Comparative enzymology of 11β-hydroxysteroid dehydrogenase type 1 from six species

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Abstract

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1), catalyzing the intracellular activation of cortisone to cortisol, is currently considered a promising target to treat patients with metabolic syndrome; hence, there is considerable interest in the development of selective inhibitors. For preclinical tests of such inhibitors, the characteristics of 11β-HSD1 from the commonly used species have to be known. Therefore, we determined differences in substrate affinity and inhibitor effects for 11β-HSD1 from six species. The differences in catalytic activities with cortisone and 11-dehydrocorticosterone were rather modest. Human, hamster and guinea-pig 11β-HSD1 displayed the highest catalytic efficiency in the oxoreduction of cortisone, while mouse and rat showed intermediate and dog the lowest activity. Murine 11β-HSD1 most efficiently reduced 11-dehydrocorticosterone, while the enzyme from dog showed lower activity than those from the other species. 7-Ketocholesterol (7KC) was stereospecifically converted to 7β-hydroxycholesterol by recombinant 11β-HSD1 from all species analyzed except hamster, which showed a slight preference for the formation of 7α-hydroxycholesterol. Importantly, guinea-pig and canine 11β-HSD1 displayed very low 7-oxoreductase activities. Furthermore, we demonstrate significant species-specific variability in the potency of various 11β-HSD1 inhibitors, including endogenous compounds, natural chemicals and pharmaceutical compounds. The results suggest significant differences in the three-dimensional organization of the hydrophobic substrate-binding pocket of 11β-HSD1, and they emphasize that species-specific variability must be considered in the interpretation of results obtained from different animal experiments. The assessment of such differences, by cell-based test systems, may help to choose the appropriate animal for safety and efficacy studies of novel potential drug candidates.

Journal of Molecular Endocrinology (2005) **35,** 89–101

Introduction

Glucocorticoids act through binding to the glucocorticoid receptor (GR), thereby transactivating or transrepressing a multitude of genes involved in various physiologic processes, including modulation of stress and inflammatory responses, maturation and differentiation of cells, and regulation of bone, carbohydrate and lipid metabolism (Barnes 1998, De Bosscher *et al.* 2003). Glucocorticoids exist in an inactive (cortisone in humans, 11-dehydrocorticosterone in rodents) and an active form (cortisol in humans, corticosterone in rodents), whereby the prereceptor enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays a key role in the control of the local amplification of glucocorticoid action by catalyzing the oxoreduction of inactive 11-keto- to active 11β-hydroxyglucocorticoids (Stewart & Krozowski 1999, Sandeep & Walker 2001). 11β-HSD1 is expressed in most organs, with high expression in liver and white adipose tissue. A second enzyme, 11β-HSD2, is expressed mainly in mineralocorticoid target tissues and catalyzes the inactivation of 11β-hydroxyglucocorticoids to 11-ketoglucocorticoids, thereby protecting the mineralocorticoid receptor (MR) from inappropriate activation by glucocorticoids (Stewart & Krozowski 1999).

An increased 11β-HSD1 oxoreductase activity results in elevated intracellular activation of GR, causing upregulation of key enzymes for gluconeogenesis. Excessive glucocorticoid action antagonizes the metabolic actions of insulin, causing insulin and leptin resistance, and leading to the development of visceral obesity, type 2 diabetes and the metabolic syndrome (Sandeep & Walker 2001). The consequences of altered 11β-HSD1 expression have been demonstrated recently in transgenic mice. Hepatic overexpression of 11β-HSD1 caused mild insulin resistance without changes in fat depot mass (Paterson *et al.* 2004). These

0952–5041/05/035–089
DOI: 10.1677/jme.1.01736
Online version via http://www.endocrinology-journals.org

animals exhibit fatty liver and dyslipidemia and are hypertensive with increased liver angiotensinogen expression. Transgenic mice overexpressing 11β-HSD1 in adipose tissue develop visceral obesity, have elevated adipose corticosterone concentrations and are susceptible to insulin-resistant diabetes, hyperlipidemia and high arterial blood pressure due to increased sensitivity to dietary salt and increased plasma levels of angiotensinogen, angiotensin II and aldosterone (Masuzaki *et al.* 2001, 2003). In contrast, mice with targeted disruption of 11β-HSD1 show, upon starvation, attenuated activation of key hepatic gluconeogenic enzymes and are resistant to hyperglycemia provoked by obesity or stress (Kotelevtsev *et al.* 1997, Morton *et al.* 2004). Moreover, in humans, 11β-HSD1 expression in subcutaneous adipose tissue is increased in acquired obesity and positively correlates to accumulation of subcutaneous and intra-abdominal fat, body-mass index, percentage of body fat and waist circumference, as well as fasting glucose, insulin levels and insulin resistance (Paulmyer-Lacroix *et al.* 2002, Rask *et al.* 2002, Lindsay *et al.* 2003, Kannisto *et al.* 2004, Valsamakis *et al.* 2004).

Therefore, 11β-HSD1 is currently considered a promising therapeutic target for the treatment of patients with metabolic and endocrine disorders (Masuzaki & Flier 2003, Chrousos 2004). Indeed, it was shown recently that the administration of a selective 11β-HSD1 inhibitor reduces blood glucose levels and increases insulin sensitivity in diabetic mice (Barf *et al.* 2002, Alberts *et al.* 2003). Furthermore, inhibition of 11β- HSD1 improved cognitive function in healthy elderly men and in type 2 diabetics (Sandeep *et al.* 2004) and lowered intraocular pressure in patients with ocular hypertension (Rauz *et al.* 2003). These findings induced a vigorous search for selective 11β-HSD1 inhibitors.

The effects of novel compounds are generally investigated in obese and diabetic mice and in other small laboratory animals. Since rodent physiology is different from human, larger animals such as dogs are commonly used in drug development for safety and efficacy assessment. Despite significant differences between human and animal glucocorticoid metabolism and the importance of 11β-HSD1, relatively little is known about the species-specific differences in substrate and inhibitor specificity of 11β-HSD1. Recently, studies from our laboratory revealed a novel function of 11β-HSD1 in the metabolism of 7-ketocholesterol (7KC), which may play a role in the development of atherosclerosis and dyslipidemia (Schweizer *et al.* 2004). While human and rat 11β-HSD1 catalyzed the stereospecific oxoreduction of 7KC to 7β-hydroxycholesterol, the hamster enzyme was not stereospecific and led to the formation of both 7α- and 7β-hydroxycholesterol, indicating significant species-specific differences in 11β-HSD1 activity. In the present study, we cloned and characterized canine 11β-HSD1; compared the specificity of 11β-HSD1 from six species for the substrates cortisone, 11-dehydrocorticosterone and 7KC; and determined the species-specific effects of various inhibitors.

