REVIEW

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Design and validation of specific inhibitors of 17β-hydroxysteroid dehydrogenases for therapeutic application in breast and prostate cancer, and in endometriosis

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Abstract

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are enzymes that are responsible for reduction or oxidation of hormones, fatty acids and bile acids *in vivo*, regulating the amount of the active form that is available to bind to its cognate receptor. All require NAD(P)(H) for activity. Fifteen 17β-HSDs have been identified to date, and with one exception, 17β-HSD type 5 (17β-HSD5), an aldo–keto reductase, they are all short-chain dehydrogenases/reductases, although overall homology between the enzymes is low. Although named as 17β-HSDs, reflecting the major redox activity at the 17β-position of the steroid, the activities of these 15 enzymes vary, with several of the 17β-HSDs able to reduce and/or oxidise multiple substrates at various positions. These activities are involved in the progression of a number of diseases, including those related to steroid metabolism. Despite the success of inhibitors of steroidogenic enzymes in the clinic, such as those of aromatase and steroid sulphatase, the development of inhibitors of 17β-HSDs is at a relatively early stage, as at present none have yet reached clinical trials. However, many groups are now working on inhibitors specific for several of these enzymes for the treatment of steroid-dependent diseases, including breast and prostate cancer, and endometriosis, with demonstrable efficacy in *in vivo* disease models. In this review, the recent advances in the validation of these enzymes as targets for the treatment of these diseases, with emphasis on 17β-HSD1, 3 and 5, the development of specific inhibitors, the models used for their evaluation, and their progress towards the clinic will be discussed.

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Introduction

Steroid-dependent diseases

Breast cancer, a major cause of death in both European and American women, occurs most frequently in post-menopausal women. After menopause, a low level of oestrogen is produced, mainly from the local conversion of androstenedione (Adione; Fig. 1a) to oestrone (E_1; Fig. 1b) by aromatase in adipose and normal and malignant breast tissues, despite the cessation of ovarian function. This oestrogen has a crucial role in supporting the growth of hormone-dependent breast cancer in these women. Much of the E_1 formed by aromatase is stored in an inactive form as E_1 sulphate, which can be reactivated within breast cells by steroid sulphatase (Stanway *et al*. 2006). Presently, various methodologies are used in the clinic to inhibit the stimulation of hormone-dependent breast cancer by oestrogen. These include oestrogen receptor (ER) antagonists, such as tamoxifen (Heel *et al*. 1978, Jordan 2003), and inhibitors of steroid synthesis, such as letrozole, an aromatase inhibitor (Bhatnagar *et al*. 1990, Bhatnagar 2006, Scott & Keam 2006) or more recently 667-COUMATE, a steroid sulphatase inhibitor (Purohit *et al*. 2003, Stanway *et al*. 2006, 2007).

Treatment of hormone-dependent prostate cancer by androgen ablation is initially usually successful, reducing primary tumour burden and increasing 5-year

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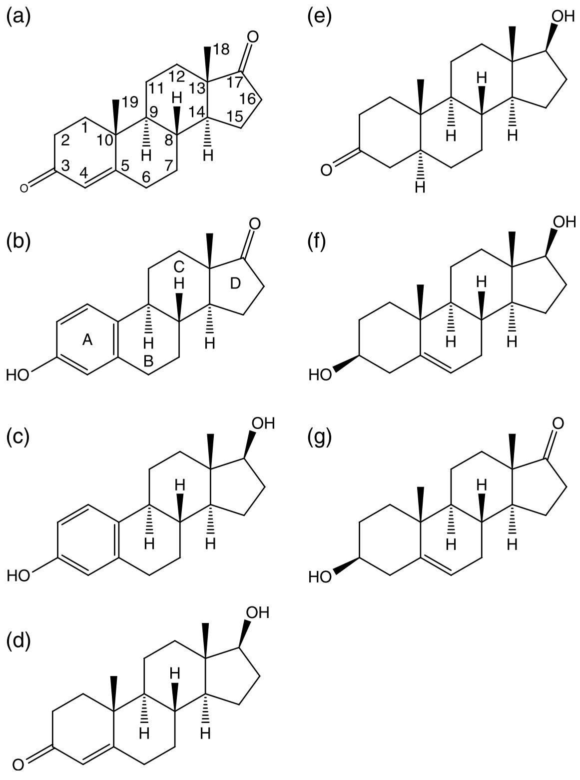

Figure 1 Steroid structures. (a) 4-Androstene-3,17-dione (Adione; with carbon positions numbered), (b) oestrone (E_1; with ring positions labelled), (c) 17β-oestradiol (E_2), (d) testosterone, (e) 5α-dihydrotestosterone (DHT), (f) 5-androstene-3β,17β-diol (Adiol) and (g) dehydroepiandrosterone (DHEA).

survival rates. Presently, androgen ablation is achieved using various approaches that include orchidectomy, androgen receptor (AR) blockers such as bicalutamide (Fradet 2004), agonists of luteinising hormone-releasing hormone such as goserelin (Akaza 2004), finasteride, an inhibitor of 5α-reductase, the enzyme that converts testosterone (Fig. 1d) to the more active androgen, 5α-dihydrotestosterone (DHT; Fig. 1e) (Rittmaster 1997), or a combination of the above.

However, prostate cancer is the third highest cause of cancer-related death in men, because tumours are often unnoticed for several years, presenting at an advanced stage in older men, by which time they have often progressed to hormone independency. At present, there are many efforts to understand the mechanisms by which prostate cancer cells develop hormone independence. These are thought to include AR up-regulation, an adaptation to the low levels of androgen present during ablation therapy or mutations to the pathways involved in the activation of the AR, such as mutations in the receptor itself, or in its co-regulators, allowing enhanced activation by the low-level androgens (Mizokami *et al*. 2004) or ligands other than androgens to activate the proliferative pathways (Rau *et al*. 2005, Pienta & Bradley 2006). It has also been suggested that many cases of recurrent androgen-independent prostate cancer may not actually be independent of androgen signalling, as high levels of testosterone and DHT have been found in the prostates of patients with recurrence during ablation therapy, suggesting that surgical or chemical castration treatments may not result in complete removal of active androgens, and that *in situ* formation of testosterone from adrenal production of androgens may continue (Titus *et al*. 2005).

Endometriosis is one of the most common causes of pelvic pain and infertility in women. In this condition, endometrial tissue grows abnormally outside the uterus, often in locations such as the ovaries, fallopian tubes and abdominal cavity. It causes adhesions and scarring, pain and heavy bleeding, and can damage reproductive organs, interfere with ovulation and inhibit implantation of the embryo. Although the specific causes of endometriosis are still undetermined, it appears that inheritable defects (Barlow & Kennedy 2005) allow for the peritoneal implantation and survival of endometrial tissue displaced by retrograde menstruation, a process initially proposed by Sampson (1927).

Treatments for endometriosis include the use of contraceptives, progestins and gonadotrophin-releasing hormone analogues (Farquhar 2007) to inhibit menstruation, a source of much of the pain associated with endometriosis, and to suppress the growth of the endometriotic tissue. Additional treatments are also necessary to relieve the effects of endometriosis, including drugs such as clomiphene citrate to improve fertility, and non-steroidal anti-inflammatory drugs (NSAIDs) and other analgesics to relieve the pain. However, although these treatments provide relief from the symptoms of endometriosis, none provides a cure, and those which alter hormone balance can result in side effects such as hot flushes, weight gain and acne.

The clinical success of inhibitors of steroid synthesis or action in both hormone-dependent breast cancer (including tamoxifen, letrozole and 667-COUMATE) and hormone-dependent prostate cancer (including bicalutamide, goserelin and finasteride) has provided justification for this approach in the treatment of these cancers. As more is becoming understood about the altered expression of steroidogenic enzymes in endometriosis, and also in endometrial cancer, it is envisaged that inhibitors of steroid action may also have a role in the clinical treatment and possible future cure of diseases of the endometrium (Hompes & Mijatovic 2007).

In all of these diseases, the clinical effect of the inhibition of the one step of hormone activation remains to be investigated (Purohit *et al.* 2006). This is the reduction of the steroids at the 17β-position, catalysed by specific 17β-hydroxysteroid dehydrogenases (17β-HSDs), to form the active steroid that binds to its specific receptor to stimulate cell proliferation. In oestrogenic activation, this results in the formation of active oestradiol (E_2; Fig. 1c) from E_1, and to a lesser degree, the production of androstenediol (Adiol; Fig. 1f) from dehydroepiandrosterone (DHEA; Fig. 1g). In androgenic activation, 17β-HSDs reduce Adione to form testosterone, and this is finally metabolised by 5α-reductase enzymes (Tindall & Rittmaster 2008) to form DHT, the active androgen.

17β-HSDs

17β-HSDs are enzymes that are responsible for reduction or oxidation of hormones, fatty acids and bile acids *in vivo*. All require NAD(P)(H) for activity. Fifteen 17β-HSDs have been identified to date, and with one exception, 17β-HSD type 5 (17β-HSD5), an aldo–keto reductase (AKR), they are all short-chain dehydrogenases/reductases (SDRs).

The major substrates for these enzymes are hormones, and the reduction or oxidation of hormones by 17β-HSDs regulates the amount of active steroid available to bind to a particular receptor. Although named as 17β-HSDs, reflecting the major redox activity at the 17β-position of the steroid, several of the 17β-HSDs are able to convert multiple substrates at multiple sites, such as at the 3 position on the steroid ring. Most also have bidirectional capabilities, catalysing either the oxidative or reductive reaction in the presence of $NAD(P)^+$ or NAD(P)H respectively, but *in vivo* appear to function unidirectionally. Although they are generally of a similar size (250–350 amino acids) and contain highly conserved motifs, such as those within the Rossman fold, overall homology across the 17β-HSDs is low (Duax *et al.* 2000, 2005, Lukacik *et al.* 2006) and the intracellular location of the enzymes is diverse. Different 17β-HSDs have been found specifically expressed in the cytosol (17β-HSD1), microsomes (17β-HSD3), mitochondria (17β-HSD10) and peroxisomes (17β-HSD4), and many have specific expression patterns across tissues and organs. These observations, along with kinetic studies, have demonstrated that although the enzymes have multifunctional capabilities, most have preferential substrate usage and directionality *in vivo*.

The 17β-HSDs: nomenclature, substrate profile and expression patterns

17β-HSD type 1 (17β-HSD1/HSD17B1; Fig. 2a) is the most well characterised of the 17β-HSDs and catalyses the reduction of E_1 to form active E_2 (Miettinen *et al.* 1996, Peltoketo *et al.* 1996), and also the reduction of DHEA to form Adiol (Lin *et al.* 2006). The reverse of these reactions, the inactivation of E_2 to E_1 and Adiol to DHEA, as well as that of testosterone to Adione, is mediated by 17β-HSD2 (HSD17B2; Fig. 2). 17β-HSD2 also activates 20α-progesterone to form progesterone but may also be involved in the oxidation of retinoids (Zhongyi *et al.* 2007). 17β-HSD3 (HSD17B3; Fig. 2b) is expressed in the testes and catalyses the reduction of Adione to testosterone (Luu-The *et al.* 1995). It is not the only 17β-HSD thought to be responsible for the formation of testosterone *in vivo*, as 17β-HSD5 (AKR1C3; Fig. 2c), which is expressed more ubiquitously, forms the testosterone produced in other steroidal tissues, such as prostate, breast, ovary and endometrium (Pelletier *et al.* 1999, Ji *et al.* 2005). However, 17β-HSD5 is a multifunctional enzyme, with additional 3α- and 20α- steroid reductase activities, including the conversion of DHT to 3α-androstanediol (Penning *et al.* 2000), and progesterone to 20α-hydroxyprogesterone (Dufort *et al.* 1999). It is also known as prostaglandin (PG) F synthase (PGFS), its 11-ketoreductase activity preferentially reducing PGD_2 to 9α,11β-PGF_2 (Matsuura *et al.* 1998), although it also forms 9α,11α-PGF_2 ($PGF_2α$) from PGH_2 (Komoto *et al.* 2004, Penning *et al.* 2006). These 17β-HSDs, 17β-HSD1, 17β-HSD2, 17β-HSD3 and 17β-HSD5, will be discussed in greater detail in later sections of this review.

