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Substrate specificities and functional properties of human short-chain dehydrogenases/reductases

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To my family

ABSTRACT

Short-chain dehydrogenases/reductases (SDRs) constitute a universal superfamily of functionally heterogeneous proteins and participate in the metabolism of steroids, prostaglandins, retinoids, aliphatic alcohols, and xenobiotics. So far, more than 3000 members including species variants and over 60 human SDR genes are deposited in databases. Several human SDR genes are involved in different steroid-dependent cancer forms and metabolic disorders by virtue of their hormone metabolizing properties, which make these SDR enzymes interesting novel targets for drug development.

The main focus of the thesis is on three human SDR enzymes, type 1 11β-hydroxysteroid dehydrogenase (11β-HSD1), type 10 17β-hydroxysteroid dehydrogenase (17β-HSD10) and the functionally unannotated protein Hep27.

11β-HSD1 was studied by analyzing its enzymological properties, substrate specificities, structure-function relationships and inhibition profiles using different mammalian orthologs. The kinetic comparison of 11β-HSD1 from the glucocorticoid resistant guinea pig versus glucocorticoid sensitive human species shows that both isoforms display Michaelis-Menten kinetics and similar kinetic constants, indicating that 11β-HSD1 is not a critical determinant of peripheral glucocorticoid resistance in guinea pig. A novel role of rodent and human 11β-HSD1 in oxysterol metabolism as a 7oxo-reductase and 7β-dehydrogenase was discovered. This novel finding indicates a possible involvement of human 11β-HSD1 in atherosclerotic lesion formation and links glucocorticoid and cholesterol metabolism. Furthermore, the active site variability in 11β-HSD1 species was investigated by performing inhibition studies, using non-selective steroid-like and selective arylsulfonamidothiazole compounds, and by determining the primary structures of 11β-HSD1 variants from several species. The results reveal significant differences within the active site architecture of 11β-HSD1 isozymes, and provide information helpful for further inhibitor design.

To understand the physiological role of the multifunctional enzyme 17β-HSD10, substrate specificities of human and *Drosophila* 17β-HSD10 were investigated. Both orthologs display similar affinities towards estrogen, androgen and hydroxyacyl-CoAs. Whereas human 17β-HSD10 catalyzes conversion of 7α-OH and 7β-OH bile acids, the *Drosophila* enzyme converts only 7α-OH steroids. In addition, 20β-OH and 21-OH activities of C21 steroids were detected for both orthologs. Homology modeling of the

enzyme variants, based on the high-resolution crystal structure of rat 17β-HSD10, and substrate docking reveal a large hydrophobic substrate pocket, able to accommodate steroids of different configurations. The mitochondrial targeting of human 17β-HSD10 was studied by using hybrid constructs with green fluorescent protein. Residues 1-34 at the N-terminal were shown to consist of a non-cleavable mitochondrial target sequence. Residues 1-15 are specific for the enzyme but not sufficient for protein import. Residues 16-34 can be replaced by similar SDR structures such as the corresponding sequence in 3β/17β-HSD. On the other hand *Drosophila* 17β-HSD10 shows a cytosolic localization pattern, due to an N-terminal sequence difference.

Cross-species comparisons revealed that Hep27 is member of a highly conserved SDR cluster found in human, *C. elegans*, *Drosophila* and *A. thaliana.* In this thesis a substrate screening was performed using a compound library, comprising a small set of steroids, retinoids, sugars and several miscellaneous xenobiotic carbonyl compounds. The results show that Hep27 is an NADPH-dependent dicarbonyl reductase, catalyzing the conversion of 3,4-hexanedione, 2,3-heptanedione and 1-phenyl-1,2-propanedione. Expression analysis reveals that Hep27 is expressed in human endothelial tissues. These results suggest that Hep27 constitutes a detoxification mechanism within the endothelium against reactive α-dicarbonyls.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Shafqat, N., Elleby, B., Svensson, S., Shafqat, J., Jörnvall, H., Abrahmsen, L. and Oppermann, U. (2003) Comparative enzymology of 11β-hydroxysteroids dehydrogenase type 1 from glucocorticoid resistant (guinea pig) versus sensitive (human) species. *J Biol Chem* **278**, 2030-2035.

II. Hult, M., Shafqat, N., Elleby, B., Mitschke, D., Svensson, S., Forsgren, M., Barf, T.,Vallgårda, J., Abrahmsen, L., Oppermann, U.: Active site variability of type 1 11β-hydroxysteroid dehydrogenase revealed by selective inhibitors and cross-species comparisons. *Manuscript.*

III. Hult, M., Elleby, B., Shafqat, N., Svensson, S., Rane, A., Björkhem, I., Jörnvall, H., Abrahmsen, L., Oppermann, U.: Human and rodent type 1 11β-hydroxysteroid dehydrogenases are 7β-hydroxycholesterol dehydrogenases involved in oxysterol metabolism. *Cell. Mol. Life Sci.* **61** (2004) 992-999.

IV. Shafqat, N., Marschall, H., Filling, C., Nordling, E., Wu, X., Björk, L., Thyberg, J., Mårtensson, E., Samina, S., Jörnvall, H. and Oppermann, U. (2003) Expanded substrate screenings of human and *Drosophila* type 10 17β-hydroxysteroid dehydrogenases reveal multiple specificities in bile acid and steroid hormone metabolism. Characterization of multifunctional 3α/7α/7β/17β/20β/21-hydroxysteroid dehydrogenase. Biochem J. **376**, 49-60.

V. Shafqat, N**.,** Shafqat, J., Eissner, G., Marschall, H., Tryggvason, K., Eriksson, U., Jörnvall, H., Oppermann, U. Hep27, a conserved member of the short-chain dehydrogenase/reductase family, is an NADPH-dependent dicarbonyl reductase expressed in vascular endothel. *Manuscript.*

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1 ABBREVIATIONS

Three- and one-letter codes for the 20 genetically encoded amino acids

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isolucine	Ile	I	Valine	Val	V

ADH	alcohol dehydrogenase
AME	apparent mineralocorticoid excess syndrome
AKR	aldo-keto reductase
AGE	advance glycation product
CBX	carbenoxolone
CDCA	chenodeoxycholic acid
cDNA	complementary deoxyribonucleic acid
DCXR	dicarbonyl/L-xylulose reductase
ERAB	endoplasmic-reticulum derived amyloid-β-peptide binding protein
GC	glucocorticoid
GR	glucorticoid receptor
GFP	green fluorescent protein
HPLC	high performance liquid chromatography
HSD	hydroxysteroid dehydrogenase
(iso)UDCA	(iso) ursodeoxycholic acid
IMAC	immobilized metal-ion-affinity chromatography
KSI	ketosteroid isomerase
MDR	medium-chain dehydrogenase/reductase
mRNA	messenger ribonucleic acid
MR	mineralocorticoid receptor
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDR	short-chain dehydrogenase/reductase
SIM	selective intracrine modulators

2 INTRODUCTION

The sequencing of the human genome is one of the most ground-breaking scientific events of this era. It offers insights into our collective history and our individual identities, and opens possibilities for diagnosis, prevention and treatment of diseases. The growing number of characterized sequences from the human genome project offers great potential to understand gene products. At present, however, a considerable number of genes identified through genome analysis are functionally uncharacterized. Consequently, efforts are directed towards analysis and understanding of "orphan" gene products.

This shift in research from genome sequence analysis to functional interpretation of the gene products includes analysis of protein functions in general. Enzymes constitute an important class of proteins, catalyzing a large variety of chemical reactions. They all divide into six different classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. The main focus of this thesis is on oxidoreductases, particularly on hydroxysteroid dehydrogenases and related enzymes, which belong to the superfamily of short-chain dehydrogenases/reductases (SDR). At present, the SDR enzyme family contains more than 3000 members annotated in databases, including those from over 60 human SDR genes. These SDR enzymes fulfil essential functions in human physiology, and are known to be involved in different metabolic diseases and hormone-dependent cancer forms [5-8].

A brief overview on some of the main enzymes involved in NAD(P)(H) dependent oxidoreductions and related to SDRs is presented in the following sections.

2.1 Alcohol dehydrogenase (ADH)

Alcohol dehydrogenases (E.C.1.1.1.1) constitute a group of enzymes, which are involved in NAD(P) dependent reversible oxidation of alcohols to aldehydes/ketones (fig. 1), and thereby provide a general detoxification system of alcohols and aldehydes in the body. These ADH enzymes are found in almost all forms of life.

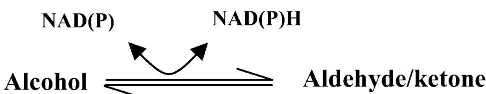

Figure 1: The ADH reaction.

Nature has provided cells with mechanisms of gene duplications and protein modifications which result in the occurrence of large protein families from single ancestral genes. Similarly, the evolutionary effects within ADH enzymes have caused them to diverge into five protein superfamilies [9]. These superfamilies are: medium-chain dehydrogenases/reductases (MDR), short-chain dehydrogenases/reductases (SDR), iron activated alcohol dehydrogenases, long-chain dehydrogenases/reductases, and aldo-keto reductases (AKR).

2.2 Medium-chain dehydrogenases/reductases (MDR)

The superfamily of medium-chain dehydrogenases/reductases constitutes oligomeric enzymes consisting of dimers or tetramers. These enzymes usually contain 350-400 amino acid residues in their polypeptide chains. The subunits consist of two domains, one catalytic and one nucleotide binding domain, often with a catalytic and a structural zinc atom, respectively. The first MDR enzyme pair recognized with different substrates was mammalian liver alcohol dehydrogenase and sorbitol dehydrogenase [10]. So far, the MDR family has been separated into eight different classes, containing around hundred different members, including species variants [9]. The sequence identity among different MDR enzymes is only ~25%. In addition to alcohol and sorbitol dehydrogenases, the MDR family also includes threonine and xylitol dehydrogenases and reductases like ζ-crystallin with quinone reductase activity. The divergence among these enzymes reflects their ancient duplicatory origin.