Materials and methods

Materials

Cell culture media were purchased from Invitrogen (Carlsbad, CA, USA); [1,2,6,7-^{3}H]-cortisone from American Radiolabeled Chemicals (St Louis, MO, USA); cortisone, 11-dehydrocorticosterone and 7KC from Steraloids (Wilton, NH, USA); and reagents for derivatization from Pierce (Rockford, IL, USA).

Radiolabeled 11-dehydrocorticosterone was produced from [1,2,6,7-^{3}H]-corticosterone, as described previously (Schweizer *et al.* 2004). Chenodeoxycholic acid (CDCA), abietic acid, flavanone, 2′-hydroxyflavanone, glycyrrhetinic acid (GA) and carbenoxolone (CBX) were from Fluka AG (Buchs, Switzerland), and were of the highest grade available. 5H-1,2,4-Triazolo(4,3-a)azepine, 6,7,8,9-tetrahydro-3-tricyclo(3·3·1·13·7)dec-1-yl-327093–42–5 (T0504) was purchased from Enamine (Kiev, Ukraine).

Construction of 11β-HSD1 expression plasmids

The cloning of human (Tannin *et al.* 1991), rat (Agarwal *et al.* 1989), mouse (Oppermann *et al.* 1995, Rajan *et al.* 1995) and guinea-pig 11β-HSD1 cDNA (Pu & Yang 2000) has been reported earlier, as has the construction of untagged and FLAG epitope-tagged human, rat and hamster 11β-HSD1 (Odermatt *et al.* 1999, Schweizer *et al.* 2004). Mouse and guinea-pig 11β-HSD1 were amplified by PCR using specific oligonucleotide primers based on available sequences from GenBank. Canine 11β-HSD1 was cloned from the liver of a poodle. Total RNA was isolated with the Trizol reagent, according to the instructions of the manufacturer (Invitrogen), followed by reverse transcription and synthesis of double-stranded cDNA. 11β-HSD1 was amplified by PCR using Taq-polymerase:Pfu-polymerase (10:1). A degenerated oligonucleotide at the 5′-end (5′-TATG GATCCAGCCAGNTCCCTGTYDGATG-3′), based on comparison with the sequences upstream of the initiation codon of 11β-HSD1 from other species, and oligo-dT_{20}, containing an XbaI site, at the 3′-end were used for initial PCR amplification. A single DNA product was obtained, cleaved with BamHI and XbaI and cloned into Bluescript vector. The sequences of 8 of 10 clones analyzed were identical. Two products contained errors introduced by the polymerase and were discarded. For the construction of 11β-HSD1 expression plasmids, all sequences were modified by PCR amplification, using an oligonucleotide at the 5′-end,

containing a BamHI endonuclease restriction site followed by a Kozak consensus sequence, the ATG initiation codon and 18–20 nucleotides of species-specific 11β-HSD1 sequence, and an oligonucleotide at the 3′-end containing specific-specific sequence, 24 nucleotides encoding the FLAG epitope, the stop codon and an XbaI endonuclease restriction site. The sequence encoding the FLAG epitope was omitted in the primer used for the construction of the untagged expression plasmid. The PCR product was cleaved with BamHI and XbaI and cloned into pcDNA3·1 expression vector (Invitrogen). All expression constructs were verified by sequencing.

Cell culture, protein expression and Western blotting

HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin, 50 mg/ml streptomycin and 2 mM glutamine, followed by transfection with FLAG-tagged 11β-HSD1 constructs (10 μg plasmid DNA per 10 cm culture dish) using the Ca^{2+}-phosphate precipitation method. Cells were washed three times 24 h after transfection with steroid-free (doubly charcoal-treated) medium and grown for another 24 h. Cells were then detached with PBS, centrifuged for 3 min at 150 ***g*** and resuspended in the appropriate volume of TS2 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 250 mM sucrose, 20 mM Tris–HCl, pH 7·4). Cells were lyzed by sonication followed by immediate analysis of enzyme activities and protein expression. Protein concentrations were determined by the Bradford assay. The expression level in different transfection experiments was determined semiquantitatively by immunoblotting. An amount of 30 μg total proteins was separated on 12% SDS-polyacrylamide gels and transferred electrophoretically to polyvinyl difluorid (PVDF) membranes (Amersham), followed by visualization with mouse monoclonal anti-FLAG antibody M2 (Invitrogen), horseradish peroxidase (HRP)-conjugated goat antimouse immunoglobulin (Ig)G and the enhanced chemiluminescence (ECL) Western detection system from Pierce (Rockford, IL, USA). Upon staining for 11β-HSD1, the membranes were stripped, and the expression of β-actin was determined. Data were analyzed by scanning densitometry with NIH Image 1·60b7 software.

Determination of 11β-HSD1-dependent oxoreduction of cortisone and 11-dehydrocorticosterone

The 11β-HSD1-dependent 11-oxoreduction of cortisone and 11-dehydrocorticosterone was determined by measuring the conversion of cortisone to cortisol, or 11-dehydrocorticosterone to corticosterone in cell lysates in the presence of radiolabeled tracer, as described recently (Atanasov *et al.* 2004, Schweizer *et al.* 2004). Briefly, lysates were incubated in TS2 buffer containing 500 μM NADPH, 30 nCi [1,2,6,7-^{3}H]-labeled tracer and various concentrations of unlabeled substrate ranging from 20 nM to 2 μM. 11β-HSD2-dependent oxidation of cortisol was measured similarly with radiolabeled cortisol and NAD^+, as described earlier (Atanasov *et al.* 2003). Samples in a final volume of 20 μl were incubated at 37 °C for 10–15 min, reactions were stopped by adding 2 mM unlabeled glucocorticoids dissolved in methanol, and steroids were separated by thin-layer chromatography (TLC) and analyzed by scintillation counting. In all reactions, the conversion of cortisone was kept below 30%. For calculation of V_{max}, the expression level of the FLAG-tagged enzyme was compared with the expression signal from β-actin as an internal control. Apparent K_m and V_{max} values of the 11β-HSD1-dependent oxoreduction of cortisone or 11-dehydrocorticosterone were calculated by nonlinear regression, assuming Michaelis–Menten kinetics. Comparable results were obtained by linear transformations (Eadie–Hofstee and Lineweaver–Burk equations). Results are expressed as mean ± S.D. and consist of three independent measurements.

