopefully be obtained in this way. *In vitro* and *ex vivo* results available so far indicate that the human adrenal may be sensitive to 3-MeSO₂-DDE toxicity (Jönsson and Lund 1994; Johansson et al. 2002; Lindhe et al. 2002).
OBJECTIVE AND AIMS

The overall objective of this thesis was to further examine and characterise the adrenocortical toxicity of 3-MeSO₂-DDE, since this substance is an environmental pollutant present in fat tissue in marine animals and humans, but also since it is suggested as a lead compound for an improved chemotherapy of adrenocortical carcinoma (ACC). In addition, the murine adrenocortical cell line Y-1 was evaluated as a possible test system for studies of 3-MeSO₂-DDE, \textit{o,p'}-DDD and other structurally similar compounds.

The specific aims were:

- to investigate and compare the pharmacokinetics of 3-MeSO₂-DDE and \textit{o,p'}-DDD in a mammalian species with large body fat stores (paper I).
- to determine the mode of 3-MeSO₂-DDE-induced cell death in the mouse adrenal cortex by investigating the expression of some caspases, heat shock protein 70 (HSP70) and some other crucial cellular events (paper IV).
- to investigate and compare CYP-catalysed irreversible binding of 3-MeSO₂-DDE and \textit{o,p'}-DDD in Y-1 cells (paper II).
- to examine and compare effects on glucocorticoid synthesis and cell toxicity in Y-1 cells exposed to 3-MeSO₂-DDE, \textit{o,p'}-DDD and some structural analogues (paper III).
- to investigate species differences in adrenocortical binding of 3-MeSO₂-DDE, and if sensitivity of species can be predicted (paper V).
MATERIALS AND METHODS

Chemicals

The chemical structures of the substances investigated are shown in Figure 4. 2-(4-chloro-3-methylsulphonylphenyl)-2-(4-chloro-[14C]phenyl)-1,1-dichloro-ethene (3-MeSO2-[14C]DDE, specific activity 13.0 μCi/μmol) and 2-(2-chlorophenyl)-2-(4-chloro-[14C]phenyl)-1,1-dichloroethane (o,p′-[14C]DDD, specific activity 11.2 μCi/μmol) were synthesized and kindly provided by Dr Åke Bergman, Department of Environmental Chemistry (Stockholm University, Sweden). o,p′-DDD was obtained from Sigma-Aldrich (St Louis, MO, USA) and 2,2-bis(4-chlorophenyl)-1,1-dichloroethene (p,p′-DDE) was from EGA-Chemie (Steinheim/Albuch, Germany). 3-MeSO2-DDE, 2-(2-methylsulphonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (2-MeSO2-DDE), 2-(4-chlorophenyl-3-sulfonyl acid)-2-(4-chlorophenyl)-1,1-dichloroethene (3-SO2OH-DDE), 2-(2-chlorophenyl)-2-(3-methyl-sulfonyl-4-chlorophenyl)-1,1-dichloroethene (3-MeSO2-o,p′-DDE), 2-(3-nitro-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-NO2-DDE), 2-(3-amino-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-NH2-DDE), 2-(4-chlorophenyl-3-sulfonyl amide)-2-(4-chlorophenyl)-1,1-dichloroethene (3-SO2NH2-DDE), 2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1-chloroethene (3-MeSO2-DDMU) and 2,2′-bis(3-methylsulphonyl-4-chlorophenyl)-1,1-dichloroethene (3,3′-(bis)MeSO2-DDE) were synthesized by Synthelec AB (Lund, Sweden). The purity of the radioactive substances was >98%. For all other substances purity was >99%.

Animals

All animal experiments were approved by the Local Ethics Committee for Research on Animals. Female C57Bl mice and female Sprague-Dawley rats were obtained from former B&K (Scanbur BK AB, Stockholm, Sweden). Female Syrian Hamsters were from Charles River (Sulzfeld, Germany) and female Dunkin Hartley guinea pigs from HB Lidköpings Kaninfarm (Lidköping, Sweden). Animals were kept with 12 h light and 12 h dark cycle,
given a standard pellet diet and tap water *ad libitum*. All experiments were preceded by at least one week of acclimatization. Ten female Göttingen minipigs (15.4-19.8 kg) were purchased from Ellegaard (Dalmose, Denmark), and kept at the Department of Clinical Sciences at the Swedish University of Agricultural Sciences (Uppsala, Sweden), housed in pens, given a standard low calorie diet (Maintenance diet for minipigs, Special Diet Services, Witham, England) and free access to water. The minipigs were acclimatised for three weeks before administration of substances.

*Figure 4.* Chemical structures of the substances investigated: a) 3-MeSO$_2$-DDE, b) $o,p'$-DDD, c) 2-MeSO$_2$-DDE, d) $p,p'$-DDE, e) 3-MeSO$_2$-$o,p'$-DDE, f) 3-NO$_2$-DDE, g) 3-MeSO$_2$-DDMU, h) 3-NH$_2$-DDE, i) 3-SO$_2$OH-DDE, j) 3-SO$_2$-NH$_2$ and k) 3,3’-(bis)MeSO$_2$-DDE.
Y-1 cell line

The mouse adrenocortical Y-1 cell line (CCL-79) was obtained from the American Type Culture Collection (ATCC). The cells were kept in a humidified atmosphere at 37°C and 5% CO₂. They were maintained in Ham’s F12K medium containing 1.2 g/L sodium bicarbonate and 2.5 mM L-glutamine (ATCC), supplemented with 15% horse serum (Sigma-Aldrich) and 2.5% fetal bovine serum (Sigma-Aldrich). The Y-1 cell line expresses several steroidogenic enzymes (e.g. CYP11B1, CYP11A1), but lacks the capability to express CYP21 (Parker et al. 1985). This means that the cells do not produce corticosterone unless the precursor 11-deoxycorticosterone (11-DOC) is provided. Also, the present Y-1 cells provided by ATCC are unresponsive to ACTH (Rainey et al. 2004), and instead the cAMP inducer forskolin is used (Seamon et al. 1981; Rice et al. 1989; Wong et al. 1989).

Irreversible binding assay (paper II)

Irreversible binding of 3-MeSO₂-DDE or o,p’-DDD to Y-1 cells was determined according to the method of Wallin et al (Wallin et al. 1981) with modifications. Y-1 cells were grown in 6-well plates until they reached about 50% confluency. The cells were stimulated with 10 μM forskolin in complete growth medium for 24 h. 3-MeSO₂-[¹⁴C]DDE or o,p’-[¹⁴C]DDD in serum-free medium were then incubated with the cells. To modify the binding, the cells were pre-incubated with CYP-inhibitors or 3-MeSO₂-DDE analogues for 30 minutes. During investigations of the binding over time, 10 μM substance was incubated for 7.5 min-8 h and for studies of concentration-dependent binding 2.5-20 μM substance was incubated for 6 h. Cells were then collected by scraping and centrifuged at 4000g for 10 minutes and the pellets were resuspended in PBS. The cell homogenates (100 μl) were transferred to microfibre filter papers (Whatman GF/C, diameter 2.5 cm). To remove not irreversibly bound substance, the filters were extracted in 95% ethanol, 2 x methanol, 2 x acetone and n-heptane. They were then dried, immersed in 10 ml Ultima Gold™ (Packard Instrument, USA) and the radioactivity was counted in a liquid scintillation analyser (Tri-Carb 1900CA, Packard Instrument, USA). Protein concentrations were measured using a BCA Protein Assay Kit (Nordic Biolabs), and the irreversible binding was expressed as pmol bound substance/mg protein.

