Steroidogenesis Studied in a Human Adrenocortical Cell Line – Effects of Single Chemicals and Mixtures

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Cover: Modified photo of the H295R cells
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Abstract

Steroidogenesis may be a target for endocrine disrupting chemicals; unfortunately data of such effects is limited. The aim of this thesis was to study effects on steroidogenesis of single chemicals and mixtures in the human adrenocarcinoma cell line H295R.

Screening for effects on steroid secretion was performed by ELISA. Mechanisms were elucidated by analysing levels of steroid intermediates, gene expression and enzyme activity. Results from 30 tested chemicals showed qualitative and quantitative differences in effect on cortisol and aldosterone secretion; inhibition, stimulation and dissimilar effects on basal *vs.* induced steroid secretion. Mechanistic studies on the fungicide prochloraz revealed dose-dependent inhibition of cortisol secretion, in contrast to a biphasic effect, with low-dose stimulation and high-dose inhibition, of aldosterone secretion. The specific effects could be explained by inhibition of CYP17A1 and CYP21A2, and by down-regulation of steroidogenic genes.

Effects of single chemicals and equimolar mixtures were compared to estimated effects from the concentration addition and independent action prediction models. The imidazole mixture of prochloraz, ketoconazole and imazalil caused additive effects similar to individual imidazole compounds, with inhibition of cortisol and biphasic effects on aldosterone secretion. A modification of the concentration addition model was required to predict the biphasic effect. The flavonoids daidzein, genistein and to a lesser extent apigenin inhibited cortisol, aldosterone and testosterone secretion. The flavonoid mixture inhibited the secretion of all tested steroids, including oestradiol, and in general acted in an additive way. The additive effects of the mixtures emphasize the need to assess chemicals together as a group.

We conclude that the H295R cell line is a promising model for both screening of effects on steroidogenesis by single chemicals and mixtures, and for mechanistic analysis. The prediction models are valuable tools for assessment of mixture effects on steroidogenesis.

Keywords: adrenal gland, H295R, steroidogenesis, cortisol, aldosterone, testosterone, oestradiol, mixtures, concentration addition, independent action

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Dedication

To my parents

Quorum pars magna fui – Of which I was a great part
Virgil (70-19 BC), Aeneid 11.6

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ullerås E., Ohlsson Å., Oskarsson A (2008). Secretion of cortisol and aldosterone as a vulnerable target for adrenal endocrine disruption – screening of 30 selected chemicals in the human H295R cell model. *Journal of Applied Toxicology* 28(8), 1045-1053.

II Ohlsson Å., Ullerås E., Oskarsson A (2009). A biphasic effect of the fungicide prochloraz on aldosterone, but not cortisol, secretion in human adrenal H295R cells – underlying mechanisms. *Toxicology Letters* 191(2-3), 174-180.

III Ohlsson Å., Cedergreen N., Oskarsson A., Ullerås E (2010). Mixture effects of imidazole fungicides on adrenal cortisol and aldosterone secretion (submitted).

IV Ohlsson Å., Ullerås E., Cedergreen N., Oskarsson A (2010). Mixture effects of dietary flavonoids on steroid hormone synthesis (submitted).

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Abbreviations

3βHSD2	3β-hydroxysteroid dehydrogenase type II
ACTH	adrenocorticotrophic hormone
Ang I	angiotensin I
Ang II	angiotensin II
AT1R	angiotensin II type 1 receptor
ATCC	American type culture collection
CA	concentration addition
CRH	corticotrophin-releasing hormone
CYP	cytochrome P450
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DMSO	dimethyl sulfoxide
DOC	11-deoxycorticosterone
EDC	endocrine disrupting compound
HPA	hypothalamic-pituitary-adrenal
IA	independent action
MoA	mode of action
OECD	Organization for Economic Cooperation and Development
RAS	renin angiotensin system
StAR	steroidogenic acute regulatory protein
USF	Ultroser SF

1 Introduction

The endocrine system is essential for communication and coordination of body function. The organs of the endocrine system are often termed glands and comprise the pituitary, the thyroid, the parathyroids, the testes, the ovary, the adrenal gland and the endocrine pancreas (Boron & Boulpaep, 2003). These glands communicate through hormones, secreted into the blood and recognized by specific, high-affinity receptors in their target tissues. The hypothalamus and the pituitary gland are central in the hierarchical regulation of the endocrine system, controlling the function of the reproductive system, growth and development, and homeostasis within the body.

The adrenal gland is recognized to be the most frequently reported toxicological target within the endocrine system (Hinson & Raven, 2006; Rosol *et al.*, 2001), but unfortunately it is also the most neglected endocrine organ in toxicology research (Diamanti-Kandarakis *et al.*, 2009; Harvey & Everett, 2003). Compounds interfering with the adrenal gland or other endocrine organs are termed endocrine disrupting compounds (EDCs) and were defined by the International Programme on Chemical Safety (IPCS) as exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (IPCS, 2002). These endocrine disrupting effects are of high importance to indentify, hence, rapid and appropriate test models to determine the effects of EDCs on the endocrine system are needed.

1.1 The adrenal gland

The adrenal gland is a small organ of 4-10 g, located at the cranial part of each kidney. The gland is histologically and functionally divided into a cortex and a medulla (Fig. 1), each part synthesising different sets of hormones. The medulla is responsible for production and secretion of catecholamines. The human adrenal cortex is divided into three distinct zones; *zona glomerulosa*, *zona fasciculata* and *zona reticularis* (Fig. 1). *Zona glomerulosa* is the outermost layer of the adrenal gland and is responsible for the production and secretion of the mineralocorticoid aldosterone. *Zona fasciculata* is located in the middle and produces cortisol, which is the main glucocorticoid in humans. The innermost layer of the cortex is *zona reticularis*. Reticularis cells produce some levels of cortisol, but primarily secrete DHEA, DHEA-S and androstenedione, which are weak androgens often used for testosterone and oestrogen synthesis in extra adrenal tissues (Boron & Boulpaep, 2003; Rosol *et al.*, 2001; Harvey, 1996).

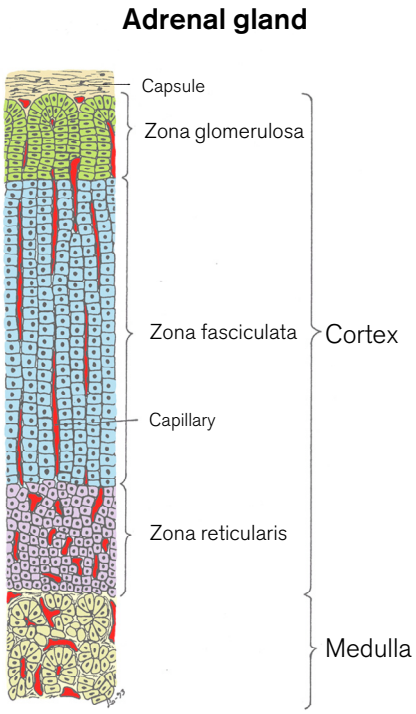

Figure 1. Adrenal histology. (Image kindly provided by Y. Ridderstråle and L. Holm.)

1.1.1 Regulation and function of the hormones of the adrenal cortex

The effects of cortisol within the body are diverse. The main effect of cortisol is carbohydrate and protein metabolism (Rang, 2007), but cortisol is also important for regulation of stress responses and immunologic mechanisms (Ruginsk *et al.*, 2009). Cortisol constitutes the main glucocorticoid in humans and the secretion is under influence of the hypothalamic-pituitary-adrenal (HPA) axis and diurnal rhythm (Fig. 2). The HPA-axis is stimulated in response to both psychological as well as physical stress, resulting in secretion of corticotrophin-releasing hormone (CRH). CRH activates pituitary corticotrophes, which leads to the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland. Activation of cortisol production is subsequently mediated through membrane bound ACTH-receptors. Upon activation, ACTH generates a signalling cascade within the fasciculata cells, mediated by cAMP and culminate in the transcription of specific genes required for the cortisol pathway (Sewer *et al.*, 2007). Secreted cortisol binds to receptors in the cells of the target tissues and acts as a ligand-dependent transcription factor (Ruginsk *et al.*, 2009). Once the stressful situation for the individual is under control negative feedback on the HPA-axis returns cortisol back to basal levels. If the stress continues, as in situations of chronic stress, cortisol levels are sustained at an elevated level (Redei, 2008).

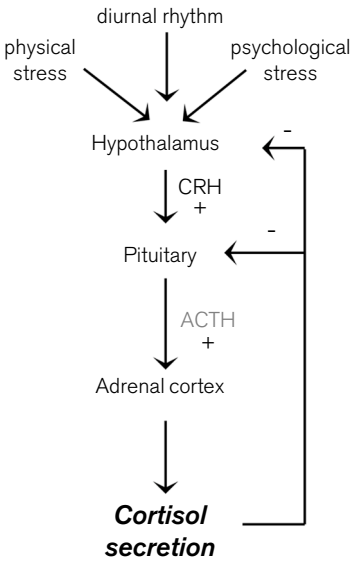

Figure 2. The HPA-axis leading to secretion of cortisol. Main cortisol activator in grey.

Aldosterone plays a fundamental role in electrolyte and water balance, by increasing reabsorption of sodium and water and increasing excretion of potassium (Rang, 2007). Thus, the effect of aldosterone increase blood volume and, therefore, increases blood pressure. Impaired aldosterone secretion may lead to hypovolaemia and circulatory collapse (Lumbers, 1999). Aldosterone release is initiated by angiotensin II (Ang II) from the renin-angiotensin system (RAS), increased potassium levels, ACTH, endothelin, neuropeptides and serotonin. RAS is the principal activator of aldosterone secretion in the adult mammals (Lumbers, 1999; Harvey, 1996) and is initiated by the release of renin from the juxtaglomerular cells in the kidney as a response to *e.g.* low sodium load or low blood pressure. Renin converts angiotensinogen from hepatocytes into angiotensin I (Ang I) (Fig. 3), which is subsequently converted into Ang II by angiotensin converting enzyme (ACE). Ang II binds to the angiotensin II type 1 receptor (AT1R) on the glomerulosa cell surface, thereby activating phospholipase C (Bassett *et al.*, 2004; Lumbers, 1999; Rainey, 1999), which result in the release of intracellular calcium storages. This calcium release activates enzymes and transcription of genes required for aldosterone secretion (Sewer *et al.*, 2007; Lumbers, 1999). Potassium also activates aldosterone secretion through increased intracellular calcium levels, but by stimulating influx of extracellular calcium by depolarizing the cell membrane (Foster, 2004). ACTH is not recognized as a major stimulator of aldosterone secretion, since glomerulosa cells are known to down-regulate aldosterone synthesis if the ACTH level is elevated for a longer period of time (Bassett *et al.*, 2004; Lumbers, 1999; Rainey, 1999). Upon release, aldosterone binds to the intracellular mineralocorticoid receptor in the target cells. The ligand-receptor complex translocates to the nucleus and interacts with DNA regulating elements to redirect gene expression of the cell (Freel & Connell, 2004). Since aldosterone promotes sodium and water retention, the biological effects of aldosterone will offset the original stimuli of aldosterone secretion.

