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REVIEW ARTICLE

The Adrenal Cortex and Steroidogenesis as Cellular and Molecular Targets for Toxicity: Critical Omissions from Regulatory Endocrine Disrupter Screening Strategies for Human Health?

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Key words: adrenal toxicity; adrenocortical function; endocrine disruption; steroidogenesis; CYP enzyme inhibitors; StAR; H295R; ovaries; testes; human; wildlife; regulatory toxicology.

Current testing strategies to assess the endocrine disrupting properties of chemicals have omitted examination of the adrenal gland and do not adequately cover the process of steroidogenesis. Steroidogenesis is critical for adrenocortical function as well as that of the testes and ovaries, and presents multiple molecular targets for toxicity, ranging from general effects on all steroidogenic tissues (e.g. via StAR protein or CYP11A1 cholesterol side-chain cleavage) through to specific targets affecting only adrenocortical function (e.g. CYP11β/18 and glucocorticoid synthesis). Numerous chemicals of environmental relevance are now being shown to affect adrenocortical function both *in vivo* in aquatic species and *in vitro* in human cell lines, and given the *vital* role of the adrenal gland to human health and development, there is a strong case for including dedicated assessment techniques in screening batteries for endocrine-disrupting chemicals, not least to assist in general data interpretation (e.g. whether adrenal hypertrophy is due to stress or to a more sinister adrenocortical insufficiency). Cell lines such as H295R (derived from a human adrenocortical adenocarcinoma) currently exist that will allow assessment of cortisol production *and* most of the major enzymes and functional proteins in the steroidogenic pathway (e.g. StAR; CYP11A1/scc; CYP11β/18; CYP17; CYP19; CYP21; 3β-hydroxysteroid dehydrogenase). Adequate assessment of adrenocortical function, as with any component of the integrated endocrine system, probably also will require the development of specific *in vivo* methodology to include effects on hypothalamo-pituitary function. Finally, although there is currently no direct evidence that environmental exposure to endocrine-disrupting (oestrogenic) chemicals has actually caused adverse human health effects, lessons have been learned on their potential from the diethylstilboestrol case. Similar evidence exists from aminoglutethimide and etomidate on the lethal impact of unpredicted chemically induced adrenal insufficiency in sensitive human subgroups, and it would seem prudent to incorporate relevant tests for adrenal function and steroidogenesis into current regulatory validation programmes. Published in 2003 by John Wiley & Sons, Ltd.

INTRODUCTION

The majority of research in the field of endocrine toxicology has focused on oestrogenicity. Historically, research in endocrine toxicology largely evolved out of reproductive toxicology where concerns centred on the potential effects of chemicals on reproduction and development. Momentum on oestrogenicity has been maintained by hypotheses that early life exposure to oestrogenic chemicals may be responsible for health effects in humans (Sharpe and Skakkebaek, 1993). Proposals for weight of evidence methodology for the ranking of chemicals based on their oestrogenic properties, and a hierarchical approach to oestrogenicity evaluation, have been developed (Ashby, 1997; Calabrese *et al.*, 1997) and structure–activity relationships have been derived for oestrogen receptor binding (e.g. Dodge, 1998; Hong *et al.*, 2002).

More recently, it has been recognized in a regulatory context that there are many processes and toxicological targets in the endocrine system, in addition to oestrogenicity, that could be subject to chemical disruption and have equally important potential consequences for human health, including development. In the USA, the Environmental Protection Agency (USEPA) Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) published its final report on endocrine testing (EDSTAC, 1998) and this and other initiatives have been discussed elsewhere (Cockburn and Leist, 1999). More recent proposals on the significance and limitations

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of 'endocrine disruption' data recognize the broader context of the endocrine system as a whole, recognize that there are gaps in current testing strategies and provide an approach to assessing the significance of the data that exists for many chemicals (Harvey and Johnson, 2002).

The importance of the EDSTAC recommendations is that, although still focusing on reproduction and development, they go beyond oestrogenicity to include other types of effect on the endocrine system, such as anti-androgenic and anti-thyroidal actions. In a general regulatory context, progress has been made in the methodology for screening anti-androgens (e.g. Ashby and Lefevre, 2000; Sohoni *et al.*, 2001) and in the extension of the OECD 407 guideline to encompass endocrine disruption, including thyroid function (e.g. Yamasaki *et al.*, 2002). However, there is currently no provision for assessment of adrenocortical function or the molecular pathways of steroidogenesis (other than limited aromatase activity) and steroidogenesis provides multiple molecular targets for toxicity, along enzymatic pathways in ovary, testis and adrenal cortex.