2.3 Aldo-keto reductases (AKR)

The aldo-keto reductases are NAD(P)(H) dependent enzymes which metabolize a wide range of substrates, including aliphatic and aromatic aldehydes, monosaccharides, steroids, prostaglandins, polycylic aromatic hydrocarbons, and isoflavonoids [11-13]. The members of this enzyme superfamily are found in mammals, amphibians, plants, yeasts, protozoa and bacteria. So far, fourteen families have been detected within this superfamily, designated AKR1-AKR14. Aldo-keto reductases adopt a $(\beta/\alpha)_8$ barrel structural fold, with the active site located in a hydrophobic cavity at the C-terminal end of the β-barrel. The C-terminal part contains the active site motif with a tetrad of catalytically important Asp, His, Tyr, and Lys residues [14, 15]. The majority of AKRs are monomeric proteins of about 320 amino acid residues, that bind to the nicotinomide cofactor without a Rossmann-fold motif [16-19].

However, multimeric forms also exist in AKR6 and AKR7. At the moment over 125 AKR genes have been identified by genome sequencing projects.

2.4 Short-chain dehydrogenases/reductases (SDRs)

2.4.1 General background

Short-chain dehydrogenases/reductases (SDRs) constitute a large family of functionally heterogeneous proteins that participate in the metabolism of steroids, prostaglandins, retinoids, aliphatic alcohols, and xenobiotics [20, 21]. The history of the SDR family starts with the characterization of *Drosophila* alcohol dehydrogenase [22], describing the family initially as insect-type alcohol dehydrogenase. With the incorporation of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase from human placenta [23] and rat liver 11β-HSD1, the family received considerable attention.

Since then, the SDR family has continued to grow enormously, showing its existence in all life forms. Today, it is considered as one of the largest protein families. Members of the SDR family are found in the cytoplasm, mitochondria, nuclei, peroxisomes, and endoplasmic reticulum. So far, more than 3000 members including species variants and over 60 human SDR genes are annotated in sequence databases [24, 25].

2.4.2 Structural features of SDR enzymes

The SDR proteins are mostly one-domain NAD(P)(H)-dependent enzymes of typically 250-350 amino acid residues, frequently with additional N or C-terminal transmembrane domains or signal peptides. The primary structure similarity between different SDR enzymes is typically around 15-25% in pair wise comparisons (fig. 2). Based on sequence alignments and differences in chain lengths, the SDR protein family can be divided into two groups, classical SDR (with ~250 residues) and extended SDR (with ~350 residues) [21, 26]. In spite of low sequence similarity among SDR enzymes, the family contains some characteristic, conserved sequence motifs. These are found at the N-terminal portion, building a nucleotide binding motif with three non-consecutive glycine residues Gly-X3-GLY-X-GLY, typical of Rossmann fold enzymes [27]. The C-terminal part contains the active site motif with a tetrad of catalytically important Asn, Ser, Tyr, and Lys residues, of which Tyr is the most conserved residue in the whole family [21, 28, 29]. The proposed reaction mechanism for SDR enzymes centers on the conserved Tyr, acting as a general base in the deprotonation process. This function is facilitated by the adjacent protonated Lys residue and the oxidized form of the

coenzyme NAD(P)$^+$, which lowers the pK_a of the phenolic Tyr-OH group. The lysine residue binds to the ribose 2′OH group of the coenzyme and fixes the coenzyme position during catalysis. The function of the serine in the catalytic tetrad is less apparent, but it could function as a stabilizing factor in the transition state or in the initial orientation of the substrate [30]. The conserved Asn residue has been suggested to be part of the active site, forming a proton relay system including the 2′OH of the nicotinamide ribose and a conserved water molecule, as supported from site-directed mutagenesis and structural studies [31, 32].

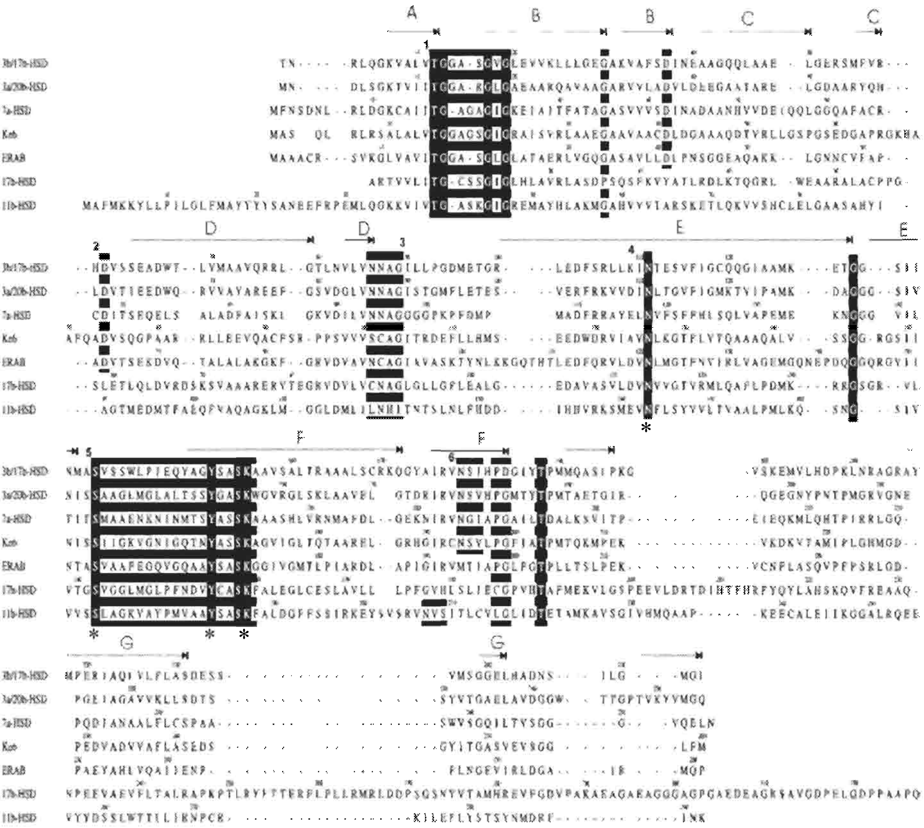

Figure 2: Structure alignment of SDR sequences. Secondary structure elements are indicated by long arrows for β-strand and small arrows for α-helices. Highly conserved sequence elements including active site residues are highlighted by black boxes and asterisks.

The first three-dimensional structures of SDRs, were those of 3α,20β-hydroxysteroid dehydrogenase [33, 34] and dihydropteridine reductase [35]. The three-dimensional structures of SDRs are not so divergent as the primary structures. They show a quaternary structure arrangement with either dimeric or tetrameric forms (fig. 3). An exception is NADPH-dependent carbonyl reductase which is monomeric [36]. Although the rate of three-dimensional structure characterizations is not comparable to that of the primary structure determinations, there has been a significant increase in known 3D-structures of SDRs in recent years, and today around 30 three-dimensional structures are known [20].

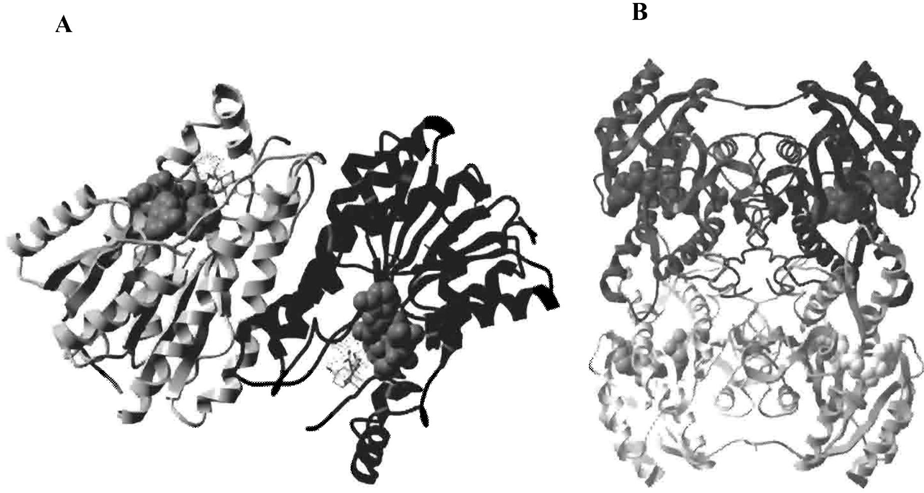

Figure 3 : 3-D structures of SDR enzymes. (A): dimeric structure of 7α-HSD (pdb code 1FMC; [37]), each subunit is shaded differently, NADH is displayed in CPK representation and substrate (glycochenodeoxycholic acid) is displayed in X-stick and surface representation. (B): tetrameric structures of 3α,20β-HSD (pdb code 2HSD; [34]), Each subunit is shaded differently, NAD^+ is displayed in space-filling representation. The figure was created using the ICM program.

2.4.3 Substrate diversity of SDR enzymes

Short-chain dehydrogenases/reductases display a diverse substrate spectrum, ranging from steroids, alcohols, sugars, and aromatic compounds to xenobiotics [24, 38]. Many SDR enzymes that act on the same endogenous substrate exhibit different subcellular localization, cofactor specificity, substrate affinity, tissue distribution or reaction direction. Several structures of different SDR substrates are shown in fig. 4. SDR enzymes catalyze critical steps of activation and inactivation of steroids, vitamins, prostaglandins, and other bioactive molecules by oxidation/reduction of hydroxyl/carbonyl groups [21].

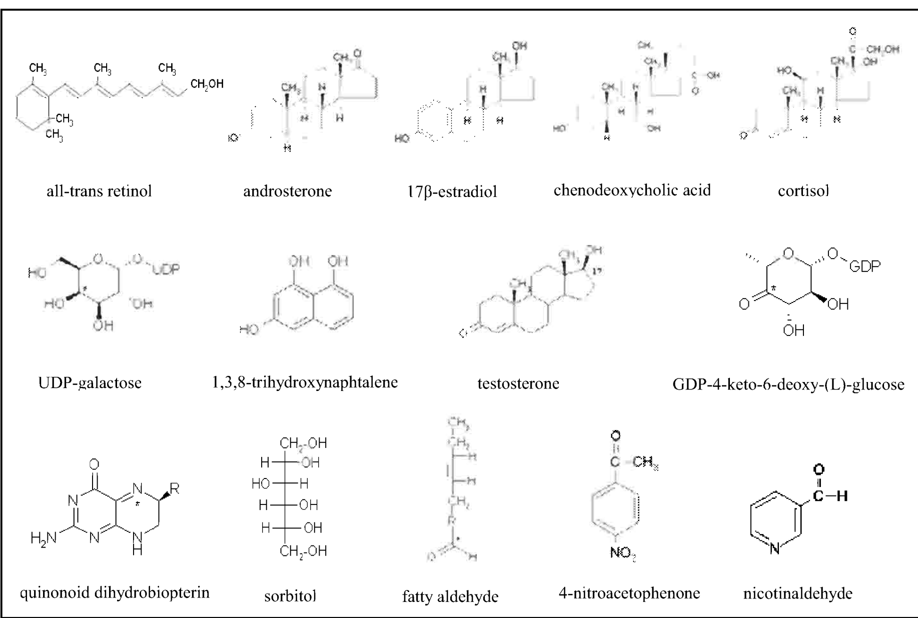

Figure 4. Broad substrate specificities of SDR enzymes exemplified by different compound classes.