The adrenal androgens, mainly dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S), but also androstenedione, are synthesised and secreted by the reticularis cells. Among the adrenal androgens DHEA-S appears to be the main secreted hormone (Belgorosky *et al.*, 2008). ACTH has been proposed as one regulator of this synthesis, but there are probably other stimulators contributing in the steroidogenic activation (Belgorosky *et al.*, 2008; Harvey, 1996). The adrenal androgens are recognised as weak stimulators of the androgen and oestrogen receptors,

but are also used as steroid substrates for testosterone and oestrogen synthesis in extra adrenal tissues.

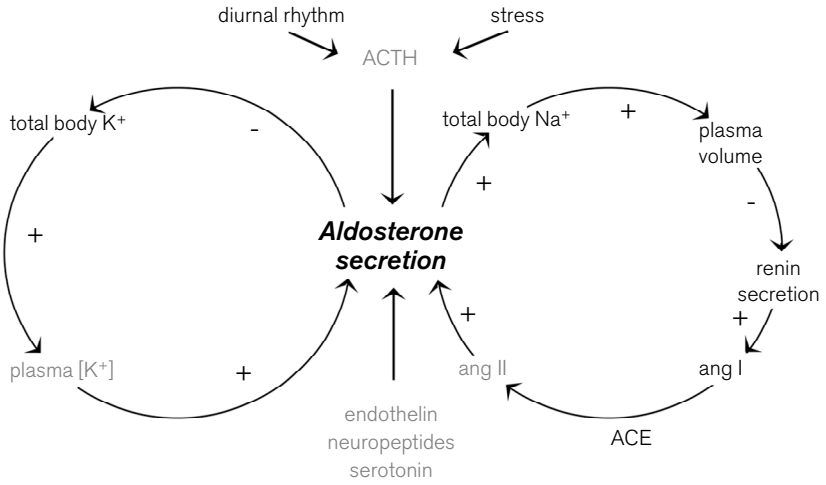

Figure 3. Activation pathways of aldosterone secretion. Activators of aldosterone secretion in grey. (Based on image from Müller (1995).)

1.1.2 Steroidogenic pathways

No steroid storages are present within the cells of the adrenal cortex, therefore up-regulation of gene expression and stimulation of enzyme activity of the steroidogenic pathway is needed for hormone secretion. The structural template of all steroids is cholesterol. Cholesterol is derived from two sources: *de novo* synthesis by acetate in the adrenal cells or by receptor-mediated uptake from plasma lipoproteins, which are stored as cholesterol acetate in neutral lipid droplets (Rosol *et al.*, 2001; Harvey, 1996). The receptor-mediated uptake in humans is mainly in the form of low-density lipoproteins (Sewer *et al.*, 2007; Harvey, 1996).

Upon stimulation of steroidogenesis, cholesterol is transported into the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) in a rate limiting step (Omura & Morohashi, 1995). Cholesterol is converted into pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A1). Depending on zone specificity pregnenolone is further converted by a number of steroidogenic enzymes, most of which are cytochrome P450 (CYP) enzymes (Fig. 4). In *zona fasciculata* pregnenolone is reduced to progesterone by the 3β-hydroxysteroid dehydrogenase type II

enzyme (3βHSD2) or hydroxylated to 17α-OH-pregnenolone by CYP17A1. 17α-OH-pregnenolone is further reduced by 3βHSD2 to 17α-OH-progesterone, and progesterone can be hydroxylated to 17α-OH-progesterone by CYP17A1 as well. Hydroxylation of 17α-OH-progesterone by CYP21A2 leads to the formation of 11-deoxycortisol, which is subsequently hydroxylated into cortisol by CYP11B1. CYP11A1 and CYP11B1 are located in the mitochondria, whereas the rest of the steroidogenic enzymes required for cortisol synthesis are located in the endoplasmic reticulum.

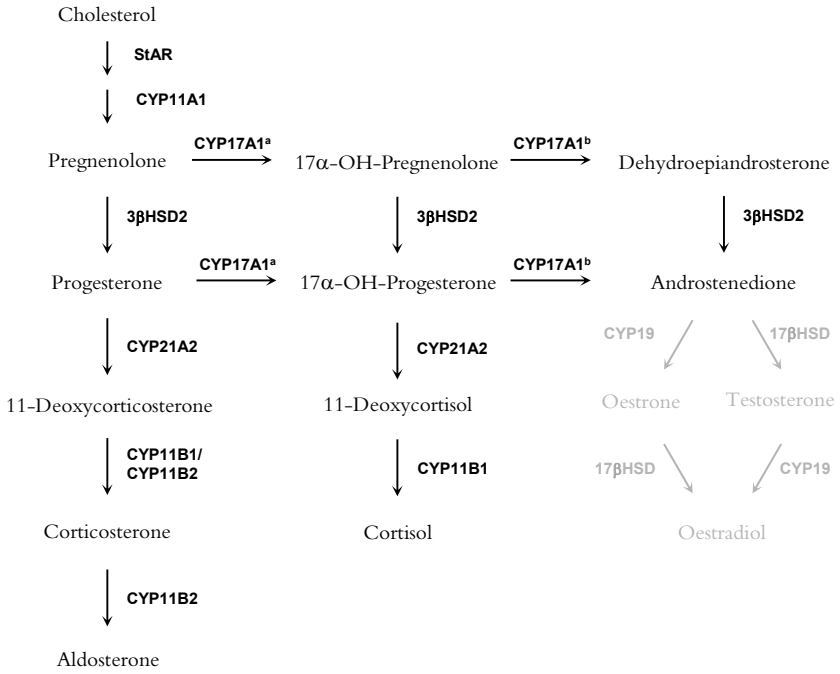

Figure 4. Steroidogenic pathways in the adrenal gland. Enzymes are indicated in **bold**. CYP17A1-activity is indicated as (a) hydroxylase activity and (b) lyase activity. Steroidogenic pathways taking place in extra adrenal tissues are written in grey.

In glomerulosa cells pregnenolone is reduced to progesterone by 3βHSD2, followed by hydroxylation by CYP21A2 into 11-deoxycorticosterone (DOC). The final step in aldosterone secretion is taking place in the mitochondria by an enzyme exclusively expressed in the glomerulosa cells, CYP11B2. It has been argued that the hydroxylation step/steps from DOC to aldosterone is either carried out solely by CYP11B2 (Lisurek & Bernhardt, 2004; Payne & Hales, 2004), or by the help of CYP11B1.

CYP11B1 is known to synthesise corticosterone, but cannot hydroxylate corticosterone into aldosterone (Hakki & Bernhardt, 2006; Payne & Hales, 2004). The zonal expression of CYP11B1 in the glomerulosa cells has not been fully resolved (Rainey, 1999).

The reticularis cells share the initial part of the steroidogenic pathway with the cortisol pathway. In contrast to *zona fasciculata*, cells of *zona reticularis* require a lyase activity of CYP17A1 to proceed with the conversion of 17α-OH-pregnenolone and 17α-OH-progesterone to DHEA and androstenedione, respectively. This lyase activity is promoted in the *zona reticularis* (Rainey & Nakamura, 2008) and is enhanced by the electron transfer protein cytochrome b_5 (CYP5) (Auchus *et al.*, 1998). CYP5 is expressed in all cells of the cortex, but to a higher extent in the reticularis cells (Rainey & Nakamura, 2008). DHEA is either reduced into androstenedione by 3βHSD2, or catalyzed to DHEA-S through the addition of a sulphate group by SULT2A1. In extra adrenal tissues adrenal androgens are converted into testosterone and oestrogens by the action of 17βHSD and aromatase (CYP19).

1.2 The H295R cell line

In 1980 a woman from the Bahamas had her right adrenal gland removed due to an adrenocortical carcinoma (Gazdar *et al.*, 1990). Parts of the tumour were used to establish the cell line NCI-H295 (H295). Characterization revealed that it secreted all of the hormones of the adrenal cortex, including the intermediate steps of the steroidogenic pathways (Rainey *et al.*, 2004; Gazdar *et al.*, 1990). It was also shown that the cell line expressed all the required enzymes for this secretion, regardless of normal *in vivo* zone specificity. In contrast to the normal adrenal gland, H295 cells also produced low levels of testosterone and oestrogens (Gazdar *et al.*, 1990). The H295 cell line is available through the American Type Culture Collection (ATCC).

Three substrains have been derived from the original H295 cell line (Rainey *et al.*, 2004), the most frequently used cell line is H295R. The H295 cells were initially grown as floating aggregates in foetal bovine serum supplemented medium. By switching to a medium supplemented with a serum substitute called Ultroser G, growth rate of the cells was enhanced while still retaining steroidogenic function (Rainey *et al.*, 2004). Selecting for cells attached to culture dishes lead to the establishment of the H295R

cell line. However, due to problems with importing Ultroser to the USA a population of the H295R cells were selected to grow in a serum substitute called Nu-Serum type I (Rainey *et al.*, 2004). The H295R cells are available through ATCC.

