Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action

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Steroid target tissues regulate the local level of steroid hormone that can bind and *trans*-activate nuclear receptors (a process known as intracrine modulation). This pre-receptor regulation can be achieved by hydroxysteroid dehydrogenases (HSDs). For each sex hormone there is a pair of HSD isoforms which act either as reductases or oxidases to convert potent steroid hormones into their cognate inactive metabolites, or vice-versa. In this manner, HSDs can function as molecular switches to regulate steroid hormone action. Because these HSDs show tissue-specific expression, inhibitors of these enzymes are predicted to cause tissue-specific responses to steroid hormones. These inhibitors would represent a new class of therapeutics called 'selective intracrine modulators' (SIMs). SIMs are expected to have the same tissue-specific effects as selective steroid receptor modulators but a different mode of action as their effects are enzyme- and not receptor-mediated. HSDs responsible for these interconversions belong to two protein superfamilies: the short-chain dehydrogenases/reductases; and the aldo-keto reductases. Crystal structures exist for HSDs in both families, making rational design of SIMs a reality. Broad-based criteria have been established which must be fulfilled to validate each HSD isoform as a potential SIM target.

Keywords: aldo-keto reductase/hydroxysteroid dehydrogenase/short-chain dehydrogenase or reductase/steroid receptor

Introduction

Steroid hormones exert their action by binding to soluble nuclear receptors (Carcon-Jurica *et al.*, 1990). Once bound, the steroid hormone–receptor complex dissociates from chaperone proteins and dimerizes. The ligand-bound dimeric receptor then binds to steroid hormone-responsive elements on the 5′-flanking (promoter) region of steroid hormone-responsive genes to cause a change in gene transcription; this process is known as *trans*-activation (Figure 1) (Carcon-Jurica *et al.*, 1990; Landers and Spelsberg, 1992; Truss and Beato, 1993). Agonist action was viewed as causing gene transcription by mimicking the action of the endogenous steroid ligand, while antagonist action was viewed as a competition with the agonist resulting in a blockade of gene transcription. This view has been supplanted by the concept that ligands alter the conformation of the receptor (Brzozowksi *et al.*, 1997; Wijayaratne *et al.*, 1999) to recruit tissue-specific co-activators (CA; e.g., SRC-1/NcoA-1) and co-repressors (CR; e.g. NcoR, SMRT) leading to a graded response (Klinge, 2000; McDonnell, 2000). Thus, antagonists are now often referred to as selective steroid hormone receptor modulators because, depending on the tissue distribution of CA and CR, steroid receptor ligands may act as agonists in one tissue and antagonists in another. For example, tamoxifen will act as an estrogen receptor agonist in the

uterus and bone, but as an antagonist in the breast (Dhringa, 1999; Jones *et al.*, 1999; Tobias, 1999). Tamoxifen and its successor raloxifene are now referred to as selective estrogen receptor modulators (SERMs) (Ibrahim and Hortobagyi, 1999).

Steroid target tissues also modulate steroid action at the pre-receptor level by regulating the amount of steroid hormone ligand available for receptor occupancy. This review will focus on the pre-receptor regulation of steroid hormone action, where ligand access to the receptor in target tissues is under enzyme control. The enzymes that are the focal point of this article are the hydroxysteroid dehydrogenases (HSDs) (Penning, 1997).

HSDs play a pivotal role in the local production of steroid hormones in target tissues (intracrine modulation) (Labrie *et al.*, 2000). For every sex hormone it is hypothesized that a pair of HSDs exist which will either convert a potent hormone into its cognate inactive metabolite, or vice-versa. HSDs often catalyse stereoselective reactions at specific positions of the steroid so that for each sex hormone there is an isoform that will either inactivate a hormone or produce an active ligand. This is achieved either by the reduction of a steroid ketone to a steroid alcohol (reductase activity) or by the oxidation of a steroid alcohol to a steroid ketone (oxidase activity). The reductive and oxidase activities of HSDs permit isoforms to function as molecular switches and turn receptor ligand occupancy 'on' or 'off'.
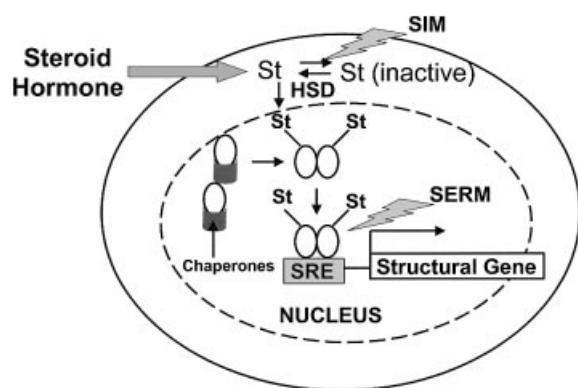
Figure 1. Mechanism of action of steroid hormones showing the site of action of selective estrogen receptor modulators (SERMs) and selective intracrine modulators (SIMs). St = steroid; SRE = steroid response element; HSD = hydroxysteroid dehydrogenase.

For example, the reductase responsible for the conversion of estrone (a weak estrogen) to 17β-estradiol (a potent estrogen) is the estrogenic type 1 17β-HSD and is the 'on' switch for the estrogen receptor (ER). The oxidase activities responsible for the reverse reaction and the inactivation of 17β-estradiol are the type 2 and type 4 17β-HSDs, and these function as the 'off' switch for the ER (Figure 2). Thus, the activity of these 17β-HSD isoforms may regulate the ligand occupancy of ERα and ERβ and their *trans*-activation in estrogen target tissues (Wu *et al*., 1993; Andersson, 1995; Labrie *et al*., 1997; 2000). Because HSD isoforms show tissue-specific expression and control intracrine levels of steroid hormones, isoform-specific HSD inhibitors will act as selective intracrine modulators (SIMs). It is likely that SERMs and SIMs will have similar pharmacological profiles but different mechanisms of action. This review will also address routes to the development of SIMs.

Intracrine formation of steroid hormones

The intracrine formation of steroid hormones is of relevance in the aetiology of steroid-dependent breast and prostate cancer (Labrie *et al*., 1997). Breast cancer is a disease that predominates in post-menopausal women. In these women the production of estrogens no longer takes place in the ovary, but occurs locally. A number of enzymes play a key role in the local production of 17β-estradiol (the most potent natural estrogen) in the breast. Dehydroepiandrosterone (DHEA) from the adrenal gland can be converted to Δ^4-androstene-3,17-dione by 3β-HSD/ketosteroid isomerase (KSI) which is aromatized by cytochrome P450 aromatase (CYP arom) to yield estrone. Much of this estrone can be stored locally in the breast as estrone sulphate, and the release of free estrone is catalysed by estrogen sulphatase (Reed *et al*., 1996). However, once free estrone (a weak estrogen) is formed it must be reduced to 17β-estradiol (a potent estrogen), and this reaction is catalysed by the estrogenic type 1 17β-HSD.

In parallel, androgen-dependent prostate cancer is a disease of the ageing male. Although Leydig cell testosterone biosynthesis wanes with age, circulating testosterone is produced in sufficient amounts so that the prostate still synthesizes 5α-dihydrotestosterone (5α-DHT; the most potent androgen) from circulating testosterone via 5α-reductase type 2. However, the dependence on the local production of androgens becomes evident when hormonal ablative treatments for this disease are considered. Following castration, the prostate maintains its ability to produce androgens locally. In this situation, adrenal dihydroepiandrosterone (DHEA) becomes the principal source of androgens in the prostate (Labrie *et al*., 2000). DHEA is converted to 5α-DHT via the concerted action of 3β-HSD/KSI, type 5 17β-HSD and 5α-reductase type 2 (Labrie *et al*., 1997).