THE ADRENAL GLAND

The adrenal gland is *vital* to health and has a role in reproduction and development. Omission of the adrenal gland in current testing guidelines for endocrine function (e.g. EDSTAC, 1998: developments in OECD 407) is surprising given that it is, in fact, the most common target for toxicity in the endocrine system (Ribelin, 1984; Colby, 1996). Most lesions involve the adrenal cortex, with fewer chemicals known to affect the adrenal medulla (Tucker, 1996). Detailed reviews of adrenal endocrinology, pharmacology and mechanisms of toxicity can be found in Harvey (1996), Hinson and Raven (1999) and Rossol *et al.* (2001). Information on the involvement of the adrenal gland and associated hormones in sexual maturation and puberty, oestrous cycles and various aspects of somatic and neurological development in rats and mice (as species and parameters assessed in various regulatory toxicology protocols) can be found in Ramaley (1978), Meijs-Roelofs and Moll (1978), Ramaley (1977), Nichols and Chevins (1981), Harvey and Chevins (1987) and Alves *et al.* (1993).

Significant progress has been made in elucidating the factors that predispose the adrenal cortex to direct toxic insult, including its disproportionately large blood supply per unit mass, lipophilicity and high concentration of cytochrome P450 (CYP) and other enzymes normally utilized in steroidogenesis but that can also bioactivate toxicants (Hinson and Raven, 1999). Indeed, the adrenal cortex is unique in its steroidogenic biosynthetic capabilities and can synthesize all major classes of steroids (androgens, oestrogens, progestogens, as well as glucocorticoids and the mineralocorticoid aldosterone; see Raven and Hinson, 1996; Hinson and Raven, 1999). Adrenocortical function is vital to health and inhibition of this process is one of the few examples whereby endocrine disruption as side-effect toxicity has resulted in confirmed human morbidity and mortality (Mann, 1996; Raven and Hinson, 1996). The versatility in the capability of the adrenal cortex would also make it a primary model to study steroidogenesis.

STEROIDOGENESIS

Toxic effects on steroidogenesis have been largely the subject of research, rather than regulatory, interest and reports of chemicals having effects on this process extend beyond measuring androgen–oestrogen conversion by aromatase (CYP19, as included in EDSTAC 1998 recommendations) and include, for example, the function of steroid acute regulatory (StAR) protein and a wide variety of other cytochrome P-450 and dehydrogenase enzymes. The range of molecular targets in steroidogenesis and the compounds known to exert actions against these targets are shown in Tables 1 and 2. It is clear from these selected examples (note that there are reports of other compounds affecting other elements of steroidogenic pathways, particularly in the adrenal cortex) that there are a number of important molecular targets that are currently not assessed in regulatory toxicology studies. The inhibition of CYP11A1 or CYP11β/18 by aminoglutethimide and etomidate, respectively, proved to be fatal in humans because of adrenocortical insufficiency (the former being the enzyme catalysing side-chain cleavage of cholesterol, which is the starting point of steroid synthesis, and the latter specifically mediating cortisol synthesis). Although these examples pertain to pharmaceuticals (the adverse drug reactions were not predicted from therapeutic category, and etomidate, as a sedative and anaesthetic induction agent, caused adverse reaction following relatively brief exposure regimes), they remain mechanisms by which other chemicals can exert effects and currently relatively little is known of structure–activity relationships to predict these actions. Nebert and Russell (2002) review human conditions (both endocrine and non-endocrine) known to result from dysfunction of cytochrome P-450.

Similar adrenocortical insufficiency is also possible by inhibition of StAR protein function (this transports cholesterol across the mitochondrial membrane for conversion to pregnenolone by CYP11A1/side-chain cleavage, and therefore StAR represents the ultimate rate-limiting step of all steroidogenesis, thereby potentially affecting testicular, ovarian and/or adrenocortical function) and there are reports of pesticides inhibiting this protein (e.g. lindane: Walsh and Stocco, 2000). Indeed, dimethoate is reported to inhibit *both* StAR gene transcription and CYP11A1/cholesterol side-chain cleavage (Walsh *et al.*, 2000c), indicating the vulnerability of the process of steroidogenesis to chemical insult (see also Ohno *et al.*, 2002: the effects of flavonoids on multiple steroidogenic enzymes in human adrenocortical cells, with 6-hydroxyflavone inhibiting four separate enzymes).