Analysis of 11β-HSD1 inhibitors

Measurements of 11-oxoreductase activity at 37 °C for 10 min were started by simultaneously adding 10 μl cell lysate and 12 μl TS2 buffer containing NADPH (400 μM final concentration), 30 nCi [1,2,6,7-^{3}H]-cortisone, unlabeled cortisone (200 nM final concentration) and the appropriate concentration of the inhibitory compound to be tested. Stock solutions of the compounds in methanol or in DMSO were diluted in TS2 buffer to yield the appropriate concentrations, whereby the concentration of methanol or DMSO in the reactions was below 0·1%. Control reactions with or without 0·1% of the solvent showed the same activity. Reactions were performed with 200 nM radiolabeled cortisone for 10 min at 37 °C followed by termination by adding 2 mM of unlabeled steroids in methanol and determination of the conversion of cortisone to cortisol as described above. The oxoreduction of 11-dehydrocorticosterone was measured similarly, using 11-dehydrocorticosterone and corticosterone respectively. All inhibitors analyzed in the present study yielded typical sigmoid dose–response curves, indicating competition at a single substrate binding site.

To measure IC_{50} values in intact cells, cells were detached 24 h after transfection, and 30 000–40 000 cells per well of a 96-well plate were incubated in standard DMEM for another 16 h. The conversion of cortisone to cortisol was then determined upon incubation in serum- and steroid-free medium for 2–4 h at 37 °C in a total volume of 50 μl medium cotaining

200 nM radiolabeled cortisone and the corresponding concentration of inhibitor in the absence of exogenous cofactor. The amount of solvent did not exceed 0·1%. Reactions were stopped by adding 25 µl methanol containing 2 mM unlabeled steroid, followed by separation of steroids by TLC.

IC_{50} values were calculated with the Data Analysis Toolbox (Elsevier MDL, San Leandro, CA, USA), assuming first-order rate kinetics. K_i values were calculated from IC_{50} values by the equation of Cheng and Prusoff (1973). Data represent mean ± S.D. from at least four independent transfections.

Determination of 11β-HSD1-dependent oxoreduction of 7KC

The 7-oxoreductase activity of 11β-HSD1 was determined as described recently (Schweizer *et al.* 2004). Briefly, freshly prepared lysates expressing 11β-HSD1 of the corresponding species were suspended in TG1 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 20% glycerol, 20 mM Tris–HCl, pH 7·4) and the reactions carried out in a final reaction volume of 1 ml in the presence of 400 µM NADPH and 800 nM 7KC for 30 min at 37 °C. Reactions were stopped by adding 7 ml dichloromethane followed by derivatization and analysis by gas chromatography-mass spectrometry (GC-MS). Data represent mean ± S.D. from three independent transfections.

Molecular modeling

Structure-based pharmacophore models for 11β-HSD1 inhibitors were derived from the recently published x-ray crystal structures 1XU7 (resolution 0·18 nm), 1XU9 (resolution 0·16 nm), and 2 BEL (resolution 0·21 nm) from the PDB (Berman *et al.* 2000). An additional model consisting only of the features common to 1XU7, 1XU9, and 2 BEL – probably the most important features for 11β-HSD1 inhibition – was created automatically by LigandScout (software available from Inte:ligand GmbH, Maria Enzersdorf, Austria, (www.inteligand.com) (Wolber & Langer 2005). The investigated inhibitors were fitted into a manually constructed pharmacophore model derived from 1XU9 and the common feature model employing the BestFit module of the software package Catalyst (Accelrys Inc., San Diego, CA, USA, Catalyst Version 4·9., www.accelrys.com).

Results

Cloning of 11β-HSD1 from dog and guinea pig and comparison with sequences from other species

In addition to the previously characterized hamster, rat and human 11β-HSD1 enzymes (Schweizer *et al.* 2004), we have now cloned 11β-HSD1 from mouse and guinea pig with oligonucleotide primers based on sequences available from GenBank (Oppermann *et al.* 1995, Rajan *et al.* 1995). The murine sequence was identical to that present in GenBank. We found three nucleotide differences for guinea-pig 11β-HSD1: T for C at position 64 (initiation codon set as position 1), resulting in Ser^{22} for Pro; T for C at position 308, resulting in Val^{103} for Ala; and C for T at position 564, not affecting the amino-acid sequence. Residue Ser^{22}, which follows the Tyr motif in the transmembrane span, is conserved throughout all known 11β-HSD1 sequences (Fig. 1). Substitution of Ser by Pro would be critical because of disturbance of the membrane helix. We have previously shown that mutations in the Tyr motif cause partial loss of enzymatic activity, probably due to disturbed folding of the protein (Odermatt *et al.* 1999). Val^{103} is also conserved in all known 11β-HSD1 sequences, except in mouse, which has the closely related hydrophobic residue Ile. The presence of a small residue such as Ala at this position is less likely. In addition to small rodents, dogs are widely used in safety and efficacy studies in the development of pharmaceutical compounds. Therefore, we cloned canine 11β-HSD1. A sequence comparison revealed 82% identity and 90% similarity of human 11β-HSD1 with that from dog and 74–79% identity and 85–89% similarity with those from hamster, guinea pig, mouse and rat, indicating that dog 11β-HSD1 is only slightly closer to the human enzyme than the corresponding rodent sequences. As shown in Fig. 1, the sequences of 11β-HSD1 from various species are highly conserved with the exception of five variable regions. The first variable region contains a potential glycosylation site in seven out of the 15 known sequences. The second variable region precedes the catalytic center of the enzyme with the highly conserved Tyr residue, and the third variable region follows downstream of the catalytic center. The highest sequence variability exists in the C-terminus with a variable region after the residues involved in dimerization and with the region of highest sequence variability at the very C-terminal end.

Expression of 11β-HSD1 from six species

The recombinant enzymes from six species were expressed at comparable levels in HEK-293 cells (Fig. 2), indicated by the similar intensities of the signals for 11β-HSD1 and β-actin on each blot. As shown in Fig. 2, human and canine 11β-HSD1 have the highest molecular mass (~35 kDa), followed by rat, mouse and hamster 11β-HSD1 (~33 kDa) and guinea pig (~32 kDa). Inspection of the sequences indicated that the differences in size may be due to distinct glycosylation. Prediction of potential glycosylation sites, using the NetNGlyc 1·0 server of the center for biologic sequence analysis at the Technical University of Denmark (www.cbs.dtu.dk/services/), suggests that human and canine 11β-HSD1

Figure 1 Alignment of the known amino-acid sequences of 11β-HSD1 from various species. Identical residues are shaded and the regions of high-sequence variability indicated with a line (regions I–V). Potential glycosylation sites are indicated by an asterisk, the N-terminal transmembrane helix flanked by charged residues determining the orientation of 11β-HSD1 is indicated by circles (positive) and diamonds (negative), the catalytic Tyr and Lys residues are indicated by (+) and the conserved glycine motif of the Rossmann fold in the cofactor-binding region is indicated by (#). Accession numbers of the 11β-HSD1 sequences are as follows: human NM_005525, chimpanzee AY410503, rhesus monkey CO726805, rat NM_017080, mouse NM_008288, hamster, AY519498, guinea pig AY535424, dog AY728264, squirrel monkey S63400, pig NM_214248, cow AF548027, sheep NM_001009395, rabbit Q7 M3I4, chicken BG710883.