Following treatment with finasteride (a 5α-reductase type 2 inhibitor), prostatic 5α-DHT levels are suppressed by only 80%, indicating that another source of 5α-DHT is available (Gormley, 1996). This source may be via the so-called back-reaction in which 5α-androstane-3α,17β-diol (3α-diol) is oxidized by 3α-HSD isoforms to yield 5α-DHT (Horst *et al*., 1975; Walsh and Wilson, 1976; McDermott *et al*., 1978). In this sequence, the source of 3α-diol is likely to arise from the hepatic metabolism of C19-steroids. Thus, in prostate disease the impact of type 5 17β-HSD and oxidative 3α-HSD isoforms may be important in sustaining 5α-DHT levels. Thus, in two principal steroid hormone-dependent cancers the local formation of potent steroid hormones by HSDs may play a key role in regulating receptor response.

Enzymes that control ligand access to steroid receptors

Enzymes other than HSDs can play important roles in controlling ligand access to steroid receptors. The importance of aromatase in making estrogens has been stressed. Similarly, 5α- and 5β-reductases can also play an important role by reducing the α,β-unsaturated ketone present in the A-ring of most steroid hormones to yield the 5α- and 5β-reduced products. Furthermore, the action of steroid sulphatases (Reed *et al*., 1996) and steroid sulpho-transferases (Falany *et al*., 1993; Luu-The *et al*., 1996) can affect the balance of active and inactive hormone available. The former will release steroids from their inactive conjugates, while the latter will eliminate the active hormone by conjugation in the presence of the sulphate donor phosphoadenosine-phosphosulphate.

However, the HSDs hold the greatest promise as drug targets. This is because the field has matured to identify the discrete HSD isoforms responsible for the formation of active androgens, estrogens and progestins and their elimination in target tissues. Crystal structures also exist for several of the important HSDs, making rational approaches to inhibitor design feasible. An additional advantage is that HSDs work as discrete entities whereas steroid receptors work in concert with a multiprotein complex that contains CA and CR. At the last count there were in excess of 50 CA and CR for the ERα alone (Klinge, 2000), suggesting that the rational targeting of these multi-protein complexes will be more difficult.

HSDs and pre-receptor regulation

11β-HSDs as prototypic SIM-targets

The differential effects of the human 11β-HSD isoforms provides a compelling example of how HSD isoforms regulate steroid hormone action at the pre-receptor level (Funder *et al*., 1988). It is described here because this is a prototypic example of how HSDs can regulate steroid hormone response. It is
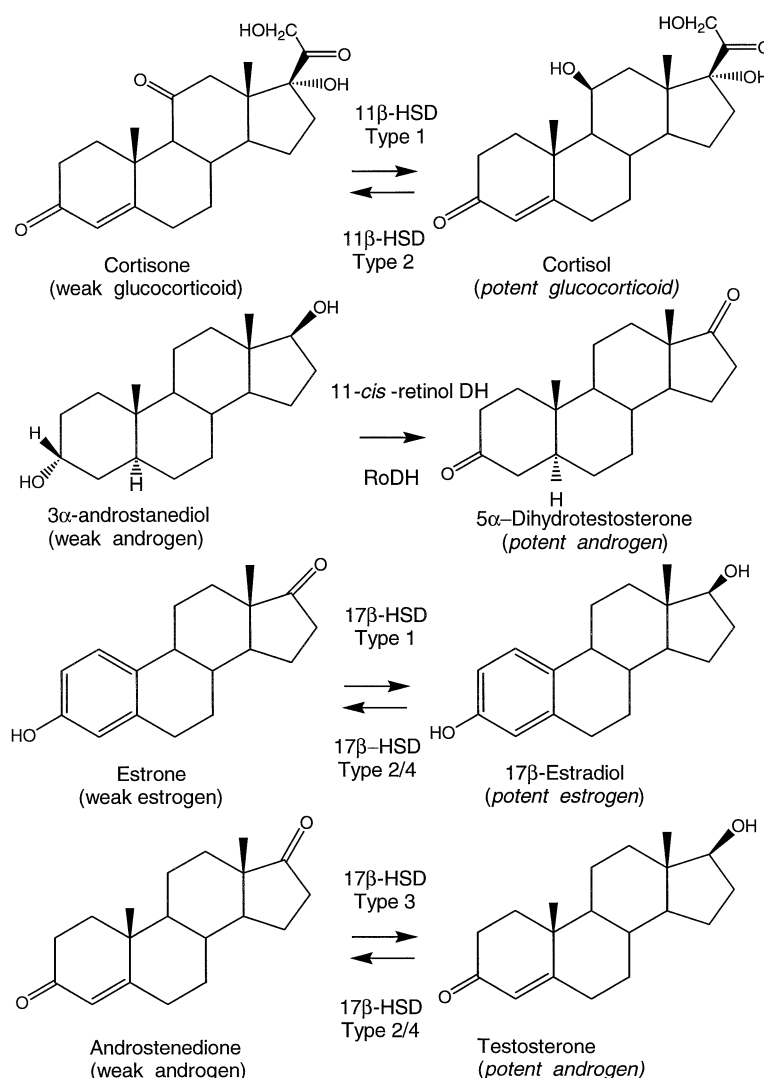
Figure 2. Short-chain dehydrogenases/reductases (SDRs) that control ligand occupancy of steroid receptors. 11-*cis*-retinol DH = 11-*cis* retinol dehydrogenase; RoDH = RoDH, 3α-HSD-like dehydrogenase.

likely that HSD isoforms involved in sex hormone inter-conversion will have similar properties.

11β-HSD type 2 functions as an NAD^{+}-dependent 11β-oxidase to convert the potent glucocorticoid (cortisol) to the inactive glucocorticoid (cortisone) (Stewart and Whorwood, 1994) (Figure 2). Importantly, cortisol has high affinity for either the glucocorticoid receptor or the mineralocorticoid receptor, and if it were not for the action of this enzyme the mineralocorticoid receptor would be flooded with excess cortisol. The protection of the mineralocorticoid receptor is achieved by discrete localization of the 11β-HSD type 2 isoform to the renal tubules, where it is co-expressed with the mineralocorticoid receptor (Rundle *et al.*, 1989; Mercer and Krozowski, 1992; Albiston *et al.*, 1994). This ensures that the receptor will respond only to fluctuating levels of aldosterone. Inherited deficiencies of 11β-HSD type 2 (Mune *et al.*, 1995; Obeyesekere *et al.*, 1995; Wilson *et al.*, 1995a; b), or 11β-HSD type 2 inhibitors derived from liquorice (glycerrhetinic acid), can cause an apparent mineralocorticoid excess (AME)

syndrome (Stewart *et al.*, 1987; Monder *et al.*, 1989), thereby demonstrating the physiological significance of this isoform in regulating steroid receptor occupancy. The importance of the 11β-HSD type 2 enzyme in regulating the mineralocorticoid receptor has since been confirmed in *11β-HSD type 2* null mice which had a phenotype characteristic of AME (Holmes *et al.*, 2001). The ability of glycerrhetinic acid to mimic AME implies that this agent would represent a founder SIM.