NP-SH determination (paper II)

The amount of non-protein sulphhydryls (NP-SH) in Y-1 cells was measured with and without a 24 h exposure to the glutathione synthesis inhibitor...
buthionine sulfoximine (BSO, 1 mM) to investigate the maximal reduction of NP-SH. Assumingly, the measured NP-SH in Y-1 cells consist of glutathione (GSH), since it has been reported that NP-SH are exclusively represented by GSH in the rat adrenal (Sedlak and Hanus 1982). The cells were grown in 25 cm² flasks and exposed to BSO or vehicle (water) for 24 h. Preparation of the cells was then performed as in Ikediobi et al. 2004 (Ikediobi et al. 2004). The proteins of the cells were precipitated with 50% trichloroacetic aid (TCA) and the samples were centrifuged (12 000g, 5 min). The concentrations of NP-SH were determined according to Sedlak and Lindsay (Sedlak and Lindsay 1968). Protein concentrations were measured using a BCA Protein Assay Kit (Nordic Biolabs). The concentration of NP-SH was expressed as nmol NP-SH/mg protein.

MTT assay (paper II, III, IV)

This cell viability assay was used in Y-1 cells, both to evaluate toxicity (paper III) and to reassure that the irreversible binding and ATP assays were carried out under conditions of no observed overt toxicity (paper II, IV). Consequently, the assay was run in parallel with other assays, using the treatment and exposure protocol of the respective assay. The assay was performed essentially as by Tada et al (Tada et al. 1986). Cell viability was determined during the last two hours of incubation with the test substances by the addition of 20 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, 5 mg/ml in PBS) to each well. The formed formazan crystals were dissolved with 100 µl 10% SDS, 0.01 M hydrochloric acid followed by an overnight incubation at 37°C, 5% CO₂.

Absorbance was measured at 560 nm in a Wallac Victor3.

11β-hydroxylation assay (paper III)

The assay measures the ability of Y-1 cells to convert the precursor 11-DOC into corticosterone, a reaction that requires 11β-hydroxylase activity mediated by CYP11B1. Y-1 cells were seeded in 24-well plates, 24 h before assay. Cells were preincubated with 10 µM forskolin or DMSO in complete growth medium for 24 h. The preincubation medium was then removed and 500 µl test solution (i.e. 3-MeSO₂-DDE, 2-MeSO₂-DDE, 3,3’-(bis)MeSO₂-DDE, p,p’-DDE, o,p’-DDD, etomidate or vehicle) was added before 24 h of incubation at 37°C, 5% CO₂. Three hours before the end of the incubation, 20 µM ³H-11-deoxycorticosterone (0.37 kBq) was added to each well. At the end of the incubation period, the medium was collected and stored at -20°C until analysis. The corticosterone analysis was performed mainly as by Bureik et al (Bureik et al. 2002b). Medium samples were thawed at room tem-
perature and extracted three times with 1 volume diethyl ether. The organic phases from the three extractions were pooled, the solvent was evaporated and samples were resuspended in 40 μl ethanol. The samples and 11-DOC and C standards (1 mM in ethanol) were loaded onto aluminium-backed silica-coated thin-layer chromatography (TLC) plates (silica gel 60 F254 20 x 20 cm). Separation was performed with chloroform:methanol:water (300:20:1) as mobile phase. The corticosterone was detected with ultra-violet light and cut out. The silica gel was scraped into plastic scintillation vials, incubated with 1 ml methanol for 3 h at 40°C and counted in 10 ml Ultima Gold™ (Packard Instrument, USA) using a liquid scintillation analyser (Tri-Carb 1900CA, Packard Instrument, USA).

ATP assay and Hoechst DNA staining (paper IV)

The ATP assay is based on the following reaction:

\[
\text{Mg}^{2+} + \text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{pyrophosphate} + \text{CO}_2 + \text{light}
\]

Y-1 cells were grown in 24-well plates in complete growth medium 24 h before assay. After various treatments of the cells, the medium was removed and the cells were washed with PBS before extraction of ATP with boiling water (Yang et al. 2002). The cell suspensions were centrifuged for 5 minutes (12 000g, 4°C) before measuring the ATP content in the supernatants using an ATP Detection Kit (Invitrogen). The luminescence of the luciferin-luciferase reaction above was measured in a Wallac Victor3.

Hoechst staining of DNA in Y-1 cells was also performed in 24-well plates. Cells were grown on glass cover slips in complete growth medium for 24 h before exposure to 3-MeSO2-DDE, staurosporine or DMSO. The cells were rinsed and fixed in 4% paraformaldehyde before staining with Hoechst 33342 (Sigma-Aldrich). Finally, the cover slips were mounted with 50% glycerol and the staining was viewed under a Leica DMRXE fluorescence microscope.

Histopathology (paper I, II, IV)

Adrenals from minipigs (paper I) or mice (paper II, IV) were excised, fixed in 4% phosphate-buffered formaldehyde (pH 7.4) and embedded in Historesin (Kulzer Histo-Technik, Germany). The adrenals were sectioned (2 μM) and stained with hematoxylin/eosin. Sections were investigated using a Leica DMRXE light microscope.
Immunohistochemistry (paper IV)

Adrenals from mice were excised and fixed in 4% phosphate-buffered formaldehyde (pH 7.4). They were embedded in low-melting temperature paraffin and sectioned (4 μm). The sections were deparaffinized with xylene, hydrated in ethanol 99.5% and ethanol 95%, washed twice in water and once in PBS. For antigen retrieval, sections were steamed (97-98°C) for 30 minutes in 10 mM sodium citrate buffer (pH 6.0). Subsequent washes in H₂O were followed by 10 minutes of incubation with 1% H₂O₂ in H₂O to inactivate endogenous peroxidases. The sections were then incubated with 5% normal goat serum in PBS for 1 h for blocking of non-specific binding. Endogenous biotin was blocked using an avidin/biotin blocking kit. The sections were incubated with the primary antibody in a humidified chamber overnight (4°C). The following morning, the sections were rinsed carefully with PBS to remove the primary antibody, before incubating with the biotinylated secondary antibody for 30 minutes, and the StreptABComplex/HRP for 30 minutes. Finally, the sections were incubated with 3,3'-diaminobenzidine (DAB). As controls, sections were incubated with only primary or secondary antibody or no antibody at all.

Antibodies used

The primary antibodies for caspase-12, caspase-9 and HSP70, were diluted 1:150 and the caspase-3 antibody was diluted 1:200. Caspase-3 is constitutively expressed in zona reticularis, which served as an internal control for caspase-3. The caspase-3 antibody only detects the cleaved large fragment of activated caspase-3 and does not recognise full length caspase-3 or other caspases. The HSP70 antibody does not cross-react with other HSPs. The caspase-9 and caspase-12 antibodies react with both the active and inactive proteins.

Adrenal tissue slice culture (paper V)

The tissue slice method for adrenals was performed as previously described (Lindhe et al. 2001). Briefly described, animals (mice, rats, guinea pigs and hamsters) were killed with CO₂, and the adrenals were rapidly excised and kept in ice-cold phosphate buffer (PBS, pH 7.4). They were embedded in 3% agarose and 200 μm slices were prepared in a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) in ice-cold PBS. The slices were then cultured in rotating 6-well plates (1 rpm) in fully supplemented Dulbecco’s modified Eagle’s medium (FDMEM, 2.5 ml) in 37°C and 5% CO₂. 3-MeSO₂-[¹⁴C]DDE (in DMSO, 3.7 MBq/ml) was added to each well, and the slices were further incubated for 24 h.
Microautoradiography and Phosphor Imaging (paper V)

Following incubation of the adrenal tissue slices, they were fixed in phosphate-buffered formaldehyde (4%) overnight and dehydrated in an ethanol series (70%, 90%, 96% and 99%). During embedding in methacrylate, the slices were oriented with the sliced plane parallel to the sectioning plane and then the slices were sectioned (2 μm) in a microtome (HM 360; Mikrom Laborgeräte GmbH, Walldorf, Germany). Glass slides carrying the sections were dipped in NTB2 liquid film emulsion (Kodak) and exposed for 6 months in the dark (4°C). The autoradiograms were developed, stained with toluidine blue and examined in a Leica DMRXE light microscope. Both dark-field and light-field photographs of the sections were taken with a digital camera (Hamamatsu Orca IIIm).