The 17β-HSD4 enzyme (HSD17B4), also known as peroxisomal multifunctional protein-2 (MFP2), is a 736 amino acid protein of ∼79 kDa. It comprises an N-terminal dehydrogenase domain, and a larger C-terminal domain, of around 45 kDa, which contains both hydratase and lipid carrier moieties (Breitling *et al.* 2001, Huyghe *et al.* 2006). Although it can have E_2 oxidoreductase activity (Adamski *et al.* 1995), *in vivo* it is involved in peroxisomal fatty acid β-oxidation (Breitling *et al.* 2001), with defects in its expression causing D-specific MFP deficiency (Huyghe *et al.* 2006). It is present in many tissues, with highest concentrations in the liver, heart, prostate and testis, and is up-regulated in prostate cancer (Zha *et al.* 2005).

17β-HSD6, initially in humans designated as 3(α→β)-hydroxysteroid epimerase (also known as RoDH-like 3α-HSD/RL-HSD), is a 36 kDa enzyme with both oxidoreductase and epimerase activities involved in androgen catabolism. The oxidoreductase

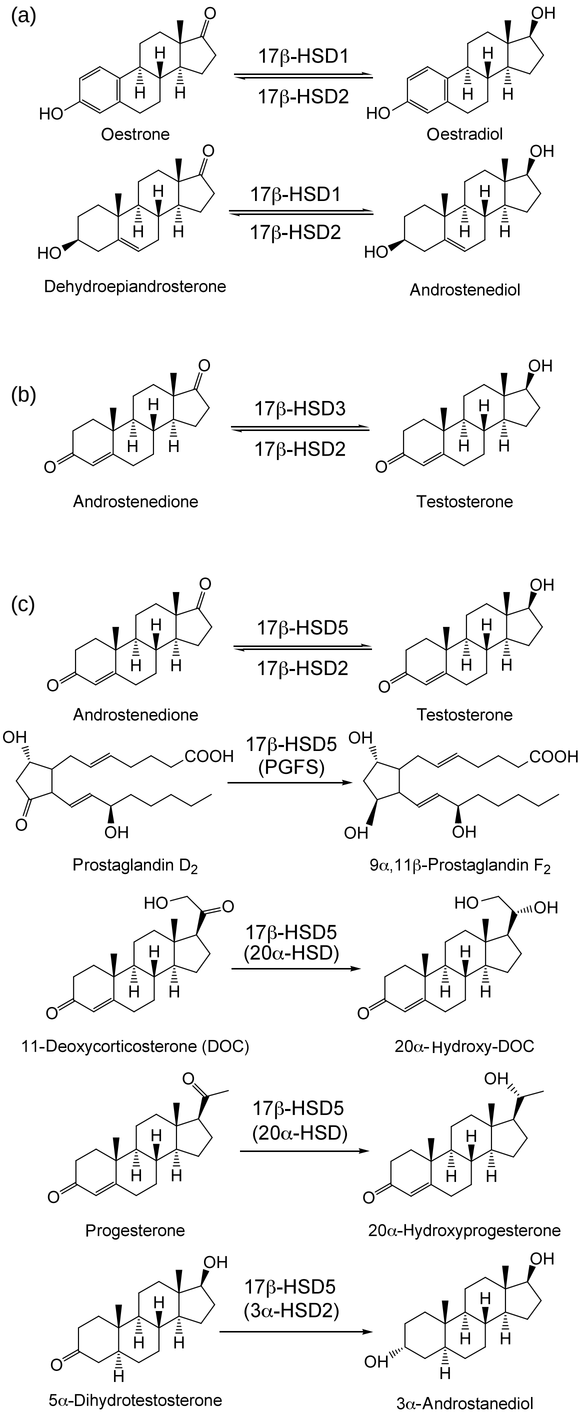

Figure 2 Activity of (a) 17β-HSD1, (b) 17β-HSD3 and (c) 17β-HSD5 (AKR1C3/PGFS/20α-HSD/3α-HSD2).

activity can oxidise 3α-Adiol to form DHT (Bauman *et al.* 2006*a*), while the epimerase activity can convert androsterone (ADT) to epiandrosterone (Huang & Luu-The 2000, 2001, Belyaeva *et al.* 2007). Despite the major, and possibly the sole, site of action of this enzyme being at the 3 position of the androgens, it is classified as 17β-HSD6 in humans as it is 71.4% homologous to rat hsd17b6 (Huang & Luu-The 2000) which does oxidise steroids at the 17 position (Biswas & Russell 1997). Recently, data have indicated that polymorphisms in the HSD17B6 gene are associated with errors in androgen metabolism in polycystic ovary syndrome (PCOS; Jones *et al.* 2006).

When prolactin receptor-associated protein (PRAP; Duan *et al.* 1996) was also identified as 17β-HSD7, it was thought to be involved predominantly in the reduction of E_1 to E_2 (Nokelainen *et al.* 1998, Krazeisen *et al.* 1999). However, sequence and promoter analysis indicate that the major role of this enzyme may be as a 3-ketosteroid reductase in cholesterol biosynthesis, reducing zymosterone at the 3 position to form zymosterol (Marijanović *et al.* 2003, Ohnesorg *et al.* 2006). Despite this, a recent study has indicate that 17β-HSD7, along with 17β-HSD1, 17β-HSD5 and other steroidogenic enzymes, is significantly up-regulated in ovarian tissue of patients with ovarian endometriosis (Šmuc *et al.* 2007).

17β-HSD8, also known as FabG (beta-ketoacyl-[acyl-carrierprotein] reductase, *E. coli*)-like (FABGL), HZ-K region expressed gene 6 (HKE6) and ring finger protein 2 (RING2), is preferentially an oxidative enzyme. Although assays of the mouse enzyme *in vitro* have demonstrated that it can use both E_2 and testosterone as substrates (Fomitcheva *et al.* 1998), sequence analysis again suggests that this enzyme may primarily be involved in the regulation of fatty acid metabolism (Pletnev & Duax 2005). It is highly expressed in murine kidney and spleen, with some expression in the ovary and testes, but is significantly down-regulated in mouse models of polycystic kidney disease (Fomitcheva *et al.* 1998). In humans, it is expressed in tissues including the liver, pancreas, kidney and skeletal muscle (Ando *et al.* 1996). A study of human genes expressed in the polymorphic human leukocyte antigen (HLA) region of chromosome 6 has indicated that 17β-HSD8 is also down-regulated in oral cavity tumour tissue compared with surrounding normal tissue (Reinders *et al.* 2007).

Mouse hsd17b9 has 17β- and 3α-HSD activities, recognising both steroids and retinols as substrates (Napoli 2001). Although it has closest homology to rat hsd17b6, it is more homologous to members of the retinol dehydrogenase family than to other 17β-HSDs (Su *et al.* 1999). A human 17β-HSD9 homologue has not been identified.

Human 17β-HSD10 was initially identified and named by several groups before its 17β-HSD activity and homology to 17β-HSD4 was recognised, resulting in its reclassification as 17β-HSD10 (He *et al.* 1999). It is also known as short-chain L-3-hydroxyacyl coenzyme A dehydrogenase II (SCHAD/HCD2/HADH2) and endoplasmic reticulum-associated binding protein (ERAB), as well as amyloid beta peptide-binding alcohol dehydrogenase (ABAD) and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD). 17β-HSD10 is a mitochondrial protein, initially isolated from rat liver (Luo *et al.* 1995) and subsequently from other species and tissues such as human brain (He *et al.* 1998). It has a wide substrate profile (Nordling *et al.* 2001, Shafqat *et al.* 2003), being involved in isoleucine degradation, β-oxidation of fatty acids and oxidation of steroids, inactivating E_2, but converting 5α-androstanediol to DHT (Yang *et al.* 2005*a*,*b*). It has been implicated in the development of Alzheimer's disease as it is overexpressed in neurones of patients with the disease, and associates with the neurotoxic peptide amyloid-β (Yan *et al.* 1997). It is expressed in other tissues including the prostate (Bauman *et al.* 2006*a*) and has been seen to be overexpressed in primary prostate cancer cell cultures (He *et al.* 2003).

17β-HSD11 (HSD17B11), also known as Pan1b, retSDR2 and DHRS8, was initially isolated by a group attempting to find enzymes homologous to the 11β-HSDs (Li *et al.* 1998). It was found to have oxidative 17β-steroid activity, metabolising 5α-androstane-3α,17β-diol to the less androgenic ADT; but despite also binding retinoids, it has no retinoid-metabolising activity (Brereton *et al.* 2001). It is expressed in both steroidogenic and non-steroidogenic tissues, including the pancreas, kidney, liver, lung, small intestine and heart, and the adrenal glands, ovary, endometrium and Leydig cells, and its expression can be down-regulated by the steroidogenic combination of cAMP with all-*trans*-retinoic acid (Chai *et al.* 2003). However, a physiological role for the enzyme in lipid metabolism is implicated: agonists of peroxisome proliferator-activated receptor-α (PPARα) induce a rapid increase in 17β-HSD11 expression in the endoplasmic reticulum and lipid droplets of mouse liver and intestine (Motojima 2004, Yokoi *et al.* 2007), and 17β-HSD11 has been identified as one of the three major proteins in lipid droplets of human liver cells that accumulate in fatty liver disease, along with adipose differentiation-related protein and acyl-CoA synthetase 3 (Fujimoto *et al.* 2004, 2006).

3-ketoacyl reductase (KAR), a protein in the membrane of the endoplasmic reticulum that uses NADPH to reduce 3-ketoacyl-CoA to 3-hydroxyacyl-CoA during the second step of fatty acid elongation (Moon & Horton 2003), has also been identified as 17β-HSD12 and has high homology to 17β-HSD3. Some recent studies have suggested that it may also be important in the reduction of E_1 to form E_2 (Luu-The *et al.* 2006, Blanchard & Luu-The 2007), although other studies have indicated that it does not efficiently catalyse this reaction (Day *et al.* 2008), despite its up-regulation in the tumours of breast cancer patients (Song *et al.* 2006). However, investigation of its expression in normal human tissues indicated that it is highly expressed in many active lipid-metabolising tissues, including liver, kidney, heart and skeletal muscle, as well as in placenta and testis, with additional expression in other steroidogenic tissues such as adrenal gland, ovary and prostate (Sakurai *et al.* 2006). This seems to support a primary role for 17β-HSD12 as a regulator of lipid biosynthesis.