In contrast, the 11β-HSD type 1 enzyme catalyses the reverse reaction. It acts predominantly as an NADPH-dependent 11-ketosteroid reductase and converts cortisone to cortisol, but may also act as a 'local-amplifier' of glucocorticoid action (Seckl and Walker, 2001). 11β-HSD type 1 is expressed in adipose tissue and in liver where it can regulate occupancy of the glucocorticoid receptor (Bujalska *et al.*, 2002). Transgenic mice overexpressing 11β-HSD type 1 in adipose tissue develop visceral obesity (Masuzaki *et al.*, 2001), while *11β-HSD type 1* null-mice have an improved lipid profile, hepatic insulin sensitization, and a potentially atheroprotective phenotype

(Morton *et al*., 2001). These studies emphasize the importance of this enzyme in controlling local cortisol action at the pre-receptor level. Thus, in the case of 11β-HSD there are two isoforms: one which acts as an oxidase to eliminate active hormone; and the other which acts as a reductase to generate active hormone. It is hypothesized that the ability of HSDs to function as molecular switches and turn receptor occupancy 'on' and 'off' will apply to those HSDs that interconvert sex steroid hormones.

HSD isoforms implicated in the regulation of sex steroid hormone receptors

HSDs that interconvert sex steroid hormones are the 3α-, 17β- and 20α-HSDs. Of these, 3α-HSD isoforms may regulate ligand access to the androgen receptor (AR) (Taurog *et al*., 1975; Jacobi and Wilson, 1976; Jacobi *et al*., 1977; Biswas and Russell, 1997). 5α-DHT (a potent androgen; K_d = 10–11 M for the AR) is converted by 3-ketosteroid reductases to 3α-diol (a weak androgen; K_d = 10–6 M for the AR) (Liao *et al*., 1973). Candidate enzymes that can catalyse this reaction are the type 2 and type 3 3α-HSD (Penning *et al*., 2000) (Figure 3). By contrast, 3α-hydroxysteroid oxidases can convert 3α-diol back to 5α-DHT. Candidate enzymes for this back-reaction are the type 3 3α-HSD, 11-*cis*-retinol dehydrogenase (11-*cis*-retinol DH) and RoDH/3α-HSD (Biswas and Russell, 1997; Penning *et al*., 2000; Chetyrkin *et al*., 2001; Huang and Lu-The, 2001a; b).

Ligand occupancy of the AR may also be controlled by the type 5 17β-HSD which acts as a 17-ketosteroid reductase and converts Δ⁴-androstene-3,17-dione to testosterone (Dufort *et al*., 1996; 1999; Lin *et al*., 1997) and by the type 2 and type 4 17β-HSD that act as a 17β-hydroxysteroid oxidase and oxidize testosterone back to Δ⁴-androstene-3,17-dione (Wu *et al*., 1993; Andersson, 1995; Adamski *et al*., 1995). The current view is that the type 3 17β-HSD, which reduces Δ⁴-androstene-3,17-dione to testosterone is not involved in regulating ligand occupancy of the AR in target tissues since it is Leydig cell-specific.

Similarly, ligand occupancy of estrogen receptor (ER)α will be regulated by the type 1 and type 5 17β-HSD which act as 17-ketosteroid reductases and convert estrone to 17β-estradiol (Peltoketo *et al*., 1988; 1999; Labrie *et al*., 1994; 1997; Penning *et al*., 2000) and by type 2 and type 4 17β-HSD which act as 17β-hydroxysteroid oxidases and oxidize 17β-estradiol back to estrone (Peltoketo *et al*., 1999) (Figures 2 and 3). Recently, 5α-androstane-3β,17β-diol (3β-diol) was identified as a ligand for ERβ (Weihua *et al*., 2002); here, the occupancy of this receptor may be governed by 3β-HSD isoforms. 3-Ketosteroid reductases will reduce 5α-DHT to 3β-diol, and 3β-hydroxysteroid oxidases will convert 3β-diol back to 5α-DHT. Whether the classical type 1 and type 2 3β-HSD/ketosteroid isomerase (KSI)s catalyse these reactions in target cells, or whether other 3β-HSD isoforms are responsible for these reactions, remains to be determined.

The ligand occupancy of the progesterone receptor (PR) will be regulated by 20-ketosteroid reductase which will reduce progesterone (a potent progestin) to 20α-hydroxyprogesterone (a weak progestin). The enzymes responsible are human 20α(3α)-HSD (Burczynski *et al*., 1998; Penning *et al*., 2000; Zhang *et al*., 2000) and type 5 17β-HSD which has strong 20α-HSD activity (Dufort *et al*., 1999). The 20α-hydroxysteroid oxidase responsible for the reverse reaction may be type 2 17β-HSD (Wu *et al*., 1993). Each of these HSDs and their classification into protein superfamilies are shown in Table I. Thus, for each sex hormone there emerge pairs of HSDs that control ligand access to the androgen receptor (AR), ER and PR.

HSDs belong to two protein superfamilies: the SDRs and AKRs

HSDs implicated in controlling ligand access to steroid receptors belong to two protein superfamilies (Jornvall *et al*., 1995; Penning, 1997). The short-chain dehydrogenase/reductase (SDR) family consists of the 11β-HSDs which control ligand access to the mineralo- and glucocorticoid receptors and most of the 17β-HSD isoforms which may control ligand access to the AR and ER (Peltoketo *et al*., 1999). There are currently 11 human 17β-HSD isoforms of which the type 1 and type 3 are the major reductive isoforms (Peltoketo *et al*., 1999). The type 1 17β-HSD is also known as the estrogenic 17β-HSD since its principal activity is to reduce estrone to 17β-estradiol (Labrie *et al*., 1997; Peltoketo *et al*., 1999). By contrast, the type 3 17β-HSD is known as the androgenic 17β-HSD and is a Leydig cell-specific enzyme which catalyses the reduction of Δ⁴-androstene-3,17-dione to testosterone (Andersson *et al*., 1995). Its gene is mutated in *17KSR3* deficiency, a condition that leads to pseudohermaphroditism, which underscores its importance in the production of testosterone in the testis (Geissler *et al*., 1994; Andersson *et al*., 1996). Because of its discrete localization it is unlikely to synthesize testosterone in target tissues for the AR. This task is performed by the type 5 17β-HSD which is an aldo-keto reductase (AKR1C3) (Lin *et al*., 1997; Dufort *et al*., 1999).

The SDRs also contain the type 2 and type 4 17β-HSDs which are the major oxidative enzymes that inactivate testosterone and 17β-estradiol by forming Δ⁴-androstene-3,17-dione and estrone respectively (Adamski *et al*., 1995; Andersson *et al*., 1995). These enzymes will deprive the AR and ER of their ligands. Of these enzymes, the type 2 may be the principal activity since the type 4 also catalysed 3-hydroxyacyl-CoA dehydrogenase activity and may function as a sterol-carrier protein (Adamski *et al*., 1995).

The AKR gene superfamily contains many of the 3α-HSD and 20α-HSD isoforms (Jez *et al*., 1997a; b). The human AKR1C family contains four human 3α-HSD isoforms (AKR1C1–AKR1C4) of interest that display varying ratios of 3-, 17- and 20-ketosteroid reductase activity and varying ratios of 3α-, 17β- and 20α-hydroxysteroid oxidase activity. This suggests that they may regulate ligand access to the AR, ER and PR respectively (Penning *et al*., 2000) (see Table I).