2.5 Human physiology and SDRs

Enzymes of the SDR family not only regulate the local concentrations of steroids, such as cortisol, estradiol, testosterone, and prostaglandins in humans [21, 39, 40], but also show involvement in retinoid metabolism [41] and xenobiotic carbonyl metabolism [42, 43]. Due to the versatility in enzymatic function, several clinically important syndromes have now been identified as derived from genetic variations in SDR enzymes (table 1). An example is 11β-HSD2, which protects the mineralocorticoid receptor from occupancy of cortisol and allow binding of aldosterone [44]. If this enzyme has too low activity from either genetic defects or because of inhibition with glycyrrhetinic acid (an 11β-HSD2 inhibitor contained in licorice) [45, 46], the high level of cortisol in the kidney will activate the mineralocorticoid receptor, leading to the syndrome of apparent mineralocorticoid excess (AME) [5, 47, 48]. This syndrome is characterized by the clinical symptoms of high blood pressure, sodium retention and hypokalemia (fig. 5).

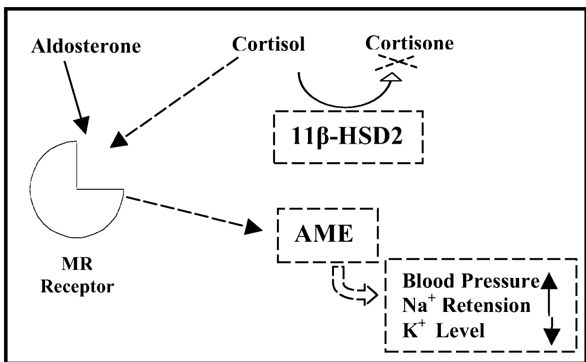

Figure 5: Malfunction of 11β-HSD type 2 will allow cortisol to bind to the mineralocorticoid receptor (MR), leading to the syndrome of AME. Dotted lines and boxes represent disorder conditions.

At present, over 60 human SDR genes have been identified [24, 25]. For many of these genes the cellular function is still unknown, but some of the functionally characterized human SDR genes are reported to be involved in different steroid dependent cancer forms, metabolic syndrome, and type 2 diabetes mellitus. These features make several SDR enzymes a prime target for the development of potentially useful inhibitors with clinical applications. A short list of several human SDR genes with their functions and links to associated diseases is shown in table 1.

Table 1: Human SDR genes and their link to associated diseases [24].

Human SDR genes	associated diseases/syndrome
3β-HSD-1	adrenal hyperplasia
3β-HSD-2	adrenogenital syndrome
11β-HSD-1	11-oxoreductase deficiency
11β-HSD-2	apparent mineralocorticoid excess syndrome
17β-HSD-1	ovarian/breast cancer risk
17β-HSD-3	male pseudohermaphroditism
17β-HSD-8	polycystic kidney disease?
17β-HSD-10	alzheimer's disease?
9*cis*/11*cis*-RDH	retinitis punctata albescens
Dihydropteridine reductase	DHPR deficiency, phenylketonuria
Dienoyl CoA reductase	dienoyl CoA reductase deficiency
UDP galactose 4´epimerase	galactosemia III
Sepiapterin reductase	tetrahydrobiopterin deficiency

2.6 Hydroxysteroid dehydrogenases (HSDs)

Hydroxysteroid dehydrogenases (HSDs) are enzymes that catalyze the oxidation/reduction of hydroxyl (-OH)/oxo groups of steroids using NAD(P)(H) as a cofactor. HSDs play a pivotal role in human physiology by regulating the biosynthesis of steroid hormones and bile acids (table 2). They also maintain intracellular levels of receptor ligands through tissue-specific expression of distinct HSD genes [14, 49]. They catalyze the stereoselective reaction at specific positions of the steroid nucleus (fig. 6), so that for each sex hormone there is an isoform that will either inactivate or produce the active ligand. In this manner, HSDs can function as molecular "switches" to regulate steroid hormone action.

Figure 6: Carbon numbering of steroids.

HSDs are implicated in controlling steroid ligand access to their receptors and mainly belong to two different protein superfamilies, the short-chain dehydrogenases/reductases (SDR) and the aldo-keto reductases (AKR) [14, 15, 20, 21, 24, 31, 50].

The HSDs that belong to the SDR family consist of 3β-HSDs which are involved in steroid synthesis, 11β-HSDs which control ligand access to the mineralcorticoid and glucocorticoid receptors, and most of the 17β-HSD isoforms which control the ligand access to the androgen receptor (AR) and estrogen receptor (ER) [51].

Table 2: Physiological roles of mammalian steroid hormones.

Chemical synthesis of major steroid hormones	**Principal target tissues**	**Hormonal function**
Cortisone (inert glucocorticoid) ⇄ 11β-HSD1 / 11β-HSD2 ⇄ **Cortisol (active glucocorticoid)**	All cells	Regulation of energy utilization
Androstenedione (weak androgen) ⇄ 17β-HSD3 / 17β-HSD2/4 ⇄ **Testosterone (potent androgen)**	Seminal vesicles Prostate Testis	Development of male sex characteristics
Estrone (weak estrogen) ⇄ 17β-HSD1 / 17β-HSD2/4 ⇄ **17β-Estradiol (potent estrogen)**	Uterus Vagina Breast Hypothalamus	Development of female sex characteristics
Pregnenolone → 3β-HSD/KSI → **Progesterone**	Uterus oviduct	Maintenance of pregnancy

In addition to the regulation of the intracellular levels of active steroid hormones (table 2), HSDs are involved in the xenobiotic phase 1 metabolism of exogeneous substances, including drugs, pesticides and carcinogens [52, 53].

2.7 HSDs as drug targets

The tissue-specific expression and their central role in steroid hormone action, make these enzymes prime drug targets. Specific inhibitors would present a new class of therapeutic compounds called “selective intracrine modulators” (SIMs). SIMs are expected to have the same tissue-specific effect as selective steroid receptor modulators but with a different mode of action since their effects are enzyme-mediated and not receptor-mediated [15].

The most compelling examples of HSDs as drug targets are 11β-HSD1 and 17β-HSDs. Inhibitors of 11β-HSD1 will possibly be helpful in the treatment of obesity, insulin resistance

and other aspects of the metabolic syndrome [54], while those for 17β-HSDs may aid in the treatment of hormone dependent cancer forms (e.g. breast, prostate and ovarian cancer).

2.8 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1)

11β-hydroxysteroid dehydrogenases (EC 1.1.1.146) are microsomal enzymes that interconvert active glucocorticoid hormones (corticosterone, cortisol) and their inactive 11-dehydro metabolites (dehydrocorticosterone, cortisone), thus regulating glucocorticoid access to intracellular receptors (fig. 7) [55-57]. At present there are two distinct isoforms of 11β-HSD described, 11β-HSD1 and 11β-HSD2 (table 3). 11β-HSD1 was isolated and cloned from liver [58-61], where it acts as a NADPH-dependent reductase to generate active glucocorticoid form from inert 11-ketosteroid. 11β-HSD1 reductase activity is widely expressed, co-localizing with glucocorticoid receptors [62, 63].

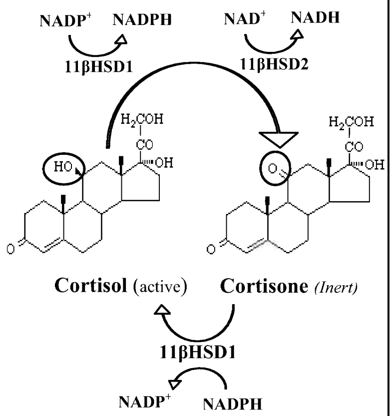

Figure 7: Enzymatic interconversion of glucocorticoids by 11β-HSD isoforms.

Table 3: Biochemical characteristics of 11β-HSD isoforms.

	11β-HSD type 1	**11β-HSD type 2**
Enzyme kinetics	*In vitro* bidirectional, *in vivo* mainly reductase. Use glucocorticoids as a substrate (K_m in µM range) NAD(P)(H) preference	Only dehydrogenase activity Use mineralocorticoid as a substrate (K_m in nM range) NAD^+ preference
Tissue expression	Liver, lung, gonads, pituitary, brain, adipocytes	Kidney, colon, salivary gland, placenta
Molecular biology	Chromosome 1 Gene: 9 Kb length, 6 exons Enzyme: 292 amino acids, 34 KDa Only 14% identity with 11β-HSD2	Chromosome 16 Gene: 6.2 Kb length, 5 exons Enzyme: 405 amino acids, 40 KDa 35% identity with 17β-HSD type 2
Subcellular localization	Endoplasmic reticulum	Endoplasmic reticulum
Function *in vivo*	Modulation of intracellular GC levels, tissue specific enhancement of GC effect	Protection of mineralocorticoid receptor (MR) against cortisol binding

At this time, no three-dimensional structure of 11β-HSD1 is available. However, primary structures from several mammalian species (human, rat, mouse, guinea pig, rabbit and squirrel monkey) have been reported, displaying 75% to 95% sequence identity among the different species variants. Besides metabolism of physiological glucocorticoid substrates, 11β-HSD1 plays an important role in the detoxification of non-steroidal carbonyl compounds like aldehydes, ketones and quinones [43, 64, 65].

2.8.1 Pharmacological importance of 11β-HSD1

The discovery of 11β-HSD1 as a drug target in the metabolic syndrome has been highlighted by several animal studies. The different effects of 11β-HSD1 gene knockout in mice are summarized in table 4. On the other hand, transgenic mice overexpressing 11β-HSD1 show visceral obesity and metabolic syndrome [66].

Table 4: Features of transgenic deletion of 11β-HSD1 in mice.