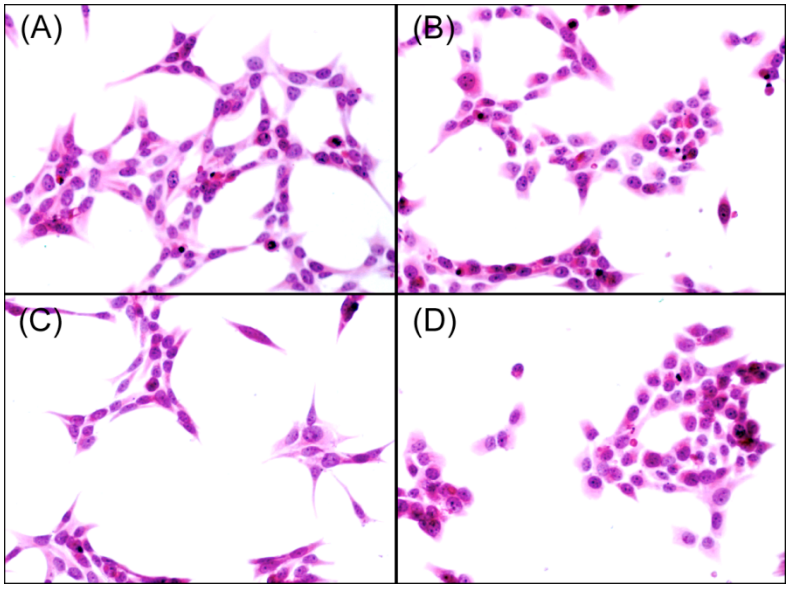

Figure 5. H295R cells cultured in the presence of (A) DMSO, (B) forskolin, 10 μM, (C) H_2O and (D) Ang II, 100 nM. 20x magnification, stained with haematoxylin and eosin.

The H295R cell line expresses the required enzymes and transport proteins needed for steroid hormone synthesis, as the parental cell line. The H295R cell line also retains the capability to produce and secrete sex hormones, which another substrain, H295A, cannot (Samandari *et al.*, 2007). In comparison to the H295A cell line H295R produce slightly lower amounts of aldosterone (Samandari *et al.*, 2007). H295R cells respond to cAMP inducers, Ang II and weakly to ACTH (Samandari *et al.*, 2007; Rainey *et al.*, 2004). Treatment with Ang II or potassium makes the H295R cells adopt a more "glomerulosa-like" behaviour with increased secretion of aldosterone. Forskolin treatment leads to a more "fasciculata-like" behaviour of the cells, with increased synthesis of cortisol and adrenal androgens (Oskarsson *et al.*, 2006; Rainey *et al.*, 2004). Some structural differences in morphology of the cultured cells can be seen following treatment with forskolin and Ang II (Fig. 5). Forskolin is a specific adenylyl cyclase activator and stimulates steroidogenesis through activation of cAMP and protein

kinase A. Forskolin is used to mimic ACTH stimulation, since the H295R cells are only weakly responsive to ACTH (Rainey *et al.*, 2004).

It has been shown that forskolin-stimulated steroidogenic gene expression in H295R cells mimics mRNA expression levels of human adrenal cells *in vivo* (Oskarsson *et al.*, 2006). Gene expression levels can successfully be analysed in H295R cells by quantitative real-time RT-PCR (Zhang *et al.*, 2005). Effects on steroidogenic gene expression levels have been reported following treatment with pesticides, natural compounds, pharmaceuticals and different food additives (Harvey *et al.*, 2007). Hence, the H295R cell line is a promising tool for the assessment of effects of chemicals on steroidogenic gene expression, as well as effects on hormone secretion.

The H295R cell line has attracted much attention as a potential screening model for toxic effects on sex hormone secretion, since the cell line secretes testosterone and oestrogens. The H295R cell line is currently undergoing a test method validation program for the Organization for Economic Cooperation and Development (OECD) as a potential tool for screening purposes of the effects on testosterone and oestradiol secretion (Hecker *et al.*, 2007). But the H295R cell line is originally derived from the adrenal cortex and may hence be a potential tool to address effects on the adrenal secretion of cortisol and aldosterone as well.

1.3 Action of endocrine disruptors on the adrenal cortex

Endocrine disrupting compounds are found among pharmaceuticals, food additives, environmental pollutants, and pesticides and are known to interfere with the endocrine system through various mechanisms. In general, the primary concern of EDCs has been receptor-mediated endocrine disruption. However, these receptor-mediated effects are often weak and there is a growing concern of the more potential effects of EDCs to cause specific effects on endocrine organs (Sharpe & Irvine, 2004). The direct interaction of EDCs with the endocrine organs may alter hormone synthesis or metabolism and hence affect homeostasis. For the adrenal gland, these direct and organ specific effects of EDCs may affect any step in the steroidogenesis, *e.g.* the function of StAR, or impair activity of steroidogenic enzymes (Harvey & Johnson, 2002). In fact, the adrenal gland has been reported as *the* most common toxicological target of all endocrine organs (Rosol *et al.*, 2001) and the need to establish effects of EDCs on adrenal steroidogenesis has been pointed out (Harvey *et al.*, 2007).

1.3.1 Toxic effects on the adrenal cortex

The structural arrangement of the adrenal gland positions almost every cell of the cortex in direct contact with a blood vessel (Harvey, 1996). Such a rich blood supply to the cortex is not only important for the rapid delivery of adrenal hormones into the blood, but also increases the risk of exposure to EDCs. Further, the high lipid content of the adrenal cortex makes it a vulnerable tissue for accumulation of lipophilic toxic compounds circulating the blood.

The most common mechanism of primary toxic action in the adrenal gland involves interaction with one or more steroidogenic enzymes (Harvey *et al.*, 2007; Rosol *et al.*, 2001; Harvey, 1996). The interaction with the steroidogenic enzymes may cause inhibition of steroidogenesis, or generate free oxygen radicals through enzymatic metabolism of chemicals present in the cells. Further, interference with gene expression levels for steroidogenic genes or receptors, such as the AT1R, have been reported for some EDCs (Hinson & Raven, 2006).

As a result of the multiple levels of control of adrenal function there are also many potential sites of actions for EDCs in extra adrenal tissues that can affect adrenal homeostasis. Any step within the HPA-axis or RAS may be susceptible for EDCs, thereby causing secondary changes to the function of the adrenal cortex.

1.3.2 Models for studies of adrenal toxicity

In adrenal toxicology *in vivo* studies are often crucial to establish effects on complex pathways such as the HPA-axis (Harvey, 1996). Species differences may, however, complicate the interpretation of results and extrapolation to human risk assessment (Harvey & Everett, 2006). For example, the main glucocorticoid in rats is corticosterone and not cortisol, since the rat (and mouse) do not express CYP17 in the adrenal gland (Payne & Hales, 2004; Harvey, 1996). The lack of CYP17 expression in rat/mouse adrenal also results in a difference of androgen secretion pattern compared to the human adrenal gland.

The recommended strategy for endocrine toxicity testing is an initial screening of effects by *in vitro* models and short-term *in vivo* studies, followed by reproduction studies *in vivo* (Harvey & Everett, 2006). Cell based models are considered promising tools for research and development in areas requiring screening of multiple compounds, such as in toxicology,

drug discovery and environmental monitoring (Banerjee and Bhunia, 2009). Rainey *et al.* (2004) presented some of the available cell lines of use in adrenal *in vitro* studies. Two of them are non-human; the murine adrenocortical cell line Y1, and bovine adrenocortical cell lines based on primary cell cultures. The murine Y1 cell model provides a good system to study both the molecular and biochemical characteristics of adrenal function, but still is a model of non-human origin. Therefore the H295/H295R cell line has been proposed as a powerful tool in adrenal toxicity. This model is not only human specific, but can also be used for the direct assessment of cytotoxicity. The OECD validation process of the H295R cells looks promising in the establishment of a screening tool for specific effects on human testosterone and oestradiol secretion.

The most obvious limitation of *in vitro* models for adrenal toxicity studies is the lack of negative feedback and crosstalk with other organs within the body. Metabolism of chemicals in extra adrenal tissues may generate potent and activated forms of a chemical, which may affect the adrenal cells differently compared to the original compound (Harvey & Everett, 2006). Further, some models are based on cell lines derived from tumors and it can be argued that these cells may act differently compared to primary cell cultures or cells *in vivo*. Despite the pros and cons of *in vitro* models, they still have advantages compared to *in vivo* studies in that it may reduce the use of exposed animals, reduce the costs for testing and give mechanistic insights.

1.4 Chemicals affecting adrenal steroidogenesis

A wide variety of chemicals has been found to cause histopathological and/or functional changes in the adrenal gland (Harvey, 1996). In this thesis a number of compounds have been used that cause, or are suspected to cause, effects on steroidogenesis. Among the tested compounds are environmental contaminants, naturally occurring dietary compounds, pharmaceuticals and pesticides.

1.4.1 Imidazole compounds

Imidazoles belong to the azoles and are recognized to inhibit lanosterol 14α-demethylase (CYP51). This enzyme is required in ergosterol biosynthesis, which is essential for maintaining the integrity of fungal cell membranes (Mason *et al.*, 1987; van den Bossche *et al.*, 1980; Siegel & Ragsdale, 1978). Hence, imidazoles are used as antifungal compounds in

agriculture or medical treatments. The enzyme inhibition is due to an interaction of the nitrogen at the basic site in the imidazole moiety with the iron atom of the CYP haem-group (White *et al.*, 1998). However, this interaction is not exclusive for CYP51 and imidazoles are known to inhibit other CYPs, including those in mammalian steroidogenesis (Ayub & Levell, 1987). In this thesis, three imidazoles have been studied; ketoconazole, prochloraz and imazalil (Fig. 6).

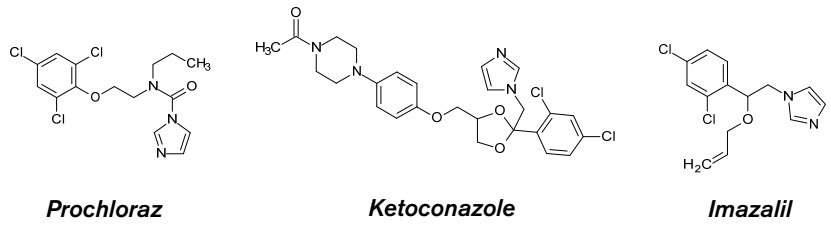

Figure 6. Chemical structure of the imidazoles prochloraz, ketoconazole and imazalil.

Ketoconazole is an imidazole originally used for antifungal treatment. Ketoconazole has been shown to inhibit cortisol secretion, both *in vitro* and *in vivo*, and is occasionally used in treatment of Cushing's disease (Engelhardt & Weber, 1994; Kong *et al.*, 1992). The impaired secretion of cortisol is due to inhibition of steroidogenic enzymes (Johansson *et al.*, 2002; Rotstein *et al.*, 1992; Ayub & Levell, 1989; Ayub & Levell, 1987; Nagai *et al.*, 1986; Loose *et al.*, 1983), but ketoconazole has been reported to down-regulate steroidogenic genes as well (Gracia *et al.*, 2006; Zhang *et al.*, 2005; Hilscherova *et al.*, 2004).