Members of the SDR and AKR superfamilies are easy to distinguish one from another (Figure 4). The SDRs are multimeric, non-metallo NAD(P)(H)-linked oxidoreductases (monomer molecular weight = 27 kDa). They are membrane-
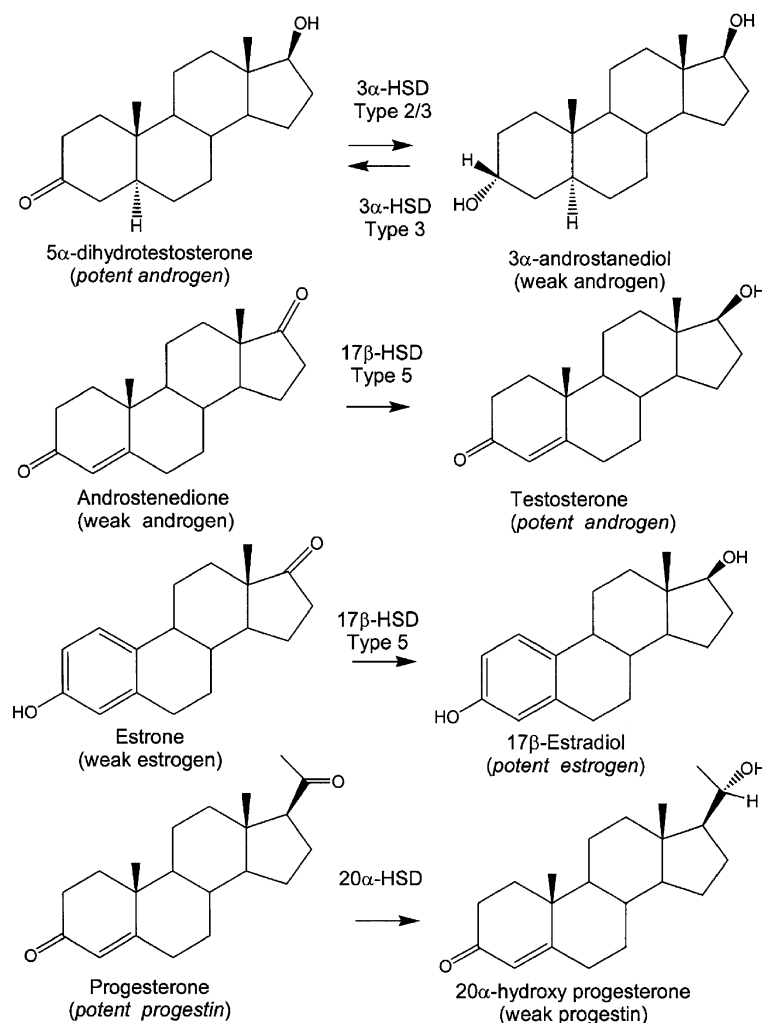
Figure 3. Aldo-keto reductases (AKRs) that control ligand occupancy of steroid receptors.

bound and often share <25% amino acid sequence identity, but available crystal structures show that disparate enzymes in this family have adopted identical protein folds. This fold has at its core a floret of 7β-strands with 3α-helices on either side of the strands (Figure 5) and is clearly evident in the crystal structure of the estrogenic type 1 17β HSD (Ghosh *et al*., 1995; Breton *et al*., 1996; Sawicki *et al*., 1999). The cofactor lies across a Rossmann fold, the nicotinamide ring and the ribose moiety are in a *syn*-conformation, and these enzymes are B-face-specific in that they will transfer the pro-*S* hydride ion from the cofactor to the ketosteroid (Figure 6). They also contain a conserved catalytic motif of Tyr-X-X-(Ser)-Lys, where tyrosine is the catalytic general acid/base (Obeid and White, 1992; Chen *et al*., 1993; Puranen *et al*., 1994). The bi-substrate reaction can display either ordered or random substrate binding, but when there is ordered binding the cofactor binds first.

The AKRs are monomeric, NAD(P)(H)-linked oxidoreductases (molecular weight = 37 kDa) and are soluble. The steroid-transforming AKRs that fall into the AKR1C family share >67% amino acid sequence identity. Available crystal struc-

tures show that the AKRs adopt the $(\alpha/\beta)_8$-barrel or TIM-barrel motif in which there is an alternating array of α-helix and β-strand that repeats itself eight times. The β-strands make up the core of a barrel, at the back of which are three large loops (A, B and C) which define steroid hormone specificity (see Figure 5). These features are clearly evident in the crystal structures of the rat and human 3α-HSDs (Bennett *et al*., 1996; 1997; Jin *et al*., 2001; Nahoum *et al*., 2001). The cofactor lies perpendicular to the steroid and is in an extended conformation in which the nicotinamide ring and the ribose are in the *anti*-conformation. The enzymes are A-face-specific and transfer the 4-pro-*R* hydride ion to the ketosteroid substrate. At the base of the barrel there is a catalytic tetrad that comprises Tyr55, Lys84, His117 and Asp50 (numbering according to rat 3α-HSD or AKR1C9) and where tyrosine is the catalytic general acid/base (Schlegel *et al*., 1998). The similarity in the catalytic residues in SDRs and AKRs led to their superimposition, and this suggested that enzymes of both families have a conserved catalytic mechanism (Bennett *et al*., 1996) (Figure 6). In this mechanism, there is a direct hydride transfer from the cofactor

Table I. Human HSD isoforms implicated in the formation and elimination of sex hormones in target tissues

Receptor	Reaction			Enzyme isoform		Gene		
				HSD	Nomenclature	Name	Size (Kb)	Chromosomal localization
ERα and ERβ	Estrone (weak estrogen)	$\rightarrow$	17β-Estradiol (potent estrogen)	Type 1 17β-HSD Type 5 17β-HSD	17-HSD/KSR1 AKR1C3	*HSD/17B1* *AKR1C3*	3.2 16.0	17q21 10.p14-15
	17β-Estradiol (potent estrogen)	$\rightarrow$	Estrone (weak estrogen)	Type 2 17β-HSD Type 4 17β-HSD	17-HSD/KSR2 17-HSD/KSR4	*HSD/17B2* *HSD/17B4*	>40.0 >100.0	16q24 1-2 5q2.3
AR	Δ^4-AD (weak androgen)	$\rightarrow$	Testosterone (potent androgen)	Type 5 17β-HSD	AKR1C3	*AKR1C3*	16.0	10.p14–15
	Testosterone (potent androgen)	$\rightarrow$	Δ^4-AD (weak androgen)	Type 2 17β-HSD Type 4 17β-HSD	17-HSD/KSR2 17-HSD/KSR4	*HSD/17B2* *HSD/17B4*	>40.0 >100.0	16q24 1-2 5q2.3
AR	5α-DHT (potent androgen)	$\rightarrow$	3α-androstanediol (weak androgen)	Type 2 3α-HSD Type 3 3α-HSD	AKR1C3 AKR1C2	*AKR1C3* *AKR1C2*	16.0 16.0	10.p14-15 10.p14-15
	3α-androstanediol (weak androgen)	$\rightarrow$	5α-DHT (potent androgen)	Type 3 3α-HSD 11-*cis*-retinol DH RoDH like 3α-HSD	AKR1C2	*AKR1C2*	16.0	10.p14-15
PR	Progesterone (potent progestin)	$\rightarrow$	20α-OH progesterone (weak progestin)	20α(3α)-HSD Type 5 17β-HSD	AKR1C1 AKR1C3	*AKR1C1* *AKR1C3*	>4.0	10.p14-15
	20α-OH progesterone (weak progestin)	$\rightarrow$	Progesterone (strong progestin)	Type 2 17β-HSD	17-HSD/KSR2	*HSD/17B2*	>40.0	16q24 1-2

ER = estrogen receptor; HSD = hydroxysteroid dehydrogenase; 5α-DHT = 5α-dihydrotestosterone (17β-hydroxy-5α-androstane-3-one).

to the 3-ketosteroid, which is then protonated by the catalytic tyrosine. This bi-substrate reaction in AKRs is strictly ordered where the cofactor must bind before steroid hormone binds to the active site.