Slides containing histological adrenal sections from each species were placed on an imaging plate (BAS-IP MP 2040S; Fuji, Japan) and exposed for two years. A semi-quantification of the tissue-bound 3-MeSO\textsubscript{2}-[1\textsuperscript{4}C]DDE was performed using the program MultiGauge (version 2.2, Fujifilm, Japan). The values were calculated from selected regions of interest (ROI) and expressed as phosphostimulated luminescence (PSL) per square millimeter (mm\textsuperscript{2}), with the background radioactivity (BG) subtracted (PSL-BG/mm\textsuperscript{2}).

Pharmacokinetic study design and data analysis (paper I)

The minipigs were divided in two groups of five and housed in two separate pens. Blood samples were collected from vena jugularis before exposure. The minipigs were administered a single oral dose of 3-MeSO\textsubscript{2}-DDE or o,p\textsuperscript{-}DDD (30 mg/kg b.w.) in corn oil. After administration, blood samples were collected at 0.5, 1, 3, 8, 24, 48 h and 4, 10, 30, 60, 90, 120 days. At 180 days after administration the study was terminated, and a blood sample was drawn from the heart after anaesthesia. At 30, 60, 90, 120, and 180 days after administration a subcutaneous fat sample was collected from the chin after local anaesthesia with mepivacain (Carbocain\textsuperscript{®} 2%, AstraZeneca AB). Samples from the adrenals, liver and abdominal fat were collected at 180 days. The concentrations of 3-MeSO\textsubscript{2}-DDE and o,p\textsuperscript{-}DDD were determined in plasma, fat and liver samples using gas chromatography, as described in detail in paper I. Pieces of adrenal and liver were embedded in methacrylate and stained with hematoxylin/eosin for light microscopic examination.

Pharmacokinetic parameters were calculated from plasma and subcutaneous fat data by non-compartmental analysis using the software program WinNonlin version 5.0.1 (Pharsight Corporation, CA, USA). The slope of the terminal phase, lambda z (\textit{\lambda}_z), was calculated with regression analysis using data points from 10-180 days, with exclusions specified in paper I. The
terminal half lives ($t^{1/2}$) were determined using $\lambda_2$. Since the bioavailabilities ($F$) of 3-MeSO$_2$-DDE and $o,p'$-DDD were unknown, the volume of distribution of the terminal phase and clearance were expressed as $V_z/F$ and $CL/F$ respectively. The area under curve (AUC) was calculated using the trapezoidal rule.

**Statistical analysis (paper II-IV)**

All data generated from the Y-1 cell line was assumed to be normally distributed. Data on irreversible binding (paper II) was analysed using Graph Pad Prism (version 3.03). Vehicle-treated cells were compared with cells treated with different substances with a paired ratio t-test. The corticosterone production results (paper III) were evaluated with Graph Pad Prism (version 4.0) using one-way ANOVA and Dunnett’s multiple comparison test. MTT data in paper III was evaluated with the program R by linear regression. The regression slopes of different compounds were compared with a one-sample t-test. To test the influence on cell viability of etomidate, the regression slopes were compared with a two-sample paired t-test. Immunohistochemistry data (paper IV) was evaluated using Graph Pad Prism (version 4.0) with one-way ANOVA and Tukeys post-hoc test. The ATP measurements (paper IV) were analysed with a paired ratio t-test using Graph Pad Prism (version 4.0).
RESULTS AND DISCUSSION

The fate of 3-MeSO₂-DDE in the body

The DDT metabolite \( p,p' \)-DDE is still the most abundant persistent halogenated hydrocarbon retained in tissues of humans and wildlife (Hagmar et al. 2001; Moore et al. 2002; Hovander et al. 2006). Despite a high resistance to metabolism, \( p,p' \)-DDE is slowly transformed to hydroxylated and methylsulphonated metabolites (Fawcett et al. 1987; Weistrand and Noren 1997). Among these metabolites, 3-MeSO₂-DDE has been detected in various tissues of marine animals, polar bears and humans (Letcher et al. 1995; Weistrand and Noren 1997; Karlson et al. 2000; Troisi et al. 2001; Chu et al. 2003; Larsson et al. 2004; Verrreault et al. 2005; Karasek et al. 2007; Lindholm et al. 2007) as well as in human milk (Newsome and Davies 1996; Noren et al. 1996). These findings could either be due to high persistency of 3-MeSO₂-DDE or a more rapid continuous metabolic formation from \( p,p' \)-DDE and a subsequent metabolism/excretion from the body. The disposition of 3-MeSO₂-[\(^{14}\)C]DDE has previously been examined with autoradiography in mice, mainly to determine the CYP-catalysed formation of an irreversibly bound metabolite in the adrenal cortex (Lund et al. 1988). To my knowledge, the distribution of administrated 3-MeSO₂-DDE in a large animal has not been examined. Also, there is no data describing the long-term kinetics of 3-MeSO₂-DDE. A pharmacokinetic study not only shows whether 3-MeSO₂-DDE can be metabolised/excreted, but also contributes to the characterisation of 3-MeSO₂-DDE as a potential therapeutical agent.

Pharmacokinetics of 3-MeSO₂-DDE and \( o,p' \)-DDD

In paper I, the behaviour of 3-MeSO₂-DDE during 6 months, following an oral administration to a large animal with physiological similarities to humans, was investigated. The Göttingen minipig was selected as a suitable animal model because of its size, body composition (large body fat depots) and since this species is often used in pharmacological and toxicological studies (Bollen and Ellegaard 1997). For comparative reasons, the pharmacokinetics of the current drug for ACC, \( o,p' \)-DDD (mitotane) was also examined. The minipigs were administered 3-MeSO₂-DDE or \( o,p' \)-DDD (30
mg/kg b.w.) as a single oral dose. Blood plasma and subcutaneous fat samples were collected up to 180 days after dosing. Blood samples taken before administration showed that no detectable amounts of 3-MeSO₂-DDE or o,p′-DDD were present. As shown in Figure 5A, the plasma concentrations of both substances increased rapidly after administration and reached maximal levels within 24 hours. The maximum plasma concentration (C_max) was about two times higher for 3-MeSO₂-DDE than for o,p′-DDD. During the following days, the substances were distributed to other tissues or were eliminated. Considerable levels of both substances were found in the subcutaneous fat samples. The concentration of 3-MeSO₂-DDE in fat was about 25 times higher than for o,p′-DDD 30 days after administration. High levels of 3-MeSO₂-DDE, but not of o,p′-DDD, were also noted in the liver at 180 days, exceeding the plasma levels about 18 times, but still about 20 times lower than in fat tissue. No histopathological lesions were observed in the adrenals or livers in any of the individuals. The concentrations of the substances in the subcutaneous and abdominal fat samples at 180 days were equal.