The imidazole prochloraz has suspected adverse effects on reproduction. Studies in rats have shown that male offspring are feminized following perinatal prochloraz exposure. This effect has partly been explained by reduced foetal production of testosterone and is associated with increased levels of progesterone (Blystone *et al.*, 2007a; Blystone *et al.*, 2007b; Laier *et al.*, 2006; Vinggaard *et al.*, 2005). Prochloraz has been shown to inhibit gonadal steroidogenesis, antagonize the androgen and oestrogen receptors and agonize the Ah-receptor (Vinggaard *et al.*, 2006). The reduced level of testosterone has been proposed to be due to inhibition of the steroidogenic enzyme CYP17A1 (Vinggaard *et al.*, 2006).

Imazalil is used as a fungicide in agriculture (EFSA, 2010; Ortelli *et al.*, 2005). Imazalil has been shown to inhibit steroidogenic enzymes (Trosken *et*

al., 2006; Mason *et al.*, 1987), which may lead to impaired oestrogen synthesis (Mason *et al.*, 1987).

1.4.2 The flavonoids

Polyphenols are abundant in various sources of plants and we are exposed to these via food (Manach *et al.*, 2004; Ross & Kasum, 2002). There are several thousand polyphenols identified in higher plants, whereof one group is the flavonoid. This group is in turn divided into six subgroups; flavonol, flavone, isoflavone, flavanone, anthocyanidin and flavanol. Compounds from two of these groups have been investigated in this thesis, the flavones and the isoflavones (Fig. 7). The chemicals of these groups contain a substituted chromon-4-one structure. The classification into different flavonoid chemicals depends on type and location of the substituents. The only difference between the isoflavones daidzein and genistein is one hydroxyl substituent in position 5 of the chrome-4-one backbone. Further, the structure of the flavone apigenin has the phenol group in position 2 instead of position 3 as in the isoflavones. Due to the flavonoid structural resemblance to 17β-oestradiol (Fig. 7) they are often called phyto-oestrogens. In plants, many of the flavonoids are found as glycosides (Manach *et al.*, 2004). These glycosides are hydrolyzed into active aglycons before absorption in the intestine (Patisaul & Jefferson, 2010).

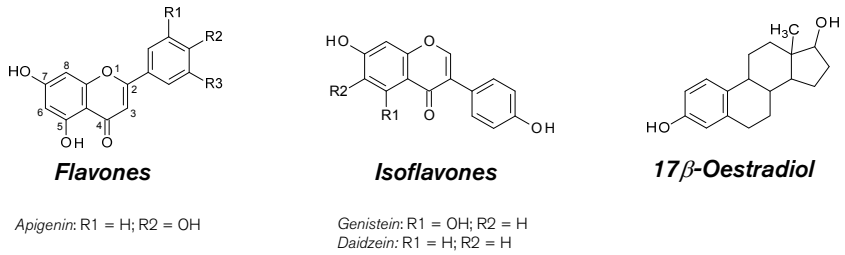

Figure 7. Chemical structure of the flavonoid subgroups flavone and isoflavone, in comparison to the chemical structure of 17β-oestradiol. Different flavonoids are identified by type of substitution in positions R1-R3.

Flavonoids can bind to the oestrogen receptors, due to the chemical resemblance to 17β-oestradiol. However, the receptor affinity for the flavonoids is one to three orders of magnitude lower than that of 17β-oestradiol (Sotoca *et al.*, 2008). A growing concern of EDCs is not the receptor-mediated effects, but the direct interference with the hormone synthesis in endocrine organs (Diamanti-Kandarakis *et al.*, 2009; Sharpe & Irvine, 2004). Flavonoids are reported to interfere with steroidogenesis by

inhibition and activation of key steroidogenic enzymes (Whitehead & Rice, 2006), which may alter hormone secretion levels.

The isoflavones are found in soybeans and soy-based food products (Mortensen *et al.*, 2009; Manach *et al.*, 2004). The main active isoflavones from soybeans are daidzein and genistein, the aglycons of daidzin and genistin respectively. Traditional Asian diets can result in an isoflavone intake of 8-50 mg/day, while a typical western diet can range from 1-3 mg/day (Mortensen *et al.*, 2009). Mean serum concentrations of isoflavones (daidzein, genistein, and equol (a daidzein metabolite)) are higher in Japanese men and women (0.8 μM) consuming a traditional diet than in Scottish men (0.05 μM) with a low consumption of soy-based food. Infants fed a soy-based diet reach very high serum concentrations (mean of 4 μM) of isoflavones (Mortensen *et al.*, 2009; Heald *et al.*, 2006). Isoflavones have been ascribed beneficial biological properties such as anticancer, anti-inflammatory, antioxidant and antiviral effects (Mortensen *et al.*, 2009; Scalbert *et al.*, 2005; Patisaul & Jefferson, 2010). However, there is no firm conclusion whether flavonoids are beneficial, or if they may in fact be harmful to human health (Patisaul & Jefferson, 2010). Isoflavones are recognized to decrease cortisol secretion *in vitro* in cell cultures (Ohno *et al.*, 2002; Mesiano *et al.*, 1999) and glucocorticoids *in vivo* in rats (Ohno *et al.*, 2003). Further, inhibitory effects on testosterone secretion have been demonstrated *in vitro* (Taxvig *et al.*, 2010) and *in vivo* (Ohno *et al.*, 2003), in contrast to increased levels of oestradiol secretion *in vitro* (Taxvig *et al.*, 2010). Daidzein and genistein have been shown to be potent inhibitors of 3βHSD2 and to a lesser extent inhibit CYP21A2 (Ohno *et al.*, 2004; Ohno *et al.*, 2002; Mesiano *et al.*, 1999). Further, daidzein and genistein have been shown to inhibit 17βHSD (Le Bail *et al.*, 2000) and genistein has been reported to stimulate activity of CYP19 (Sanderson *et al.*, 2004).

The flavone apigenin is the aglycon of apiin and most often found in food such as parsley, celery and citrus (Meyer *et al.*, 2006; Manach *et al.*, 2004). Apigenin is, as the isoflavones, recognized with beneficial biological effects such as anticancer, anti-inflammatory, antioxidant and anti-proliferative properties (Patel *et al.*, 2007; Meyer *et al.*, 2006). Inhibitory effects on cortisol secretion have been demonstrated for apigenin *in vitro* (Ohno *et al.*, 2002) and by decreasing glucocorticoid levels in rats (Yi *et al.*, 2008). Inhibitory effects of apigenin on steroidogenic enzyme activity have been reported for 3βHSD2 (Ohno *et al.*, 2004; Ohno *et al.*, 2002) and CYP19 (Sanderson *et al.*, 2004).

1.5 Chemical mixtures

Risk assessment of chemical exposure has generally been performed by addressing effects of single chemicals. However, exposure of single chemicals is not the likely way of exposure to EDCs (Diamanti-Kandarakis *et al.*, 2009). By assessing risk from single chemicals separately, the total risk of chemical exposure may be underestimated. This is not only due to rare combination effects displaying synergism, but simply through additive effects of large numbers of chemicals. This fact has brought about an increasing interest in mixtures and their toxicological effects in human risk assessment.

Chemical mixtures are defined as simple or complex mixtures. Simple mixtures consist of rather few compounds, ten or less (Groten *et al.*, 2001). The composition of a simple mixture is often known, both qualitatively as well as quantitatively. Pesticides are a good example of simple mixtures, where some compounds are combined to make the product more efficient. In contrast, the chemical composition of a complex mixture is often not fully known, which may not be surprising when a complex mixture can contain tens to thousands of chemicals (Groten *et al.*, 2001). The most likely type of mixture humans and animals are exposed to is complex mixtures, however, testing for all kinds of complex mixtures are virtually impossible. Therefore a number of mathematical prediction models have been brought forth to facilitate the evaluation of mixture effects. These models are often based on some sort of assumption regarding the mode of action (MoA) of a specific compound, either by acting by the same or different MoA.

The definition of MoA in mixture toxicology is not distinct. In pharmacology, and toxicology, mechanism of action denotes the molecular events of a chemical leading to a specific biological response in a target organ. In contrast, MoA gives a more general description of the effect of a chemical, referring to the type of response (Borgert *et al.*, 2004). Two of the most frequently used prediction models are concentration addition (CA) (Loewe & Muischnek, 1926), also known as dose addition, and independent action (IA) (Bliss, 1939), also known as response addition. CA is based on the assumption that the chemicals in the mixture act through the same MoA, whereas IA assumes the chemicals of the mixture to act through different MoA. Both models are based on the assumption that a chemical in a mixture do not interact with the other chemicals.

By comparing an observed effect with the CA and/or IA predicted effects of the mixture it is possible to establish how the chemicals in a mixture act in

combination. In mixture research chemicals are often defined to act through additive, synergistic or antagonistic effects. In contrast to the mathematical use of "additivity" this term in toxicology is ascribed chemicals that act together to produce an effect without enhancing or diminishing the effect of the other compound (Kortenkamp, 2007). Deviation from additivity is regarded as an interaction between the effects of the chemicals and is defined as synergism if the observed effect is greater than additive, or as antagonism if the observed effect is lower than expected (Groten, 2000; Berenbaum, 1989). To make these distinctions it is crucial to chose the correct model based on the correct assumptions about MoA, otherwise the model erroneously may ascribe the compounds with synergistic or antagonistic effects (Kortenkamp, 2007).

2 Aim of thesis

The overall aim of this thesis was to investigate the effects of single chemicals and mixtures on steroidogenesis in the human adrenocarcinoma cell line H295R, by performing screening and mechanistic studies.

3 Material and Methods

The following is a brief description of the material and methods used in this thesis. For detailed information the reader is referred to the material and methods in each paper.

3.1 Chemicals, solubility and cell viability

In this thesis, chemicals with known or suspected effects on adrenal steroidogenesis have been used. Most of these chemicals have been dissolved in dimethyl sulfoxide (DMSO), except for cadmium, lithium, arsenite, and Ang II, which have been dissolved in H_2O.