are glycosylated at Asn^{123}, Asn^{162} and Asn^{207}. As a control, human 11β-HSD1 was transcribed and translated *in vitro*, using rabbit reticulocyte lysate and dog pancreas microsomes. Unglycosylated enzyme (band at ~29 kDa) and enzyme glycosylated at one, two or all three sites was detected (Fig. 2). The band observed upon expression of human 11β-HSD1 in HEK-293 cells corresponds to the fully glycosylated product. Asn^{123} is

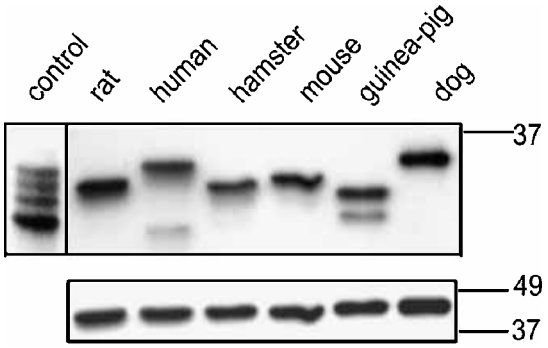

Figure 2 Expression of FLAG-tagged 11β-HSD1 from various species. FLAG-tagged 11β-HSD1 constructs from various species were expressed in HEK-293 cells. An amount of 30 μg total protein was separated by 12% SDS–PAGE, transferred to PVDF membranes and detected with monoclonal anti-FLAG antibody, as described in Materials and methods. After stripping of the membrane, the expression of β-actin was detected as an internal control for the amount of protein. The upper panel shows the expression of 11β-HSD1 from various species. The first lane shows 11β-HSD1 protein after *in vitro* transcription and translation in the rabbit reticulocyte system, using dog pancreas microsomes as a control. Expression of β-actin in the same samples is depicted in the bottom panel. The size of protein standard (kDa) is indicated.

located in a region with high sequence variability and is replaced by Gln in rat and mouse and by Tyr in hamster and guinea pig. Asn^{162} is conserved in all 11β-HSD1 sequences except guinea-pig (Gln), sheep (Ser) and the partial chicken sequence, and Asn^{207} is present in all known 11β-HSD1 sequences (Fig. 1).

Variability in substrate specificity

To identify species-specific substrate variability in 11β-HSD1, we compared the 11-oxoreduction of cortisone and 11-dehydrocorticosterone by 11β-HSD1 from human, dog, mouse, rat, hamster and guinea pig. As shown in Table 1, there were no significant differences in kinetic parameters of human, hamster and guinea-pig 11β-HSD1 with the substrate cortisone. Compared with the human enzyme, a reduced V_{max} was measured for murine 11β-HSD1 and a decreased substrate affinity for rat 11β-HSD1, resulting for both enzymes in a 50% lower catalytic efficiency (V_{max}/K_m). Despite the closest sequence similarity to human 11β-HSD1, the canine enzyme showed both lower substrate affinity and V_{max}, resulting in fivefold lower catalytic efficiency. As with cortisone, human, hamster and guinea-pig 11β-HSD1 showed comparable catalytic activities for the oxoreduction of 11-dehydrocorticosterone. The rat enzyme displayed lower affinity for 11-dehydrocorticosterone but higher V_{max}, while the dog enzyme had both lower affinity and V_{max}, resembling the pattern obtained with cortisone. Unlike with cortisone, mouse 11β-HSD1 more efficiently catalyzed the oxoreduction of 11-dehydrocorticosterone compared with the human enzyme. Recently, we demonstrated a novel function for 11β-HSD1 by catalyzing the 7-oxoreduction of 7KC (Schweizer *et al.* 2004). Here, we confirm the finding that human and rat 11β-HSD1 stereospecifically reduce 7KC to 7β-hydroxycholesterol, whereas the 7-oxoreductase activity of the hamster enzyme results in formation of both 7α- and 7β-hydroxycholesterol (Fig. 3). We extend the previous findings by further showing that mouse, dog and guinea-pig 11β-HSD1 also stereospecifically lead to the formation of 7β-hydroxycholesterol, whereby, the guinea-pig and dog enzymes have very low 7-oxoreductase activities.

Species-specific effects of 11β-HSD1 inhibitors measured in cell lysates

The significant species-specific variability in the catalytic efficiency of 11β-HSD1 for its substrates led us to analyze the inhibitory potency of various compounds, including endogenous inhibitors, natural compounds and pharmaceutical chemicals (Fig. 4). The best-known inhibitors of 11β-HSD enzymes are glycyrrhetinic acid

Table 1 Kinetic parameters of 11β-HSD1 from various species. FLAG-tagged 11β-HSD1 cDNAs from six species were each expressed in HEK-293 cells. Cells were then incubated in steroid-free medium for 24 h, lysates were prepared and the conversion of radiolabeled cortisone to cortisol or 11-dehydrocorticosterone to corticosterone was measured immediately as described in Materials and methods. Data were analyzed by nonlinear regression and represent mean ± S.D. from three independent experiments.

	Oxoreduction of cortisone			Oxoreduction of 11-dehydrocorticosterone		
	K_m [nM]	V_{max} [nmol×h^{-1}×mg^{-1}]	V_{max}/K_m	K_m [nM]	V_{max} [nmol×h^{-1}×mg^{-1}]	V_{max}/K_m
Species						
Human	519 ± 71	7·8 ± 1·3	0·015	420 ± 87	2·1 ± 0·4	0·005
Dog	1571 ± 304*	4·5 ± 0·8*	0·003*	571 ± 89	1·5 ± 0·2*	0·003*
Rat	1362 ± 87*	9·5 ± 1·0	0·007*	852 ± 122*	3·9 ± 1·2*	0·005
Mouse	515 ± 45	4·1 ± 0·5*	0·008*	384 ± 28	3·0 ± 0·9	0·008*
Hamster	568 ± 99	8·6 ± 0·6	0·015	449 ± 67	2·9 ± 0·5	0·006
Guinea pig	638 ± 109	6·8 ± 1·3	0·011	425 ± 48	2·7 ± 0·6	0·006

*$p < 0·05$ compared with human 11β-HSD1.