11β-HSD1 deficient mice	**References**
Lower blood glucose after overfeeding and after stress	[1]
Impaired activation of gluconeogenesis on fasting	[1]
Hypercorticosteronaemia	[2]
Protected from age dependent cognitive decline	[3]
↑ HDL cholesterol, ↓ LDL cholesterol, ↓ triglycerides	[4]

These results suggest that specific 11β-HSD1 inhibitors could lower intrahepatic and intra-adipose cortisol concentrations and thereby enhance insulin sensitivity, reduce gluconeogenesis and potentially reduce adiposity. This approach would be applicable to patients with insulin resistance, hyperglycemia, type II diabetes mellitus, visceral obesity and metabolic disorders [54].

2.9 17β-hydroxysteroid dehydrogenases (17β-HSDs)

17β-Hydroxysteroid dehydrogenases (EC 1.1.1.62) are enzymes involved in the activation and inactivation of androgens and estrogens, by catalyzing the reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids [51, 67-69]. The enzyme activities associated with the different 17β-HSD isoforms are widespread in human tissues. So far, 11 different types of 17β-HSD have been reported from different species (table 5).

Table 5: Different types of 17β-HSD with their function and reaction direction.

Type	Protein family	Species found	Reaction direction	Function
1	SDR	Human, rat, mouse	Reductase	Estradiol production Testosterone production
2	SDR	Human, rat, mouse	Dehydrogenase	Estradiol, testosterone inactivation 20α-progesterone activation
3	SDR	Human, mouse	Reductase	Testosterone production
4	SDR	Human, rat, mouse, chicken, procine, guinea pig	Dehydrogenase	β-oxidation of fatty acids, estradiol inactivation
5	AKR	Human Mouse	Reductase	Testosterone, bile acids production Testosterone production
6	SDR	Rat	Dehydrogenase	5α-dihydrotestosterone inactivation
7	SDR	Human, rat, mouse	Reductase	Estradiol production
8	SDR	Human, mouse	Dehydrogenase	Estradiol, androgen inactivation
9	SDR	Mouse, human	Dehydrogenase	Estradiol, androgen inactivation
10	SDR	Human, rat, mouse, *Drosophila*	Dehydrogenase?	β-oxidation of fatty acids, estradiol inactivation
11	SDR	Human, mouse	Dehydrogenase	Estradiol, androgen inactivation

With the exception of type 5 17β-HSD, which belongs to the AKR family, all other 17β-HSDs belong to the SDR protein family (table 5). These 17β-HSDs are involved in the last step of the biosynthesis of sex steroids and therefore constitute an interesting target for the control of estrogens and androgen levels [70-72].

2.9.1 17β-hydroxysteroid dehydrogenase type 10 from human and *Drosophila*

The human type 10 17β-HSD (17β-HSD10) is a tetrameric enzyme with identical subunits of 261 residues as inferred from the crystal structure of the rat ortholog [73]. The enzyme displays a broad substrate and positional specificity including 17β-OH dehydrogenation of oestrogen, the 3α-OH dehydrogenation of androgens and the hydroxacyl-CoA oxidation of fatty acids and branched-chain amino acids [74-77]. Because of this functional versatility, different names have been assigned to this enzyme, e.g. 17β-hydroxysteroid dehydrogenase (17β-HSD10), short-chain hydroxyacyl-CoA dehydrogenase type II (SCHAD II), 3-hydroxyacyl-CoA dehydrogenase type II (HADH II), and amyloid β-peptide-binding alcohol dehydrogenase (ABAD) [74, 78]. It was initially described as an endoplasmic-reticulum derived amyloid-*β*-peptide binding protein (ERAB) [73] and its mitochondrial localization was determined subsequently [73, 79]. It is expressed in mitochondria of liver, gonads and brain. The importance of this enzyme was demonstrated in studies showing over-expression of 17β-HSD10 in neurons affected in Alzheimer's disease, and in studies showing elevated expression in the azoospermic w/w^v mouse model [73, 80-84].
The essential *Drosophila* gene *scully* [85] is homologous to human 17β-HSD10. Its amino acid sequence identity with other mammalian 17β-HSD10 enzymes is approximately 70%. However, the N-terminal signal sequence is shorter than that of the mammalian orthologous proteins (fig. 8).

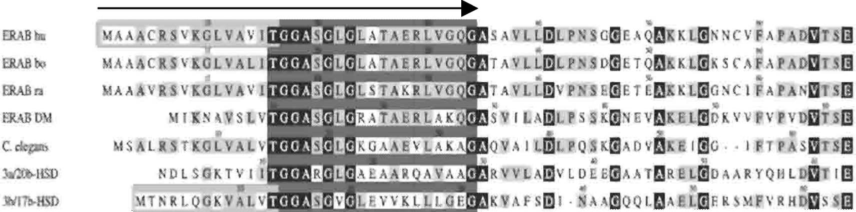

Figure 8: N-terminal sequence alignments of 17β-HSD10 from different species, arrow indicates mitochondrial signal sequences.

The wild type expression pattern of *Drosophila* 17β-HSD10 was reported for many tissues, including the central nervous system, but its high expression pattern in ovaries and testes suggest an involvement in germ line formation [85].

2.10 Hep27, an orphan SDR

Hep27 is a conserved member of the short-chain dehydrogenase/reductase protein family and was first isolated from the nuclear fraction of a hepatoblastoma (HepG2) cell line. It was reported to be synthesized in the G1 phase of HepG2 cells [86, 87]. The expression pattern of Hep27 is not only restricted to malignant cells with growth arrest in G1 phase, but it is also widely expressed in tissues such as liver, parotid gland, ovary, placenta, spleen, heart, skeleton muscle, kidney, breast, testis and monocyte-derived dendritic cells [88, 89]. Primary structure properties of Hep27 were studied earlier by a cross-species comparison between human, *C. elegans*, *Drosophila* and *A. thaliana* which shows that Hep27 belongs to a highly conserved cluster of SDR enzymes, present in all of these four genomes [25]. To this point, the functional role of Hep27 either in cancerous cells or in normal body tissues is not known.

3 EXPERIMENTAL

The methods used are described in detail in the original articles I-V. Only an overview on the central methodology is presented in this section.

General Methods in molecular biology

Some of the methods used, such as DNA sequencing, DNA purification, RNA isolation, gene cloning, PCR, plasmid DNA isolation and transformation, in *vitro* transcription and translation, Southern, Northern and Western blot analysis were common techniques which were carried out according to established protocols [90, 91]. Protein purification was carried out mainly by using IMAC (immobilized metal-ion-affinity chromatography) on His-bind resin (Novagen) by use of an FPLC system (Amersham Pharmacia Biotech). Protein sequence analysis was performed using Procise N and C terminal sequencers (PE Biosystems).

Enzyme Kinetics

Initial velocity measurement with a number of steroids, bile acids, oxysterols and dicarbonyl compounds were performed on heterologously expressed SDR enzymes. All measurements were carried out in the linear range of product formation *versus* reaction time and enzyme concentration, and conditions were chosen with no more than 20% of substrate conversion. Kinetics constant were calculated using the Prism (Graphpad), Sigmaplot (Systat) or Grafit (Erithacus) software packages by linear or non-linear regression analysis and by fitting to different models describing either the Michaelis-Menten kinetics ($V = V_{max} \times S^h/(K_M^h + S^h)$; $h = 1$) or a cooperative kinetic behavior ($h \neq 1$), respectively.

Enzyme inhibition

Inhibition constants were determined at varied inhibitor and substrate concentration and the data obtained were analyzed by fitting to models describing different inhibitor types (reversible vs. tight binding) by using the SigmaPlot software package.

Enzymatic Assays

Three different methods were applied for monitoring the catalytic reactions:

1) UV spectrophotometric analysis: The activities were measured by following the conversion of NAD(P)(H) at 340 nm (Cary 300Bio instrument), using a molar absorption coefficient (ε) of 6.22 $mM^{-1}.cm^{-1}$.

2) HPLC analysis: Steroid and oxysterol metabolites were separated by reverse-phase HPLC on a C_{18} column and monitored by UV detection as described in detail in papers I, II and III.

3) GC/MS analysis: Steroids were converted into volatile methyl ester trimethylsilyl (TMS) ether derivatives, as described [92]. Compounds were separated isothermically at 280°C on a fused capillary column and products were identified by comparisons with authentic compounds and retention indices as described in detail in paper IV.

Active site titration:

Fractional velocities of 11β-HSD1 were measured in the presence of increasing amounts of the arylsulfonamidothiazole inhibitor compound BVT.24829 or CBX. The data obtained were fitted by non-linear regression to the Morrison equation using GraphPad software program, as described in detail in paper I.

Morrison equation:

$$vi/vo = 1- ((E + I + Ki^{app})- ((E+ I + Ki^{app})^2- 4 \times E \times I)^{1/2}) \times (2 \times E)^{-1}$$

Cell culture:

The mammalian cell lines COS7, HEK297, HepG2 and HMEC were grown in appropriate cell media supplemented with 10% (v/v) FCS (fetal-calf serum) and 100 units/ml penicillin or 100 mg/l streptomycin at 37°C in a humidified CO_2 atmosphere of 5%. Transfection experiments were performed with varied amount of plasmid DNA using the FuGENE 6 transfection reagent (Roche molecular Biochemicals). After transfection cells were either fixed and permeabilized with methanol or paraformaldehyde/Triton X-100 for microscopic

analysis, or were subjected to incubation with MitoTracker dyes for vital mitochondrial staining and latter analysed by fluorescent or immunoelectron microscopy.

Kinetic analysis of human and guinea pig 11β-HSD1 in mammalian COS-7 cells.

COS-7 cells were transfected with 1.4 μg of guinea pig or human 11β-HSD1 plasmid DNA subcloned in the mammalian expression vector pcDNA3.1. Cells (0.5 x 10^6 in 6-well plate) were grown in 3 ml of medium/well, and 11β-HSD1 activities were analyzed 24 hours after transfection (~80-90% cell confluency). Finally, cells were incubated for 1-3 hours in the presence of unlabeled cortisone or cortisol and appropriate glucocorticoid tracer (200,000 cpm, ^{3}H-labeled). Supernatant was extracted on solid phase (Oasis columns, Waters) and further analyzed by thin layer chromatography and liquid scintillation counting [93] to determine the fractional conversion of substrate. Background activities (ranging from 0.9 to 2% of total recovered tracer radioactivity) were subtracted from the respective measurement.