Despite solubility in DMSO some chemicals had a tendency to precipitate after dilution in cell culture medium. Effective concentrations of a chemical may depend on this solubility; hence much effort was put into determining the concentration where no precipitation was shown. This maximum concentration was later used as a starting point for cytotoxicity screening of each chemical. Precipitation tests were carried out in 96-well plates in cell culture medium used for chemical treatment. Precipitation could easily be detected by microscope examination (Fig. 8). None of the tested concentrations of the chemicals in paper I were limited by precipitations.

Cell viability assays were performed in 96-well plates. Viability was measured with a MTS reduction test, wherein a tetrazolium compound is transformed to a coloured formazan product by mitochondrial activity in viable cells. After a one-hour incubation absorbance at 490 nm was measured for each well. The absorbance was proportional to the number of living cells in the culture. The concentration to use in paper I was determined based on maximum 10% reduction of cell viability (IC_{max10}). If a concentration was regarded as toxic according to this definition, lower

concentrations were tested to establish a non-toxic concentration. Thus, the concentrations used in this thesis are believed to cause specific effects on steroid secretion without inducing cytotoxic effects.

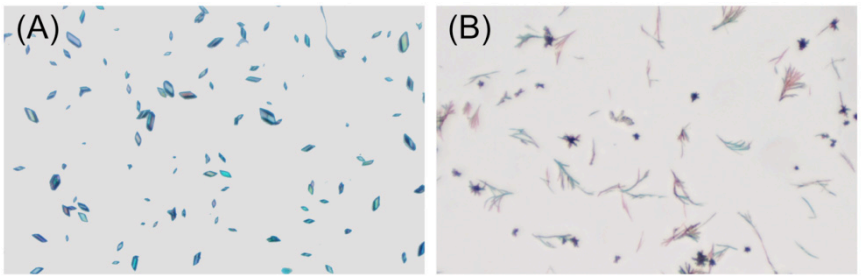

Figure 8. Precipitation of chemicals in serum-free cell culture medium following 24 hours incubation. (A) Ketoconazole, 70 μM and (B) 6-hydroxyflavone, 40 μM. 10x magnification.

Tests of cell viability were always run concurrently with the chemical experiments.

3.2 Cell treatment

H295R cells were seeded 72 h before exposure, in order to get the cells attached and about 70-80% confluent before treatment with the chemical. If different types of cell-culture plates were used (*i.e.* different number of wells in the plate), adjustments were done to maintain the same proportion of cells per volume and surface area. This resulted in:

- *6-well plates*: 5.0×10^5 cells per well, 2.5 ml medium
- *12-well plates*: 2.0×10^5 cells per well, 1.0 ml medium
- *96-well plates*: 1.7×10^4 cells per well, 0.1 ml medium

On the day of exposure culture medium was removed and serum-free medium containing the test chemical was added to each well in an appropriate volume. Treatments were performed for 24 h.

Stimulation of fasciculata and/or glomerulosa like behaviour of the H295R cells, with increased cortisol and aldosterone secretion, was achieved by co-treating the cells with 10 μM forskolin and 100 nM Ang II, respectively.

3.3 Enzyme activity assays

There are different ways to measure enzyme activity within a system. In this thesis an excess of steroid substrates and test chemical have been added to the cells and the level of the end product, or intermediate steps of a specific steroid pathway, has been measured by ELISA. For the CYP17A1-activity assay trilostane was used to block the activity of 3βHSD2, redirecting the steroid substrates away from mineralo- and glucocorticoid synthesis to DHEA production (Fig. 9). Thus, effects on CYP17A1-activity could be determined by measuring the level of DHEA in the cell culture medium.

Emphasis has been put into establishing a substrate and 3βHSD2-inhibitor concentration that did not saturate the measured pathways.

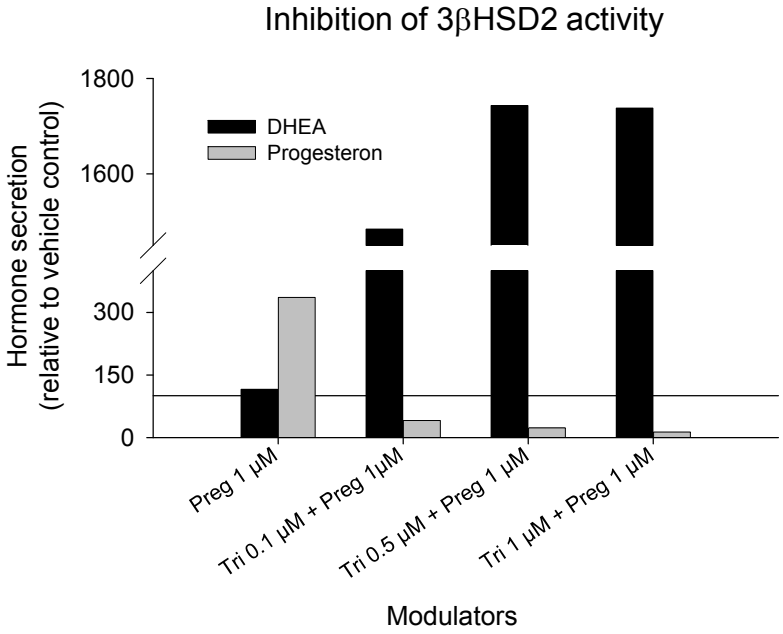

Figure 9. Effects on progesterone and DHEA secretion following treatment of H295R cells with increasing concentrations of trilostane (Tri) in order to block the activity of 3βHSD2. Pregnenolone (Preg) was added as a steroid substrate. Effect of vehicle control (DMSO) is indicated with a horizontal line in the figure at 100%.

3.4 Mixture predictions

3.4.1 Concentration addition

The concept of CA was suggested by Loewe and Muischneck (1926). It is applied in cases where the chemicals of a mixture are known, or suspected, to act through a common MoA. The only difference between the compounds is the individual potency, hence it is believed that one compound in a mixture can be partly or fully replaced with another compound of similar potency, without influencing the original effect of the mixture. The effect of the mixture is predicted based on the dose-response curves of the individual compounds within the mixture.

Fitting a curve to observed responses of the individual chemicals in the mixture can be done by a log-logistic dose-response model:

$$y = \frac{100\%}{1 + (x/e)^b} \quad (1)$$

, where the data is fitted with a common upper limit of 100% and a lower limit set to zero. The x represents concentration, e is the 50% effect concentration (EC_{50}) and b is proportional to the slope of the curve around e (Steribig et al., 1993).

The generated curve can then be applied in the model of CA as it is described by Backhaus *et al.* (2004b), which states that:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1 \quad (2)$$

, where ECx_i gives the concentration of chemical i needed to provoke the effect x when applied singly and c_i denotes the concentration of that chemical in the mixture, when the total mixture concentration provokes the effect x. Knowing the shapes of the dose-response curves of the single chemicals and their relative contribution to the mixture, the expected effect of any concentration of the mixture can be calculated. This is done by first retrieving a range of *ECx* values for all the individual chemicals in the mixture. Then, for each *ECx* value, the mixture concentration giving the effect x is calculated by substituting c_i in equation 1 with $p_i z$, where p_i is the proportion of chemical i ($0 < p_i < 1$) of the mixture concentration called z:

$$\sum_{i=1}^{n} \frac{p_i z}{EC_{x_i}} = 1 \tag{3}$$

Solving the equation for z gives that,

$$z = \frac{1}{\left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}} \right)} \tag{4}$$

A drawback with the CA model is that it does not take non-monotonic responses into consideration. A single endpoint, for example hormone secretion, may be either activated or inhibited by different compounds at different concentrations, making dose-response relationships of their activity non-monotonic. Recent research has emphasized that non-monotonic dose-response relationships are occasionally observed in toxicology with low-dose effects distinct from effects observed at high dose (Calabrese, 2008). To predict these types of mixture effects a modified CA model has been proposed (Belz *et al.*, 2008). To describe a non-monotonic curve a log-logistic dose-response model, including a term for the stimulatory response, is used:

$$y = \frac{100\% + fx}{1 + \left(x/e\right)^{b}} \tag{5}$$

The data is fitted with a common upper limit of 100% and a lower limit set to zero. In this model the parameter f is proportional to the size of the response increase, while e denotes the infliction point of the decreasing part of the dose-response curve (Brain & Cousens, 1989).

In the modified CA model it is assumed that the magnitude of stimulation follows a linear model, stated as:

$$\mu_P = \mu_A \cdot \frac{p}{100} + \mu_B \cdot \frac{100 - p}{100} \tag{6}$$

, where μ_p is the expected maximum of the mixture, based on the maximum stimulation of compound A tested alone (μ_A) and the percent proportion of A in the mixture, given as (p), and the maximum stimulation of compound B (μ_B) and the proportion of B (100-p) in the mixture. The model by Beltz *et al.* (2008) was proposed for binary mixtures, as reflected in equation 6, but can theoretically be extended to an infinite amount of compounds.

Knowing the concentration of the maximum secretion level and the curve from the level of 0-100% effects, these data can be used to model the non-monotonic response according to equation 5 to yield the final model prediction.

For most chemicals there are numerous potential sites of action, unfortunately often not known. CA can in some cases overestimate an antagonistic effect when the basic assumptions cannot be met (Goldoni & Johansson, 2007) and competition for sites of action may influence the effect of the compounds.

3.4.2 Independent action

The concept of IA was proposed by Bliss (1939) and assumes that two or more compounds of a mixture act in an independent way. Therefore the MoA of one chemical has no direct impact on the MoA of another chemical in the mixture (Lambert & Lipscomb, 2007). IA was first designed for modulation of quantal responses, such as live or dead, but has been successfully used for graded responses (Backhaus *et al.*, 2004a):

$$E_{mix} = 1 - \left((1 - E_1) \cdot (1 - E_2) \cdot \ldots (1 - E_n) \right) = 1 - \prod_{i=1}^{n} (1 - E_n) \quad (7)$$

E_{mix} is the fraction affected by the mixture and E_1, $E_2 \ldots E_n$ are the fractions affected by the individual chemicals. IA can also be expressed in terms of the unaffected fraction, R, which by definition is $1\text{-}E$:

$$R_{mix} = R_1 \cdot R_2 \cdot \ldots R_n = \prod_{i=1}^{n} R_n \quad (8)$$

, where R_{mix} is the response of the mixture given as the unaffected fraction, while R_1, $R_2 \ldots R_n$ are the responses of the individual chemicals in the mixture. In contrast to the standard probabilistic IA model, here we define the response to be the relative hormone secretion compared to the control set to 100%.