Terminal half-life (t½)/mean residence time (MRT) of 3-MeSO₂-DDE and o,p′-DDD in plasma were calculated to 50/56 and 28/12 days respectively. In contrast to terminal t½, the mean residence time (MRT) was calculated from the whole plasma vs time curve. The terminal t½ is often overestimated if the measured concentrations are close to the detection limit, as was the case for o,p′-DDD in plasma. Therefore, MRT, which is less influenced by this factor, could be a more appropriate parameter in this particular case. The shorter plasma t½ of o,p′-DDD was reflected by the CL/F, which was 60 times larger than for 3-MeSO₂-DDE. Both substances had large V₀/F values, especially o,p′-DDD. This indicated a higher lipophilicity and higher relative accumulation in lipophilic compartments of o,p′-DDD than of 3-MeSO₂-DDE. In subcutaneous fat, t½ was estimated to 52 days for 3-MeSO₂-DDE and 22 days for o,p′-DDD.

Figure 5. A: Concentrations in plasma during the first 10 days after a single oral dose of 30 mg/kg b.w. of 3-MeSO₂-DDE (■) or o,p′-DDD (○) to minipigs. B: The influence of body weight (●) on the plasma concentration in one individual minipig given 3-MeSO₂-DDE (■).
The elimination of both 3-MeSO₂-DDE and o,p'-DDD in the minipigs was slow. However, there are known biotransformation pathways for o,p'-DDD, such as formation of hydroxylated products (Reif et al. 1974), o,p'-DDA which is readily excreted in urine (Sinsheimer et al. 1972), o,p'-DDE (Andersen et al. 1999) and a methylthio-containing metabolite (Inouye et al. 1989). No elimination pathway has yet been shown for 3-MeSO₂-DDE, but one possibility could be non-biliary intestinal excretion, i.e. direct transfer from blood to the gut lumen, which several other highly lipophilic compounds undergo (Page and Carlson 1994).

Considering the use of o,p'-DDD as a drug for over 40 years, the existing pharmacokinetic data for this substance is surprisingly limited. Besides plasma monitoring of ACC patients, most pharmacokinetic information originates from one single study published in 1961 (Moy 1961). The present study in minipigs is the first long-term study of o,p'-DDD following a single dose. It is interesting to note that a single dose of this drug, which is considered to be completely eliminated within days and with an elimination t½ of 2-3 h (Moolenaar et al. 1981), still remains in plasma and subcutaneous fat of the minipigs after 6 months. ACC patients are often administered high doses o,p'-DDD, normally 2-6 g/day for months in order to reach therapeutical plasma levels (Allolio et al. 2004; EMEA 2005). The fat tissue of such patients is loaded with considerable o,p'-DDD concentrations and the resulting terminal plasma t½ varies from 18-159 days (Moolenaar et al. 1981; van Slooten et al. 1982). During monitoring of the weight of the minipigs, we noted a weight loss in 4 pigs given 3-MeSO₂-DDE and in one pig given o,p'-DDD, two months after administration. At the same time point, the plasma concentrations in these pigs reached a second peak. It was evident that there was a correlation between plasma concentrations of the substances and the amount of body fat. This effect is illustrated in Figure 5B. Loss of weight in a minipig gave rise to a simultaneous plasma concentration peak of o,p'-DDD. Subsequently, a rapid weight loss in a patient with a lot of o,p'-DDD stored in fat tissue could result in large amounts of substance released from fat into the blood stream. This could certainly have toxic consequences for the patient due to the narrow therapeutical window of the drug. Previous human data shows that 3-MeSO₂-DDE and o,p'-DDD are accumulated in body fat (Bergenstal et al. 1960; Weistrand and Noren 1997; Chu et al. 2003).

High levels of 3-MeSO₂-DDE were detected in the livers of the minipigs. This accumulation in the liver could be due to protein binding, since the methylsulphonyl group of several xenobiotics seems to give rise to tissue-selective binding (Brandt and Bergman 1987). For example MeSO₂-PCBs are known to associate with ligand-binding proteins in different organs of experimental animals (Brandt et al. 1985; Lund et al. 1985). Considerable amounts of 3-MeSO₂-DDE have previously been detected in mammalian liver calculated on a lipid weight basis (Karlson et al. 2000; Chu et al. 2003;
Larsson et al. 2004). This has also been interpreted as retention of 3-MeSO₂-DDE in the liver due to protein binding, since the levels in the liver based on lipid weight exceed the fat concentrations manifold.

The detected 3-MeSO₂-DDE in tissues of marine mammals and humans could probably be a result of continuous formation from \( p,p' \)-DDE in the body. It can, however, be concluded from the present study that 3-MeSO₂-DDE is very persistent in itself due to lack of rapid elimination pathways and since 3-MeSO₂-DDE remained for months in the minipigs, despite absence of \( p,p' \)-DDE.

The fate of 3-MeSO₂-DDE in the adrenal gland

The knowledge about the cellular events between the metabolic activation, of 3-MeSO₂-DDE and adrenocortical cell death is limited. Also, there are known species differences regarding the adrenocorticolytic activity of 3-MeSO₂-DDE. While the mouse is the most sensitive species studied, no toxicity of 3-MeSO₂-DDE has been detected in the rat adrenal cortex (unpublished data from the group). Toxicity of 3-MeSO₂-DDE has also been shown in the chicken adrenal cortex (Jönsson et al. 1994). Consequently, the mechanisms of toxicity, species differences and effects of 3-MeSO₂-DDE were further investigated.

Bioactivation and irreversible binding of reactive intermediate

Generally, lipophilic xenobiotics are enzymatically transformed into hydrophilic compounds by phase I and phase II enzymes and readily excreted from the body. However, if these enzymatic reactions instead form a toxic reactive intermediate (which may interact with DNA or proteins), this is termed bioactivation. 3-MeSO₂-DDE is bioactivated by CYP11B1 in the mouse adrenal cortex (Lund and Lund 1995), but the cellular events following this bioactivation, leading to cell death, are unknown. The small size, influences from other organs and feed-back regulatory mechanisms in the body make \textit{in vivo} investigations of the adrenal cortex complicated and the results difficult to interpret. Therefore, to enable further exploration of the cellular mechanisms preceding 3-MeSO₂-DDE-induced cell death, we investigated the usefulness of the murine adrenocortical Y-1 cell line as a test system. The first step in this evaluation was to reproduce and further characterise the CYP11B1-catalysed irreversible binding reported \textit{in vivo}, \textit{ex vivo} (tissue slice) and \textit{in vitro} (adrenal homogenates), using a modified version of a method originally developed by Wallin and co-workers (Wallin et al. 1981). As a comparison, \( o,p' \)-DDD which does not bind to the mouse adrenal cortex \textit{in vivo} was examined (Lund et al. 1986).
The irreversible binding of 3-MeSO₂-DDE observed in vivo, was successfully reproduced in Y-1 cells (paper II). This binding was both time- and concentration-dependent as shown in Figure 6A and B. Surprisingly, also o,p'-DDD was irreversibly bound to Y-1 proteins, despite that this compound is not irreversibly bound to the adrenal cortex in vivo (Lund et al. 1986). Although the binding of o,p'-DDD and 3-MeSO₂-DDE reached a similar level (Figure 6A), the profiles of the binding curves differed markedly. As shown in Figure 6B, the binding of 3-MeSO₂-DDE started within minutes and increased rapidly, reaching almost a maximum binding after about one hour of incubation. In contrast, the binding of o,p'-DDD increased slowly during the eight hours of incubation. A difference was also found for the concentration-dependent binding. According to the concentration-binding curve, the binding of 3-MeSO₂-DDE seemed to be saturated at lower concentrations than that of o,p'-DDD (Figure 6A).

Figure 6. Irreversible binding of 3-MeSO₂-[¹⁴C]DDE (■) and o,p'-[¹⁴C]DDD (○) to forskolin-stimulated Y-1 cells at different concentrations (A) and time points (B). (Figures modified from paper II).