Criteria to implicate HSDs in pre-receptor regulation of steroid hormone action

A number of criteria have been established that must be fulfilled by all HSDs if they are to be involved in the pre-receptor regulation of steroid hormone action. First, the HSD must function in a unidirectional manner; that is, it must act as a reductase or oxidase within a cellular context. Second, the HSD must be co-expressed with the steroid receptor that it is predicted to regulate. Third, the HSD and steroid receptor must be co-localized within the same cell type if the HSD is to effect intracrine modulation of the receptor. Fourth, HSDs should modulate *trans*-activation of steroid hormone receptors by their ligands in reporter gene assays; that is, HSDs should attenuate *trans*-activation of the receptor by active hormone or stimulate *trans*-activation of the receptor by inactive hormone (Figure 7). Fifth, HSDs should show differential expression in hormonal dependent cancers; for example, AR- positive prostate cancer and ER-positive breast cancer. Sixth, inactivation of HSD isoforms by either targeted gene disruption, anti-sense oligonucleotides or small interfering RNA (si-RNA) should cause an altered steroid response at the receptor level.

Many HSDs, when characterized *in vitro*, behave as oxidoreductases and will freely interconvert steroid ketones with steroid alcohols. However, when they are transiently or stably transfected into null-environments they act

predominantly as reductases or oxidases (Luu-The *et al.*, 2001). It has been proposed that this may be predicted by their pyridine nucleotide preference. Those enzymes that prefer NADPH will act as reductases as they will use the major reductive cofactor in cells, whereas those that prefer NAD$^+$ will act as oxidases as they will use the major oxidative cofactor found in cells. This may be an oversimplification however, as directionality will also be governed by the K_{eq} for the forward and reverse reactions, and by the redox state that will govern the ratio of reduced versus oxidized pyridine nucleotide cofactor. Another powerful influence may be product inhibition by the opposing cofactor. In recent experiments, the NADPH-dependent reduction of 5α-DHT catalysed by AKR1C2 was found not to be affected by increasing concentrations of NAD$^+$. By contrast, the NAD$^+$-dependent oxidation of 3α-diol to 5α-DHT catalysed by AKR1C2 was potently inhibited by low (micromolar) concentrations of NADPH. This reflects the very tight binding of NADP(H)—which is a characteristic property of AKRs—and suggests that these enzymes will function as reductases in a cellular context when low micromolar concentrations of NADPH prevail (Rizner *et al.*, 2003a).

Evidence that SDR isoforms regulate sex steroid hormone receptors

How well do SDRs that interconvert sex steroid hormones satisfy the criteria to regulate steroid hormone receptors? SDRs most implicated in regulating the ER are the type 1 and
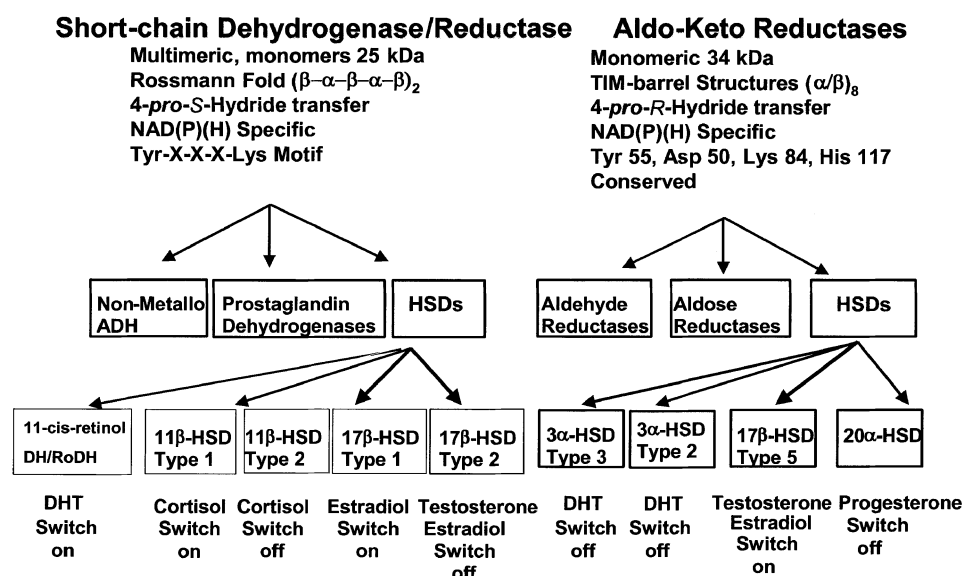
Short-chain Dehydrogenase/Reductase

Multimeric, monomers 25 kDa
Rossmann Fold (β–α–β–α–β)$_2$
4-*pro*-*S*-Hydride transfer
NAD(P)(H) Specific
Tyr-X-X-X-Lys Motif

Aldo-Keto Reductases

Monomeric 34 kDa
TIM-barrel Structures (α/β)$_8$
4-*pro*-*R*-Hydride transfer
NAD(P)(H) Specific
Tyr 55, Asp 50, Lys 84, His 117
Conserved

Non-Metallo ADH	Prostaglandin Dehydrogenases	HSDs

Aldehyde Reductases	Aldose Reductases	HSDs

11-cis-retinol DH/RoDH	11β-HSD Type 1	11β-HSD Type 2	17β-HSD Type 1	17β-HSD Type 2
DHT Switch on	Cortisol Switch on	Cortisol Switch off	Estradiol Switch on	Testosterone Estradiol Switch off

3α-HSD Type 3	3α-HSD Type 2	17β-HSD Type 5	20α-HSD
DHT Switch off	DHT Switch off	Testosterone Estradiol Switch on	Progesterone Switch off