Presently, little is known about 17β-HSD13, 14 and 15, as they have only recently been identified. 17β-HSD13 is found at 4q22.1 and is also known as short-chain dehydrogenase/reductase 9. It has 78% homology with 17β-HSD11, also located at 4q22.1, and is highly expressed in the liver, apparently within the cytoplasm (Liu *et al.* 2007). 17β-HSD14 was originally named retSDR3 as it was first discovered in the retina, and is also known as DHRS10. It is a cytosolic enzyme with high levels of expression in the brain, liver and placenta, and can oxidise E_2, testosterone and Adiol using NAD^+ as cofactor (Lukacik *et al.* 2007). Transfection of human MCF-7 and SKBR3 breast cancer cell lines with 17β-HSD14 significantly decreased E_2 levels, and in an RT-PCR study of 131 breast tumours, patients with ER-positive tumours that highly expressed 17β-HSD14 showed significantly better recurrence-free survival and breast cancer-specific survival prognoses (Jansson *et al.* 2006) indicating that 17β-HSD14 may well have a role in E_2 inactivation *in vivo*. 17β-HSD15, the most recently discovered of the 17β-HSDs, may play a role in androgen biosynthesis (Luu-The *et al.* 2008).

Inhibition of 17β-HSD enzymes

The selectivity of each of the 17β-HSD enzymes for their preferred substrates and directional redox activities, combined with their tissue-specific localisations, contributes greatly to the fine tuning of the endocrine system. This selectivity of action also suggests that many of the 17β-HSDs would provide good targets for modulation of the endocrine response in disease states, especially in those diseases in which they or other steroidogenic enzymes are being abnormally expressed.

Early work on inhibitors of these enzymes was reviewed by Penning & Ricigliano (1991), again by Penning (1996), and most recently and comprehensively by Poirier (2003). Several of these enzymes have now been validated as targets for the treatment of endocrine-related diseases. Progress in the design and development of inhibitors of specific 17β-HSD enzymes for use in the treatment of various disorders, including steroid-dependent diseases such as breast and prostate cancer, and endometriosis, has advanced greatly in recent years, and will be discussed in the course of the rest of this review.

17β-HSD1 inhibition

Application of 17β-HSD1 inhibitors in breast cancer and endometriosis

Oestrogens have a crucial role in supporting the growth of hormone-dependent breast cancer in post-menopausal women. Although both 17β-HSD1 and 17β-HSD2 are present in healthy pre-menopausal subjects, several studies have indicated that the ratio of 17β-HSD1 to 17β-HSD2 is increased in the tumours of post-menopausal patients with hormone-dependent breast cancer (Suzuki *et al.* 2000, Miyoshi *et al.* 2001). This results in an increased level of E_2 that drives the proliferation of the tumour tissue via the ER. Several studies have indicated that patients with tumours that have high 17β-HSD1 expression have significantly shortened disease-free and overall survival (Gunnarsson *et al.* 2005, Salhab *et al.* 2006, Vihko *et al.* 2006), suggesting that compounds which inhibit the activity of this enzyme may be of therapeutic benefit in the treatment of hormone-dependent breast cancer in post-menopausal patients (Reed & Purohit 1999, Purohit *et al.* 2006, Sasano *et al.* 2006).

In endometriotic tissue, although data are conflicting, there seems to be a change in the expression of steroidogenic enzymes, resulting in the presence of a high concentration of E_2 that stimulates proliferation of the tissue (Bulun *et al.* 2000). Aromatase, responsible for the formation of E_1 from Adione, is negligible in normal endometrium, but is up-regulated in endometriotic tissue (Gurates & Bulun 2003). It has been suggested that the expression of 17β-HSD2, the enzyme that inactivates E_2 to E_1, is down-regulated in endometriosis (Bulun *et al.* 2006), although in two studies of mRNA expression in the endometriotic tissue down-regulation of 17β-HSD2 was not seen (Matsuzaki *et al.* 2006, Carneiro *et al.* 2007). A recent study, however, indicated that there is a down-regulation of 17β-HSD2 mRNA expression in endometriotic samples, while both aromatase and 17β-HSD1 are up-regulated in comparison with normal endometrium (Dassen *et al.* 2007). The down-regulation of 17β-HSD2 in endometriosis is thought to be due to a lack of progesterone receptor B (PR-B) expression and a very low level of PR-A expression in the endometriotic tissue, resulting in the resistance to progesterone, which in normal endometrium stimulates the expression of 17β-HSD2 (Bulun *et al.* 2006). In another recent RT-PCR study, the authors found no change in the expression of 17β-HSD2 mRNA between normal and endometriotic tissue (Šmuc *et al.* 2007), but did find an increase in the expression of 17β-HSD1, 17β-HSD7, steroid sulphatase and ERβ mRNA. It has also been suggested that there is a link between a 17β-HSD1 polymorphism, Ser312Gly, and endometriotic risk and severity (Tsuchiya *et al.* 2005). This polymorphism has previously been associated with higher E_2 levels in some women (Setiawan *et al.* 2004).

Inhibitors of 17β-HSD1

As both hormone-dependent breast cancer and endometriosis are oestrogen-dependent diseases, with an increase in the ratio of 17β-HSD1 to 17β-HSD2 expression implicated in many studies, it has been suggested that this enzyme is a good target for inhibition in the treatment of both of these diseases. A recent 17β-HSD1 transgenic mouse study indicated that 17β-HSD1 is also capable of causing a significant amount of androgen activation *in vivo*, suggesting that 17β-HSD1 inhibitors may also have a role to play in women with diseases related to androgenic dysfunction (Saloniemi *et al.* 2007). There are now many different groups working to find selective inhibitors of 17β-HSD1.

Until recently, there have only been two major methods by which the activity and inhibition of 17β-HSD1 are assayed. In whole cells and lysates, as for many other steroidal enzymes, 17β-HSD1 activity is usually measured using radiometric assays, often with tritiated substrate at physiological concentrations (Singh & Reed 1991). Substrate and product require extraction, followed by separation by either thin layer chromatography (TLC) or reverse phase high performance liquid chromatography (HPLC). In assays using purified enzyme, however, the redox state of the cofactor, NAD(P)(H), can be used to determine the progress of the reaction using spectrophotometric measurement at 340 nm (Chin & Warren 1975). The radiometric TLC and HPLC methods allow for sensitive determination of changes in activity, but are very time consuming; whereas the spectrophotometric method, though less sensitive, is faster, but cannot be used for analysis of 17β-HSD1 activity in tissue samples as it requires purified enzyme. ELISA-based assays are also used for determination of steroid levels in blood and other tissues, but again there are problems with the sensitivity requirements for their use. Recently, novel methods have been developed to improve the determination of 17β-HSD1 activity. These include homogeneous proximity (Kokko *et al.* 2006) and fluorescence resonance energy transfer (FRET)-based assays (Kokko *et al.* 2007) for high throughput *in vitro* screening, and sensitive HPLC-based methods for tissue sample analysis, such as that of Delvoux *et al.* (2007), which can be used to determine the activity of several different steroidogenic enzymes in one tissue sample.

The elucidation of the crystal structure of 17β-HSD1 provided a good basis for the design of initial inhibitors. After optimisation of the crystallisation conditions for 17β-HSD1 (Zhu *et al.* 1993, 1994), a homodimer, structural data revealed a Rossman fold and an active site containing a Tyr-X-X-X-Lys sequence, both characteristic of short-chain dehydrogenases (Duax *et al.* 2000). 17β-HSD1 also contains three α-helices and a helix-turn-helix motif, which along with a histidine residue, influence active site availability and thus substrate specificity (Ghosh *et al.* 1995). When crystallised in the presence of E_2 (Azzi *et al.* 1996), and also as a ternary complex in the presence of both E_2 and the cofactor $NADP^+$ (Breton *et al.* 1996), data indicate that the histidine residue, His221, and another residue, Glu 282, form hydrogen bonds with the hydroxyl group at the 3 position of E_2, while two other residues in the narrow active site, Ser142 and Tyr155, are involved in hydrogen bond formation with the oxygen atom at the 17 position of E_2. There are further hydrophobic interactions between the steroid and several other residues (Lin *et al.* 1996, Ghosh & Vihko 2001).

Mutational analysis of recombinant 17β-HSD1 indicated that Lys159 forms the catalytic triad with Ser142 and Tyr155, and that all of these residues are essential for activity (Puranen *et al.* 1997). In a mechanism postulated to be conserved across the SDR and AKR superfamilies (Bennett *et al.* 1996), it is thought that Ser142 and Lys159 lower the pKa of the Tyr155 hydroxyl proton for donation to the C17 keto oxygen, while a hydride is transferred to the C17α position from the nicotinamide ring of the cofactor (Ghosh & Vihko 2001). Site-directed mutagenesis has also helped to establish the importance of other residues in substrate and cofactor recognition by 17β-HSD1. Altering Ser12 for a positively charged residue considerably increases the preference of 17β-HSD1 for NADPH over NADH (Huang *et al.* 2001), whereas the substitution of Leu36 for a negatively charged residue changes its preference from NADPH to NADH, resulting in a lower specificity for E_1 (Gangloff *et al.* 2001). Substitution of His221 for Ala lowers the affinity of the enzyme for E_1 (Huang *et al.* 2001).

Crystallisation studies with the enzyme in complex with 20α-hydroxyprogesterone, DHT and DHEA (Lin *et al.* 1999, Han & Lin 2000, Han *et al.* 2000), indicated that although all of these steroids can be accommodated in the binding site of 17β-HSD1, they bind in the reverse orientation to E_2 (Gangloff *et al.* 2003), and it is Leu149 that is involved in the discrimination between E_2 and C19 steroids, resulting in a far greater affinity for E_2. Val225 has been shown to act on the α-face of E_2, in concert with Leu149 on the β-face, to sandwich the A-ring in place and sterically hinder binding of C19 steroids. The carboxamide group of NADP(H) forms a hydrogen bond with the peptidic amide group of Val188, stabilising the binding of the cofactor, but in C19 steroid complexes this bonding is disturbed, destabilising the ternary structure (Shi & Lin 2004).

Modelling studies of 17β-HSD1 place E_2 in the same binding area within the cleft as the crystallographic data, with His221 and Glu282 forming hydrogen bonds to the C3-OH end of E_2, and Ser142 and Tyr155 binding to C17-OH, and also interacting with the cofactor. These mechanisms of selectivity over C19 steroids, steric hindrance at the A-ring and hydrogen bonding of the hydroxyl groups at either end of the molecule, are also seen in the binding of E_2 to ERα (Nahoum *et al.* 2003). Modelling has also confirmed that cofactor and substrate may bind in either order (Zhorov & Lin 2000), as was seen in early kinetic activity studies (Betz 1971).

Before 17β-HSD1 was successfully crystallised, the inhibitory potency of other steroids and similar non-steroidal compounds was assessed to study the binding characteristics and specificity of the enzyme (Blomquist *et al.* 1984). Substrate and cofactor analogues were developed to explore the involvement of amino acid residues of the active site in the binding mechanism. These included alkylating agents such as 3-chloroacetylpyridine-adenine dinucleotide, an NAD^+ analogue (Fig. 3a; Biellmann *et al.* 1976), 16α-bromoacetoxyoestradiol 3-methyl ether, an E_2 analogue (Fig. 3b; Chin & Warren 1975) and 6β-bromoacetoxyprogesterone, a progesterone analogue (Fig. 3c; Thomas & Strickler 1983). C16,C17-substituted pyrazole and isoxazole E_1 derivatives (Fig. 3d) have also been shown to be competitive inhibitors of 17β-HSD1 (Sweet *et al.* 1991).