ASSESSMENT OF ADRENOCORTICAL FUNCTION AND STEROIDOGENESIS: DATA GAPS, CURRENT STATUS OF METHODOLOGY AND FINDINGS

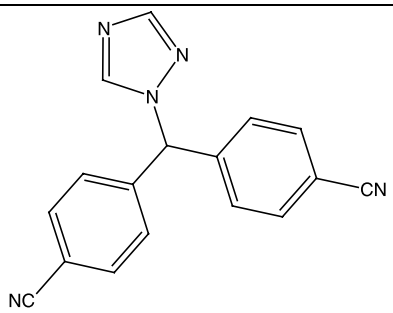
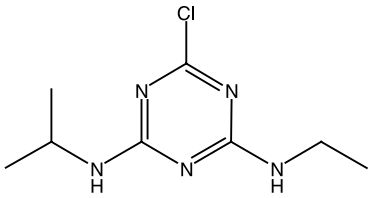
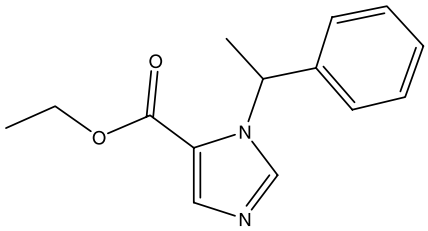
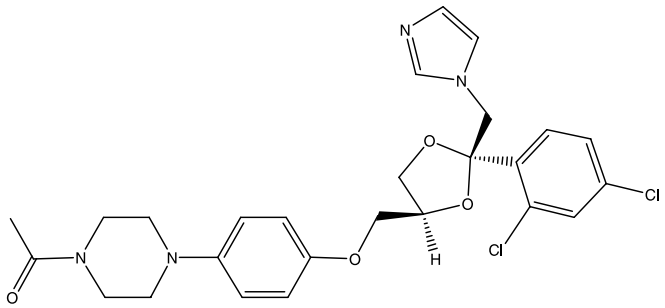
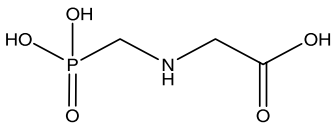
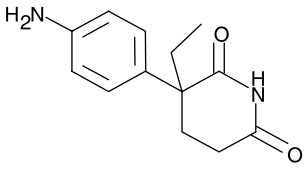
In order to assess adequately a compound for *endocrine* disruption, it is necessary to examine the *entire* endocrine system (i.e. all the glands, hormonal target tissues, receptors, synthesis enzymes, hormones and carrier

Table 1—Major toxicological targets in steroidogenesis: steroid acute regulatory protein, cytochrome P-450 (CYP) and dehydrogenase enzymes and agents of toxicity

Steroidogenic pathway target	Function	Compound	Reference
Steroid acute regulatory (StAR) protein	Transports cholesterol across mitochondrial membrane for the first step in steroidogenesis: conversion to pregnenolone	Econazole, Miconazole Lindane Dimethoate	Walsh *et al.* (2000a) Walsh and Stocco (2000) Walsh *et al.* (2000c)
CYP11A1 (CYP side-chain cleavage; CYPscc)	Conversion of cholesterol to pregnenolone	Aminoglutethimide Dimethoate	Camacho *et al.* (1967) Walsh *et al.* (2000c)
CYP17 (17α-hydroxylase/17,20-lyase)	Conversion of pregnenolone to 17α-hydroxypregnenolone and onto dehydroepiandrosterone Conversion of progesterone to 17α-hydroxyprogesterone and onto androstenedione	Spironolactone Ketoconazole	Kossor *et al.* (1991) Loose *et al.* (1983)
3-Hydroxysteroid dehydrogenase Δ 4,5 isomerase	Conversion of pregnenolone to progesterone Conversion of 17α-pregnenolone to 17α-hydroxyprogesterone	Cyanoketone Trilostane	McCarthy *et al.* (1996) Potts *et al.* (1978)
17β-Hydroxysteroid dehydrogenase	Conversion of androstenedione to testosterone and oestrone to oestradiol	Di(2-ethylhexyl) phthalate	Akingbemi *et al.* (2001)
CYP21 (21-hydroxylase)	Conversion of progesterone to 11-deoxycorticosterone Conversion of 17α-hydroxyprogesterone to 11-deoxycortisol	RU486	Albertson *et al.* (1994)
CYP11B1 CYP11β/18 (11β/18-hydroxylase)	Conversion of 11-deoxycorticosterone to corticosterone and 11-deoxycortisol to cortisol	Metyrapone Mitotane Etomidate	Liddle *et al.* (1958) Hornsby (1989) Raven and Hinson (1996)
CYP19 (aromatase)	Aromatization of testosterone to oestradiol and androstenedione to oestrone	Prochloraz Fenarimol	Andersen *et al.* (2002)
CYP11B2 (aldosterone synthase)	Conversion of 11-deoxycorticosterone to aldosterone	Guanabenz-related amidinohydrazones	Soll *et al.* (1994) Hinson and Raven (1996)