Exposure of Y-1 cells to forskolin (10 μM) for 24 h increased the binding of both 3-MeSO₂-DDE and o,p'-DDD about 3 times. This indicated that the binding was preceded by bioactivation of the substances by a forskolin-inducible enzyme. It was further shown that the binding of both substances was CYP-catalysed, since the CYP-inhibitors etomidate and ketoconazol decreased the binding extensively. Metyrapone inhibited the binding of 3-MeSO₂-DDE, but not significantly that of o,p'-DDD. Two CYPs are known to be induced by forskolin in Y-1 cells; CYP11A1 and CYP11B1 (Wong et al. 1989), and one or both of these enzymes should therefore be involved in the bioactivation of the test substances. Previous data shows that mouse CYP11A1 is unable to metabolise 3-MeSO₂-DDE (Lund and Lund 1995). Consequently, CYP11B1 is most probably the only CYP enzyme catalysing the bioactivation of 3-MeSO₂-DDE in Y-1 cells.

There are several factors that may explain the discrepancy of irreversible binding of o,p'-DDD to Y-1 cells, but not to the mouse adrenal cortex in vivo. For example, the o,p'-DDD concentration could be too low, or there
could be a discrepancy of bioactivating CYP isoforms in the mouse adrenal in vivo compared to Y-1 cells.

CYP11B1 is located in mitochondria, and the initial damage induced by 3-MeSO₂-DDE is observed in this organelle (Jönsson et al. 1991). Also, 3-MeSO₂-[¹⁴C]DDE has been found to bind fifty times more to mitochondrial than to microsomal/cytosolic fractions prepared from mouse adrenal homogenates (Jönsson et al. 1991). It can be assumed that mitochondrial proteins are the primary target for the reactive intermediate formed from 3-MeSO₂-DDE. In a preliminary study, this assumption was addressed in Y-1 cells. Y-1 cells were incubated with 3-MeSO₂-[¹⁴C]DDE and homogenised. The homogenate was centrifuged at 300g, then 9000g and finally 12000g. The 9000g pellet was presumed to contain mainly mitochondria and the 12000g supernatant was presumed to contain mainly microsomal/cytosolic proteins. A 2.4 times higher binding of 3-MeSO₂-[¹⁴C]DDE (pmol/mg protein) to the 9000g pellet than to the 12000g supernatant was observed, indicating that the reactive intermediate was mainly bound close to the bioactivation site in Y-1 cells. Using the same procedure, the binding of o,p’-[¹⁴C]DDD was 3.7 times higher to the 9000g pellet than to the 12000g supernatant. This preliminary data from the Y-1 cells, indicates that both bioactivated substances were mainly bound in the mitochondria, but were also transported from this organelle and bound to other proteins.

Bioactivation and species differences

A contributory cause to the previously shown species differences in 3-MeSO₂-DDE-induced adrenocortical toxicity could be the ability of each species to bioactivate and irreversibly bind 3-MeSO₂-DDE. The CYP11 enzymes catalysing 11β-hydroxylase activity in different species differ in amino acid sequences (Bureik et al. 2002a), and these enzymes could therefore differ in substrate specificity. In paper V, the adrenal binding of 3-MeSO₂-[¹⁴C]DDE in four rodent species; hamster, guinea pig, mouse and rat was investigated using the adrenal tissue slice technique. Microautoradiography and phosphor imaging were used for localization and quantification of binding of radioactivity in the adrenal sections. Extensive binding of 3-MeSO₂-[¹⁴C]DDE was found in the hamster adrenal cortex, confined to zona fasciculata, but intense spots of radioactivity were also detected in the adrenal medulla. As expected, substance was also bound to the mouse adrenal cortex, specifically zona fasciculata. In contrast, both rat and guinea pig adrenals were devoid of binding. Unlike the mouse adrenal, the hamster adrenal medulla contains clusters of cortical cells (Tucker 1996), which may express functional steroidogenic enzymes like CYP11B1. This may explain the binding in the hamster adrenal medulla.

The distribution of 3-MeSO₂-[¹⁴C]DDE in one hamster and one guinea pig has also been studied in vivo by autoradiography 48 h after an i.p. injec-
tion of 3-MeSO₂-[¹⁴C]DDE (0.075μCi/g b.w.) (unpublished data). At 48 h after administration, the radioactivity was located to fat tissue, liver, intestinal contents and the adrenal cortex in both species. Extractions of the tissue sections in a series of organic solvents aimed to reveal non-extractable, i.e. irreversibly bound substance. After extractions, only a very faint binding was left in the adrenal cortex of the guinea pig sections. In contrast, most of the substance remained in the hamster adrenal cortex after extractions. This discrepancy further supports the results from the studies in tissue slices ex vivo presented in paper V. If the species differences could be explained by identifying crucial amino acid sequences in the active site of CYP11B1, a prediction of sensitive species would be possible.

Effects on hormone production

The bioactivation of 3-MeSO₂-DDE by the corticosterone-producing enzyme CYP11B1 has previously been shown to cause disturbances in the corticosterone production both in mice in vivo (Jönsson 1993) and in Y-1 cells (Johansson et al. 1998). In paper III, an 11β-hydroxylation assay was modified from Bureik et al (Bureik et al. 2002b) and evaluated in the Y-1 cell line by testing etomidate, a potent CYP11B1 inhibitor. Also, the effect of forskolin was examined. Stimulation with forskolin (10 μM) for 24 h increased the corticosterone production (assumed CYP11B1 activity) nearly seven-fold compared to basal (not forskolin-induced) production. As shown in Figure 7A, etomidate efficiently blocked the forskolin-induced corticosterone production. Significant inhibition of basal corticosterone production was also observed.

![Figure 7. Production of corticosterone in forskolin-induced Y-1 cells after incubation with (A) p,p'-DDE (●), 2-MeSO₂-DDE (▼), 3,3'-(bis) MeSO₂-DDE (○), etomidate (●) or (B) 3-MeSO₂-DDE (■) or o,p'-DDD (○). The graphs show the mean±SE based on three independent experiments. Data was analysed by one-way ANOVA with Dunnett’s multiple comparison test. *=p<0.05 and **=p<0.01.](image-url)
Results of paper III revealed a significant concentration-dependent reduction of forskolin-induced corticosterone production by 3-MeSO₂-DDE in the Y-1 cell line (Figure 7B). Corticosterone was reduced to 28±6% (mean±SE) compared to controls by 3-MeSO₂-DDE (20 μM). In comparison, 20 μM o,p’-DDD reduced corticosterone production to 15±1% (mean±SE) of controls. Also, a concentration-dependent significant decrease of basal corticosterone production was noted by both 3-MeSO₂-DDE and o,p’-DDD (data not shown). The decreased corticosterone levels observed were probably not due to cytotoxicity, since no reduced cell viability (as shown by MTT assay) was present in the Y-1 cells. The inhibition of corticosterone production by these two substances is compatible with that they both bind irreversibly to Y-1 cells (paper II). The results supported that CYP11B1 participated in the bioactivation of both substances in Y-1 cells.

In addition, three 3-MeSO₂-DDE analogues were examined: 2-MeSO₂-DDE, p,p’-DDE and 3,3’-(bis)MeSO₂-DDE. The effects of these analogues on forskolin-induced corticosterone production are shown in Figure 7A. It is obvious from the graphs in Figure 7 that none of these analogues was as potent inhibitor of corticosterone production as 3-MeSO₂-DDE. Nevertheless, a significant concentration-dependent decrease of both basal and forskolin-induced corticosterone production by 3,3’-(bis)MeSO₂-DDE was noted. In forskolin-induced cells, maximal inhibition was to 67±2% of the concentration in control cells. 2-MeSO₂-DDE slightly reduced forskolin-induced corticosterone production (maximally to 75±4% of the concentration in control cells) but not basal production. The parent compound of 3-MeSO₂-DDE, p,p’-DDE, did not affect the corticosterone production.