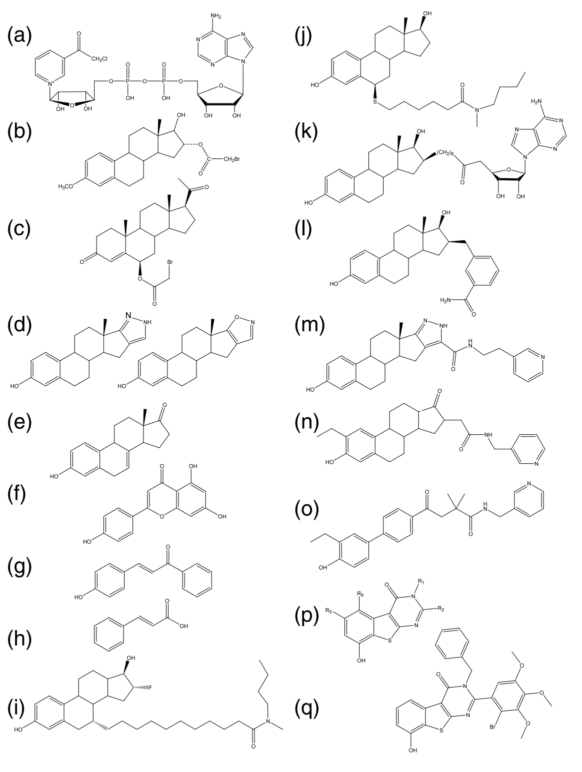

Figure 3 17β-HSD1 inhibition. (a) 3-chloroacetylpyridine-adenine dinucleotide, (b) 16α-bromoacetoxy-E_2 3-methyl ether, (c) 6β-bromoacetoxyprogesterone, (d) 16,17-pyrazole-/-isoxazole-E_1, (e) equilin, (f) apigenin, (g) 4-hydroxychalcone, (h) cinnamic acid, (i) EM139, (j) 6β-(thiaheptanamide)-E_2, (k) EM1745, (l) 16β-*m*-carbamoyl benzyl-E_2, (m) C5′-pyridylethylamide-16,17-pyrazole-E_1, (n) STX1040 (2-ethyl-16β-*m*-pyridyl methyl amido-methyl-E_1), (o) non-steroidal STX1040 mimic, (p) pyrimidinone core and (q) 3-benzyl-2-(2-bromo-3,4,5-trimethoxy-phenyl)-8-hydroxy-3H-benzo[4,5]thieno[2,3-d]pyrimidin-4-one.

Later, equilin (Fig. 3e), an equine oestrogen that is a major component of Premarin, used in hormone replacement therapy, was also shown to inhibit 17β-HSD1 ($IC_{50} < 1$ μM), and the crystal structure of the 17β-HSD1 homodimer in a ternary complex with $NADP^+$ and equilin was solved (Sawicki *et al*. 1999). Equilin binds at the substrate binding site, with the substrate entry loop in a closed conformation. In the equilin complex, the 17-keto group is a greater distance from the C4 atom of the cofactor due to the C7=C8 double bond of equilin, and this results in inhibition of the catalytic hydride transfer.

Many flavonoids and other phytoestrogens also inhibit 17β-HSD1 (Mäkelä *et al*. 1998, Hoffrén *et al*. 2001). Flavonoids with a hydroxyl group in position 7 of the A-ring, which mimics the D-ring of steroids, such as apigenin, chrysin, genistein and naringenin, inhibit 17β-HSD1, apigenin being most potent ($IC_{50} < 1$ μM; Fig. 3f; Le Bail *et al*. 1998). Derivatives of flavonoids, the chalcones, also inhibit 17β-HSD1 and aromatase when a hydroxyl substitution is present on position 4 of the A-ring (e.g. 4-hydroxychalcone, $IC_{50} = 16$ μM; Fig. 3g), equivalent to position 7 of the flavonoids (Le Bail *et al*. 2001). Phytoestrogens have also been found to inhibit fungal *Cochliobolus lunatus* 17β-HSD (Kristan *et al*. 2005, Sova *et al*. 2006), used as a model enzyme for the SDR family, as do cinnamic acid (Fig. 3h) and its derivatives (Gobec *et al*. 2004, Kristan *et al*. 2006, Sova *et al*. 2006), suggesting that they would be good inhibitors of 17β-HSD1. Two residues that appear crucial for binding of inhibitors in the fungal enzyme active site, Asn154 and Tyr212, can be matched by their counterparts, Arg258 and Tyr218, in 17β-HSD1. The cofactor NADP also occupies the same position in both enzymes. However, there are no residues in the fungal enzyme which correspond to Glu282 and His221 of human 17β-HSD1, and these appear to determine selectivity for 4′-hydroxyflavones.

Unfortunately, although many of these steroids and phytoestrogens are potent inhibitors of 17β-HSD1, they are not useful as therapeutic inhibitors as they are often oestrogenic, or are not specific for 17β-HSD1 inhibition, having inhibitory effects on other steroidal enzymes and receptors (Deluca *et al*. 2005). These include other 17β-HSDs, for example, 17β-HSD5 (Brožič *et al*. 2006); aromatase, as the 7-hydroxy group of the flavonoids mentioned above is also essential for aromatase inhibition (Le Bail *et al*. 2001); 3β-HSD (Arlt *et al*. 2004) and the ER, often resulting in stimulatory effects (Usui 2006, Turner *et al*. 2007). However, as we have seen, their use has been invaluable in the understanding of the mechanism of catalysis and inhibition of 17β-HSD1.

From these studies, several important factors have been determined for the design of potent inhibitors: a planar hydrophobic ring core structure, such as E_2, lacking a C19 group, to fit into the narrow hydrophobic binding region; β-oriented electron withdrawing groups to form hydrogen bonds with catalytically essential amino acids such as Tyr155 and Ser142; α-oriented hydrophobic groups at C17 or C16 to block the cofactor from binding; and the availability of space to accommodate substituents at the 7α-position of the steroid (Han *et al*. 2000, Owen & Ahmed 2004, Alho-Richmond *et al*. 2006).

A series of compounds designed as pure anti-oestrogens for the treatment of hormone-dependent breast cancer had additional 17β-HSD1 inhibitory properties in *in vivo* mouse studies (Labrie *et al*. 1992). The competitive inhibitors, steroidal derivatives possessing both a 7α-undecanamide group and either a halogen atom at C16 or a double bond at C14–C15 or

C15–C16, inhibited both E_1-stimulated uterine growth, an anti-oestrogenic effect, and the conversion of E_1 to E_2, and A to T, in the mice, when dosed at concentrations as low as 3 μg twice daily. 17β-HSD1 was crystallised with one of these dual-site inhibitors, EM139 (Fig. 3i), which although larger than E_2, binds at the same position (Zhu *et al.* 1999). These first dual-site inhibitors had only weak 17β-HSD1 inhibitory activity but were optimised with the aims of improving 17β-HSD1 inhibitory potency and specificity and reducing intrinsic oestrogenicity, while also maintaining the ER antagonist activity (Tremblay & Poirier 1998).

Many steroid derivatives, including those of E_2, E_1, progesterone and Adione substituted at C16 with reactive halogenated functional groups, such as bromoacetoxy and bromoacetamido groups, were found to be irreversible inhibitors of 17β-HSD1 as the halogenated group covalently bonds with an amino acid residue, permanently inactivating the enzyme (Poirier *et al.* 1998). Several oestratriene derivatives with fluorine substitutions at C17 inhibit 17β-HSD1 with micromolar potencies, but most have activities against other 17β-HSD enzymes, including types 2, 5 and 7 (Deluca *et al.* 2006). 16α-iodopropyl and bromopropyl substituted E_2 derivatives are potent inhibitors, with IC_{50} values of 420 nM (Sam *et al.* 1998) and 460 nM (Sam *et al.* 1998, Tremblay & Poirier 1998) respectively in assays using partially purified 17β-HSD1 from human placenta.

Reversible E_2 derivatives with β-oriented thiaalkanamide side chains at C6 show enhanced inhibitory activity, with the most potent, 6β-(thiaheptanamide) E_2 (Fig. 3j), having an IC_{50} of 170 nM in assays using 17β-HSD1 partially purified from human placenta (Poirier *et al.* 1998). This compound, however, was found to be oestrogenic, and attempts to improve it by removing the 3-hydroxy group, changing the 6β-substitution for a 6α-substitution, changing the amide group of the side chain for a methyl or changing the thio-ether for an ether bond, all reduced the potency of the compound, despite the ether bond improving the oestrogenic profile (Tremblay *et al.* 2005).

These structure-activity relationships (SAR) studies indicate that 17β-HSD1 potency and specificity may be optimised by the rational design of compounds that interact with both the cofactor-binding and substrate-binding regions of the enzyme. A series of E_2 derivatives with 16β-propylaminoacyl substitutions were designed containing hydrophilic and hydrophobic moieties to interact with the cofactor- and substrate-binding regions of the enzyme respectively (Tremblay *et al.* 2001). Although these compounds are non-oestrogenic, having no interaction with the ER, they also fail to inhibit 17β-HSD1. Derivatives of gossypol, which potently inhibits lactate dehydrogenase by targeting the Rossmann fold, inhibit 17β-HSD1 in the micromolar range, also by binding in the Rossmann fold from the cofactor binding site across to the substrate-binding region (Brown *et al.* 2003). Active site modelling suggested that their potency and specificity may be improved by the incorporation of a substrate analogue into their structure.

E_2-adenosine-based compounds were designed to specifically target both the substrate and cofactor binding sites. The most potent of these hybrid inhibitors is EM1745 (Fig. 3k), a reversible competitive inhibitor with an IC_{50} of 52 nM, in which the steroid is linked to the adenosine moiety by a 16β-oriented side chain containing eight methylene groups, allowing it to bind to Leu96 and Val196 (Qiu *et al.* 2002). Two compounds, which each have only one of the components of EM1745, 16β-nonyl-E_2 and 5-nonanoyl-*O*-adenosine, do not inhibit the enzyme (Poirier *et al.* 2005). Simplified versions of these hybrids, containing adenosine mimics to improve the stability and bioavailability of these compounds, are less potent than EM1745 (Bérubé & Poirier 2004), as are C16-substituted aryl E_1 and E_2 derivatives, such as 16β-benzyl E_2, which has an IC_{50} of ∼800 nM using purified enzyme (Poirier *et al.* 2006). However, further modification of this structure resulted in 16β-*m*-carbamoylbenzyl-E_2, an inhibitor with an IC_{50} of 44 nM (Fig. 3l) in which the *m*-carbamoylbenzyl group mimics the nicotinamide ring of the cofactor. Although this compound is weakly oestrogenic (Laplante *et al.* 2008), its oestrogenic profile is improved by modifications at the C2, C3 and C7 positions; however, these substitutions lead to a decrease in potency.

Other inhibitors have also been designed to bind across the substrate binding site towards that of the cofactor by substituting the steroid scaffold at the C16 position, both alone and in combination with C2, C6 and C17 substitutions. One class of compounds resulting from this approach is the E-ring pyrazole amides (e.g. C5′-pyridylethylamide-16,17-pyrazole-E_1; Fig. 3m), inhibitors that selectively inhibit 17β-HSD1 with sub-micromolar IC_{50} values in a whole cell assay (Fischer *et al.* 2005, Allan *et al.* 2006*a*). Of the others, which include C16-substituted alkenyl, alkyl and carboxyl, C6-oxo and C6 and C16 and C17-oxime E_1 and E_2 derivatives, the most active inhibitors are those containing an *m*-methylene carboxamide functionality extending from the C16β position (Lawrence *et al.* 2005, Allan *et al.* 2006*b*, Vicker *et al.* 2006).