Most chemicals probably have some actions that are not fully independent from those of other chemicals. This is one limitation with the IA model, since chemicals with overlapping MoA cannot be properly tested (Goldoni & Johansson, 2007).

4 Results and discussion

The aim of this thesis was to screen the human adrenocarcinoma cell line H295R for effects of single chemicals and mixtures on steroidogenesis. The chemicals were tested during basal or stimulated conditions, and mechanisms behind observed effects were investigated.

Effects of chemicals on hormone secretion were measured by ELISA. More detailed information about mechanisms behind the observed hormone secretion patterns was achieved by using gene expression analysis and ELISA based enzyme activity assays. In general, the H295R cell line appears to be a promising tool to address effects on steroidogenesis following chemical exposure.

4.1 The human H295R cell line as a screening tool for effects of chemicals on steroid secretion – paper I

The chemicals tested in this thesis range from pharmaceuticals and pesticides to environmental contaminants. The 30 chemicals tested in paper I were selected based on potential effect on steroidogenesis. The chemicals were tested at the maximum concentration causing max 10% unspecific cytotoxicity (IC_{max10}). Thus, the used concentrations are believed to cause specific effects on steroid secretion without inducing cytotoxic effects. Cells were treated at basal and forskolin- and Ang II-stimulated conditions for 24 hours, followed by measurements of cortisol and aldosterone secretion levels with ELISA.

According to the results, the 30 chemicals in paper I can be divided into four categories based on the IC_{max10} potency: chemicals with no effect on steroid secretion, chemicals with an intermediate inhibitory effect, chemicals

with a strong inhibitory effect on hormone secretion and chemicals with stimulatory effects on aldosterone secretion.

4.1.1 Effects of chemicals on the stimulated cortisol and aldosterone pathway

The cells of the adrenal cortex respond to ACTH or Ang II for activation of cortisol and aldosterone secretion, respectively. It was shown that the addition of forskolin, to mimic ACTH stimulation, increased cortisol secretion in H295R cells 2.7-fold. The addition of Ang II increased aldosterone secretion from the cells 2.7-fold. In general, the effects of the chemicals were similar for both basal and stimulated hormone secretion. However, in some cases the degree or even direction of effects differed between the basal and induced hormone secretion. Metyrapone, a compound used in diagnosis and treatment of adrenocortical disease, displayed increased aldosterone secretion at basal conditions but inhibited Ang II-stimulated aldosterone secretion. Similar effects were seen for dibromoacetic acid, a drinking water disinfection by-product, and silymarin, a flavonolignan compound with antioxidant properties. In general, analyzing and comparing the effects on basal with induced hormone secretion may provide additional information suggestive of possible mechanisms. However, for rapid screening purposes basal conditions seem adequate to detect most of the potential effects of chemicals on cortisol and aldosterone secretion.

4.1.2 Inhibition and stimulation of hormone secretion

The results in paper I revealed a number of potent inhibitors of cortisol and aldosterone secretion. The most potent steroidogenic inhibitor of the 30 tested compounds, based on IC_{max10}, was etomidate, originally used as an anaesthetic. Comparing the chemical effects on cortisol and aldosterone secretion showed that a majority of the observed effects was similar for the two pathways. Since both pathways, to a large extent, share and depend on the same set of enzymes (except CYP17A1 and CYP11B2), this would suggest that the tested chemicals act on one or more of the steroidogenic synthesis steps that are common to the two pathways. Some of the other potent inhibitors of cortisol and aldosterone secretion were mitotane (o,p'-DDD), aminoglutethimide, the flavonoids daidzein and 6-hydroxyflavone, and the imidazoles prochloraz, ketoconazole and imazalil. However, as previously indicated for the effects on basal and forskolin/Ang II induced steroidogenesis, some compounds also stimulated hormone secretion in H295R cells. Among these were metyrapone, silymarin and dibromoacetic acid. The effects of the various compounds used in this thesis shows that the

test system is capable of detecting up-regulation as well as down-regulation of steroid secretion.

4.1.3 Similar chemical structures cause similar effects on steroid secretion

It was shown that chemicals with structural similarities appear to display the same type of effect. The flavonoids daidzein and 6-hydroxyflavone displayed marked inhibitory effects on hormone secretion. As mentioned above, the imidazoles prochloraz, ketoconazole and imazalil displayed similar inhibitory effect on cortisol and aldosterone secretion at the tested concentrations. This apparent relation between effect and chemical structure is of interest in a screening perspective.

In summary, we showed that the adrenal steroidogenesis is sensitive to chemical disruption, and that the H295R cell line is a promising tool to detect these types of effects on cortisol and aldosterone secretion.

4.2 Mechanistic studies of effects on steroidogenesis – paper II

In paper II we were interested to use the H295R cell line as a model for mechanistic studies on steroidogenesis. Based on the results from the screening in paper I we focused on the mechanisms behind the effect of the imidazole prochloraz. Prochloraz is a fungicide with anti-androgenic effects, including inhibition of testosterone secretion. The effect on testosterone secretion was suggested to be due to inhibition of CYP17A1 activity (Vinggaard *et al.*, 2006). Addressing the effects of this compound on cortisol and aldosterone secretion is hence of great interest, since CYP17A1 is also required for cortisol secretion. Effects on enzymatic activity as well as gene expression levels could explain the specific effects on cortisol and aldosterone secretion, and this was investigated in this paper.

4.2.1 Mechanistic evaluation based on hormone secretion patterns

The complexity of the steroidogenic pathways, with shared enzymes and transporter proteins, complicates the evaluation of the mechanisms of effects of chemicals on steroidogenesis. One way to address effects on the steroidogenic pathways is to measure the end products, and in addition the intermediate steps of the pathways. This was done in paper II. H295R cells were treated with five concentrations of prochloraz, and the levels of cortisol, aldosterone, progesterone, corticosterone and 17α-OH-progesterone were determined by ELISA.

A biphasic dose-response, with increased hormone secretion at lower concentrations followed by inhibition at higher concentrations, was observed for aldosterone secretion. Maximum 2.0-fold secretion was noted at a concentration of 0.10 μM prochloraz. Aldosterone secretion following treatment with prochloraz at 10 μM was inhibited to 23% of control levels. In contrast, cortisol secretion was dose-dependently inhibited. Cortisol levels were reduced to 55% of control levels at 0.10 μM prochloraz, the same concentration where the maximum stimulation was reached for aldosterone secretion.

To investigate the mechanisms behind observed effects further ELISA analyses were performed for selected intermediate steps of the two pathways. Biphasic dose-responses, with increased hormone secretion at lower concentrations followed by inhibition at higher concentrations, were observed for progesterone, corticosterone, and 17α-OH-progesterone following prochloraz treatment. Highest fold-induction of hormone secretion was noted for progesterone with a 5.3-fold stimulation at a prochloraz concentration of 1.0 μM. The biphasic pattern for corticosterone was almost identical to the observed effect for aldosterone, with maximum secretion of 2.1-fold noted at 0.10 μM prochloraz. Biphasic effect was also shown for 17α-OH-progesterone secretion with a maximum of 1.3-fold at 0.30 μM prochloraz. All results except for the biphasic effect shown for 17α-OH-progesterone were in favour of a hypothesis implying an inhibition of CYP17A1. An inhibition of CYP17A1-activity would have explained the decrease in cortisol secretion and the increase in aldosterone, corticosterone and progesterone secretion, since the steroid substrates would be directed down the aldosterone pathway if CYP17A1 was blocked (Fig. 4). The biphasic effect on 17α-OH-progesterone was unexpected and hence required an adjusted hypothesis. We therefore speculated that the observed effects could be caused by an additional inhibition of CYP21A2. A subsequent inhibition of CYP21A2-activity would explain the observed decrease in hormone secretion of corticosterone, aldosterone and 17α-OH-progesterone at higher concentrations of prochloraz treatment. An inhibition of CYP21A2 would also explain why progesterone secretion increased (accumulated) up to a concentration of 1.0 μM prochloraz, since progesterone could not be further synthesised.

4.2.2 Mechanistic studies based on gene expression

Chemicals interfering with the transcription of steroidogenic genes may influence steroid synthesis and secretion. It was, however, demonstrated that gene expression only partially could explain the observed effects on hormone secretion pattern following prochloraz treatment in paper II. The gene expression analysis revealed that prochloraz caused a dose-dependent down-regulation of all analysed steroidogenic genes, except *CYP11B2*. *CYP11B2* showed no effect on gene expression levels except for a slight down-regulation at 0.3-1.0 μM and a marked up-regulation at 10 μM prochloraz. This pattern of *CYP11B2*-expression for prochloraz did not explain the observed effect on aldosterone secretion. The expression of *CYP17A1* was inhibited even at the lowest concentration of prochloraz, supporting the hypothesis that this enzyme is involved in the effects on cortisol and aldosterone secretion observed at low concentrations. Since the most common mechanism of action in adrenal toxicology involves interaction with one or more steroidogenic enzymes (Harvey *et al.*, 2007; Rosol *et al.*, 2001; Harvey, 1996) it may not be so surprising that gene expression levels cannot fully explain the observed effects on steroidogenesis.

4.2.3 Mechanistic studies based on enzyme activity assays

To verify that prochloraz inhibits CYP17A1 and CYP21A2, as suggested by the hormone secretion patterns, we used a combined CYP21A2- and CYP11B1-activity assay, a CYP11B1-activity assay, and modified a CYP17A1-activity assay to investigate the effects on the implied enzymatic steps. The activity of the enzymes was tested by adding the preceding steroid substrate and measure the level of cortisol and DHEA secretion. The results indicated that CYP11B1-activity was not influenced by prochloraz treatment. Prochloraz was shown to dose-dependently inhibit the activity of CYP17A1 and CYP21A2. The overall inhibition or inhibitory trend of steroid secretion at 10 μM prochloraz may be ascribed to unspecific inhibition of imidazoles at higher concentrations (Ayub & Levell, 1989). Taken together, the study performed in paper II demonstrated that prochloraz caused a dose-dependent inhibition of CYP17A1 and CYP21A2-activity. These effects on enzyme activity explained the observed hormone secretion patterns following prochloraz treatment. The results also showed that the H295R cell line appear to be a promising tool for mechanistic studies on steroidogenesis.