Additional details of enzymes in steroidogenesis can be found in Raven and Hinson (1996) and Hinson and Raven (1999). Additional examples are given in the text and selected structures are given in Table 2. Note that compounds may have more than one molecular target (see text; Walsh *et al.*, 2000c; Ohno *et al.*, 2002), indicating the vulnerability of steroidogenesis to toxic insult.

Table 2—Structures of selected compounds with actions on cytochrome P-450 (CYP) enzymes and other proteins involved in steroidogenesis in the adrenal cortex and/or testes or ovaries

Compound	Structure	Action	Reference
Letrozole (medicine)		Selective steroidogenesis (aromatase CYP19) inhibitor	Assikis and Buzdar (2002)
Atrazine (pesticide)		Anti-androgen Selective aromatase (CYP19) *inducer*	Danzo (1997) Hayes *et al.* (2002) Sanderson *et al.* (2000, 2002)
Etomidate (medicine)		Selective steroidogenesis (CYP11β/18) inhibitor Potent selective adrenocortical inhibitor	Raven and Hinson (1996) Weber *et al.* (1993)
Ketoconazole (medicine)		Selective steroidogenesis (CYP17) inhibitor	Raven and Hinson (1996) Weber *et al.* (1993)
Roundup (pesticide)		Complete steroidogenesis (StAR protein) inhibitor	Walsh *et al.* (2000b)
Amino-glutethimide (medicine)		Complete steroidogenesis (CYP11A1,scc) inhibitor Adrenocortical inhibitor	Raven and Hinson (1996)

Additional examples are given in Table 1 and text. Note that steroidogenic CYP enzymes can be inhibited or induced (cf. letrozole and atrazine). Additional structures of compounds active against other targets in the endocrine system (e.g. anti-androgens, anti-thyroidal peroxidase inhibitors and CYP19 inhibitors) are given in Harvey and Johnson (2002).

proteins, etc. that comprise the system as a whole; Harvey *et al.*, 1999a,b; Harvey and Johnson, 2002). However, specific tests for adrenocortical function and steroidogenesis are generally lacking from major toxicity testing guidelines. Current regulatory mammalian toxicology protocols will detect most types of toxicity, but there are particular dangers in the interpretation of endocrine findings without additional dedicated test parameters incorporated into existing protocols. For example, adrenal hypertrophy is often disregarded as being merely an indirect stress effect. This *may* be the case but there could be other reasons for excessive stimulation of the adrenal cortex by adrenocorticotrophin (ACTH), not least because of loss of negative feedback inhibition due to an inhibitory effect on glucocorticoid synthesis (the mechanism by which aminoglutethimide and etomidate killed patients) and supporting studies would be required to exclude positively any more sinister mechanisms. Elucidation of the mechanism of toxicity is critical in endocrine toxicology in order to understand the relevance of findings. Because several rodent and non-rodent test species are used in regulatory toxicology packages, it is important to note that there differences in adrenal function due to species, strain, gender, reproductive status, age and a variety of environmental factors (see Harvey, 1996).

At present, not enough is known about endocrine disruption to state conclusively whether human health is being affected adversely by exposure to chemicals with endocrine-modulating actions (Harrison, 2001). It is, however, clearer that wildlife first started to show the effects of endocrine disruption (due to, for example, exposure to inherently oestrogenic chemicals) and that these effects can seemingly occur at *environmentally relevant* exposure levels (see Hayes *et al.*, 2002 for recent data on atrazine and feminization of frogs, possibly via CYP19 induction). If aquatic wildlife are considered to be general sentinel species (the reproductive defects detected in aquatic species raised concern over oestrogenic chemicals and human health) because of their constant exposure in contaminated media, then the emerging data on the effects on adrenal function deserve serious consideration.