The most potent of the *m*-methylene carboxamide compounds, with an IC_{50} of 27 nM in a whole cell assay, is STX1040 (2-ethyl-16β-*m*-pyridyl methyl amido-methyloestrone; Fig. 3n; Lawrence *et al.* 2005). Fig. 4 shows STX1040 docked in place of E_2 in the 17β-HSD1 crystal structure, protein database (PDB) entry 'IFDT', in complex with $NADP^+$ using the docking programme GOLD (Jones *et al.* 1997). The high potency of STX1040 may be explained by its interactions with the cofactor. In 1FDT, the nicotinamide carbonyl and amide nitrogen of the cofactor form hydrogen bonds with Val188 and Thr140 respectively. In the inhibitor complex, it appears that there may be an interaction between the nicotinamide amide moiety and the amide carbonyl of the 16β side chain. The pyridyl nitrogen of the 16β side chain may interact with an oxygen atom 3.16 Å away in the phosphate group of the cofactor. A C2 ethyl group, included to eliminate oestrogenicity as it interferes with hydrophobic interactions with the ER (Vicker *et al.* 2006), also contributes to inhibitory activity by interacting with Leu262 and Phe259. Hydrophobic interactions with Leu149, Val225, Phe226 and Phe259, observed when substrate is docked into the enzyme, are maintained in the inhibitor complex.

Non-steroidal E_1 mimics are also active as 17β-HSD1 inhibitors (Allan *et al.* 2008). In these inhibitors, the E_1 moiety is replaced by aryl ring-containing scaffolds that retain both a hydroxyl group equivalent to that at the 3 position of E_1 and a ketone functionality at the 17-position equivalent. The most potent of these, with IC_{50} values of 3.7 and 1.7 μM in a whole cell assay, are the biphenyl ethanones and biphenyl indanones respectively. Substitution of the biphenyl ethanone scaffold to form mimics of STX1040 results in improved activity, with the most potent compound having an IC_{50} of 1.8 μM (Fig. 3o).

The potency of STX1040 was assayed using T47D cells to measure 17β-HSD1 inhibition, while the high selectivity of STX1040 for the 17β-HSD1 enzyme over 17β-HSD2 was determined using MDA-MB-231 cells, as these breast cancer cell lines have high 17β-HSD1 and 17β-HSD2 activities respectively (Day *et al.* 2006*a*, Purohit *et al.* 2006). The T47D cell line was also used to develop an *in vitro* proof of concept assay for the inhibition of E_1-stimulated proliferation of hormone-dependent breast cancer cells by 17β-HSD1 inhibitors such as STX1040, as it is an ER-positive breast cancer cell line whose proliferation is dependent on oestrogens in the medium. For this reason, it is also an appropriate cell line to use to establish whether such inhibitors are oestrogenic *in vitro* (Day *et al.* 2006*b*, 2008, Laplante *et al.* 2008). Using this model, and dosing E_1 from 1 nM to 1 μM, STX1040 at 5 μM is seen to be non-oestrogenic while significantly inhibiting the E_1-dependent proliferation of T47D cells (Day *et al.* 2006*b*, 2008).

The low inter-species homology of the 17β-HSD1 enzyme has made the development of animal models for the assessment of the inhibitors difficult. Mouse 17β-HSD1 is only 63% homologous to human 17β-HSD1 at the amino acid level, and this is reflected in its different substrate affinity, as, in addition to E_1 to E_2 activity, it also converts Adione to testosterone (Nokelainen *et al.* 1996). The rat enzyme has 93% homology to that of the mouse, but only 68% to human 17β-HSD1 (Ghersevich *et al.* 1994). STX1040 has an IC_{50} of ∼100 μM against the rat enzyme when it is expressed in 293-EBNA (Invitrogen, Paisley, UK) cells (unpublished results), indicating that inhibition by STX1040 is specific to the human enzyme. In female rodents, the major expression of 17β-HSD1 is in the ovary (Peltoketo *et al.* 1999), with low levels in the uterus and some expression in the sebaceous glands of the skin (Nokelainen *et al.* 1996, Pelletier *et al.* 2004). To limit endogenous steroid enzyme expression and oestrogen synthesis ovariectomised mice are often used in animal models of steroidogenic enzyme

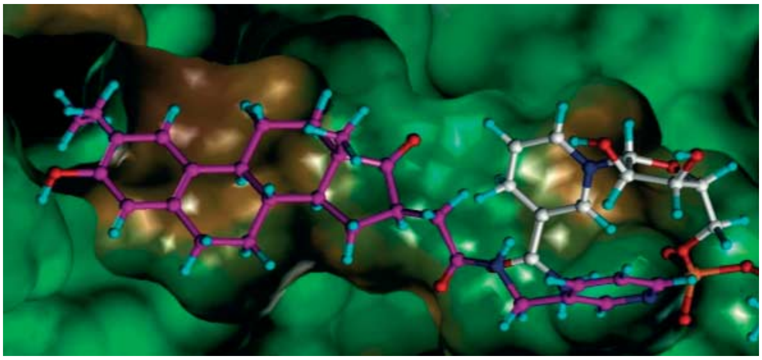

Figure 4 STX1040 docked in place of E_2 in the 17β-HSD1 crystal structure (reproduced with permission from: Day *et al.* 2008).

inhibition, resulting in similar oestrogen levels to those of post-menopausal women (Yue *et al*. 1994). Human tumour xenograft growth can then be stimulated by exogenous doses of steroid, dependent on expression of the required enzyme by the human tumour cells for conversion to the more active steroid.

STX1040 was shown to be efficacious *in vivo* using a novel ovariectomised nude mouse model based on the principles above (Day *et al*. 2006*b*, 2008). As T47D cells had been successful in *in vitro* model development, this cell line was also used to develop the *in vivo* model in which growth of the T47D tumours was stimulated by low doses of E_1, dependent on the human 17β-HSD1 expressed by the tumours for conversion to E_2, the more active oestrogen. Tumours stimulated with E_1, either daily by s.c. injection (0.05–0.1 μg/day) or by use of a time-release pellet (0.1–0.28 μg/day), as unconjugated oestrogens have a short half-life *in vivo* (Ruder *et al*. 1972), were significantly larger than those of the control animals. Treatment of the mice with STX1040 caused a significant decrease in their tumour volumes and plasma E_2 levels in comparison with those dosed with E_1 alone. STX1040 was confirmed to be non-oestrogenic *in vivo* using a standard rat uterotrophic model.

Despite the number of groups working towards the development of 17β-HSD1 inhibitors for clinical use, only one other group has reported activity *in vivo* using inhibitors optimised from those with core structures which mimic those of the natural inhibitors, coumestrol and kaempferide. From various non-steroidal flavone-, tetrahydrochromanoquinoline-, chromenone- and pyrimidinone-based structures, the pyrimidinone core was selected for further optimisation (Fig. 3p; Messinger *et al*. 2006) as selectivity and solubility problems were observed with the other classes of compounds. Several compounds were selected for synthesis and evaluation from those modelled using published crystal structures. The most potent of these (Fig. 3q) has an IC_{50} of 5 nM in assays using purified recombinant 17β-HSD1, although in whole cell assays, its efficacy is much lower than that of STX1040 (inhibition at 1 $\mu M < 67\%$). It is selective for 17β-HSD1 over 17β-HSD2, is non-oestrogenic and is efficacious *in vivo* using an intact nude mouse model bearing MCF-7 cells transfected with 17β-HSD1 (Husen *et al*. 2006*a*,*b*).

Although there are differences between the two *in vivo* models, in both studies treatment of the mice with E_1 caused mouse uterine weight to increase significantly. Use of the inhibitors, however, although resulting in a decrease in E_1-stimulated tumour size, did not significantly decrease the E_1-stimulated uterine weight. Despite this, E_2 levels in the T47D tumour study (Day *et al*. 2006*b*, 2008) were shown to be significantly decreased after treatment with STX1040, suggesting that tumour 17β-HSD1 expression is a major E_2 source in these models. The lack of an effect of the 17β-HSD1 inhibitors on uterine weight in both models may therefore be due to either the higher sensitivity of the uterus than the tumour to circulating oestrogens or maximal stimulation of uterine growth over the initial few weeks.

The demonstration of E_1-dependent tumour growth inhibition *in vivo* by 17β-HSD1 inhibitors provides justification for the many years of research into inhibition of the enzyme, and suggests that it may indeed be a valid target for the treatment of hormone-dependent breast cancer. Although models for the use of these inhibitors in other hormone-dependent diseases, such as endometriosis, are yet to be developed, the observed decrease in the plasma level of active hormone after STX1040 treatment of animals carrying the human enzyme indicates that these inhibitors may well be effective for the treatment of these other diseases of hormone metabolism. The successful *in vivo* application of inhibitors of 17β-HSD1, the most studied of the 17β-HSDs, also suggests that inhibition of other 17β-HSDs known to be involved in disease states may well prove clinically beneficial in the future.

17β-HSD3 inhibition

Application of 17β-HSD3 inhibitors in prostate cancer

DHT is the main intracellular androgen in the prostate and stimulates the growth of hormone-dependent prostate tumours via its interaction with the AR. It is formed from testosterone by 5α-reductases 1 and 2. 17β-HSD3, microsomally expressed almost exclusively in the testes (Geissler *et al*. 1994, Luu-The *et al*. 1995), specifically converts non-androgenic Adione (Laplante & Poirier 2008) to active circulating testosterone in the presence of NADPH. It has not been reported to have any other activities. However, it is not the only enzyme that provides testosterone to the body, as 17β-HSD5 (AKR1C3), whose activities and inhibition will be discussed later in this review, converts Adione to testosterone in the prostate and other tissues.

A defect in the expression of 17β-HSD3 causes the autosomal recessive genetic disorder, male pseudohermaphroditism (Geissler *et al*. 1994), in which the individual is usually reared as a female, often having been born with female external genitalia and the absence of a prostate, despite having testes and Wolffian duct-derived male internal genitalia (Andersson & Moghrabi 1997). Diagnosis is assisted by the presence of a high Adione to testosterone ratio, distinguishing the disorder from the clinically similar androgen insensitivity syndrome. The phenotype can vary in severity, even in individuals with the same mutation (Lee *et al*. 2007), but subjects often become virilised at puberty. The mechanism by which this occurs is not fully understood, although it has been suggested that either this is due to incomplete impairment of testes testosterone production, or due to peripheral conversion of Adione to testosterone (Andersson *et al*. 1996), perhaps by the action of 17β-HSD5.

Although 17β-HSD3 is expressed almost exclusively in the testes, there have been some reports of its expression in other tissues. One report indicated that expression of 17β-HSD3 mRNA increased over 30-fold in cancerous prostate biopsies (Koh *et al*. 2002). In this study, the authors also found a corresponding decrease in 17β-HSD2 mRNA expression, indicating that the reductive formation of testosterone is favoured, but found no change in the expression of AKR1C3 mRNA. Expression of 17β-HSD3 was up-regulated in an AR-positive prostate cell line, LNCaP, after it was treated for 48 h with dutasteride (Biancolella *et al*. 2007), an inhibitor of 5α-reductases 1 and 2. A polymorphism in the HSD17B3 gene, G289S, has also been linked to an increased susceptibility to prostate cancer (Margiotti *et al*. 2002). Microarray and subsequent RT-PCR and functional analysis indicated that 17β-HSD3, along with 17β-HSD12, is also expressed in human blood platelets and megakaryocytes. While 17β-HSD12 is up-regulated >25-fold in essential thrombocythemia, a rare myeloproliferative disorder, 17β-HSD3 is down-regulated ~4.5-fold (Gnatenko *et al*. 2005).