Involvement of the GSH detoxification pathway

The tripeptide glutathione (GSH) (glutamate, cysteine, glycine) is involved in several cellular functions, such as acting as a co-factor for glutathione peroxidase and maintaining cytosolic proteins in a reduced state. The ratio between GSH and the oxidized form glutathione disulfide (GSSG) is normally >100. GSH conjugation is also one of the most important pathways of detoxification of xenobiotics and protects against oxidative stress (Anderson 1998). The adrenal gland has been reported to contain high GSH levels, in rats even exceeding the levels of the liver (Hornsby and Crivello 1983b). In the Y-1 cell line, GSH seemed to be important for the detoxification of the reactive intermediate of 3-MeSO₂-DDE, but had no effect on the irreversible binding of o,p’-DDD (paper II). This was shown by treating the cells with buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase and subsequently GSH synthesis. Following BSO treatment, the irreversible binding of 3-MeSO₂-DDE was doubled in Y-1 cells, while the binding of o,p’-DDD remained unchanged. These results are in accordance with earlier indications that GSH may be important to protect the adrenal cortex from
covalent binding and toxicity of 3-MeSO₂-DDE (Lund et al. 1988). In the adrenal cortex, o,p’-DDD, via a CYP-catalysed reaction, forms a reactive acyl chloride, which can bind covalently to proteins (Martz and Straw 1980; Cai et al. 1995b). We conclude that the acyl chloride was not detoxified by GSH conjugation in Y-1 cells.

The presumed GSH conjugation of the reactive 3-MeSO₂-DDE metabolite could decrease the available GSH and cause changes in the GSH/GSSG ratio. A correlation between GSH depletion and induction of stress proteins, particularly HSP70 has been shown (Filomeni et al. 2005; Fratelli et al. 2005; Hassen et al. 2007). Heat shock proteins are up-regulated following cellular stress and are key proteins in regulation of cell death (Jolly and Morimoto 2000; Garrido et al. 2001), as will be discussed below.

Cellular reactions and necrotic cell death

The cell death in zona fasciculata in mice following i.p. administration of 3-MeSO₂-DDE is well documented histopathologically, but less is known concerning what kind of cell death machinery is activated and the underlying mechanisms.

Using the MTT assay, a significant concentration-dependent cytotoxicity of 3-MeSO₂-DDE was observed in the Y-1 cell line (paper III). 3-MeSO₂-DDE (20 μM) was shown to reduce cell viability to 12±5% (mean±SE) of control levels (Figure 8A). In comparison, o,p’-DDD did not cause any changes in cell viability compared to control cells at doses <10 μM (Figure 8A). Only at the highest dose (20 μM) the viability was found to be slightly lowered compared to control cells. However, this decrease in cell viability was not significant.

Figure 8. (A) Cell viability of Y-1 cells after 72 h of incubation with 3-MeSO₂-DDE (■) or o,p’-DDD (○). The graph shows the mean ± SE based on four independent experiments. (B) ATP content in Y-1 cells after incubation with 10 μM 3-MeSO₂-DDE. The graph shows mean ± SD based on five independent experiments.
The CYP11B1 inhibitor etomidate counteracted the cytotoxicity from 3-MeSO₂-DDE, confirming the crucial interaction between CYP11B1 and 3-MeSO₂-DDE. Since we were able to reproduce CYP-dependent irreversible binding, corticosterone inhibition and cell death in Y-1 cells, this cell line was considered appropriate for studies of the mechanisms behind the 3-MeSO₂-DDE-induced toxicity.

Mechanisms of cell death have been widely discussed in the literature and there are several pathways described. The two classical modes of cell death are apoptosis and necrosis. Necrosis includes random DNA fragmentation, cell blebbing and rupture of the cell membrane, often followed by inflammation in surrounding tissue. This kind of cell death occurs due to for example oxidative stress or ATP depletion and has been described as a random, non-programmed kind of cell death. In contrast, apoptosis has generally been described as a programmed form of cell death, initiated by stimuli such as oxidative stress, death receptor activation or DNA damage (Malhi et al. 2006). Initiator caspases (caspase-2, -8, -9, -10, -12) are activated, resulting in activation of effector caspases (caspase-3, -6, -7) (Ashe and Berry 2003). A characteristic sign of apoptosis is chromatin condensation resulting from DNA fragmentation by caspase-activated endonucleases. Other cellular and nuclear proteins are degraded by caspases, but the cell membrane remains intact, avoiding an inflammatory response in surrounding tissue. The cell shrinks and a hallmark of apoptosis is the formation of apoptotic bodies, which are finally phagocytosed. The involvement of some crucial proteins in mitochondria-dependent apoptosis is illustrated in Figure 9.

Figure 9. Schematic illustration of some crucial steps in the mitochondrial pathway of apoptosis. Cyt C = cytochrome c
Mitochondria have previously been suggested as primary targets for 3-MeSO₂-DDE-induced toxicity in the mouse adrenal cortex. Therefore it was hypothesized that the 3-MeSO₂-DDE-induced cell death might be a result of mitochondria-dependent apoptotic signalling. Electron microscopy has earlier shown severe vacuolization of the mitochondria of zona fasciculata cells 6 h after an i.p. injection of 25 mg/kg b.w. 3-MeSO₂-DDE (Jónsson et al. 1991). This insult could cause a subsequent release of cytochrome c (Cyt C) from the mitochondrial intermembranal space. In the cytosol, Cyt C forms a complex (apoptosome) with the apoptotic protease activating factor-1 (Apaf-1). The interaction between Apaf-1 and Cyt C requires energy, ATP (Hu et al. 1999). The apoptosome further activates procaspase-9, which in turn can cleave the effector caspase-3.

In paper IV, we examined the immunohistochemical expression of three caspases (caspase-9, caspase-12 and caspase-3) 12, 24 and 48 h after an i.p. injection of 3-MeSO₂-DDE (50 mg/kg b.w.) in mice. An increase in caspase-9 staining was noted in the outer part of zona fasciculata, particularly in cell nuclei, 24 h after injection. This finding indicates that early apoptotic signals were initiated. However, a very weak but at no time point significant increase in caspase-3 staining in zona fasciculata was observed. Interestingly, in the inner zona reticularis (where no cell death has been observed) a marked increase in caspase-3 expression was evident. The lack of significant caspase-3 activation in zona fasciculata indicates that the apoptotic signalling was interrupted downstream the presumed Cyt C release and upstream caspase-3 activation. The ATP dependent apoptosome formation might be a critical factor. ATP measurements in the adrenal cortex in vivo are difficult to perform, without also measuring ATP from adrenal medulla cells and erythrocytes. The early effect on ATP levels was therefore determined in Y-1 cells following exposure to low concentrations (2.5-10 μM) of 3-MeSO₂-DDE (paper IV). The ATP levels decreased in the exposed cells, maximally to about 80% of control cells after exposure to 10 μM 3-MeSO₂-DDE for 6 h (Figure 8B). This implies that 3-MeSO₂-DDE rapidly reduces ATP levels and further apoptosome formation. HSP70 has been suggested to inhibit apoptosis by blocking both the oligomerization of Apaf-1 and the activation of procaspase-9 by a direct interaction with Apaf-1 (Saleh et al. 1999; Beere et al. 2000). A pronounced increase of HSP70 in the cytosol of the whole adrenal cortex was found, particularly in zona fasciculata. In this zone, HSP70 seemed to accumulate in the cell nuclei. HSP70 nuclear translocation has earlier been observed due to cellular stress (Welch and Feramisco 1984; Ohtsuka and Laszlo 1992; Abe et al. 1995; Knowlton and Salfity 1996; Gibney et al. 2004). As mentioned above, a GSH depletion by 3-MeSO₂-DDE could possibly contribute to the observed HSP70 induction. No increase in caspase-12 staining could be observed at any time point. Caspase-12 is normally activated during ER insult and then activates both procaspase-9 and procaspase-3 (Morishima et al. 2002; Szegezdi et al. 2003).
A typical morphological feature of apoptosis is the DNA fragmentation by endonucleases, which can be seen as characteristically condensed chromatin. Y-1 cells were exposed to the apoptosis-inducer staurosporine or a high concentration of 3-MeSO₂-DDE (20 μM) and the DNA fragmentation pattern was studied using Hoechst staining (paper IV). DNA in staurosporine-exposed cells was condensed in a typical apoptotic mode as shown in Figure 10C. In contrast, DNA in the majority of cells dying from 3-MeSO₂-DDE exposure appeared as condensed, intensely stained spots as shown in Figure 10B. This DNA pattern was interpreted to reflect necrotic cell death.