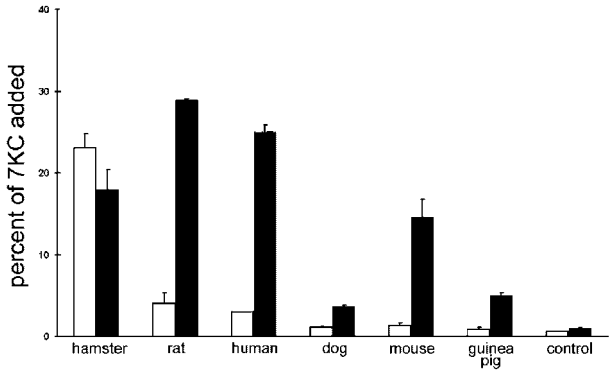

Figure 3 Metabolism of 7-ketocholesterol (7KC) by recombinant 11β-HSD1 in cell lysates. Enzymatic activities of recombinant human, dog, rat, mouse, hamster and guinea-pig 11β-HSD1 were determined in freshly prepared HEK-293 cell lysates upon incubation with 800 nM 7KC for 30 min at 37 °C, as described in Materials and methods. Background activity is shown in untransfected HEK-293 cell lysates (control); 7α-hydroxycholesterol, white bars; 7β-hydroxycholesterol, black bars. Results are expressed as a percentage of initially supplied 7KC.

(GA) and its synthetic analog carbenoxolone (CBX). Analysis of human 11β-HSD1 and 11β-HSD2 revealed that both enzymes are efficiently inhibited by GA and CBX, whereby GA preferentially inhibited the latter enzyme (Table 2). A species comparison revealed that GA most potently inhibited the oxoreduction of cortisone by mouse and dog 11β-HSD1 with two- to threefold lower K_i than 11β-HSD1 from human, rat, hamster and guinea pig. The closely related synthetic compound CBX inhibited mouse, rat and human 11β-HSD1 with K_i values very close to those obtained with GA. In contrast, CBX was about three times less effective than GA in inhibiting dog, hamster and guinea-pig 11β-HSD1. CBX inhibited murine 11β-HSD1 nine to ten times better than that of hamster and guinea pig. Abietic acid, a naturally occurring diterpenoid, widely used in cosmetics, that inhibits both 11β-HSD1 and 11β-HSD2 (Schweizer *et al.* 2003), most potently inhibited rat 11β-HSD1; showed similar effects on human, dog, mouse and hamster enzymes; but was a very weak inhibitor of guinea-pig 11β-HSD1. In contrast, flavanone and 2′-hydroxyflavanone, present in yellow and red fruits and vegetables, both selectively inhibited 11β-HSD1, but not 11β-HSD2, and showed efficient inhibition of the hamster and guinea-pig enzymes, with only weak effects on 11β-HSD1 from human, dog, rat or mouse. In addition, we compared the inhibitory effect of the bile acid CDCA on the 11β-HSD1-dependent oxoreduction of cortisone from the six species and observed dramatic differences (Table 2). CDCA potently inhibited rat and canine 11β-HSD1 with K_i values of 550 and 770 nM, had intermediate effects on murine and human 11β-HSD1 with fivefold higher K_i values, and showed very weak inhibitory effects on hamster and guinea-pig 11β-HSD1 with 50-fold higher K_i values.

Currently, there is considerable effort in the pharmaceutical industry to develop highly potent and selective inhibitors for 11β-HSD1. To investigate whether such compounds have distinct effects on 11β-HSD1 from various species, we have chosen compound T0504 from a compound library of Enamine Ltd, which is described by Merck in patent WO 03/065983. This compound displayed approximately 100-fold selectivity for the inhibition of human 11β-HSD1 (K_i 15 nM) compared with human 11β-HSD2 (K_i 2 μM). Although 11β-HSD1 from all species tested were potently inhibited, analysis of the six species revealed significant differences. Hamster 11β-HSD1 was most potently inhibited with a K_i of 6 nM, followed by human 11β-HSD1 with a K_i of 15 nM. The inhibitory effect on rat 11β-HSD1 was seven times lower and for dog, mouse and guinea-pig 11β-HSD1 over 10 times

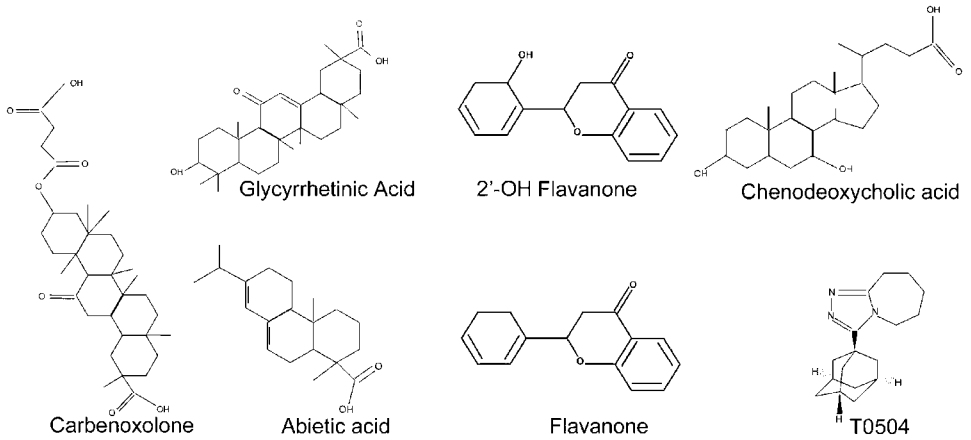

Figure 4 11β-HSD1 inhibitors used in the present study.

Table 2 Determination of the inhibitory effects of various compounds on the activities of 11β-HSD1 from six species and of human 11βHSD2 in cell lysates. 11β-HSD activities were measured in cell lysates as described in Materials and methods. IC_{50} and K_i values are in μM. Data represent mean ± S.D. from at least four independent experiments