Inhibitors of 17β-HSD3

Because of its unique expression and substrate specificity, 17β-HSD3 provides a good target for the inhibition of testosterone formation in the treatment of androgen-dependent diseases such as hormone-dependent prostate cancer. Although its crystal structure has not yet been solved, 17β-HSD3 has undergone various mutational analyses as pseudohermaphroditism results from the effects of deleterious mutations on 17β-HSD3 activity (Lee *et al*. 2007). This has given an insight into the catalytic importance of various residues, such as ARG80, which are involved in the binding and selectivity of the enzyme for the cofactor, NADPH (McKeever *et al*. 2002).

The first demonstrated inhibition of 17β-HSD3 activity was in canine testicular microsomes by two steroids, 4-estrene-3,17-dione (Fig. 5a) and 5-androstene-3,17-dione (Fig. 5b), which are structurally very similar to the substrate, Adione (Pittaway 1983). These studies indicated that a non-aromatic A-ring and C17 carbonyl group were important for inhibition. Using human testicular tissue, the similarly structured atamestane (1-methyl-3,17-dione-androsta-1,4-diene; Fig. 5c), a potent aromatase inhibitor, was also shown to inhibit 17β-HSD3 (Lombardo *et al*. 1993).

As expression of 17β-HSD3 is specific to the testes, attempts have been made to find a more readily available source of the enzyme for the screening of

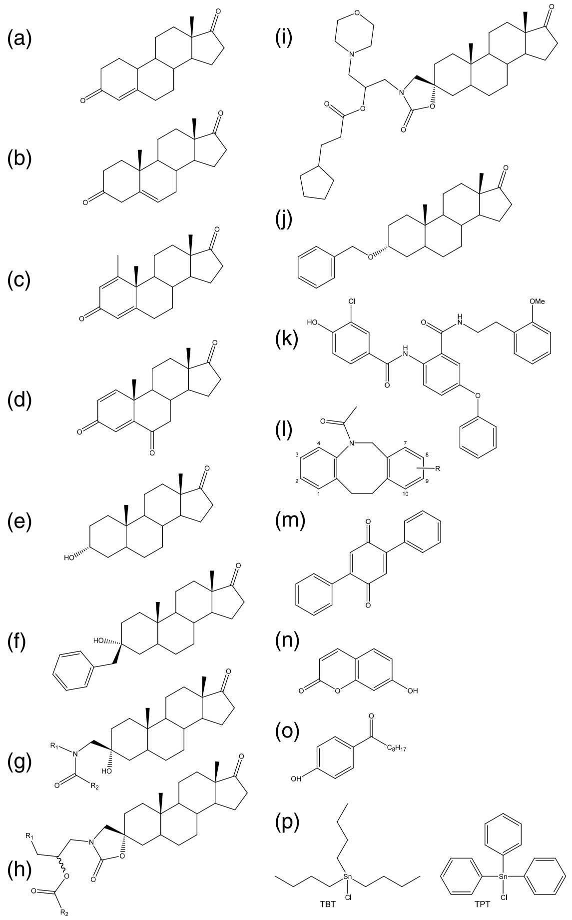

Figure 5 17β-HSD3 inhibitors. (a) 4-estrene-3,17-dione, (b) 5-androstene-3,17-dione, (c) atamestane, (d) 1,4-androstadiene-3,6,17-trione, (e) androsterone (ADT), (f) 3β-phenylmethyl-ADT, (g) 3β-amidomethyl-ADT derivatives, (h) 3-carbamate-ADT derivatives, (i) 3R-spiro-{3′-[3″-*N*-morpholino-2″-(3‴-cyclopentylpropionyloxy) propyl]-2′-oxo-oxazolidin-5′-yl}-5α-androstan-17-one, (j) 3-*O*-benzyl-androsterone, (k) BMS-856, (l) 8/9-substituted tetrahydrodibenzazocine (THB), (m) diphenyl-*p*-benzoquinone, (n) umbelliferone, (o) 1-(4-hydroxyphenyl)-nonan-1-one, (p) tributyltin chloride (TBT) and triphenyltin chloride (TPT).

potential inhibitors of 17β-HSD3. Microsomes isolated from rat testicular tissue were tested, but the enzyme was found to differ from the human enzyme in optimum reaction pH, in sensitivity to inhibition by candidate compounds, and in substrate specificity, efficiently reducing E_1 as well as Adione (Le Lain *et al.* 2001). Most groups working on the inhibition of 17β-HSD3 now use enzyme from cells transfected with 17β-HSD3 cDNA, in purified form, as microsomes, or in whole cell assays. Recently, the pig 17β-HSD3 enzyme has been sequenced, cloned and expressed. As it has a similar substrate profile to the human enzyme and a higher amino acid homology to the human enzyme, at 82%, than that of rats and mice, microsomes from pig testicular homogenates may also have the potential to be used as a ready source of 17β-HSD3 for inhibitor studies (Ohno *et al.* 2006).

Using transfected cell microsomes, 1,4-androstadiene-3,6,17-trione (Fig. 5d) potently and selectively inhibited 17β-HSD3 activity (Luu-The *et al.* 1995). Another steroid, the weak androgen ADT (Fig. 5e), was found to be twice as potent as the substrate Adione, with an IC_{50} of 330 nM in transfected cell microsomes. Several libraries of ADT derivatives were synthesised, and their inhibitory potencies and androgenicity compared (Tchédam Ngatcha *et al.* 2000, 2005, Maltais *et al.* 2001, 2002). ADT derivatives substituted at the 16 position, although proving non-androgenic, demonstrated only weak inhibition of 17β-HSD3 activity (Tchédam Ngatcha *et al.* 2002). 3β-Substituted alkyl and aryl derivatives of ADT were potent inhibitors, with IC_{50} values of 57–147 nM, and were found to be specific for 17β-HSD3 over 17β-HSD1 and 17β-HSD5. 3α-Ether-3β-substituted ADT derivatives had a lower inhibitory activity than the 3β-substituted ADT analogues, with the exception of 3β-phenylethyl-3α-methyl-*O*-ADT and 3β-phenylethyl-ADT, which had IC_{50} values of 73 and 99 nM respectively. The most potent of these 3β derivatives was 3β-phenylmethyl-ADT ($IC_{50}=57$ nM; Fig. 5f; Tchédam Ngatcha *et al.* 2000, 2005), but unfortunately it was found to be as androgenic as DHT. However, a somewhat less potent ($IC_{50}=227$ nM) ADT derivative synthesised using parallel solid-phase techniques, 3β-peptido-3α-hydroxy-5α-androstan-17-one, was found to be tenfold less androgenic (Maltais *et al.* 2001).

Further understanding of the SAR for the ADT derivatives was achieved using 3β-amidomethyl-ADT (Fig. 5g) and 3-carbamate-ADT (Fig. 5h) libraries generated by liquid-phase parallel syntheses. Long alkyl chains at R_1 and R_2 were found to be badly tolerated, with preference for a shorter R_2 chain than that at R_1, and tertiary amides were more active than secondary amines. Adding polarity to the chains did not generally improve their inhibitory potential, but adding rigidity at R_1 did. The most potent of nearly 300 amidomethyl compounds, with an IC_{50} of only 35 nM, was 3β-[(*N*-adamantylmethyl-*N*-butanoyl)aminomethyl]-3α-hydroxy-5α-androstan-17-one, but this compound was found to be almost as androgenic as 3β-phenylmethyl-ADT. The most potent of the 25 3-carbamate-ADT compounds was 3*R*-spiro-{3′-[3″-*N*-morpholino-2″-(3‴-cyclopentyl propionyloxy)-propyl]-2′-oxo-oxazolidin-5′-yl}-5α-androstan-17-one (Fig. 5i), with an IC_{50} of 74 nM, and this compound was not significantly androgenic, even at 1 μM (Maltais *et al.* 2002).

Bisubstrate compounds from the same group, incorporating components that bind in both the substrate and cofactor binding sites, similar to the hybrid inhibitors developed for the inhibition of 17β-HSD1, were also tested as inhibitors of 17β-HSD3. Activity in homogenated cells overexpressing 17β-HSD3 was 78% inhibited by 1 μM Adione substituted at the 17α-position with a 12 methylene spacer esterified to adenosine, although this compound was less potent in whole cells (Bérubé *et al.* 2006). Attempts to improve the potency of this class of compound by phosphorylation of the adenosine group, to mimic the preferred cofactor NADPH over NADH, did not prove successful, suggesting that either this moiety is not interacting with the cofactor binding site, possibly due to prior binding of the cofactor itself, or that its phosphate group interaction is not optimised (Bérubé & Poirier 2007).

A 3-substituted ADT derivative, 3-*O*-benzylandrosterone (Fig. 5j), was used by Bristol Myers Squibb (BMS) to initiate a large-scale screening programme (Spires *et al.* 2005). Inhibition of 17β-HSD3 activity in transfected cell microsomes was assayed using a 96-well format scintillation proximity assay (SPA) with a testosterone-specific antibody for higher throughput of compounds than the usual radiolabelled TLC-based assay (Luu-The *et al.* 1995). 3-*O*-benzylandrosterone and 18β-glycyrrhetinic acid both had inhibitory IC_{50}s of ∼90 nM in the SPA assay. Inhibition in whole cells was measured using an AR stimulation-dependent chemiluminescent secreted alkaline phosphatase (SEAP) reporter assay in AR-positive cell lines transfected stably with 17β-HSD3 and transiently with a prostate serum antigen (PSA)-SEAP reporter plasmid. The inhibitory IC_{50} values for 3-*O*-benzylandrosterone and 18β-glycyrrhetinic acid were, as expected, much higher in this whole cell assay, at 1.6 and 3.8 μM respectively (Spires *et al.* 2005).

Of over 200 000 compounds screened by BMS, a series of anthranilamide-based compounds substituted at position 1 with an amide-linked chlorophenol group were identified as inhibitors of 17β-HSD3 activity, and these were most potent when substituted at the 4 position, further carboxamide substitutions at the 2 position (e.g. BMS-856; Fig. 5k; IC_{50} values of 60 and 300 nM respectively in the enzyme and whole cell assays). Dibenzothiazocines (DBTs) and tetrahydrodibenzothiazocines (THBs) were also discovered to be highly active in these assays (Fink *et al.* 2006). Although DBTs were most potent, especially when substituted with chlorine at position 2 or 3, the THBs were chosen for further SAR study as the aryl sulphur atom of the DBT compounds renders them susceptible to metabolic degradation. Unlike the DBTs, substitution of the THBs at the A-ring did not improve their potency, although substitution at positions 8 and 9 of the C-ring did (Fig. 5l), with an aryl ring substitution at position 8 providing significant improvement. *Ortho*-substitution of the 8-aryl-substituted ring with electron donating groups, including methyl ester, methyl ketone and trifluoromethyl and aldehyde groups, resulted in the highest potencies in both the enzyme and cellular assays (IC_{50} values of around 20 and 500 pM respectively). However, no data on the selectivity of these inhibitors for 17β-HSD3 over other 17β-HSDs has been published.