4 AIMS OF THE THESIS

In order to understand functional aspects and structure-function relationships of SDRs in general, the biochemical properties of the following three human SDR enzymes were investigated in this thesis:

- 11β-HSD1, an enzyme mediating glucocorticoid activation.
- 17β-HSD10, a mitochondrial enzyme with broad substrate specificity towards androgens, estrogens and fatty acids.
- Hep27, a functionally uncharacterized member of the SDR enzyme family, synthesized and overexpressed in human hepatoblastoma (HepG2) cells.

In particular, three aspects were of importance and were studied in more detail:

1) Structure-function relationships, including kinetics and inhibition profiles of a medically important SDR enzyme, the 11β-HSD type 1 (papers I, II).

2) Substrate specificities of three different SDR enzymes, the 11β-HSD1, 17β-HSD10 and Hep27 (papers III, IV, V).

3) Subcellular localization of 17β-HSD10 (paper IV).

5 RESULTS AND DISCUSSION

5.1 Kinetic analysis of 11β-HSD1 (paper I)

To investigate the involvement of 11β-HSD1 in glucocorticoid resistance, a kinetic analysis of 11β-HSD1 from glucocorticoid resistant (guinea pig) *versus* sensitive (human) species was performed. Both orthologs were successfully expressed as full-length enzymes in yeast and COS7 cells, and as soluble, transmembrane deleted constructs in *E.coli* [94]. The kinetic results reveal that both 11β-HSD1 isozymes display low micromolar K_m values for 11-oxo reduction in yeast homogenates (table 6), which are in intact cells lowered by about one order of magnitude, allowing corticosteroid activation at physiological glucocorticoid levels. Similar results were obtained for the purified, soluble enzymes showing K_m values of 0.8 μM (human) and 0.6 μM (guinea pig), close to the values obtained from intact cells (table 6). Importantly, the kinetic analysis shows no indication of enzyme cooperativity, as reported earlier [95]. Instead our results suggest that both 11β-HSD1 isozymes display Michaelis-Menten kinetics (fig. 9), with nearly identical k_m values in homogenates and in purified material. In this study, we also report for the first time active site titration experiments with highly purified human 11β-HSD1 enzyme using the specific arylsulfonamidothiazole inhibitor BVT.24829, showing 40-50% active sites per mol of total enzyme material.

Table 6: Kinetic constants form human and guinea pig 11β-HSD1, K_m in μM, V_{max} in pmol × min^{-1} × mg^{-1}, for intact cells in pmol × (10^6 cells)$^{-1}$ x h^{-1}, for purified enzyme nmol × min^{-1} × mg^{-1}. Hepatic microsome data: [a] data taken from [96], [b] data taken from [97].

Source	K_m	V_{max}
guinea pig		
Pichia pastoris	2.2 ± 0.7	42.0 ± 1.9
E.coli purified	0.6 ± 0.3	18.8 ± 2.5
COS intact	0.28 ± 0.029	173 ± 10
COS extract	8.2 ± 1.9	221 ± 15.9
liver microsomes[a]	1.2 ± 0.1	4670 ± 150
human		
Pichia pastoris	1.9 ± 0.2	30.3 ± 8.5
E.coli purified	0.8 ± 0.2	21.8 ± 5.1
COS intact	0.33 ± 0.045	191 ± 38
COS extract	8.9 ± 2.3	150 ± 66
liver microsomes[b]	2.5 ± 0.1	70.0 ± 3.0

Comparison of the kinetic data indicates no significant differences in substrate affinities (table 6) and hence speaks against involvement of 11β-HSD1 as a modulating factor for the glucocorticoid resistance observed in guinea pigs [98]. Instead, altered glucocorticoid receptor affinities, ACTH-mediated cortisol secretion, and lowered plasma protein binding secretion are suggested factors mediating resistance to glucocorticoids [99-103]. Our histochemical data also suggest that by high expression of 11β-HSD1 in the Zona glomerulosa of the guinea pig adrenal gland this enzyme is involved in regulation of mineralocorticoid synthesis in this hypercortisolic species.

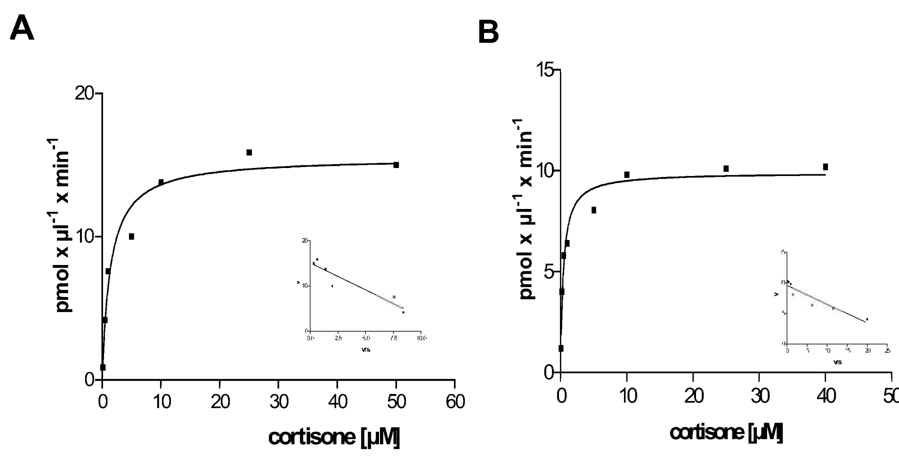

Figure 9: Kinetic analysis of soluble human and guinea pig 11β-HSD1. Panel A: human, Panel B: guinea pig. Inserts show linear regression analysis through Eadie-Hofstee plots.

5.2 Analysis of structure-function relationships of 11β-HSD type 1 (paper II)

To understand structure-function relationships of 11β-HSD type 1, the primary structure from several mammalian species (cat, hamster, cynomolgus, chimpanzee, and dog) were determined in this study and were compared with the known 11β-HSD1 isozymes. In total, there are now 13 11β-HSD1 primary structures available from different mammalian species, presented in the sequence alignment (fig. 10). Inspection of the sequence alignment reveals that all important SDR structural motifs are present in 11β-HSD1, except the "NNAG" sequence motif, which is located at the nucleotide binding site. This variable motif is replaced by LNHI (or LNHV in guinea pig; position 118-121 in the human sequence). Besides the high

sequence similarity among different 11β-HSD1 species, we identified 6 variable regions designated V1-V6 (fig. 10).

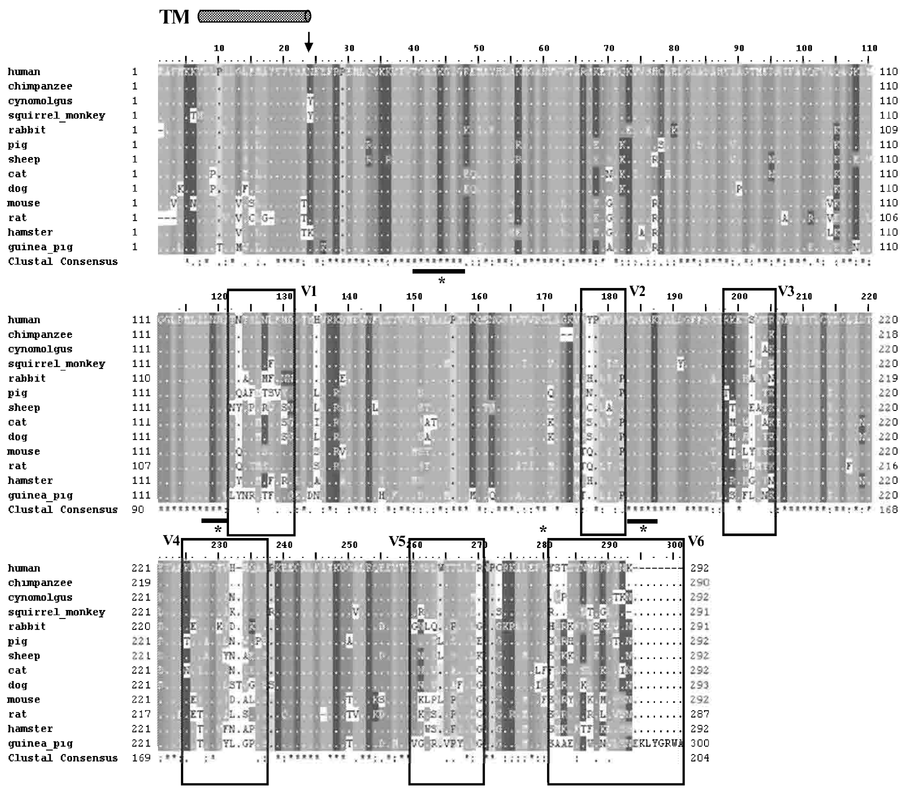

Figure 10: Primary structure comparison of mammalian 11β-HSD1 forms.

Segments V1-V3 are located within the central SDR core, whereas sections V4-V6 are found in the C-terminal sequence portion, which is a highly variable region in all SDR forms. Furthermore active site variability in 11β-HSD1 species was studied by performing inhibition studies on the human, rat, mouse and guinea pig 11β-HSD1 isozymes, by using the non-selective compound carbenoxolone (CBX) and several selective arylsulfonamidothiazole inhibitors (fig. 11). These data reveal that carbenoxolone is a potent inhibitor versus all species, except for guinea pig. Different arylsulfonamidothiazoles inhibitors display distinct inhibition profiles against different mammalian species tested, with several compounds

displaying tight binding inhibition for the human enzyme ($K_i \approx 50$ nM), intermediate for the mouse and guinea pig enzymes, and weak or no inhibiton for the rat enzyme ($K_i > 3$ μM).

Figure 11: Chemical structures of arylsulfonamidothiazole compounds and carbenoxolone, an inhibitor of 11β-HSD1.

Taken together, this study shows that significant differences exist in the active site architecture of 11β-HSD1 enzyme forms. The differences are revealed by determination of variable and constant segments in the primary structure, and by studying inhibition patterns of different, highly selective arylsulfonamidothiazole compounds. Consequently, this study provides information to understand the structure-function relationships of 11β-HSD1 and shows a line for further investigations, allowing improvement of inhibitor potency directed against this novel and promising drug target.

5.3 Novel oxysterol metabolizing properties of the rodent and human 11β-HSD1 isozyme (paper III)

This study was based on reports from Song et al. describing isolation of an $NADP^+$-dependent 7α-OH cholesterol dehydrogenase (7α-HSD) from hamster liver [104, 105]. These studies together with the molecular cloning of hamster 11β-HSD1 from our laboratory (paper II) suggest identity between these two enzymes and possible interactions between oxysterol and glucocorticoid metabolic pathways.