Figure 4. Comparison of SDRs and AKRs that control steroid hormone action. For TIM-barrel structures, see text.
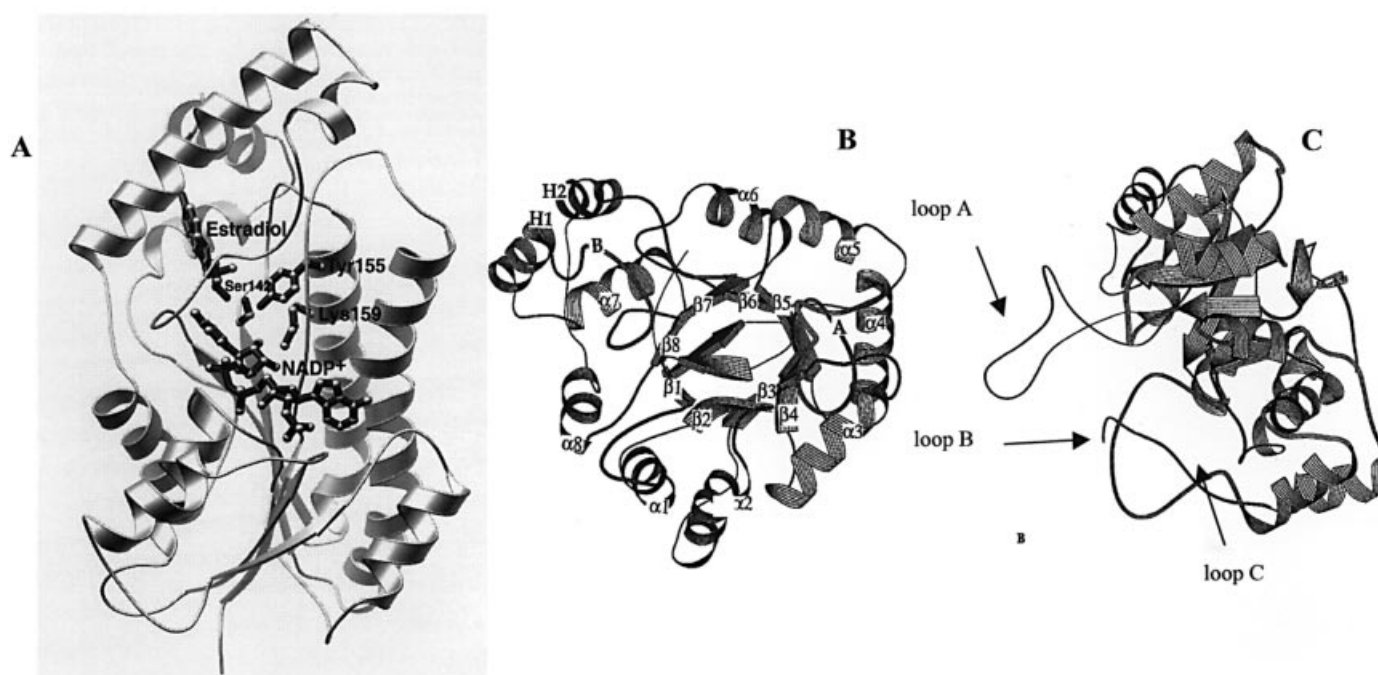

Figure 5. Structural motifs of SDRs and AKRs. Panel A: ternary complex of type 1 17β-HSD containing NADP$^+$ and 17β-estradiol (after Breton *et al.*, 1996). Panel B: α/β-barrel fold of an AKR. Panel C: loops A, B and C of an AKR (after Bennett *et al.*, 1996).

type 2/type 4 17β-HSDs. Transfection of type 1 17β-HSD into human embryonic kidney (HEK) cells showed that this enzyme acts almost exclusively as a reductase in a cellular context, producing 17β-estradiol (Labrie *et al.*, 1997). The enzyme is also highly overexpressed in mammary gland, and immunohistochemistry co-localizes the enzyme with ERα and ERβ in human breast epithelial cells (Miettinen *et al.*, 1996; Jarvinen *et al.*, 2000). The type 1 17β-HSD isoform was also elevated 20-fold in epithelial and stromal cells of human breast tumours (Spiers *et al.*, 1998). It displayed marked

elevated expression in invasive lobular carcinoma by immunohistochemistry, and its overexpression correlated with the overexpression of aromatase (Sasano *et al.*, 1996). Although 17β-HSD, aromatase, ER and PR are co-expressed in mammary gland, a direct correlation between type 1 17β-HSD and ER expression has yet to be observed (Sasano *et al.*, 1996).

By contrast, transfection of the type 2 17β-HSD into human embryonic kidney cells (HEK) 293 showed that this isoform preferentially oxidized 17β-estradiol back to estrone, and therefore this enzyme would eliminate estrogens in a cellular context (Wu
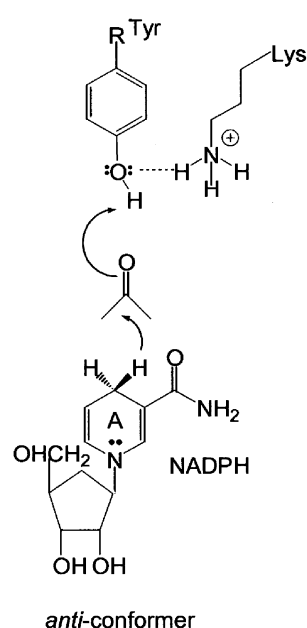
199
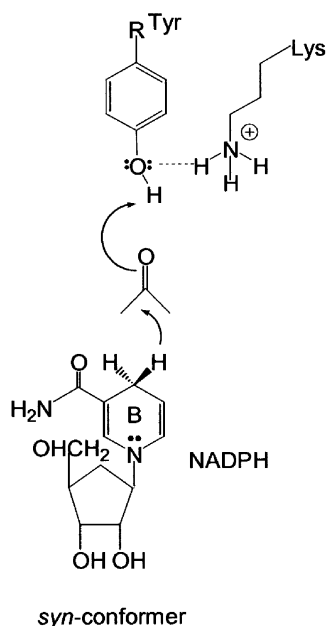
Figure 6. Conserved reaction mechanism for HSDs. SDRs and AKRs use opposing faces of the nicotinamide ring (B or A, respectively) to deliver the hydride ion. Therefore, SDRs and AKRs catalyse pro-*S* and pro-*R* hydride transfers via identical mechanisms. A catalytic tyrosine and its interaction with a lysine residue is shown for simplicity. In AKRs, the catalytic tyrosine acts as a general base because its pK_a is lowered by the lysine (oxidation direction), but it also acts a general acid because its pK_b is increased by an adjacent histidine (reduction direction) (see Schlegel *et al*., 1998). In SDRs, the catalytic tyrosine acts as a general base because its pK_a is lowered by the lysine (oxidation direction), but it also acts as a general acid and the mechanism by which tyrosine assumes this role is uncertain.
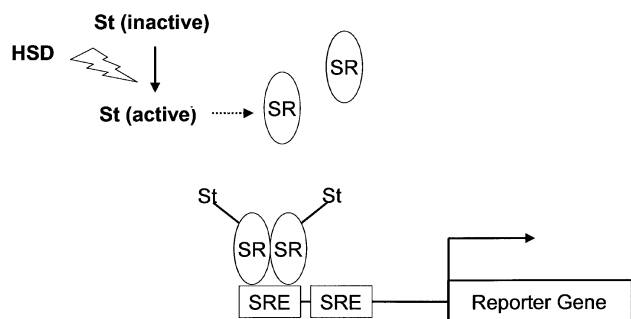

Figure 7. HSDs modulate *trans*-activation of steroid hormone receptors. HSD = hydroxysteroid dehydrogenase (SDR or AKR); St = steroid hormone; SR= steroid receptor; SRE = steroid response element.

et al., 1993). Under these conditions, the K_m for 17β-estradiol was 0.21 μM and the V_{max} was 38 nmol oxidized/min/mg. Additionally, the type 4 17β-HSD catalysed the NAD$^+$-dependent oxidation of 17β-estradiol with a K_m <1.0 μM when expressed in the same cells, but the conversion rate was in pmol/min and a specific activity was not reported (Adamski *et al*., 1995).

Other 17β-HSD isoforms which are SDRs may also be implicated in the control of ligand access to steroid receptors. 17β-HSD type 7 (prolactin receptor-associated protein) also catalyses the formation of 17β-estradiol and is expressed in the breast and prostate (Krazeisen *et al*., 1999). Other 17β-HSD isoforms (types 8, 10 and 11), although important, are not expressed in breast and prostate cancer and are therefore unlikely to control ligand access to steroid receptors in these diseases.