Screening of a range of compounds by another group (Le Lain *et al.* 2001) revealed that other non-steroidal compounds, such as diphenyl-*p*-benzoquinone, phenyl-*p*-benzoquinone (Fig. 5m), 7-hydroxyflavone, baicalein and biochanin A, with IC_{50} values of 2.7, 5.7, 9.0, 9.3 and 10.8 μM respectively, are also able to inhibit 17β-HSD3. The 7-hydroxycoumarins, umbelliferone (Fig. 5n) and 4-methylumbelliferone are more potent inhibitors of human testes microsomal 17β-HSD3, with IC_{50} values of 1.4 and 1.9 μM respectively (Le Lain *et al.* 2002). However, many of these compounds, such as the flavones, as already discussed in regard to 17β-HSD1 inhibition, are non-selective for 17β-HSD3 inhibitory activity, having effects on other 17β-HSDs, other steroidal enzymes and on steroid receptors. 4-Hydroxyphenyl ketones with attached straight chain alkyl groups inhibit 17β-HSD3 activity in rat testicular microsomes with IC_{50} values in the low micromolar range. The best of those tested was 1-(4-hydroxyphenyl)-nonan-1-one ($IC_{50}=2.86$ μM; Fig. 5o; Lota *et al.* 2006), containing an octyl chain, thought to mimic the steroid backbone of Adione while the 4-hydroxy group forms hydrogen bonds at the active site. In this study, baicalein was shown to be far less potent (IC_{50} value of 186 μM) than in the human microsome study discussed above ($IC_{50}=9.3$ μM; Le Lain *et al.* 2001), suggesting that the alkyl-substituted 4-hydroxyphenyl ketones may prove more potent if tested on the human enzyme.

Organotin compounds are known to be endocrine-disrupting marine pollutants. Two of these compounds, tributyltin chloride and triphenyltin chloride (TBT and TPT respectively; Fig. 5p), were found to be potent inhibitors of 17β-HSD3 in microsomes from pig testes, with IC_{50} values of 7.2 and 2.6 μM respectively. The compounds did not affect expression of the enzyme, suggesting that these effects are due to direct inhibition of 17β-HSD3 activity. Surprisingly, however, in whole cultured Leydig cells, the potency of both compounds increased ∼60-fold to an IC_{50} of 114 nM for TBT and 48 nM for TPT. The unusual increased activity in the whole cell assay was suggested to be either due to the fat solubility of organotins resulting in their high concentration throughout the membrane structure of Leydig cells, in which 17β-HSD3 is located, or due to effects on other enzymes involved in the production of testosterone, such as those involved in transcription and signal transduction (Ohno *et al.* 2005).

Despite the many groups now working on inhibitors of 17β-HSD3, the crystal structure of the enzyme has not been published and there are no formal reports of the efficacy of these inhibitors in *in vivo* models. Groups such as Schering–Plough (WO2004060488) and Sterix Ltd (WO2007003934) have published patents pertaining to the development of 17β-HSD3 inhibitors, with Schering–Plough indicating efficacy of non-steroidal inhibitors in mouse Shionogi tumour (Minesita & Yamaguchi 1965) and monkey models in conference abstracts (American Association of Cancer Research annual meeting 2005); however, data from these studies have not yet been published in peer-reviewed journals.

The development of successful cost-effective *in vivo* models to ascertain the efficacy of 17β-HSD3 inhibitors may prove complex due to the low homology between the human and mouse enzymes. Although use of the Shionogi model, a transplantable androgen-dependent AR-positive mouse breast tumour (Minesita & Yamaguchi 1965) may give an indication of efficacy, the effect would depend on inhibition of testosterone formation by mouse 17β-HSD3, and thus would not truly reflect the use of inhibitors against the human enzyme. Despite the more accurate representation of the human situation by primate models, the cost and regulations involved in their use are prohibitive for most groups and for screening of more than a few compounds. To overcome the problem of the lack of homology between human and rodent

17β-HSD3, the development of models using castrated mice with human 17β-HSD3-expressing androgen-dependent tumour xenografts may prove useful.

17β-HSD5 inhibition

Application of 17β-HSD5 inhibitors in prostate cancer

17β-HSD5 (AKR1C3/PGFS) also catalyses the reduction of Adione to form testosterone, but unlike 17β-HSD3 it is expressed far more ubiquitously in tissues including the prostate, breast, ovary and endometrium (Pelletier *et al*. 1999, Penning *et al*. 2000, Ji *et al*. 2005). It has additional 3α- and 20α-steroid reductase activities (Dufort *et al*. 1999, Penning *et al*. 2000), including the protective inactivation of deoxycorticosterone in mineralocorticoid tissues (Sharma *et al*. 2006). However, its other major activity is as PGFS: it has PGD_2 11-ketoreductase activity that reduces PGD_2 to 9α,11β-PGF_2 (Matsuura *et al*. 1998) and also PGH_2 9,11-endoperoxide reductase activity that reduces unstable PGH_2 to $PGF_{2\alpha}$ (Komoto *et al*. 2004). AKR1C3 is the only 17β-HSD that is not a short-chain dehydrogenase. It is an AKR and is highly homologous to three other AKR enzymes, AKR1C1, AKR1C2 and AKR1C4. Although multifunctional, AKR1C2 and AKR1C4 primarily have 3α-HSD activities, while the major activity of AKR1C1 is as a 20α-HSD (Penning *et al*. 2004).

17β-HSD5/AKR1C3 has been seen to be up-regulated in prostate cancer. Elevated expression of 17β-HSD5 protein has been demonstrated in the epithelium of malignant prostate tissue as well as in non-neoplastic processes such as benign prostatic hyperplasia and inflammation (Bauman *et al*. 2006*b*, Fung *et al*. 2006). Its expression is increased in advanced prostate cancer (Nakamura *et al*. 2005) and has been correlated with Gleason grade (Wako *et al*. 2008), a measure of tumour aggressiveness. In a microarray analysis of bone marrow metastases of hormone-independent prostate cancer, the expression of 17β-HSD5 was more than fivefold higher than in primary androgen-dependent tumours, and this was confirmed by both RT-PCR and immunoblotting (Stanbrough *et al*. 2006). Remarkably however, although other androgen synthesis genes were also up-regulated, and expression of the AR was 5.8-fold that in primary tumours, there was no correlation between the increased expression of 17β-HSD5 and AR in the samples, suggesting the involvement of different mechanisms of androgen independence.

Although there are no reports of increased expression of 17β-HSD5 in the ovaries of hyperandrogenised individuals with PCOS, in one study a single nucleotide polymorphism in the AKR1C3 promoter that increases its affinity to nuclear transcription factors, SNP-71G was found in around 10% of affected individuals (Qin *et al*. 2006). Conversely, in a separate study, there was no association between PCOS or testosterone levels and the occurrence of either SNP-71G or four other AKR1C3 polymorphisms (Goodarzi *et al*. 2008). There was also no association between four common AKR1C3 polymorphisms and precocious puberty, a hyperandrogenic condition thought to be a risk factor for PCOS (Petry *et al*. 2007). 17β-HSD5 is also expressed in normal (Pelletier *et al*. 1999, 2001) and malignant breast tissue (Amin *et al*. 2006). Its expression is significantly up-regulated in breast cancer and is associated with a poor prognosis (Vihko *et al*. 2005).

However, as previously mentioned, 17β-HSD5 is also known as PGFS. It is thought that it may also exert a proliferative signal in cancer by reducing PGD_2 to 9α,11β-PGF_2. 9α,11β-PGF_2 is a proliferative PG that stimulates the MAP kinase pathway, preventing the formation of the PPARγ ligand, PGJ_2, a PG involved in differentiation signalling (Desmond *et al*. 2003).

Inhibitors of 17β-HSD5

The earliest patents for the inhibition of AKR1C1–AKR1C4 (US5439943, US5399790, US5187187, US5118621 and US5068250) were published by the University of Pennsylvania from 1992–1995. Novel non-steroidal suicide inhibitors were described for potential therapeutic use in potentiating the action of androgens, in androgen replacement therapy for hypogonadism of pituitary and testicular origin, and in the maintenance of male fertility. Suicide substrates mimic the physiological substrate and are transformed by the enzyme to highly reactive alkylating agents which then inactivate it by forming a covalent bond at the active site. They can be highly selective as they are themselves inactive until they are transformed by the target enzyme. These patents described the activity of monocyclic-aromatic allylic and acetylenic alcohols as highly selective inhibitors that are transformed by the 3α-HSD activity to monocyclic-aromatic vinyl and acetylenic ketones that alkylate the pyridine nucleotide binding site of the enzyme (e.g. 1-(4′-nitrophenyl)-2-propen-1-ol is transformed to 1-(4′-nitrophenyl)-2-propen-1-one by the action of the 3α-HSDs; Fig. 6a).

Since then, as 17β-HSD5 has been shown to have activities other than as a 3α-HSD, interest in its

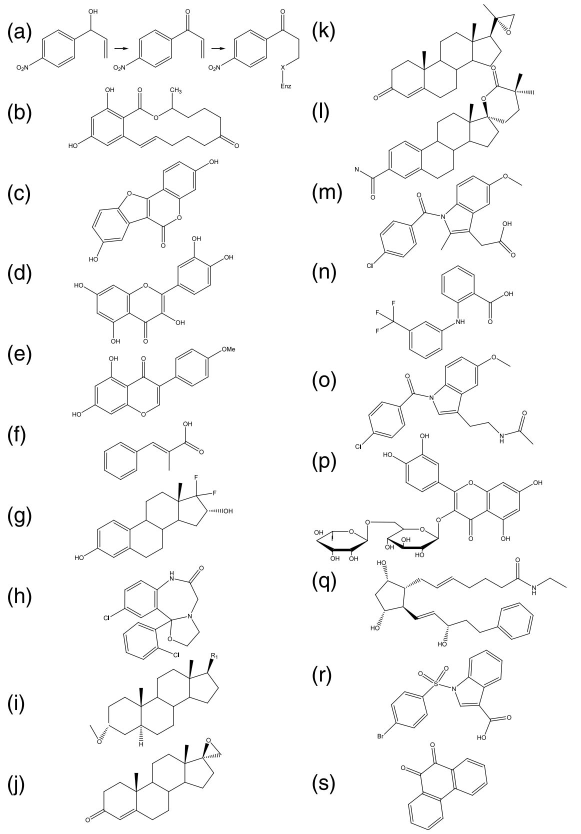

Figure 6 17β-HSD5 inhibitors and substrate analogues. (a) 1-(4′-nitrophenyl)-2-propen-1-ol to 1-(4′-nitrophenyl)-2-propen-1-one that alkylates the enzyme, (b) zearalenone, (c) coumestrol, (d) quercetin, (e) biochanin A, (f) α-methylcinnamic acid, (g) J2404, (h) cloxazolam, (i) 3α-spiro-oxirane, (j) 17β-spiro-oxirane, (k) 20α-spiro-oxirane, (l) EM1404, (m) indomethacin, (n) flufenamic acid, (o) *N*-(4-chlorobenzoyl)-melatonin, (p) rutin, (q) bimatoprost, (r) Ex144 and (s) 9,10-phenanthrenequinone.

inhibition for anti-cancer therapy has been growing. Several environmental dietary phytoestrogens, such as zearalenone, coumestrol, quercetin and biochanin A (IC_{50}=2–14 μM; Fig. 6b–e), have been shown to inhibit 17β-HSD5 (Krazeisen *et al*. 2001). The potency of these compounds increases with increasing number of hydroxylated sites, apparently due to binding to the hydrophilic cofactor-binding pocket of the enzyme. Derivatives of *trans*-cinnamic acids, α/β-unsaturated plant carboxylic acids that are natural precursors of structurally related 17β-HSD5-inhibiting flavonoids, have also been evaluated as 17β-HSD5 inhibitors (Brožič *et al*. 2006). The best inhibitor in the series tested was α-methylcinnamic acid (Fig. 6f; IC_{50}=6.4 μM). Parallel tests indicated that these compounds have no inhibitory effects on fungal 17β-HSD, suggesting that they may be selective for 17β-HSD5 over the 17β-HSDs from the short-chain dehydrogenase family. A C17-difluorinated oestratriene derivative, J2404 (Fig. 6g), inhibited 17β-HSD5 by 72% at 2 μM, with no effect on the other SDR 17β-HSD enzymes tested, including types 1, 2, 4 and 7 (Deluca *et al*. 2006).