4.3 Dose-response effects of single chemicals and mixtures on steroidogenic pathways – paper III & IV

Mixtures are of a growing concern and have attracted an increasing interest in toxicology research. In paper III and IV the single and mixture effects of three imidazoles and three naturally occurring flavonoids, respectively, were addressed on steroid secretion. The dose-response of the individual chemicals was compared with the observed dose-response of the mixture. Since prediction models are of increasing interest in the assessment of mixtures, we found it relevant to compare the observed mixture effects on steroid secretion in H295R cells with the predicted mixture effects from two well recognized prediction models, CA and IA.

4.3.1 Effects of single chemicals and mixtures of imidazoles on steroidogenesis – paper III

In paper I it was shown that prochloraz, ketoconazole and imazalil were potent inhibitors of cortisol and aldosterone secretion. In paper II it was shown that prochloraz displayed a monotonic dose-dependent inhibition of cortisol secretion, but caused a biphasic aldosterone secretion pattern, with low-dose stimulation and high-dose inhibition. The question emerged whether this was a specific effect for prochloraz or if this was a general effect for other imidazoles. Further, since mixtures are of increasing interest in toxicology the combined effect of prochloraz, ketoconazole and imazalil was investigated and compared with the predicted mixture effect according to the CA and IA model.

H295R cells were treated with nine different concentrations of the individual imidazoles. A ternary mixture based on a 1:1:1 ratio of the imidazoles was tested at eight different concentrations. Binary mixtures were tested at two concentrations, where the imidazoles contributed equally. Following treatments, levels of cortisol and aldosterone were determined by ELISA.

Effect of prochloraz, ketoconazole, imazalil, and their mixtures on cortisol and aldosterone secretion

It was shown that prochloraz, ketoconazole and imazalil, as well as the ternary imidazole mixture, inhibited cortisol secretion in a similar monotonic order. The inhibition was almost complete at an imidazole concentration of approximately 10 μM and was evident even at sub-micromolar concentrations. The EC_{50} values for cortisol secretion following treatment with prochloraz, imazalil and the ternary mixture were identical

(0.13 μM), ketoconazole had a slightly higher EC_{50} value (0.15 μM). This indicated that the three imidazoles act in an additive manner over the tested concentration range.

In contrast to the effects on cortisol secretion, prochloraz, ketoconazole and the ternary mixture caused biphasic effects on aldosterone secretion. Maximum secretion, with 2.1-fold stimulation for prochloraz and 1.3-fold for ketoconazole, was shown at 0.06 and 0.23 μM, respectively. In contrast to prochloraz and ketoconazole, imazalil caused a monotonic dose-dependent inhibition of aldosterone secretion. This indicated that the biphasic response is not general for all imidazoles. The effect of ketoconazole and imazalil will require further study to explain the mechanisms behind the observed aldosterone secretion pattern for these imidazoles. Most likely, the noted difference in effect for the three single imidazoles is due to their different selectivity and potency regarding effects on specific CYP enzymes (Rotstein *et al.*, 1992; Ayub & Levell, 1989; Drummond *et al.*, 1988). The biphasic pattern for the ternary mixture displayed a 1.7-fold stimulation of aldosterone secretion at a concentration of 0.11 μM. The magnitude of the stimulatory response was lower compared to prochloraz alone, but larger than the effect of ketoconazole. However, at concentrations above 1.0 μM all three imidazoles and the ternary mixture inhibited aldosterone secretion, although with lower potency than the inhibitory effect on cortisol.

Predicting effects of the imidazole mixture based on the CA and IA models

The CA and IA predictions excellently, and equally well, described the observed effects of the imidazole mixture on cortisol secretion. Since IA and CA equally well described cortisol secretion following mixture treatment, no conclusion could be drawn regarding which model should be preferred in this case.

Currently, there is no generally accepted model to predict effects displaying non-monotonic dose-responses. Belz *et al.* (2008) have proposed a modified CA model to circumvent this. The modified CA model by Belz *et al.* (2008) was applied to the effects of the imidazoles and was shown to predict the stimulatory effect of the mixture quite well. The modified model is, however, not mechanistically founded since the mechanism behind stimulatory responses is not generally understood. Therefore, it remains to be determined to what extent the modified CA model is generally applicable for non-monotonic responses.

The IA prediction model is based on quantal responses and has not been used for non-monotonic dose-responses. The modified IA model used in this thesis resulted in a marked overestimation of the stimulatory effect and an underestimation of the inhibitory effect of the imidazoles. Thus, the modified IA model failed to predict the non-monotonic dose-response.

Observed and predicted effects of binary imidazole mixtures

Rather than establishing full dose-response curves for binary mixtures, we compared the CA and IA predictions with data from two imidazole concentrations where we observed big differences between cortisol and aldosterone secretion. As for the ternary mixture, CA and IA predictions of cortisol secretion were overlapping and the observed binary effect of the mixture generally aligned very well with the predicted curves. The situation for aldosterone secretion was more complex. Modified CA and IA prediction curves including prochloraz displayed a biphasic pattern, while the predicted curve for the ketoconazole-imazalil mixture was close to monotonic. The modified CA model predicted the observed effect of the binary mixtures well, whereas the modified IA model failed once more in describing the biphasic effect on aldosterone secretion.

Effects of single imidazoles and binary mixtures on gene expression

Gene expression analysis was performed for the single imidazoles and the binary mixture to address possible mechanisms behind the effects. Only a modest down-regulation following treatment with the individual imidazoles and their binary combination at 0.03 μM were displayed. Most prominent were the effects for *3βHSD2* and *CYP21A2*, which were down-regulated to 55% of control levels. However, as previously described in paper II, effects on gene expression alone cannot satisfactorily explain the distinct patterns in cortisol and aldosterone secretion following imidazole treatment.

Taken together, the results in paper III indicated that the three imidazoles prochloraz, ketoconazole and imazalil inhibited cortisol secretion in a monotonic, additive and predictive way. The biphasic aldosterone secretion pattern was shown to be specific for the tested imidazoles and could not easily be predicted by the modified prediction models. The modified CA prediction model looks promising, but requires further refinement and empirical support.

4.3.2 Effects of single chemicals and a mixture of flavonoids on steroidogenesis – paper IV

Flavonoids are potent inhibitors of cortisol (Ohno *et al.*, 2002; Mesiano *et al.*, 1999) and aldosterone secretion, as indicated in paper I, but also affect secretion of testosterone (Taxvig *et al.*, 2010; Ohno *et al.*, 2003) and oestradiol (Taxvig *et al.*, 2010). Thus, in paper IV we wanted to explore the effect on cortisol, aldosterone, testosterone, and oestradiol secretion in H295R cells following treatment with three naturally occurring flavonoids, daidzein, genistein and apigenin. These compounds are very similar in chemical structure and the isoflavones daidzein and genistein differs only in a hydroxyl group in position 5 (Fig. 7). Difference in chemical structure is also seen between the flavones and isoflavones. The flavone apigenin has the phenol group in position 2, and the isoflavones have the phenol group in position 3. Since these flavonoids are naturally present as mixtures in food we found it of high interest to study the effects of a mixture, and to compare the observed effects of the mixture with predictions from the CA and IA model. The individual flavonoids were tested at five different concentrations and the mixture was based on a 1:1:1 ratio of each flavonoid tested at six concentrations. Hormone secretion levels from H295R cells were measured by ELISA.

Effect of daidzein, genistein, and apigenin on cortisol, aldosterone, testosterone and oestradiol secretion

All three flavonoids displayed dose-dependent inhibition of cortisol, aldosterone and testosterone secretion, with daidzein and genistein showing higher potency than apigenin. In contrast, oestradiol secretion was only inhibited by apigenin. The most sensitive targets of the flavonoids were the secretion of cortisol and testosterone, where daidzein and genistein presented EC_{50} values below 1.0 μM. In contrast, apigenin had an EC_{50} value of 2.0 μM for cortisol secretion and 5.5 μM for the inhibition of testosterone secretion. A decrease in cortisol secretion has previously been demonstrated in H295R cells following treatment with daidzein (Ohno *et al.*, 2002; Mesiano *et al.*, 1999), genistein (Ohno *et al.*, 2002; Mesiano *et al.*, 1999) and apigenin (Ohno *et al.*, 2002). These *in vitro* results indicate a direct effect of flavonoids on steroid biosynthesis. The results are in agreement with *in vivo* data from rats treated with genistein, showing a significantly decreased serum corticosterone concentration and a tendency of decreased testosterone levels (Ohno *et al.*, 2003). Decrease in testosterone secretion in H295R cells, following daidzein and genistein treatment, has been reported by Taxvig *et al.* (2010).

In paper IV it was shown that aldosterone secretion was dose-dependently inhibited by the three flavonoids. Daidzein had an EC_{50} value of 1.6 μM on aldosterone secretion, genistein had a slightly higher EC_{50} of 2.2 μM. Apigenin was a less potent inhibitor and displayed an EC_{50} value of 8.7 μM. A previous study in orchidectomized rats treated with genistein has indicated decreased levels of aldosterone and corticosterone in blood (Ajdzanovic *et al.*, 2009). Our *in vitro* results are in line with the trend in the *in vivo* data presented by Ajdzanovic *et al.* (2009).

The observed effects on cortisol, aldosterone and testosterone secretion may be ascribed to potent effect of daidzein and genisteins as 3βHSD2-inhibitors and their effect on CYP21A2-activity (Ohno *et al.*, 2002; Mesiano *et al.*, 1999). Flavonoids with the phenolic ring in position 3 in the chemical backbone (daidzein and genistein, not apigenin) have been suggested as 3βHSD2- and/or 17βHSD-inhibitors (Le Bail *et al.*, 2000), which may explain the more potent testosterone inhibition of daidzein and genistein, compared to the effect of apigenin.

Taxvig *et al.* (2010) reported increased oestradiol secretion following daidzein and genistein treatment of H295R cells, which we did not find. This disagreement in observed effect may be due to differences in experimental design. The lack of inhibition of oestradiol for daidzein and genistein compared to the inhibition of testosterone secretion, could be due to stimulation of CYP19-activity in H295R cells (Sanderson *et al.*, 2004), thereby compensating for the inhibition of upstream enzymes. Le Bail *et al.* (2000) suggested that flavonoids with the phenolic ring in position 2 of the chemical backbone (*i.e.* apigenin) were more potent inhibitors of oestradiol secretion, and Sanderson *et al.* (2004) have reported apigenin to inhibit CYP19-activity. These studies are in line with our results, where apigenin was shown to be a potent inhibitor of oestradiol secretion with an EC_{50} of 6.4 μM.