	GA	CBX	ABAC	FLAV	2′-OH- FLAV	CDCA	T0504
Inhibition of 11β-HSD2-dependent oxidation of cortisol							
Human							
IC_{50}	0·031 ± ·007[a]	0·65 ± ·024	12 ± 2[a]	>200[a]	>200[a]	22 ± 2[b]	2·0 ± 0·7
K_i	0·028	0·59	11	>200	>200	20	1·8
Inhibition of 11β-HSD1-dependent oxoreduction of cortisone							
Human							
IC_{50}	0·40 ± 0·08	0·47 ± 0·11	5·4 ± 0·4	21 ± 3	24 ± 3	4·1 ± 0·6	0·021 ± 0·003
K_i	0·29	0·34	3·9	15	17	3·0	0·015
Dog							
IC_{50}	0·13 ± 0·02*	0·32 ± 0·06	5·8 ± 0·4	39 ± 8*	109· ± 1·2*	0·87 ± 0·06*	0·097 ± 0·011*
K_i	0·12	0·28	5·2	35*	9·7*	0·77*	0·080*
Rat							
IC_{50}	0·50 ± 0·09	0·40 ± 0·12	2·4 ± 0·3*	62 ± 14*	57 ± 16*	0·63 ± 0·11*	0·050 ± 0·007*
K_i	0·44	0·35	2·1*	54*	50*	0·55*	0·43*
Mouse							
IC_{50}	0·16 ± 0·04*	0·15 ± 0·03*	8·9 ± 0·8*	47 ± 9*	26 ± 2	3·4 ± 0·3	0·114 ± 0·018
K_i	0·12*	0·11*	6·4*	34*	19	2·5	0·082*
Hamster							
IC_{50}	0·34 ± 0·11	1·27 ± 0·36*	8·3 ± 0·6*	1·7 ± 0·4*	2·7 ± 0·8*	47 ± 9*	0·008 ± 0·001*
K_i	0·25	0·94*	6·1*	1·2*	2·0*	35*	0·006*
Guinea pig							
IC_{50}	0·43 ± 0·11	1·30 ± 0·15*	35 ± 4*	2·4 ± 0·3*	2·5 ± 0·8*	32 ± 5*	0·107 ± 0·01*
K_i	0·33	0·99*	27*	1·8*	1·9*	24*	0·081*
Inhibition of 11β-HSD1-dependent oxoreduction of 11-dehydrocorticosterone							
Human							
IC_{50}	0·41 ± 0·10	0·33 ± 0·04	9·7 ± 0·8*	43 ± 5*	6·7 ± 1·8*	3·0 ± 0·3*	0·035 ± 0·005*
K_i	0·30	0·23	7·0*	31*	4·9*	2·1*	0·025

[a]Values were taken from (Schweizer *et al.* 2003); [b]value was taken from (Stauffer *et al.* 2002).
*$p < 0·05$ compared with the cortisone reduction by human 11β-HSD1.

lower, confirming the existence of huge species-specific differences of inhibitory compounds on 11β-HSD1 activity. Next, we determined the dependence of the inhibitory effect from the substrate by measuring the inhibition of the oxoreduction of 11-dehydrocorticosterone by human 11β-HSD1 in the presence of various concentrations of the inhibitors. The compounds showed similar inhibitory effects when cortisone and 11-dehydrocorticosterone were used as substrates with rather modest differences. Importantly, flavanone and 2′-hydroxyflavanone comparably inhibited 11β-HSD1-dependent activity with cortisone, but the latter compound was sixfold more potent with 11-dehydrocorticosterone, indicating that subtle differences in the molecular structures of both substrate and inhibitor are critical for the competition with stabilizing interactions in the hydrophobic binding pocket.

Inhibitory effects in intact cells

To assess the inhibitory potency of these compounds in intact cells, we transiently expressed FLAG-tagged 11β-HSD1 constructs from the six species in HEK-293 cells and measured the oxoreduction of cortisone in the presence of increasing concentrations of inhibitor. The most potent inhibitor was the uncharged compound T0504 (IC_{50} 0·157 ± 0·037 μM). Both flavanone and 2′-hydroxyflavanone were more potent in intact cells than in cell lysates, with IC_{50} of 0·62 ± 0·08 μM and 3·08 ± 1·49 μM respectively, suggesting accumulation of these inhibitors. GA inhibited the oxoreduction of cortisone with an IC_{50} 1·64 ± 0·69 μM, while CBX was less potent (IC_{50} 10·4 ± 2·0 μM), indicating that the additional carboxy group hinders the entry of CBX into the cell. An unexpected inhibitory effect was observed for the bile acid CDCA. At concentrations up to 12 μM, a twofold activation of the 11β-HSD1-dependent oxoreduction of cortisone was measured, followed by a typical inhibition curve with an IC_{50} of 230 μM. The inhibitory effect at these high concentrations was not due to detergent effect, since similar concentrations of cholic acid did not inhibit 11β-HSD1 and the micellar concentration is in the millimolar range.

We then assessed the species-specific effect in intact cells for the most potent inhibitor T0504 and the relatively weak inhibitor abietic acid on 11β-HSD1-dependent oxoreduction of cortisone, to see whether the relative inhibitory potency observed in experiments with cell

Table 3 Determination of the inhibitory effects of various compounds on 11β-HSD1 in intact cells. 11β-HSD1-dependent oxoreduction of cortisone was measured in intact, transiently transfected HEK-293 cells, as described in Materials and methods. IC_{50} values are in μM. Data represent mean ± S.D. from four independent experiments.

	IC_{50} [μM]	
	ABAC	T0504
Species		
Human	15·4 ± 2·6	0·157 ± 0·037
Rat	10·9 ± 1·4*	0·274 ± 0·054*
Mouse	38·6 ± 10·3*	1·66 ± 0·42*
Hamster	n.d.	0·101 ± 0·014*
Guinea-pig	103 ± 15*	0·805 ± 0·138*

n.d., not determined.
*$p < 0·05$ compared with human 11β-HSD1.

lysates can be reproduced in intact cells. As shown in Table 3, the relative inhibitory strength of both compounds measured in intact cells followed that obtained in cell lysates. Hamster 11β-HSD1 was over 10-fold more sensitive to T0504 than the mouse enzyme, while rat 11β-HSD1 was inhibited at 10-fold lower abietic acid concentrations than the guinea-pig enzyme.

Discussion

In the present study, we demonstrate significant species-specific variability of the enzymatic properties of 11β-HSD1 for its substrates cortisone and 11-dehydrocorticosterone as well as for its alternative substrate 7KC. These differences have to be considered in trying to extrapolate results obtained from animal experiments to humans. Corticosterone is the major glucocorticoid in mice and rats, whereas cortisol is the major glucocorticoid in humans, thus limiting the use of these most commonly used rodents to study effects on glucocorticoid metabolism and action. Cortisol concentrations reach approximately 30% of the levels of corticosterone in hamsters (Ottenweller *et al.* 1985). In guinea pig, cortisol is the predominant glucocorticoid (Dalle & Delost 1976); however, this animal is relatively resistant to glucocorticoids and shows a reduced affinity of cortisol to GR compared with humans (Keightley *et al.* 1998). Although some species use cortisone (human, dog, guinea pig) and others 11-dehydrocorticosterone (hamster, rat, mouse), we did not find a correlation between substrate use and affinity. In the assessment of the safety and efficacy of drugs, dogs are often used as a second animal model in addition to rats or mice. Our finding of significantly lower catalytic efficiency of canine than human 11β-HSD1, due to both reduced substrate affinity and lower maximal activity, is somewhat surprising and indicates potential limitations of the dog as a suitable model for the development of therapeutic 11β-HSD1 inhibitors.