![Figure 10](image.png)

*Figure 10.* Typical features of the DNA Hoechst staining in cells exposed to (A) DMSO, (B) 3-MeSO₂-DDE (20 μM) or (C) the apoptosis-inducer staurosporine (1 μM).

The results of paper IV indicate that apoptotic signalling was triggered by 3-MeSO₂-DDE, but that the cells failed to complete apoptosis perhaps due to factors like ATP depletion, as well as prevention of apoptosome formation and caspase-activation by anti-apoptotic HSP70. Alternatively, reactions between 3-MeSO₂-DDE and thiol groups of the caspases could have occurred, yielding inactive caspases. These results show the complexity in determining the mode of cell death caused by a chemical. They are also in accordance with the accumulating amount of literature highlighting apoptosis and necrosis as two extreme forms of cell death and that there are several overlapping mechanisms (Leist and Nicotera 1997; Raffray and Cohen 1997), or even that both kinds of cell death may occur within the same cell (Fiers et al. 1999). Concepts like postapoptotic necrosis and necrapoptosis have been introduced (Lemasters 1999; Lauber et al. 2003). There are examples that cells can switch from apoptotic signalling to necrosis because of ATP deprivation (Leist et al. 1997; Leist et al. 1999; Yaglom et al. 2003; Nicotera and Melino 2004). Caspase activity has been reported in necrosis (Schwab et al. 2002) and necrosis has been suggested to be a regulated mode of cell death (Proskuryakov et al. 2003; Zong et al. 2004). Also, apoptosis can be completed without caspase-activation (Borner and Monney 1999).
3-MeSO₂-DDE analogues

The ability of 3-MeSO₂-DDE to specifically interact with CYP11B1 favours its use as a therapeutical agent or a PET tracer. However, the slow elimination of 3-MeSO₂-DDE, shown in paper I, could cause challenges in designing a suitable dosing regime of this substance. Optimally, a substance with the same adrenocorticolysis characteristics but with a more rapid elimination would be desirable. To find such a new candidate substance, it is important to evaluate 1) what kind of characteristics of the chemical structure are important for adrenocorticolysis activity 2) how the t½ can be reduced, i.e. how elimination can be facilitated.

In paper II, eight substances, analogous to 3-MeSO₂-DDE, were screened for adrenocorticolysis in the mouse adrenal gland in vivo. The substances were 2-MeSO₂-DDE, 3-SO₂OH-DDE, 3-MeSO₂-o,p’-DDE, 3-NO₂-DDE, 3-NH₂-DDE, 3-SO₂NH₂-DDE, 3-MeSO₂-DDMU and 3,3’-(bis)MeSO₂-DDE. The purpose was to study the structure specificity of the adrenal toxicity of 3-MeSO₂-DDE and possibly find a substance which is more easily metabolised. None of the compounds caused any histological lesions to the mouse adrenal cortex 24 or 72 hours after a single i.p. injection of 100 mg/kg b.w. The analogous substances seemed to be harmless to the adrenal cortex, indicating that they were not bioactivated at a sufficiently high rate. Seven of the 3-MeSO₂-DDE-analogues were also examined regarding their ability to interact with the CYP-catalysed binding of 3-MeSO₂-[¹⁴C]DDE to Y-1 proteins, reflecting an interaction with CYP11B1. Only two substances, 2-MeSO₂-DDE and 3-MeSO₂-o,p’-DDE, could significantly decrease the binding. Assuming that this inhibition reflects the ability of the test compounds to interact with CYP11B1, it further confirms the narrow substrate specificity of the enzyme. Accordingly, 2-MeSO₂-DDE slightly decreased the corticosterone production, without any reduced cell viability, in the Y-1 cells (paper III). Interestingly, 3,3’-(bis)MeSO₂-DDE, which previously has been shown not to accumulate and bind in the adrenal cortex of mice (Lund et al. 1988), caused a concentration-dependent cytotoxicity as well as a reduction of corticosterone production (paper III). Previously, it has been indicated that the methylsulphone moiety is crucial for the bioactivation and adrenocortical toxicity of 3-MeSO₂-DDE. The results from this thesis further support this assumption, but since several MeSO₂-containing compounds were still non-toxic in mice in vivo, other factors should be decisive as well.
CONCLUSIONS AND FUTURE PERSPECTIVES

Both for 3-MeSO₂-DDE as a possible drug lead compound and for the toxicological risk assessment of this compound, valuable information has emerged from the work of this thesis. Although 3-MeSO₂-DDE and \( o,p' \)-DDD are lipophilic and structurally similar compounds, some important discrepancies regarding their bioactivation/binding in the Y-1 cell line and their kinetics have been revealed. The main conclusions of this thesis are:

- 3-MeSO₂-DDE and \( o,p' \)-DDD had highly different kinetic profiles in minipigs, since \( o,p' \)-DDD was more readily eliminated, whereas there are no known excretion pathways for 3-MeSO₂-DDE. Both compounds were extensively accumulated and retained in fat tissue. Unlike \( o,p' \)-DDD, 3-MeSO₂-DDE was found in high levels in the liver.
- Cell death caused by 3-MeSO₂-DDE in the mouse adrenal cortex was probably due to necrosis, despite that the toxic lesion originates in mitochondria. It seemed like apoptotic signalling was triggered, but interrupted due to ATP depletion and expression of heat shock protein 70.
- Although both 3-MeSO₂-DDE and \( o,p' \)-DDD were found to bind irreversibly to Y-1 cells, the initial rate of binding seemed to be strikingly higher for 3-MeSO₂-DDE. This discrepancy could explain why \( o,p' \)-DDD is not irreversibly bound in the mouse adrenal cortex \textit{in vivo}.
- Concentration-dependent toxicity was induced in Y-1 cells by 3-MeSO₂-DDE but not by \( o,p' \)-DDD. Both substances also caused a concentration-dependent reduction of corticosterone production without reduced cell viability in this cell line.
- The Y-1 cell line proved to be a useful test system for studies of the cellular mechanisms preceding the toxicity of 3-MeSO₂-DDE and, unexpectedly, \( o,p' \)-DDD. Also, the results suggest that this cell line is suitable for screening for irreversible binding, cytotoxicity and hormone (i.e. corticosterone) disruption of other lipophilic compounds.
- 3-MeSO₂-DDE was irreversibly bound to the adrenal cortex tissue in hamsters and mice, but not in guinea pigs and rats \textit{ex vivo} using the
adrenal tissue slice method. These results emphasise the large species differences of adrenal irreversible binding of 3-MeSO\(_2\)-DDE, a factor to be considered when performing a complete toxicological risk assessment of this widespread environmental pollutant.