To investigate if 11β-HSD1 is involved in oxysterol metabolism, different 11β-HSD1 species variants (human, rat, mouse) were expressed and purified either as recombinant full-length enzymes in yeast or as transmembrane-deleted enzymes in *E. coli* [106, 107]. These 11β-

HSD1 recombinant clones and human liver microsomes were incubated in the presence of NADP(H) with either 7α-OH cholesterol, 7β-OH cholesterol or 7-ketocholesterol. The *in vitro* data obtained from liver microsomes and from different 11β-HSD1 enzyme versions reveal that 7-ketocholesterol is reduced exclusively to 7β-OH cholesterol, without detectable formation of 7α-OH cholesterol. Similarly, only 7β-OH cholesterol is oxidized to the 7-oxo derivative, but no conversion with 7α-OH cholesterol was detected. Furthermore these oxysterol activities of 11β-HSD1 were confirmed by inhibition studies, using the non-specific inhibitor CBX and the specific compound BVT.24829 which shows close to complete inhibition of 7-oxysterol metabolite formation, suggesting a possible identity of human hepatic 11β-HSD1 with 7β-OH cholesterol dehydrogenase. Taken together, this study demonstrate that beside 11β-HSD1 involvement in glucocorticoid metabolism [44, 54, 108] and in the carbonyl detoxification process [43, 64, 65], 11β-HSD1 is involved in oxysterol metabolism as well. This novel finding establishes the enzymatic origin of endogenous 7β-OH cholesterol in humans, and points to a possible involvement of 11β-HSD1 in atherosclerosis.

5.4 Novel substrate specificities of human and *Drosophila* 17β-HSD10 (paper IV)

To understand the physiological role of the multifunctional enzyme 17β-HSD10, substrate specificities of human and *Drosophila* orthologs were studied by performing substrate screening assays using a wide range of different steroids and lipids. The results obtained revealed that beside known reactions of human 17β-HSD10, i.e., 17β-OH dehydrogenation of estrogen, 3α-OH dehydrogenation of androgen and oxidation of branched chain fatty acids (table 7), both isozymes catalyze conversions of distinct bile acids and iso bile acids. In particular, oxidative conversion of 7α-OH bile acids (cholic acid, chenodeoxycholic acid) was detected with human but not with Drosophila 17β-HSD10, whereas both forms convert 7β-OH groups in ursodeoxycholic or isoursodeoxycholic acid (table 7).

Table 7: Kinetic constants for dehydrogenase and reductase activities of 17β-HSD10 from human and *Drosophila*. K_M values are given in 10^{-6}M, k_{cat} values in min^{-1} and k_{cat}/K_M values in $10^6 min^{-1} M^{-1}$. For coenzyme k_{cat}/K_M values in 10^{-6} M. Abbreviations: nd: not determined and na: no activity detected. [b]values from [74], [c]values from [75].

	Human				*Drosophila*			
Substrate	K_m	k_{cat}	k_{cat}/K_m	$K_{mNAD(H)}$	K_m	k_{cat}	k_{cat}/K_m	$K_{mNAD(H)}$
Acetoacetyl-CoA/NADH	25.7 ± 0.9	1430 ±70	55.6	30.6	33.7 ± 8	1660 ±187.5	50.2	32.5
β-hydroxybutyryl-CoA/NAD$^-$	85.2 ± 7.2	290.0 ± 10	3.4	42.3	101 ± 8	68.0 ± 5.5	0.67	64.4
Androsterone/NAD$^-$	45 ± 9.3[b]	0.66 ± 0.08[b]	0.014[b]	242[b]	37.3 ± 2	12.6 ± 0.65	0.33	124
Androsterone/NAD$^-$	41 ± 14	0.04 ± 0.005	0.0011	nd	-	-	-	-
5α-Dihydrotestosterone/NADH	112 ± 18[c]	1.94 ± 0.21[c]	0.017[c]	nd	12.3 ± 1	4.6 ± 0.85	0.37	nd
17β-Estradiol/NAD$^-$	43 ± 2.1[b]	0.66 ± 0.01[b]	0.015[b]	50[b]	11.1 ± 2.3	0.52 ± 0.15	0.048	nd
5α-Pregnan-20β-ol-3-one	5 ±1	0.25 ± 0.035	0.053	nd	9 ± 2	0.076 ± 0.01	0.0082	nd
Isoursodeoxycholic acid	219 ± 20	0.054 ± 0.012	0.0002	nd	3 ± 0.3	0.014 ± 0.003	0.005	nd
Chenodeoxycholic acid	36.4 ± 5.1	0.034 ± 0.003	0.001	nd	na	-	-	-
Dedydrocorticosterone	1.7 ± 0.02	0.03 ± 0.0001	0.018	nd	nd	-	-	-
20-Hydroxyecdysone/NAD$^-$	na	-	-	-	9.8 ± 4	1.28 ± 0.15	0.15	nd

These results establish 17β-HSD10 as a novel human liver dehydrogenase that is active in oxidizing bile acids with either a 7α- or a 7β-hydroxyl group. Thus, 7-keto bile acids such as 7-keto-CDCA may be formed in the liver and serve as a source for UDCA. Furthermore, using GC-MS or HPLC we detected 20β-OH and 21-OH activities of C21 steroids like 5α-pregnane, 20β-ol, 3-one and glucocorticoids, e.g. cortisol, cortisone or dehydrocorticosterone, respectively (table 7). The 20β-OH and 21-OH dehydrogenase activities observed with C21 steroids suggest a general role of 17β-HSD10 in controlling the levels of progesterone and glucocorticoid hormone levels. Therefore 17β-HSD10 might participate in the production of highly polar glucocorticoid C21-carbonic acids in conjunction with aldehyde dehydrogenase [109, 110].

These novel substrate specificities were explained by homology modeling of human and *Drosophila* 17β-HSD10, using as template the high-resolution crystal structure of rat 17β-HSD10 (PDB 1E6W). Inspection of the active site reveals a large hydrophobic cavity, similar as that described for the rat enzyme [111]. Within this active site, substrate docking with pregnanolone, isoUDCA, CDCA and cortisol as representative substrates was carried out, and we obtained atomic distances compatible with enzyme catalysis (table 8, fig. 12). Accordingly, the active site of 17β-HSD10 is built up to accommodate the steroid molecule in different orientations in relation to the active site residues Ser155 and Tyr168 and the

nucleotide cofactor, which explains the multiple steroid specificities observed in our substrate screen.

Table 8: Molecular distances (in Å) obtained from docking results of human and *Drosophila* 17β-HSD10. Distances between atoms involved in catalysis are given.

Complex	H-O (Tyr)	O-H (Ser)	H-C4 (NAD)
HSD10_hu: isoUDCA	2.2	2.1	2.3
HSD10_Dro: isoUDCA	2.1	2.1	2.1
HSD10_hu: CDCA	2.2	1.8	2.4
HSD10_hu: pregnanolone	1.8	1.8	2.2
HSD10_Dro: pregnanolone	1.7	1.9	2.2
HSD10_hu: cortisol	2.2	2.0	2.2
HSD10_Dro: cortisol	2.1	2.0	2.2

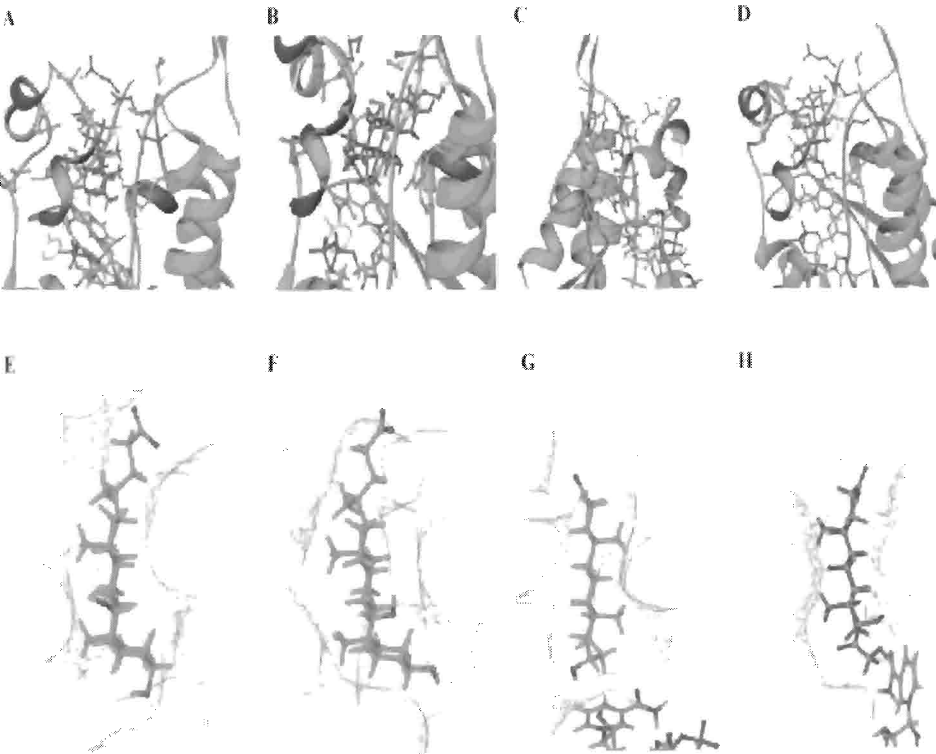

Figure 12: Binding modes for substrates at the active site of human 17β-HSD10 in relation to residues necessary for catalysis and the coenzyme molecule. Catalytic residues (Ser138, Tyr151 side chains shown in orange, A-D) and residues forming van der Waals contact with the substrates are shown. The complementarity between the different substrate molecules and parts of the accessible surface area of human 17β-HSD10 (panels E-H) is shown. Panels A, E: 7β-HSD configuration, substrate isoUDCA. Panels B, F: 7α-HSD configuration, substrate: CDCA. Panels C, G: 20β-HSD, substrate: 5α-pregnane, 20β-ol, 3-one. Panels D, H: 21-HSD configuration, substrate cortisol. The figure was created with RIBBONS.