Observed and predicted effects of the flavonoid mixture

The flavonoid mixture inhibited secretion of all steroid hormones, including oestradiol, in a dose-dependent way. Cortisol secretion had an EC_{50} value of 0.6 μM, aldosterone secretion 3.1 μM, testosterone secretion 0.9 μM, and oestradiol secretion 5.5 μM. The CA and IA prediction models described the observed effects on cortisol, aldosterone and testosterone secretion well. This indicated that the three flavonoids act in an additive way at the tested concentrations. Both models, however, failed to predict the observed effect

on oestradiol secretion. In general, the IA and CA prediction models equally well described the effects of the flavonoid mixture.

Taken together, the results in paper IV indicated that the three flavonoids daidzein, genistein and apigenin inhibited steroid secretion, with the exception for oestradiol secretion following daidzein and genistein treatment. The three flavonoids in general displayed additive effects on steroid secretion. The observed mixture effect on cortisol, aldosterone and testosterone secretion was described well by both CA and IA prediction models, while both models underestimated the effect on oestradiol secretion.

5 Concluding remarks

The focus of this thesis has been effects of chemicals on the human adrenal steroidogenesis. A human *in vitro* model, based on the adrenocarcinoma cell line H295R, would more appropriately reflect effects on the human adrenal steroidogenesis than animal models. The H295R cell line is already undergoing a validation process by the OECD as a screening tool to address effects of chemicals on testosterone and oestradiol secretion.

It was concluded that the H295R cell line is a promising tool to address effects on human steroidogenesis and production of cortisol and aldosterone, in addition to testosterone and oestradiol. Chemicals inhibited as well as stimulated hormone secretion, showing that the H295R cell line is able to detect both up-regulation as well as down-regulation of steroid secretion. Further, the H295R cells responded to a cAMP-inducer and Ang II by increasing cortisol and aldosterone secretion, respectively. It was shown that the cell model could differentiate between specific effects of chemicals on basal steroid secretion compared to effects on stimulated steroid secretion.

The H295R cells synthesise and secrete all steroids of the steroidogenic pathway, which makes this cell line a good screening tool where quantitative as well as qualitative information on several steroids can be obtained from one test. One way to further develop the model would be to simultaneously determine the level of all steroid hormones and intermediates by new high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS), excluding the need for steroid specific ELISAs. We have just initiated an investigation using this method for analysis.

It was concluded that imidazoles displayed chemical specific effects on cortisol and aldosterone secretion, and mechanistic data from the H295R

cells facilitated the explanation of these effects. Since the H295R cell line proved to be a promising tool for mechanistic studies, further research is encouraged to explore the mechanisms behind the effects of ketoconazole and imazalil.

The three natural flavonoids tested in this thesis inhibited cortisol, aldosterone, testosterone and oestradiol secretion in concentrations similar to the levels detected in human plasma. The effect of flavonoids on steroidogenesis indicates that natural compounds may be more potent disruptors of the endocrine system, than synthetic compounds. It was concluded that the isoflavones (daidzein and genistein) were more potent inhibitors of steroidogenesis than the flavone (apigenin), except for oestradiol secretion where the opposite was shown. This difference in effect between the isoflavones and the flavone may be due to chemical specific effects on enzyme activity.

In this thesis effects of chemical mixtures on steroidogenesis have been addressed by treating the H295R cells with mixtures of imidazoles and flavonoids. Prediction models such as CA and IA was shown to be valuable tools in the assessment of mixture effects. However, the imidazole mixture highlighted the problem with predicting effects of biphasic dose-response patterns. A modified CA model could partially describe the biphasic effect for the imidazole mixture on aldosterone secretion. This modified model requires further assessment, in order to evaluate its potential to predict biphasic effects.

Further research into effects of mixtures is encouraged, since mixtures are the most likely way of exposure to humans and animals. It would be of interest to investigate the mixture effect of compounds with established mechanisms of action, *e.g.* chemicals with dissimilar MoA.

An *in vitro* model for effects on adrenal steroidogenesis does not exclude the need for *in vivo* studies. *In vitro* models can provide detailed information about the direct effects of a chemical, or mixture, on the adrenal steroidogenesis. The adrenal steroid secretion is under influence of the HPA-axis and/or RAS, which is not easily assessed with an adrenal *in vitro* model. We have tried to mimic stress and salt regulated activation of the adrenal steroidogenesis by stimulating the cells with a cAMP inducer and Ang II. Still, animal models are required to study adaptive responses to effects on steroidogenesis *in vivo*. It could be argued that down-regulation of

steroidogenesis may be compensated for by activation of the HPA-axis and RAS. However, it may be harder for the endocrine system to adapt to stimulation of steroidogenesis by chemicals.

The liver is the main organ responsible for metabolism of dietary compounds and chemicals. Combining the H295R model with a metabolising system would make it possible to detect effects from metabolites as well, providing further insights to the possible effects of single chemicals and mixtures on the steroidogenic pathway.

It is recommended to consider the H295R cell line as a screening tool for effects on cortisol and aldosterone secretion, in addition to the effects on testosterone and oestradiol secretion that the OECD is evaluating this model for. In this thesis we have shown that the H295R cell line also can be applied for investigation of mechanistic studies.

Populärvetenskaplig sammanfattning

Binjuren är ett endokrint organ som producerar och utsöndrar steroidhormoner såsom kortisol och aldosteron. I denna avhandling har vi undersökt olika kemikaliers effekt på steroidproduktionen i binjurebarken.

Hormonstörande ämnen är ett samlingsnamn för kemikalier, enskilda eller blandningar, som påverkar funktionen av ett hormonsystem. Detta kan leda till effekter på vår hälsa, påverka fortplantningsförmågan, samt störa den tidiga utvecklingen. Hormonstörande ämnen har länge ansetts utöva sin effekt genom att binda till receptorer, som normalt känner igen signaler från olika hormoner i kroppen. Men, utöver dessa receptorförmedlade effekter har man börjat uppmärksamma direkta effekter av dessa substanser på hormonproduktionen. Dessa direkta effekter kan också få till följd att vår hälsa påverkas, då hormonnivåerna i kroppen kan förändras på grund av kemikalierna. Hormonstörande ämnen återfinns bland ett flertal grupper av kemikalier och vi utsätts dagligen för dessa genom till exempel läkemedel, naturligt förekommande ämnen i mat, samt bekämpningsmedel eller miljöföroreningar. Många av dessa ämnen utsätts vi för samtidigt, som en blandning av olika kemiska föreningar.

Binjurebarken producerar och utsöndrar huvudsakligen tre typer av steroidhormoner, nämligen kortisol, som reglerar metabolismen av kolhydrater och protein, stress samt immunförsvar; aldosteron, som reglerar saltbalansen och därmed indirekt även blodtrycket; samt så kallade svaga androgener/könshormon. Kemikalier som påverkar produktionen av dessa hormon kan störa grundläggande funktioner i kroppen och orsaka sjukdom. Det är viktigt att det finns bra verktyg, så som olika cellmodeller eller djurförsök, för att upptäcka dessa typer av effekter. I denna avhandling har

en human binjurecellmodell, H295R, använts för att studera de hormonstörande ämnenas effekter på produktionen av steroidhormoner.

Flera kemikalier med misstänkta effekter på steroidproduktionen studerades genom att utsätta cellerna för dessa ämnen i cellodlingsmediumet. Mängden utsöndrad hormon efter kemikalieexponeringen bestämdes sedan i mediumet. Huvudsakligen har effekter på produktionen och utsöndringen av kortisol och aldosteron studerats, men även effekter på produktionen och utsöndringen av könshormonerna testosteron och östradiol, samt vissa mellansteg i steroidproduktionen. Normalt producerar inte binjuren könshormon, men cellinjen som använts i denna avhandling kan producera och utsöndra alla typer av steroidhormoner. Bland det trettiotal kemikalier som studerats uppvisades tydliga skillnader i effekt på utsöndringen av kortisol och aldosteron. Vanligast var att kemikalierna hämmade utsöndringen av kortisol och aldosteron, medan ett fåtal av kemikalierna ökade utsöndringen.

En kemikalie som studerats närmare är prokloraz. Denna kemikalie används som svampbekämpningsmedel inom växt- och livsmedelsproduktionen. När cellerna utsattes för prokloraz hämmades kortisolutsöndringen. Utsöndringen av aldosteron var stimulerad vid låga koncentrationer av prokloraz, men hämmades vid höga koncentrationer. En hämning av aktiviteten av enzymerna CYP17A1 och CYP21A2 kunde förklara varför utsöndringen av kortisol gick ned medan utsöndringen av aldosteron gick upp vid låga koncentrationer av prokloraz.

Eftersom vi utsätts för många potentiella hormonstörande ämnen samtidigt studerade vi även effekter av blandningar och inte bara enskilda kemikalier. Två grupper av kemikalier valdes ut för blandningsstudier, imidazoler och flavonoider. Till imidazolerna räknas prokloraz, ketokonazol och imazalil, vilka alla har en svampdödande effekt och används inom jordbruket eller inom medicin. Flavonoider är naturligt förekommande ämnen som vi får i oss via föda. Tre flavonoider har studerats närmare, daidzein, genistein (finns i sojabönor) och apigenin (finns i persilja och selleri). På grund av svårigheten att testa alla blandningar vi utsätts för har det tagits fram matematiska modeller för att beräkna en blandningseffekt utifrån kunskap om de enskilda kemikaliernas effekter. Vi har jämfört våra resultat från kemikalieblandningarna med resultat från matematiska modeller. Generellt sett visade det sig att modellerna fungerar bra för både imidazol- och flavonoidblandningarna, dock förekom vissa avvikelser. Mest problematiskt

blev det att beräkna effekten av imidazolblandningen på produktionen av aldosteron, där det krävdes modifierade matematiska modeller.

Sammanfattningsvis visade vi att steroidproduktionen är känslig för påverkan av kemikalier och att cellinjen H295R verkar vara en lovande modell för att studera hormonstörande ämnens effekt på binjurebarkens hormon-produktion. De matematiska modeller som använts i denna avhandling är värdefulla redskap i utvärderingen av kemikalieblandningar.

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