Impaired adrenocortical responses have been reported in brown trout from metal-contaminated rivers (Norris *et al.*, 1999) and several other fish species collected from sites contaminated with polyaromatic hydrocarbons and polychlorinated biphenyls (Hontela *et al.*, 1992). In the laboratory, impaired cortisol responses have been produced in tilapia by feeding PCB 126 (Quabius *et al.*, 1997), and in *in vitro* studies using rainbow trout adrenocortical steroidogenic cells, the pesticides atrazine, mancozeb, diazinon and endosulfan inhibited cortisol secretion in response to challenge by ACTH or dibutyryl-cAMP (Bisson and Hontela, 2002). The importance of adrenocortical inhibition is that it can be fatal to an animal existing in a stressful environment, and any associated reduced survival fitness in wild populations may be too general and less obvious for causal diagnosis compared with, for example, the overtly structural developmental defects associated with other mechanisms of endocrine disruption (e.g. deformities in frogs, as reported by Hayes *et al.*, 2002).

Turning to implications for human risk assessment, proper assessment of adrenocortical function will probably require the development of relevant validated *in vivo* (to assess the integrated function of the hypothalamo-pituitary-adrenocortical axis) regulatory toxicology protocols in the same way that protocols are now being developed to cover additional thyroid and reproductive endpoints (e.g. OECD 407 developments: Yamasaki *et al.*, 2002; Andrews *et al.*, 2001). However, there are *in vitro* techniques that can be used to screen and provide supportive mechanistic data. The H295R cell line is derived from human adrenocortical adenocarcinoma cells and has been used to assess the effects of toxicants on both cortisol production and a range of steroidogenic enzymes and processes. Using this cell line, Ohno *et al.* (2002) report reduced cortisol production following treatment with a range of flavonoid phytochemicals. They examined major steroidogenic enzymes (including CYP11A1/scc, CYP11β/18, CYP17, CYP21 and 3β-hydroxysteroid dehydrogenase) and found that 6-hydroxyflavone inhibited CYP17 and CYP11β and, together with several other flavonoid chemicals, also inhibited CYP21 and 3β-hydroxysteroid dehydrogenase. That one compound can affect so many different steroidogenic targets illustrates the potential vulnerability of the process. Additionally, inhibition of only a single enzyme could have serious consequences for the steroidogenic process as a whole, because of its reliance on sequential biotransformations. The H295R cell line has been used to assess both inhibition and induction of CYP19 aromatase activity: the pesticides imazalil and prochloraz were potent inhibitors (Andersen *et al.*, 2000; Sanderson *et al.*, 2002) whereas atrazine, simazine, propazine and vinclozolin were CYP19 inducers (Sanderson *et al.*, 2000, 2002). To summarize, the H295R cell line is derived from human tissue, it expresses most of the molecular targets in the steroidogenic pathway, it has demonstrable responses to a number of environmentally relevant chemicals and it therefore could be viewed as a potential model for validation as an *in vitro* screen for both adrenocortical toxicity and steroidogenesis, which is relevant to data provision for human hazard and risk assessment purposes.

CONCLUSIONS

The adrenal gland is currently not investigated in regulatory strategies for endocrine disrupters and the process of steroidogenesis is inadequately assessed. These are considered to be significant data gaps. However, they can probably be readily addressed by existing techniques. Without dedicated adrenal endocrine toxicology studies, data from general toxicology studies can be misinterpreted.

It is considered that for any realistic assessment of chemicals for endocrine disrupting effects, the endocrine system as an integrated functional whole must be examined: as a minimum, each of the major glands require specific examination, as does the process of steroidogenesis, because each step and enzyme represents a molecular target for toxicity from a growing number of environmentally relevant toxicants (see Table 1). The dependency of steroidogenesis on sequentially ordered molecular biotransformation steps also contributes to the vulnerability of the process as a whole, and the adrenal

cortex, because of its unique universal steroidogenic capability and role in response to environmental (and toxic) insult, is certainly the most common and perhaps the most vulnerable endocrine target.

There is already evidence of adrenal toxicity and cortisol deficits in fish following exposure to chemicals in the aquatic environment, with supportive evidence of mediation by selective effects on steroidogenesis, and these species have been sentinels previously for other types of endocrine disruption. Given the lack of certainty of the potential effects of exposure to environmental endocrine-disrupting chemicals on human health in general, coupled with the confirmed knowledge of the human conditions caused by adrenal dysfunction, iatrogenic or otherwise, it would seem prudent to devote some effort into the incorporation of tests of adrenocortical function and steroidogenesis (as clear potential targets for chemical disruption) into current regulatory test validation programmes.

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