5.5 Functional characterization of an orphan SDR (paper V)

Primary structure properties of Hep27 were studied earlier by a cross-species comparison between human, *C. elegans*, *Drosophila* and *A. thaliana* (fig. 13) which shows that Hep27 belongs to a highly conserved cluster of SDR enzymes, present in all of these four genomes [25]. Other human representatives of this cluster are SRL, a peroxisomal protein (annotated as retinol dehydrogenase), and DCXR, a mitochondrial dicarbonyl reductase/xylulose reductase [112]. From this SDR sequence alignment, human Hep27 is predicted to have a cofactor specificity towards NADP(H), and falls into the cP2 classification of SDR enzymes [113], as identified through the highly conserved residue Arg68 (fig. 13).

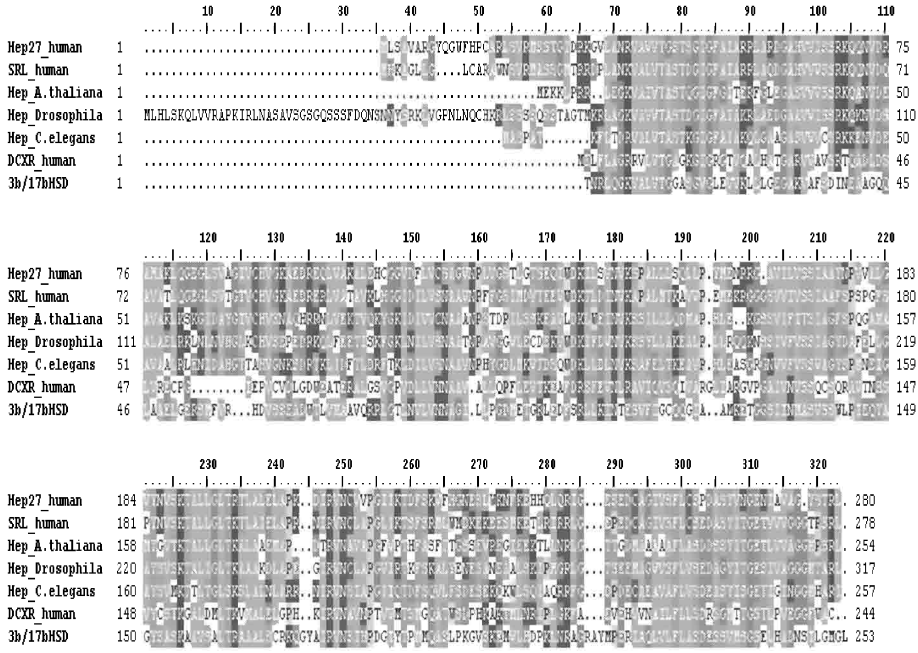

Figure 13: Sequence alignment of human Hep27 and related SDR enzymes. Boxing indicates sequence motifs and conserved residues found in SDR enzymes.

Purified, recombinant Hep27 was subjected to a substrate screen using a compound library (containing about 100 compounds) of possible SDR substrates. This collection comprises different steroids, retinoids, sugars and several miscellaneous xenobiotic carbonyl compounds. The substrate screening was carried out using different assay formats (UV/VIS, HPLC, GC/MS). Initially the cofactor absorption change was monitored, and dependent on the results, secondary analysis of product formation was carried out by using HPLC based or GC/MS assays. This screen shows that Hep27 has a minor 7β-OH dehydrogenase (isoUDCA) activity with steroids, however, no other steroid dehydrogenase activity was detectable. This makes it unlikely that the function of Hep27 is to control hormone levels in a manner analogous to that of known steroid dehydrogenases. Furthermore, no activity was detected with retinoids. However, Hep27 shows NADPH-dependent substrate specificities overlapping with DCXR. The substrates found to be converted include several reactive dicarbonyl compounds such as 3,4-hexanedione, 2,3-heptanedione and 1-phenyl-1,2-propanedione, displaying k_{cat} values 11.7 min^{-1}, 40 min^{-1}, 15.2 min^{-1} respectively (table 9), but in contrast to DCXR, Hep27 shows no activity towards aldehydes such as methylglyoxal or towards sugar substrates like xylulose or threose.

Table 9: Kinetic constants for human Hep27. The kinetic constants for the substrates were determined with 200 µM NADPH.

Substrate	K_M (mM)	K_{cat} min^{-1}	K_{cat}/K_M M^{-1} min^{-1}
1-Phenyl-1,2-propanedione	0.3 ± 0.04	15.2 ± 1.5	5.0 x 10^4
2,3-Heptanedione	1.1 ± 0.5	40.0 ± 8.9	3.6 x 10^4
3,4-Hexanedione	0.8 ± 0.3	11.7 ± 1.8	1.5 x 10^4

Expression analysis shows that Hep27 is expressed in liver, kidney and several cell lines, like HEK, MOLT4 or Hela, in agreement with previously reported data [88, 89]. Surprisingly, we discovered expression of Hep27 in the microvascular human endothelial cell line HMEC by Northern blot and RT-PCR analysis (fig. 14C, A). Western blot analysis with anti-human Hep27 antiserum reveals that Hep27 is also detectable as protein product, both in the endothelial cell line and in primary endothelial cells isolated under surgery or delivery (fig.

14B). Based on the substrate specificity and on the expression in endothelial tissues, it is suggested that Hep27 constitutes a detoxification mechanism against reactive α-dicarbonyls and presumably provides the first line of defence within the endothel.

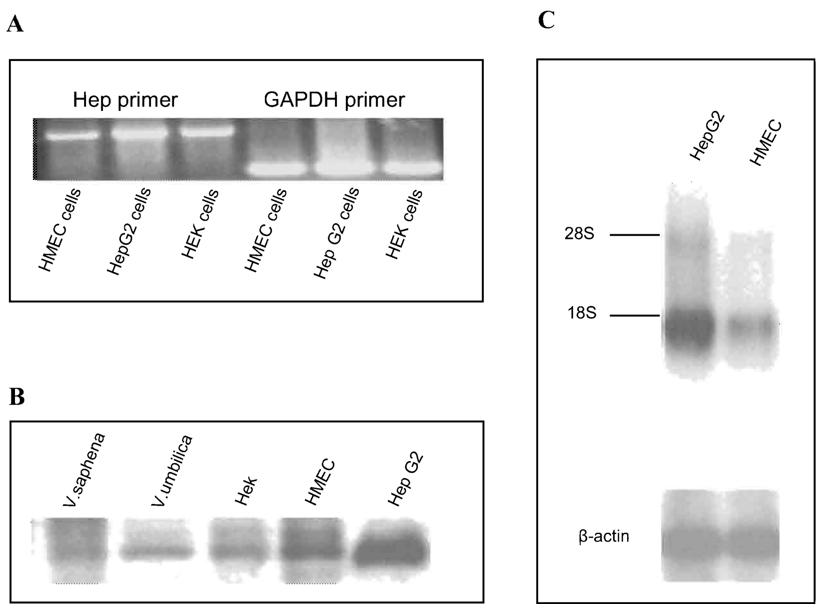

Figure 14: Expression analysis of Hep27 in endothelial tissue and different cell lines. Panel A: Reverse-transcriptase PCR and detection of Hep27 in HMEC, HepG2 and HEK cells. As control, GAPDH primers were used. B: Western blot of different cell homogenates probed with anti-human Hep27 antibodies. C: Northern blot analysis and transcript comparison of HepG2 and HMEC cells. As loading control the blot was hybridized with a labeled probe detecting actin.

5.6 Subcellular localization of human and *Drosophila* 17β-HSD10 (paper IV)

To analyze the mitochondrial targeting motif in human 17β-HSD10, the N-terminal sequences between human 17β-HSD10 (residues 1-15) and the related enzyme 3β/17β-hydroxysteroid dehydrogenase from *Comamonas testosteroni* (residues 1-12) were exchanged to yield constructs with N-terminal 17β-HSD10 and core 3β/17β-HSD sequences (17β-HSD10/3β) and vice versa (3β/17β-HSD10) (fig. 15).

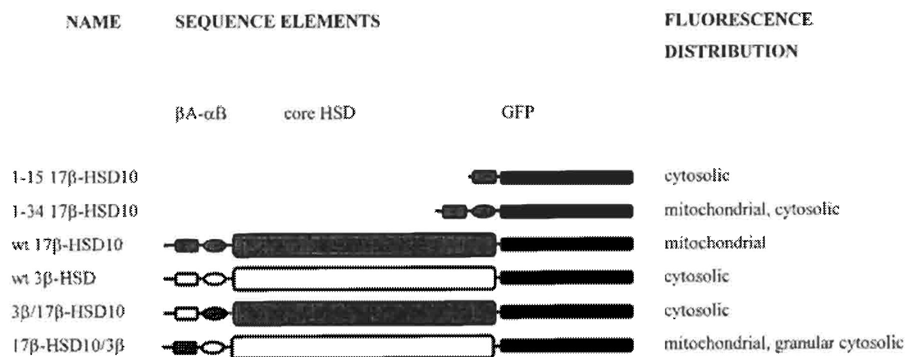

Figure 15: Graphical representation and subcellular distribution of wild-type, deletion and hybrid GFP constructs with human 17*β*-HSD10 used in this study. Secondary structure elements *β*A and *α*B are depicted as rectangles and ellipses, respectively, with the core SDR protein displayed as larger rectangles (17*β*-HSD10 in grey, 3*β*-HSD in white). These constructs were fused to the GFP passenger protein (displayed as the black rectangle). Subcellular distribution and specific mitochondrial localization, determined by fluorescence microscopy are indicated.

The N-terminal part 1-34 of 17β-HSD10 was determined to contain a noncleavable mitochondrial signal sequence (fig. 16). This contains part 1-15 that is specific for 17β-HSD10 but not sufficient for mitochondrial import, and part 16-34 that can be replaced by similar structures as the corresponding region in 3β/17β-HSD. These constructs were fused to green fluorescent protein (GFP) as a reporter molecule.