Other SDRs implicated in regulating sex-steroid hormone receptors are the 11-*cis*-retinol-DH and RoDH/3α-HSD. These microsomal enzymes exhibit 3α-hydroxysteroid oxidase activity and convert 3α-diol to 5α-DHT (Biswas and Russell, 1997; Huang and Luu-The, 2001a). Transfection studies showed that in HEK cells, 11-*cis*-retinol-DH will act almost exclusively as an oxidase (Huang and Luu-The, 2001a). These enzymes are expressed in the prostate gland. Co-transfection of a pCMV-11-*cis* retinol-DH expression vector, a luciferase reporter gene construct driven by a prostate-specific antigen (PSA) promoter, and the human AR (as pCMV-hAR) showed that in the presence of 3α-diol expression from the PSA promoter was elevated 5- to 6-fold—an effect that was abolished by 4-hydroxy-flutamide (an AR antagonist) (Huang and Luu-The, 2001b). This is the first demonstration that a HSD can modulate *trans*-activation of a sex steroid receptor.

Based on the criteria that must be satisfied to implicate SDRs in the regulation of sex steroid hormone receptors, the strongest case can be made for type 1 17β-HSD regulating the ER. However, experiments demonstrating that this isoform can regulate *trans*-activation of the ER are lacking, and modulation of estrogen response using anti-gene or si-RNA targeting this HSD have yet to be performed.

Evidence that AKR isoforms regulate sex steroid hormone receptors

There are at least four human aldo-keto reductases that may be involved in regulating ligand access to nuclear receptors. These were originally identified as 3α-HSD isoforms and correspond to:

AKR1C1 20α(3α)-HSD; AKR1C2 (type 3 3α-HSD, and bile acid-binding protein); AKR1C3 (type 2 3α-HSD which is identical to type 5 17β-HSD); and AKR1C4 (type 1 3α-HSD) (Stolz *et al.*, 1993; Khanna *et al.*, 1995; Hara *et al.*, 1996; Lin *et al.*, 1997; Burczynski *et al.*, 1998; Dufort *et al.*, 1999). Characterization of the recombinant enzymes showed that each isoform catalysed the reduction of 3-, 17- and 20-ketosteroids; and that each isoform catalysed the oxidation of 3α-, 17β- and 20α-hydroxysteroids to varying extents (Penning *et al.*, 2000). Product profiling indicated that each isoform will reduce 5α-DHT to 3α-diol, Δ⁴-androstene-3,17-dione to testosterone, estrone to 17β-estradiol and progesterone to 20α-hydroxyprogesterone. Product profiling also indicated that each isoform will oxidize testosterone to Δ⁴-androstene-3,17-dione, 17β-estradiol to estrone and 20α-hydroxprogesterone to progesterone. Only one isoform, type 3 3α-HSD (bile acid-binding protein) was able to oxidize 3α-diol to 5α-DHT and is therefore a candidate enzyme to catalyse the back-reaction previously described. Combined AKR1C1–AKR1C4 catalyse the appropriate reactions to control ligand access to the AR, ER and PR (Penning *et al.*, 2000).

The bimolecular rate constants (k_{cat}/K_m) for 3-keto-, 17-keto- and 20-ketosteroid reduction and 3α-, 17β- and 20α-hydroxysteroid oxidation indicate that these AKR1C enzymes catalyse these reactions to varying extents. For example, AKR1C1 prefers to function as a 20α-HSD; AKR1C2 is a 3α-HSD with a low k_{cat}/K_m; AKR1C3 prefers to act as a 17β-HSD; and AKR1C4 is a 3α-HSD with a high k_{cat}/K_m. How well do these enzymes satisfy the criteria to be involved in regulating ligand occupancy of steroid hormone receptors?

Transient transfection studies have addressed the directionality of AKR1C1–AKR1C3 in a cellular context. Transfection of AKR1C1 into COS-1 cells shows that this enzyme will reduce progesterone to 20α-hydroxyprogesterone, but the oxidation reaction is barely detectable (Zhang *et al.*, 2000; T.L.Rizner and T.M.Penning, unpublished results). Thus, the enzyme is tailored to eliminate progesterone. Transient transfection of AKR1C2 into HEK and COS-1 cells (Dufort *et al.*, 1996; Rizner *et al.*, 2003b) and stable expression of AKR1C2 into PC-3 cells shows that this enzyme will preferentially reduce 5α-DHT to 3α-diol, but that the reverse reaction is minor (Ji *et al.*, 2003; Rizner *et al.*, 2003a; b). Whether this back-reaction can produce sufficient 5α-DHT to activate the AR in a cellular context has yet to be determined.

Transient transfection of AKR1C3 into HEK cells showed that its preferred reaction was the reduction of Δ⁴-androstene-3,17-dione to testosterone. This activity was considered labile because the identical reaction was difficult to monitor in HEK cell lysates (Dufort *et al.*, 1999). By contrast, the bacterially expressed enzyme was stable (Lin *et al.*, 1997; Burczynski *et al.*, 1998). Thus, data are emerging which suggest that AKR1C1 will inactivate progesterone, AKR1C2 and AKR1C3 will inactivate 5α-DHT, while AKR1C3 can form testosterone in a cellular context.

The discrete tissue distribution of these AKR isoforms suggests that they may regulate steroid hormone action at the pre-receptor level. Quantitative isoform-specific RT-PCR showed that AKR1C2 and AKR1C3 are highly overexpressed in human prostate. Using cDNA probes for the coding region and the 3′-UTR of AKR1C3, high expression was observed in primary epithelial cells from patients with benign hypertrophy of the

prostate and prostate cancer, whereas expression in stromal cells was less (Lin *et al.*, 1997). Although these probes cannot discriminate between AKR1C isoforms, they do indicate that they are more highly expressed in cells known to contain the AR.

Immunohistochemistry using a peptide-derived monoclonal antibody for AKR1C3 showed that this isoform is localized to the basal cells of the prostate and may provide a source of testosterone for the luminal cells to make 5α-DHT for the AR (El-Alfy *et al.*, 1999). RT-PCR also demonstrated high expression of AKR1C2 in human prostatic epithelial cells from prostate cancer patients, but expression is markedly attenuated in normal cells (Lin *et al.*, 2000; Rizner *et al.*, 2003). These data imply that AKR1C3 and AKR1C2 are expressed in regions of the prostate where they will control the local concentration of androgen available for the AR. The 17-ketosteroid reductase activity of AKR1C3 may contribute to the local production of testosterone, while the 3-ketosteroid reductase activities of AKR1C3 and AKR1C2 may lead to the elimination of 5α-DHT.

AKR1C3 is the most abundant AKR isoform expressed in human mammary epithelial cells when measured by RT-PCR (Penning *et al.*, 2000). It has also been detected in these cells by immunohistochemistry using a peptide-derived antibody (Pelletier *et al.*, 1999). Its unique product profiling suggests that it may contribute to a pro-estrogenic state in the breast. This enzyme will reduce estrone to 17β-estradiol; and it will reduce Δ⁴-androstene-3,17-dione to testosterone, which can then be aromatized to 17β-estradiol by CYP arom. AKR1C3 can also reduce progesterone to the inactive progestin (20α-hydroxyprogesterone). When these reactions are combined, AKR1C3 might be responsible for the unopposed actions of estrogens in the breast.

More still needs to be done to characterize the role of AKR1C1 in progesterone action. One hypothesis is that AKR1C1 plays a role in diseases of the endometrium. Endometriosis and endometrial cancer are both diseases that may arise due to the action of estrogens when unopposed by progestins (Attia *et al.*, 2000; Arnett-Mansfield *et al.*, 2001; Bulun *et al.*, 2002; Chwalisz *et al.*, 2002; Rice, 2002). In the normal female, this could occur if there were a decrease in PR expression and/or an increase in the elimination of progesterone at the target tissue. In this instance, overexpression of AKR1C1 in these diseases might be anticipated.