17β-HSD5 is also found in the brain, where it is believed to be involved with AKR1C1 and AKR1C2 in the metabolism of neurosteroids, which act on the γ-aminobutyric acid type A ($GABA_A$) receptors. Benzodiazepines are sedatives that modulate the activity of the $GABA_A$ receptors. However, several of them have been found to inhibit AKR1C1–AKR1C3 (17β-HSD5), and one, cloxazolam (Fig. 6h) is a potent and specific inhibitor of 17β-HSD5 with an IC_{50} of 2.5 μM (Usami *et al*. 2002).

Studies of the catalytic mechanism of the enzyme by Penning *et al*. (2001), based on studies of a 69% homologous rat 3α-HSD (AKR1C9), indicated that an ordered bi-mechanism operates in which the substrate binds to the enzyme once the pyridine nucleotide cofactor, NADPH, is bound. After the reduction of the substrate through an oxyanion transition state, the product is released before the release of the cofactor. This mechanism suggests that steroidal-based inhibitors that compete with the steroid product are desirable as they would act as uncompetitive inhibitors. This led to the suggestion that transition state analogues, such as steroid carboxylates and pyrazoles, and mechanism-based inactivators, such as 3α-, 17β- or 20α-spiro-oxiranyl steroids (Fig. 6i–k) and oxiranyl non-steroids, may be potent 17β-HSD5 inhibitors. Endorecherche, Inc. (Quebec, Canada) has published several patents pertaining to the inhibition of 17β-HSD5 by steroidal and spirolactone compounds for the treatment of androgen-dependent diseases including prostate cancer (e.g. US2004082556, AU2004200173, MXPA00008868, ZA9901924, EP1321146).

One of the most potent of the Endorecherche, Inc. compounds is an E_2 derivative, EM1404 (Fig. 6l), a strong competitive inhibitor with an IC_{50} of 3.2 ± 1.5 nM using 100 nM Adione as substrate, a higher potency than that of any other reported inhibitor. The lactone ring of EM1404 is located at the base of the substrate binding site, and the amide group is oriented towards the surface of the enzyme, despite the fact that the inhibitor occupies only part of the binding cavity (Qiu *et al*. 2007). Although this compound is specific for 17β-HSD5 over 17β-HSD1, 17β-HSD2 and 17β-HSD3 (IC_{50}>10 μM; patent WO9946279), its specificity over other members of the AKR1C family and cyclooxygenase 1 and 2 (COX1/COX2) remains to be assessed.

17β-HSD5 is also inhibited by NSAIDs, including indomethacin (Fig. 6m) and flufenamic acid (Fig. 6n), at similar concentrations and the same order of potency as for the inhibition of COX1/COX2 (Bauman *et al.* 2005, Byrns *et al.* 2008). In HL-60 promyelocytic leukaemia cells, the inhibition of 17β-HSD5 by indomethacin is thought to lead to a decrease in proliferation and a corresponding increase in differentiation via the PPARγ signalling pathway due to the presence of higher concentrations of PGJ_2 and lower concentrations of $PGF_{2\alpha}$ (Desmond *et al.* 2003). Using the extensive SAR knowledge generated during the development of COX1 and COX2 inhibitors, derivatives of NSAIDs with increased 17β-HSD5 potency and selectivity for 17β-HSD5 inhibition over COX1 and COX2 inhibition are being developed, with structures based on *N*-phenylanthranilic acids, cholanic acids, and on *N*-acylanthranilic acids, 2-benzoylbenzoic acids, benzophenones, and phenoxybenzoic acid (Bauman *et al.* 2005, Gobec *et al.* 2005, Penning *et al.* 2006). One of the most specific is *N*-(4-chlorobenzoyl)-melatonin (Fig. 6o), a derivative of indomethacin that inhibits the reduction of 5 μM Adione to testosterone by 17β-HSD5 with similar potency ($IC_{50} = 11.4$ μM) to indomethacin ($IC_{50} = 8.5$ μM), but which does not inhibit COX1, COX2, AKR1C1 or AKR1C2 (Byrns *et al.* 2008).

The rational design of these inhibitors has been greatly assisted by the elucidation of the crystal structure of 17β-HSD5, complexed as a ternary structure with the cofactor NADP(H), and with either the substrates PGD_2 (Komoto *et al.* 2004) or Adione (Qiu *et al.* 2004), the product testosterone (Qiu *et al.* 2004) or potential inhibitors, such as the NSAIDs, indomethacin and flufenamic acid (Lovering *et al.* 2004), rutin (Fig. 6p; Komoto *et al.* 2004), a $PGF_{2\alpha}$ analogue, bimatoprost (Fig. 6q; Komoto *et al.* 2006) and EM1404 (Qiu *et al.* 2007).

A patent for the use of *N*-sulphonylindole derivatives as selective 17β-HSD5 inhibitors was recently published by Astellas Pharma Inc. (Tokyo, Japan, WO2007100066). Derivatives in which a carbon atom in the indole group is substituted by a carboxy group, a carboxy-substituted lower alkyl group or a carboxy-substituted lower alkenyl group were found to potently inhibit 17β-HSD5 with selectivity over 17β-HSD3 inhibition ($IC_{50} > 10$ μM). The most potent of these inhibitors have IC_{50} values below 100 nM (e.g. Ex144; Fig. 6r).

In all of these studies, the potency of the inhibitors has been assayed using cell lines transfected with 17β-HSD5. The expressed enzyme has then either been assayed *in situ* in whole cell assays using radiolabelled steroid substrate or purified for direct enzyme assay, measuring the decrease in absorbance at 340 nm as NADPH is oxidised to $NADP^+$, using either steroid substrate or 9,10-phenanthrenequinone (Fig. 6s) as a test substrate. The purified enzyme has also been used for crystallisation studies, as previously mentioned, to determine the structure of the active site and the residues that are important to its activity and inhibition. Although there are several groups presently working on these inhibitors, there have been no reports of the development of *in vivo* models of 17β-HSD5 inhibition. This may well be due to the ubiquitous expression of 17β-HSD5 throughout the tissues, its substrate plasticity and the initial lack of specificity of these inhibitors. However, in order to understand the contribution of the various catalytic activities of 17β-HSD5 to the development of hormone-dependent prostate cancer and its progression to hormone independence, and to other cancers, analysis of the effect of inhibition of 17β-HSD5 *in vivo* is necessary. Until these models have been developed and the effects of these inhibitors on both androgen and PG production have been tested *in vivo*, the value of the development of these inhibitors and their future application in the treatment of prostate or other cancers cannot be fully established.

Inhibition of other 17β-HSDs

Applications and inhibitor development

17β-HSD2 is the enzyme responsible for the oxidation of active E_2 and testosterone to their inactive forms, E_1 and Adione. As it is involved in inactivation of active steroids, there has been less interest in the development of 17β-HSD2 inhibitors than in those of 17β-HSD1, 17β-HSD3 and 17β-HSD5. However, partly for use as a research tool and partly as inhibitors of 17β-HSD2 may prove beneficial for conditions in which the concentration of active steroid is too low, some effort has been made to find inhibitors of this enzyme. In 2002, Bayer Pharmaceuticals published patent WO0226706 for the use of pyrrolidinones, pyrrolidin-thiones and 1-methyl-4-phenylpyrrolidin-2-ones (Fig. 7a i) in the treatment of osteoporosis. Another group has explored the use of 17-spiro-lactones attached to either an E_2 nucleus (Sam *et al.* 2000, Bydal *et al.* 2004) or other steroid nuclei (Tremblay *et al.* 1999, Poirier *et al.* 2001) as 17β-HSD2 inhibitors. These compounds are selective for 17β-HSD2 inhibition over 17β-HSD1 and 17β-HSD3, with IC_{50} values in the nM range. However, the most active of these compounds, the spiro-δ-lactone C17β-O/C17α-δ-lactone (Fig. 7a ii), which has an IC_{50} of 6 nM for 17β-HSD2 inhibition, also inhibits 17β-HSD5 with an IC_{50} of ∼10 nM and has some oestrogenicity (Bydal *et al.* 2004).

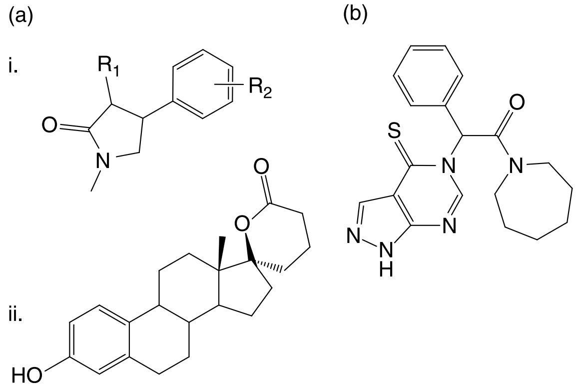

Figure 7 Inhibitors of other 17β-HSDs. (a) 17β-HSD2: (i) 1-methyl-4-phenyl-pyrrolidin-2-ones and (ii) 17-(spiro-δ-lactone)-E_2. (b) 17β-HSD10: AG18051 (1-azepan-1-yl-2-phenyl-2-(4-thioxo-1,4-dihydropyrazolo-[3,4-d]pyrimidin-5-yl)-ethanone).

Presently, there is little research into the development of inhibitors of the remaining, less well characterised, 17β-HSDs. However, as their number continues to increase, and the substrate specificity, directional activity and expression pattern of each of the enzymes are elucidated, the potential of specific inhibitors of each of these enzymes for the treatment of various diseases, of both steroidal and non-steroidal origin, will become clear. Indeed, the inhibition of 17β-HSD10 is already being explored for the treatment of Alzheimer's disease, and a potent inhibitor, AG18051 (1-azepan-1-yl-2-phenyl-2-(4-thioxo-1,4-dihydropyrazolo[3,4-d]pyrimidin-5-yl)-ethanone; Fig. 7b), with an IC_{50} of 92 nM, has been identified (Kissinger *et al.* 2004). As this enzyme also converts 5α-androstanediol to DHT (Yang *et al.* 2005*a*,*b*) and has been seen to be overexpressed in primary prostate cancer cultures (He *et al.* 2003), it would also be interesting to see whether inhibitors of 17β-HSD10 may also have a role in the treatment of prostate cancer.

Summary

Despite the success of inhibitors of steroidogenic enzymes in the clinic, such as those of aromatase and steroid sulphatase, the development of inhibitors of 17β-HSDs is at a relatively early stage. At present, none of these inhibitors has reached clinical trials, and only recently has efficacy been demonstrated for the first of the enzymes in *in vivo* disease models. However, great advances in both the understanding of the function of these enzymes, and in techniques for elucidation of protein structure and computer-aided SAR have been made over the last decade. As the precise substrate and cofactor specificity, directional activity and expression pattern of each of the 15 17β-HSDs in normal and diseased states becomes clearer, it can be seen that the development of specific inhibitors could provide a great opportunity to fine-tune pathways in the therapeutic intervention of steroidogenic and other metabolic disorders. Presently, the development of inhibitors of 17β-HSD1 for the treatment of hormone-dependent breast cancer and endometriosis, and 17β-HSD3 for the treatment of hormone-dependent prostate cancer, are most advanced.

Declaration of interest

Prof M J Reed and Dr A Purohit are consultants to Ipsen Ltd. Prof M J Reed is a director of Sterix Ltd.

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