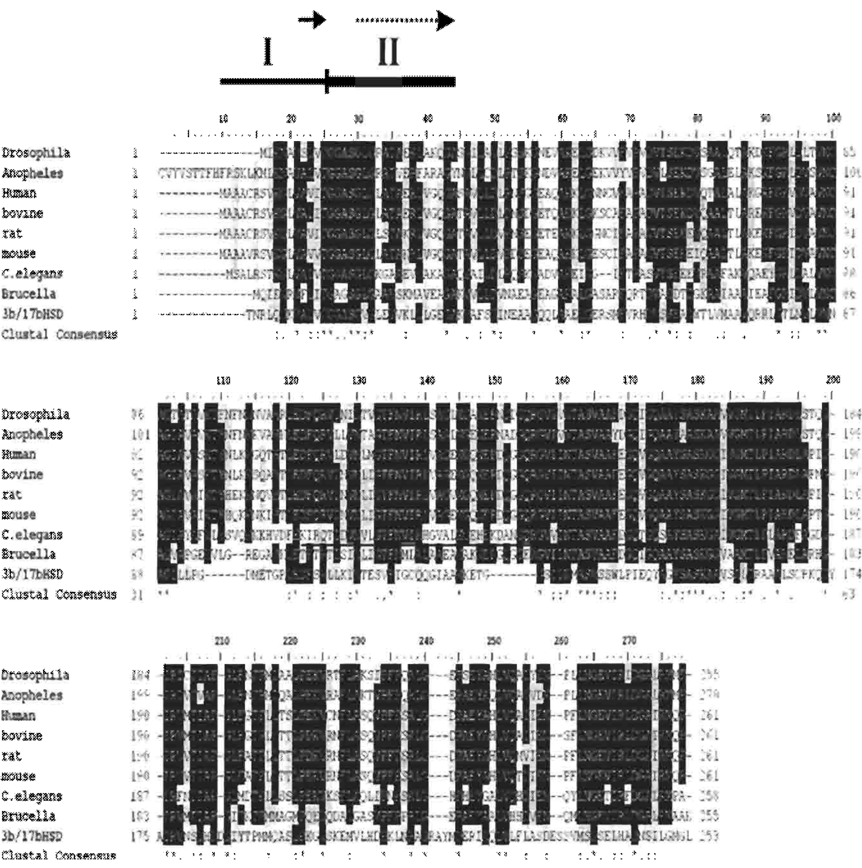

Figure 16: Sequence alignment of 17*β*-HSD10 orthologues and a related bacterial HSD (3*β*/17*β*-HSD from C. testosteroni). Highly conserved sequence elements of the primary structures are highlighted by black and grey shading. The N-terminal segments (I and II) exchanged in this study between human 17*β*-HSD10 and bacterial 3*β*/17*β*-HSD from C. testosteroni are marked. The arrows indicate secondary-structure elements within the targeting sequence, the solid arrow depicts strand *β*A, and the dotted arrow shows helix *α*B.

Fluorescence microscopy analysis revealed that 17β-HSD10/3βHSD indeed is retained substantially within mitochondrial structures (fig. 17 J-L). In contrast, the fluorescence of the 3β/17β-HSD10 construct is distributed over the cytoplasmic compartment without any notable mitochondrial enrichment (fig. 17 G-I). These results suggest that the sequence of the first 34

amino acid residues of the 17β-HSD10 structure is sufficient to direct and translocate 17β-HSD10 to mitochondria. On the other hand, *Drosophila* 17β-HSD10 shows a cytosolic localization pattern, possibly due to the N-terminal sequence difference that in human 17β-HSD10 constitutes a mitochondrial targeting signal (fig. 18).

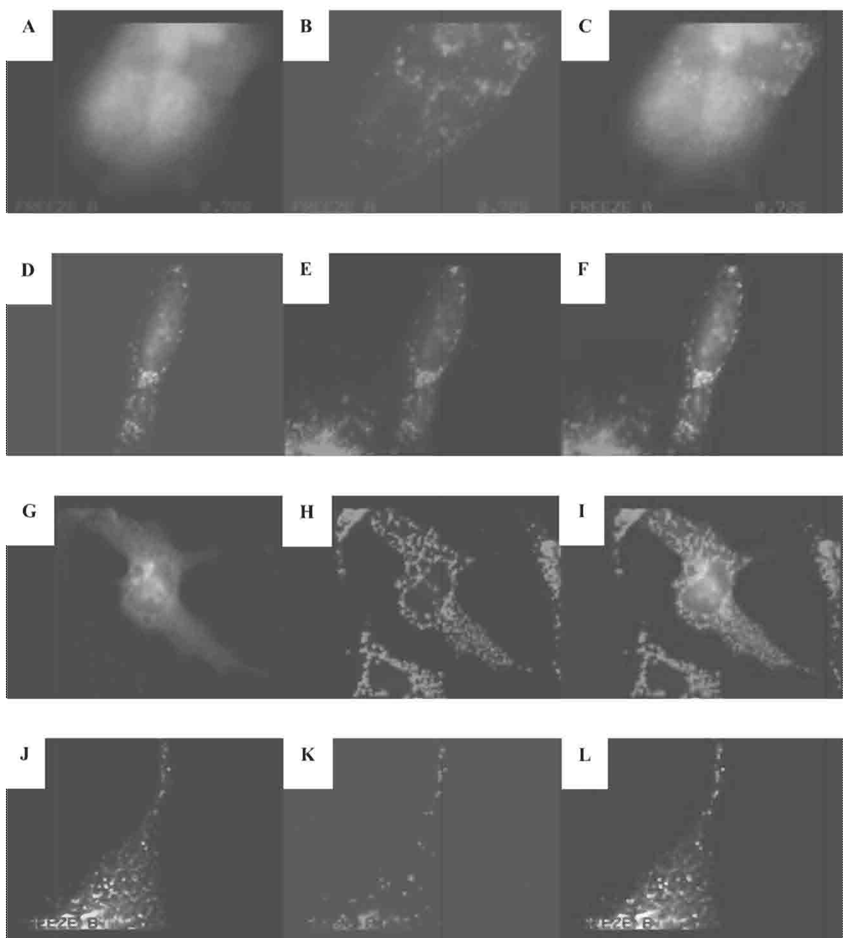

Figure 17: Mitochondrial targeting analysis of human 17β-HSD10 derived peptides fused to GFP.
Construct 1–15 (A–C) and construct 1–34 (D–F). (A and D) GFP fluorescence; (B and E) MitoTracker analysis; (C and F) overlay of (A)+(B) and (D)+(E) respectively. No mitochondrial targeting is observed with the 1–15 construct, whereas mitochondrial co-localization is detected with the 1–34 hybrid. Co-localization of GFP hybrids with exchanged N-terminal motifs is shown: (G–I) 3β/ERAB hybrids; (J–L) ERAB/3β hybrids. (G and J) Fluorescence of hybrid GFP constructs. (H and K) mitochondrial co-localization using Grp75 antibodies; (I and L) overlays of (G)–(H) and (J)+(K) respectively. No mitochondrial localization is observed with the 3β/ERAB (G) construct, whereas some of the ERAB/3β-GFP fluorescence is clearly co-localized to mitochondria (J), indicating a weak mitochondrial import signal.

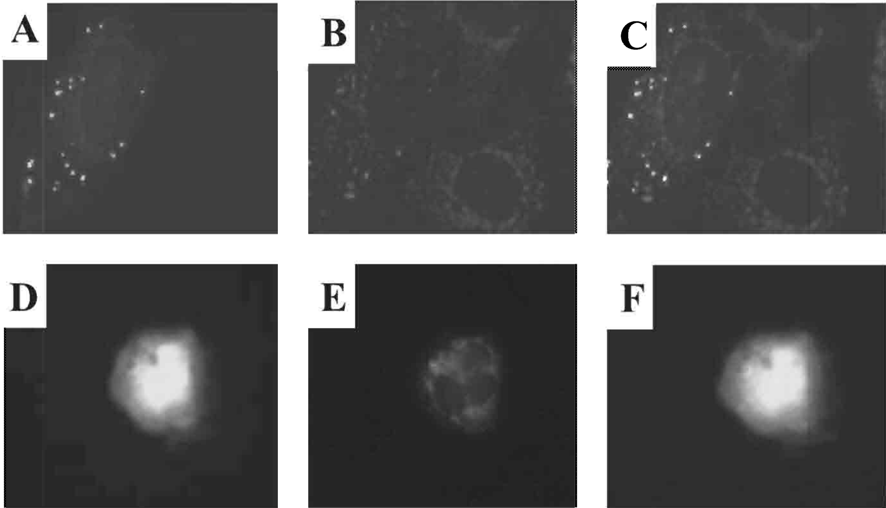

Figure 18: Subcellular localization of human and Drosophila 17β-HSD10, using GFP reporter constructs in COS-7 cells. Mitochondrial staining in (B) was obtained through MitoTracker dye staining. Panels C and F represent overlays of A+B and D+F, respectively. Clear mitochondrial colocalization is observed with the human form (A-C), whereas no colocalization is detected with Drosophila 17β-HSD10 (D-F).

6 CONCLUSIONS

Kinetic analysis of 11β-HSD1

- 11β-HSD1 is not a modulating factor for the glucocorticoid resistance observed in the hypercortisolic guinea pig. Instead, the expression of 11β-HSD1 in the Zona glomerulosa of the guinea pig adrenal gland suggests a role of this enzyme in mineralocorticoid synthesis in this species.
- Full-length and transmembrane-deleted variants show comparable kinetic characteristics, whereas in intact cells K_m values are lower. Both human and guinea pig 11β-HSD1 isozymes display Michaelis-Menten kinetics and no sign of sigmoidal kinetic behavior was observed.

Structure-function relationships of 11β-HSD1

- Revealed from 11β-HSD1 primary structures comparison, several variable and constant segments were identified within the active site architecture of 11β-HSD1 isozymes. Possibly these structural differences reflect the differences observed in the inhibitor characteristics of 11β-HSD1 isozymes.

Novel substrate specificities of different SDR enzymes

- The involvement of 11β-HSD1 in oxysterol metabolism reveals an enzymatic origin of endogenous 7β-OH cholesterol in humans, and points to a possible involvement of 11β-HSD1 in atherosclerosis.

- The novel substrate specificities of 17β-HSD10 orthologs demonstrate that 17β-HSD10 is a versatile catabolic enzyme which is involved in degradation pathways of glucocorticoids and sex steroids, and in epimerization of bile acids, besides the oxidation of fatty acids and branched chain amino acids.

- The characterization of Hep27 as an NADPH dependent dicarbonyl reductase and its expression in endothelial tissues suggest an involvement of this enzyme in a

detoxification mechanism against reactive α-dicarbonyls, which are shown to be involved in covalent modifications of essential cellular components.

Subcellular localization of human and *Drosophila* 17β-HSD10

- Confocal and electron microscopy reveals that the human 17β-HSD10 is localized to mitochondria, whereas the *Drosophila* 17β-HSD10 shows a cytosolic localization pattern, possibly due to an N-terminal sequence difference that in the human form constitutes a mitochondrial targeting signal reaching into parts of the Rossmann-fold.

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