We recently provided evidence that 11β-HSD1 plays a role in the metabolism of 7KC (Schweizer *et al.* 2004). 7KC is the major dietary oxysterol formed during processing of cholesterol-rich food and is the major oxidation product of cholesterol found in human atherosclerotic plaques (Lyons & Brown 1999). It is not clear whether the long-term treatment of humans with specific 11β-HSD1 inhibitors may lead to an accumulation of 7KC and have a negative impact on health by promoting the development of atherosclerosis and dyslipidemia. To assess a potential risk from accumulation of 7KC upon long-term treatment with 11β-HSD1 inhibitors, experiments in suitable animal models have to be performed. Because of their low 7-oxoreductase activities, guinea pigs and dogs are not suitable models for this purpose. In these animals, alternative pathways, such as 27-hydroxylase (Brown *et al.* 2000), may be responsible for the metabolism of 7KC and compensate for the lack of 11β-HSD1-dependent 7KC reduction. Our finding of very low 7-oxoreductase activity of guinea-pig 11β-HSD1 explains previous observations by Maeda *et al.* (2002), who reported background conversion of 7-oxycholesterols in guinea-pig liver microsomes. Due to the lack of stereospecificity of hamster 11β-HSD1, this animal model may also have its limitations, since the formation of 7α-hydroxycholesterol, the first step in the elimination of cholesterol through the bile acid pathway, may lead to a more rapid removal of 7KC. Rats and mice efficiently convert 7KC (Hult *et al.* 2004, Schweizer *et al.* 2004) and may be the most suitable animal models to study the interference of 11β-HSD1 inhibitors with the detoxification of 7KC.

Furthermore, our results show significant species-specific variability of the inhibitory effects of terpenoids, flavanoids, bile acids and other synthetic chemicals on 11β-HSD1 11-oxoreductase activity, further emphasizing that care should be taken when extrapolating results obtained from one animal model to other animals or to humans. The interpretation of data obtained from different species is most critical for inhibitors with a relatively low selectivity. CBX, an 11β-HSD inhibitor widely used in research, shows only a slight preference to inhibit human 11β-HSD1 (IC_{50} 0·47 μM) compared with human and mouse 11β-HSD2 (IC_{50} 0·65 μM and 0·60 μM). However, CBX preferentially inhibits murine 11β-HSD2 (IC_{50} 0·15 μM). Thus, CBX may preferentially inhibit 11β-HSD1 in mouse disease models, whereas it is expected to cause more pronounced side effects in humans than in mice through inhibition of 11β-HSD2, which results in cortisol-dependent activation of the mineralocorticoid receptor and hypertension (Frey *et al.* 2004).

CDCA has been used to treat cholesterol cholelithiasis in humans (Dyrszka *et al.* 1975, Okun *et al.* 1982), without significant toxicity. In contrast, lesions developed in rats treated with comparable concentrations of CDCA. There is controversy about the inhibitory effect of the bile acid CDCA on 11β-HSDs. Diederich *et al.* (2000) suggested that CDCA acts as a selective 11β-HSD1 inhibitor, with an IC_{50} of 2·8 μM obtained with human hepatic microsomes. Morris *et al.* (2004) reported inhibition of the dehydrogenase activity of 11β-HSD1 with IC_{50} values of 0·2–7 μM, but only 37–200 μM for the oxoreductase activity in rat liver microsomes. They found weak inhibition of 11β-HSD2 from sheep kidney, with an IC_{50} of 70 μM. The use of different tissues and species makes it difficult to interpret these results and compare them with others. We have recently reported an IC_{50} value for CDCA of 22 μM for the inhibition of human 11β-HSD2, demonstrated CDCA-dependent, cortisol-induced activation of the mineralocorticoid receptor in intact cells, and provided evidence for bile acid-dependent inhibition of 11β-HSD2 in patients with cholestasis (Quattropani *et al.* 2001, Stauffer *et al.* 2002). The comparison of K_i values of human 11β-HSD1 and 11β-HSD2 revealed sevenfold selectivity of CDCA to inhibit 11β-HSD1. The comparison of the six species shows that CDCA is a potent inhibitor of rat and canine 11β-HSD1, with over 50-fold higher potency than hamster and guinea-pig 11β-HSD1. Our measurements with intact HEK-293 cells transiently expressing human 11β-HSD1 emphasize that the relative inhibition of 11β-HSD1 and 11β-HSD2 seen in cell lysates cannot be directly extrapolated to intact cells. In intact HEK-293 cells, CDCA showed a biphasic effect with activation at low concentrations followed by inhibition at high concentrations. Although CDCA was able to inhibit 11β-HSD2, which is oriented into the cytoplasm, and mediates cortisol-dependent activation of mineralocorticoid receptor (Stauffer *et al.* 2002), it was unable to block the 11β-HSD1-dependent conversion of cortisone in the endoplasmic reticulum lumen and prevent subsequent GR activation (not shown). The huge variability found with different species and assay systems may explain some of the controversial findings, and it further emphasizes the necessity of the appropriate use of suitable cell systems and animal models.

In contrast to CDCA and abietic acid, the two selective 11β-HSD1 inhibitors flavanone and 2′-hydroxyflavanone (Schweizer *et al.* 2004) most efficiently inhibited hamster and guinea-pig 11β-HSD1, but were very weak inhibitors of the rat and mouse enzymes, suggesting differences in the binding of flavanoids, compared with bile acids and diterpenoids, to the hydrophobic pocket. The subtle difference of the presence of a single hydroxyl group enhanced the potency of 2′-hydroxyflavanone to inhibit the oxoreduction of cortisone by twofold in mouse and fourfold in canine 11β-HSD1. Furthermore, the distinct inhibition of the oxoreduction of the two substrates cortisone and 11-dehydrocorticosterone by flavanone and 2′-hydroxyflavanone suggests that such compounds may exert different physiologic effects, depending on whether cortisone- or 11-dehydrocorticosterone-based species are used in the assessment of inhibitory effects. The pharmaceutical compound T0504 is a highly potent inhibitor of human 11β-HSD1 and shows 100-fold selectivity toward human 11β-HSD2. The significant species effects were also observed for this compound, with the lowest K_i value for 11β-HSD1 from hamster, a threefold higher K_i for human 11β-HSD1 and over 10-fold higher K_i values for mouse and dog 11β-HSD1. These species-specific effects may become relevant in investigating the inhibition of 11β-HSD1 *in vivo*. If human and mouse 11β-HSD2 were similarly inhibited by T0504 (remains to be determined), the selectivity to inhibit 11β-HSD1 would be limited to 10-fold in mouse and side effects would be more likely to occur.