Co-localization of AKR1C1–AKR1C3 with the PR, AR and ERα and ERβ in different target tissues has yet to be performed systematically. In addition, the ability of these enzymes to modulate the *trans*-activation of the AR, ER and PR using reporter gene constructs is yet to be elucidated. The murine homologues of these AKR1C enzymes have as yet not been completely identified; therefore, the generation of *AKR1C* null/mice has not been attempted. Thus, much needs to be done to validate the role of AKR1C isoforms in the pre-receptor regulation of steroid hormone action.

Development of SIMs

HSDs which are SDRs and AKRs are clearly potential drug targets. Development of SIMs requires validation of the target, and the most important targets in the SDR superfamily would appear to be 11β-HSD type 1 and type 2 and estrogenic type 1 17β-HSD. There is sufficient validation of these targets to warrant inhibitor design. Inhibitors of type 1 11β-HSD would
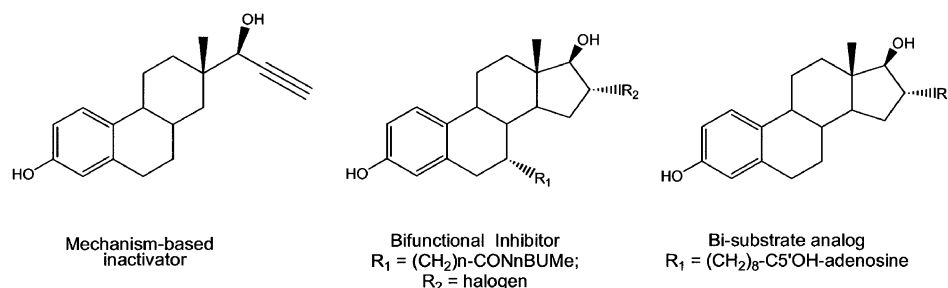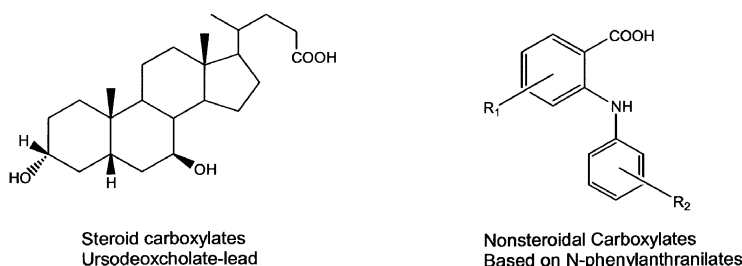
Figure 8. Potential SIMs; lead compounds.

prevent cortisone conversion to cortisol and could be used to control visceral obesity. Alternatively, inhibitors of type 2 11β-HSD would enhance mineralocorticoid activity and would provide a method of treating hypotension. Progress towards these inhibitors will not be discussed since the emphasis of this review is on HSDs and their pre-receptor regulation of sex steroid receptors.

Emphasis has been placed on the development of steroid-based inhibitors of estrogenic type 1 17β-HSD based on three strategies. These include the development of mechanism-based inactivators (Penning and Ricigliano, 1991; Penning, 1994), the synthesis of bifunctional ligands, that will inhibit 17β-HSD and antagonize ERα (Labrie *et al.*, 1992), and bi-substrate analogues which contain portions of both the steroid and cofactor substrates (Qiu *et al.*, 2002) (Figure 8). Mechanism-based inactivators are substrate mimics which are innocuous by themselves but require transformation by the target enzyme to an electrophilic species that will cause enzyme inactivation via alkylation. A series of latent Michael-acceptors were designed which all involved the enzyme-catalysing conversion of a steroid alcohol to yield an α,β-unsaturated ketone which would then covalently modify the target. The problem with this approach was that the reactive species is produced as a result of oxidation when, in a cellular context, type 1 17β-HSD prefers to function as a reductase. This area was recently reviewed (Penning, 1996).

Bifunctional ligands have been developed with the knowledge that the ER is best antagonized when there is a large substituent on the B-ring and 17β-HSD will tolerate substituents at the 16α-position. A series of compounds which was developed to meet these criteria included 7α-alkylamide 16α-halogenated estradiol (E$_2$) analogues (EM-139) (Labrie *et al.*, 1992) and 6β-butyl-methylalkylamides (Poirier *et al.*, 1998).

Bi-substrate analogues have been designed which contain portions of both substrates. Thus, hybrids of 17β-estradiol-adenosine have been synthesized and evaluated (Qiu *et al.*, 2002). EM-1745, which contained a linker of 8-methylene groups between the two moieties, was found to be a potent competitive inhibitor of type 1 17β-HSD (K_i = 3.0 nM). A crystal structure of the 17β-HSD·EM-1745 binary complex, obtained at 1.6 Å provided atomic resolution of the strong interactions that occur between the bi-substrate analogue with the steroid and cofactor binding sites of the enzyme (Qiu *et al.*, 2002).

Potential drug targets in the AKR1C family would be AKR1C1, AKR1C2 and AKR1C3. Inhibitors of AKR1C1 could prevent progesterone metabolism, and help maintain pregnancy. Inhibition of AKR1C3 may be important to prevent the local formation of testosterone in the prostate and the formation of a pro-estrogenic state in the breast. Two approaches are being exploited. One involves the development of steroid carboxylates, while the other is based on non-steroidal anti-inflammatory drug (NSAID) analogues. Steroid carboxylates are desirable because the active site is character-ized by an oxyanion hole that has (TyrOH$_2^+$) character (Jin *et al.*, 2001). Also, although AKR1C1 and AKR1C2 differ by seven amino acids only the latter is potently inhibited by bile acids (C-24 carboxylates) yielding IC$_{50}$ values in the nanomolar range (Hara *et al.*, 1996). These findings indicate the requirement for steroid carboxylates as inhibitors and indicate that discrimination between AKR1C isoforms can be achieved.

NSAID analogues are desirable for several reasons. First, classical NSAIDs have been found potently to inhibit AKR1C isoforms in rank-order of their anti-inflammatory potency (Penning and Talalay, 1983). Second, classical NSAIDs all

contain carboxylate functionalities which should tether the analogues to the catalytic tyrosine. Third, *N*-phenylanthranilic acid, 1-methyl-pyrrole acetic acids and arylpropionic acids can be made by simple coupling chemistries so that a large number of analogues are either available or can be synthesized by combinatorial means (Acheson, 1973; Scherrer and Beatty, 1980). Fourth, an examination of the structure–activity relationships for classical NSAIDs indicates which substituents abolish their ability to inhibit prostaglandin H synthase, providing important leads to inhibitors that can distinguish between AKR1C isoforms and prostaglandin H synthase.

Conclusions

It is hypothesized that HSDs regulate steroid hormone action at the pre-receptor level. Enzymes exist that may regulate the AR, ER and PR occupancy and their *trans*-activation. Each enzyme requires further validation as a drug target. Currently, the most convincing evidence is that the type 1 17β-HSD can regulate ER and is therefore a drug target. These HSDs belong to two protein superfamilies, the SDRs and the AKRs, and available crystal structures for enzymes of both families suggest that rationale design of isoform selective inhibitors should be possible.

Acknowledgements

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