As mentioned, 3-MeSO\(_2\)-DDE is a proposed lead compound for development of an improved chemotherapy for ACC and Cushing’s syndrome in humans. It is also a candidate PET imaging agent for diagnosis of adrenocortical disorders. However, the large species differences demonstrated, cause difficulties to predict the toxic potency of this substance in the human adrenal gland. The indications available so far do imply that the human adrenal cortex might be sensitive to 3-MeSO\(_2\)-DDE. The information about the actions of 3-MeSO\(_2\)-DDE in the human adrenal is very limited, but still in full agreement with the findings in mice. The strategy adopted to investigate the in vitro/ex vivo/in vivo correlations in the mouse to finally approach the human adrenal gland via the human adrenocortical H295R cell line continues, as illustrated in Figure 3. There are some crucial studies determining the future of 3-MeSO\(_2\)-DDE as a drug lead compound. The knowledge from the Y-1 cell line is currently applied in the H295R cell line, for example by measuring the irreversible binding of 3-MeSO\(_2\)-DDE and o,p\(^\prime\)-DDD to H295R cells. The remaining tissue samples from the minipigs will be further examined to determine the metabolite pattern of 3-MeSO\(_2\)-DDE. Also, identification of the reactive intermediate(s) and localisation of the target protein(s) are projects that need to be carried out. In conclusion, further studies are in progress to evaluate 3-MeSO\(_2\)-DDE as a human adrenal toxicant. The work presented in this thesis reveals both advantages and disadvantages for 3-MeSO\(_2\)-DDE as a future drug, but the results are derived from other species and the effects in humans are indefinite with regard to the species differences in bioactivation and toxicity.
Miljögiftet DDT finns i höga koncentrationer i vävnader från människor och marina djur, numera främst i form av sin nedbrytningsprodukt \( p,p' \)-DDE. 3-MeSO\(_2\)-DDE kan i sin tur bildas från \( p,p' \)-DDE. I möss omvandlas 3-MeSO\(_2\)-DDE av enzymet cytochrom P45011B1 (CYP11B1) till en reaktiv metabolit. Denna binds irreversibelt i binjurens bark och ger upphov till omfattande celldöd, specifikt i den zon som bildar kortisonlika hormoner. CYP11B1 katalyserar normalt det sista steget i bildningen av dessa hormoner. På grund av den höga och vävnadsspecifika toxiciteten har 3-MeSO\(_2\)-DDE föreslagits som potentiellt cellgift vid binjurebarks cancer och som en ”tracer” för diagnostisering av binjurebarkstumörer med så kallad positronemissionstomografi (PET). Syftet med denna avhandling var att vidare utreda kinetiken, celldödsmekanismerna, artskillnaderna och de binjuretoxiska effekterna av 3-MeSO\(_2\)-DDE.

Farmakokinetiken för 3-MeSO\(_2\)-DDE studerades i minigris, en djurart som uppbär en ansenlig mängd fettväv och fysiologiskt liknar människan. Minigrisarna gavs en oral engångsdos av 3-MeSO\(_2\)-DDE eller det befintliga läkemedlet för binjurebarkscancer \( o,p' \)-DDD. Prover från blod och underhudsfett togs vid upprepade tillfällen under 6 månaders tid. Vid försökets slut togs även lever- och binjureprover. Resultaten visade att båda substanserna lagrades i fettväv, men att \( o,p' \)-DDD eliminerades snabbare än 3-MeSO\(_2\)-DDE. Den terminala halveringstiden (t\(\frac{1}{2}\)) i plasma beräknades till 52 dagar för 3-MeSO\(_2\)-DDE och 22 dagar för \( o,p' \)-DDD, vilket visar att även \( o,p' \)-DDD stannar kvar betydligt längre i kroppen än vad som tidigare varit känt. Möjligheterna för elimination av \( o,p' \)-DDD och svårigheten att eliminera 3-MeSO\(_2\)-DDE avspeglades i substansernas beräknade clearancevärden. Till skillnad från \( o,p' \)-DDD ansamlades 3-MeSO\(_2\)-DDE i höga halter i levern. Resultaten antyder att en lämplig doseringsregim för 3-MeSO\(_2\)-DDE kan vara svår att hitta eftersom substansen har en så lång uppehållstid i kroppen. En modifierad substans med samma vävnadsspecifika effekt, men med en snabbare elimination skulle således vara önskvärd. Åtta analoga substanser till 3-MeSO\(_2\)-DDE studerades, med avseende på binjurebarkstoxicitet. Ingen av analogerna gav upphov till binjuretoxicitet 72 timmar efter dosering. Både 3-MeSO\(_2\)-DDE och \( o,p' \)-DDD visades binda irreversibelt, både tids- och koncentrationsberoende till den murina binjurebarkscellinjen Y-1. Eftersom \( o,p' \)-DDD inte binder till binjurebark hos levande möss var detta ett oväsentat fynd. Bindningen av båda substanserna visades vara CYP-
katalyserad då den effektivt hämmades med CYP-hämmarna ketoconazol och etomidat. Vi fann även att den endogena tripeptiden glutation (GSH) var av betydelse för avgiftningen av den förmodade reaktiva intermediären av 3-MeSO₂-DDE, men ej för den av o,p′-DDD. Även här testades sju av de åtta analogerna, för att genom påverkan på bindningen av 3-MeSO₂-DDE påvisa om de kunde konkurrera om bindningen till CYP11B1. Två analoger, 2-MeSO₂-DDE och 3-o,p′-MeSO₂-DDE, visades kunna hämma bindningen av 3-MeSO₂-DDE till Y-1 proteiner. 2-MeSO₂-DDE kunde även hämma hormonproduktionen i Y-1 celler, dock ej lika effektivt som 3-MeSO₂-DDE och o,p′-DDD och utan att reducera cellernas viabilitet. Trots att de testade substansernas strukturer är välålig den hos 3-MeSO₂-DDE, verkar effekten av 3-MeSO₂-DDE vara mycket strukturspecifik.

Toxiciteten av 3-MeSO₂-DDE är artsspecifik. Detta kan bero på möjligheten för olika arter CYPar, i synnerhet CYP11B1, att bioaktivera substansen. Förmågan att binda 3-MeSO₂-DDE undersöks i två nya arter, hamster och marsvin, genom att inkubera binjuresnitt med 3-MeSO₂-[^14C]DDE. Bunden 3-MeSO₂-DDE återfanns i hamsterns binjurebark och i förmodade kortikala celler som låg insprängda i binjuremärgen. Bindningen till marsvinbinjure var däremot försumbar. Möjligtvis är det små olikheter i aminosyrasammansättning i de aktiva sätet hos CYP11B1 i olika arter som gör skillnaden.


I denna avhandling visades att 3-MeSO₂-DDE är persistent och lagras i fettvävnad. Artskillnader vad gäller irreversible bindning i binjurebark av 3-MeSO₂-DDE har belyst genom att påvisa en ny känslig och en okänslig art, hamster respektive marsvin. 3-MeSO₂-DDE-inducerad celldöd i binjurebarkens i mus föreslogs vara nekrotisk. Slutligen visades Y-1 celllinjen vara en fungerande in vitro-modell för fortsatta studier av mekanismerna och effekterna av denna substans. Avhandlingsarbetet lägger grund för fortsatta studier i den humana binjurecancercelllinjen H295R.
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