The experimental data suggest that the chemicals analyzed in the present study competitively inhibit 11β-HSD1. Figure 5A shows a pharmacophore model based on the solved crystal structure of 11β-HSD1 (1XU9) with cortisone in the binding pocket. CBX, GA and CDCA fitted well into the 1XU9 model consisting of five hydrogen bond acceptor features and three hydrophobic features, and all three inhibitors seem to occupy competitively the cortisone-binding pocket (Fig. 5B). Abietic acid, flavanone, 2′-hydroxyflavanone and T0504 are comparably small compounds and do not possess enough chemical features to fit into the pharmacophore; therefore, they were fitted into the common feature model consisting of three hydrophobic features and one hydrogen bond acceptor feature. The common features represent hydrophobic regions in close vicinity to Leu^{217} (A), Ala^{172} (B) and Leu^{126}/Ala^{226} (C) as well as a hydrogen bond to Tyr^{177}. All four inhibitors fitted into the common feature model of 11β-HSD1, indicating that they are able to occupy sites of the ligand-binding domain important for enzyme inhibition (Fig. 5C). The modeling approach showed that the presented inhibitors are all able to fit into the ligand-binding domain of 11β-HSD1; however, as this pharmacophore describes the interaction sites between a ligand and the protein qualitatively, no quantitative information about binding strength can be gained from such a model. From the common feature model, it can be concluded that three hydrophobic sites and one hydrogen bond play essential roles in enzyme inhibition. To gain further insight into inhibition mechanisms of 11β-HSD1, expanded molecular modeling studies will be required.

In conclusion, our results revealed significant species-specific differences in the inhibition of 11β-HSD1 by

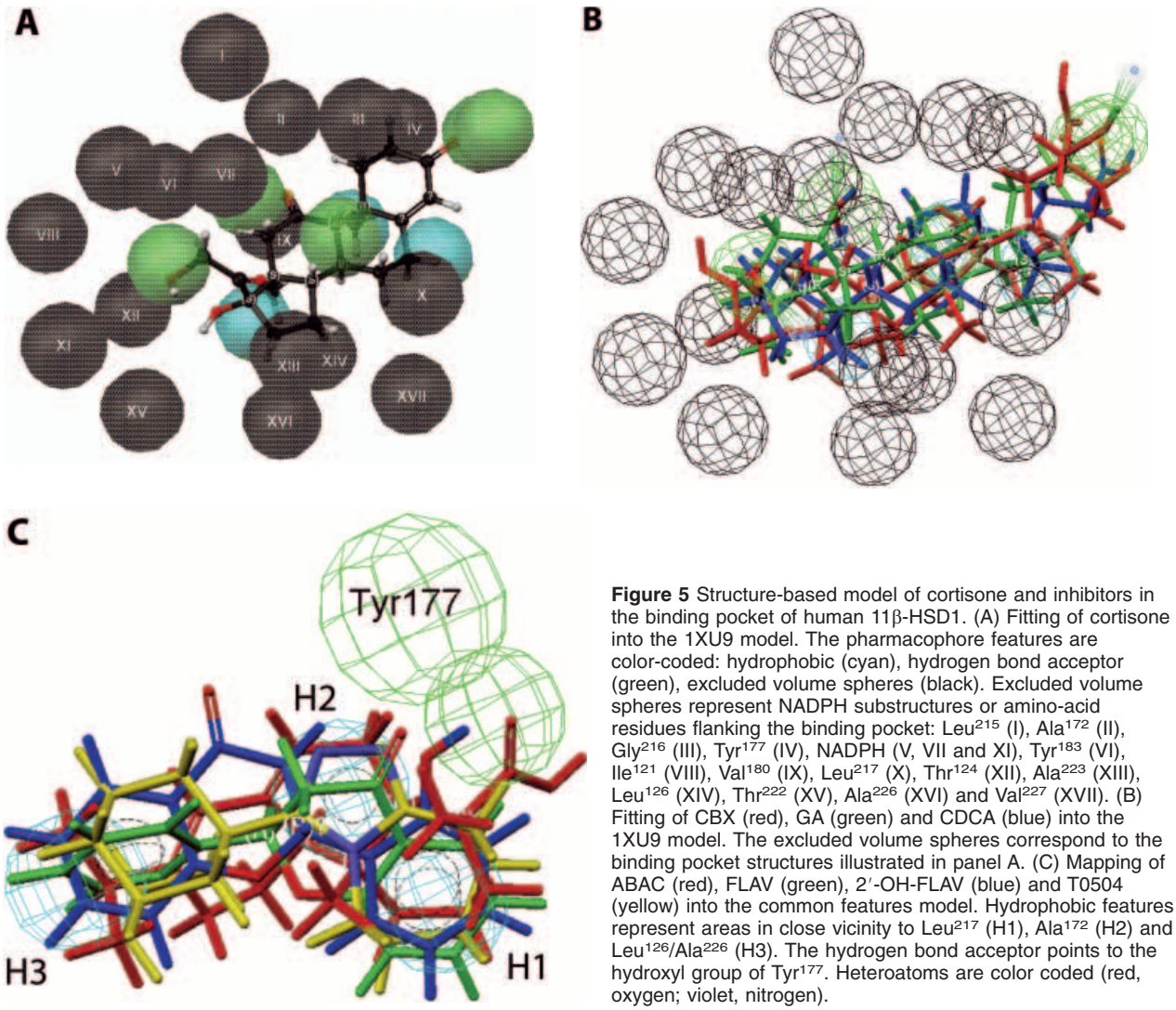

Figure 5 Structure-based model of cortisone and inhibitors in the binding pocket of human 11β-HSD1. (A) Fitting of cortisone into the 1XU9 model. The pharmacophore features are color-coded: hydrophobic (cyan), hydrogen bond acceptor (green), excluded volume spheres (black). Excluded volume spheres represent NADPH substructures or amino-acid residues flanking the binding pocket: Leu^{215} (I), Ala^{172} (II), Gly^{216} (III), Tyr^{177} (IV), NADPH (V, VII and XI), Tyr^{183} (VI), Ile^{121} (VIII), Val^{180} (IX), Leu^{217} (X), Thr^{124} (XII), Ala^{223} (XIII), Leu^{126} (XIV), Thr^{222} (XV), Ala^{226} (XVI) and Val^{227} (XVII). (B) Fitting of CBX (red), GA (green) and CDCA (blue) into the 1XU9 model. The excluded volume spheres correspond to the binding pocket structures illustrated in panel A. (C) Mapping of ABAC (red), FLAV (green), 2′-OH-FLAV (blue) and T0504 (yellow) into the common features model. Hydrophobic features represent areas in close vicinity to Leu^{217} (H1), Ala^{172} (H2) and Leu^{126}/Ala^{226} (H3). The hydrogen bond acceptor points to the hydroxyl group of Tyr^{177}. Heteroatoms are color coded (red, oxygen; violet, nitrogen).

several chemicals from different classes that all occupy the hydrophobic binding pocket. We suggest that after *in vitro* testing and selection of potential 11β-HSD1 inhibitors for therapeutic applications, the effect of such inhibitors on the recombinant enzyme from the corresponding species be determined before initiating extensive *in vivo* tests.

Acknowledgements

We thank Drs Roger Clerc and Kurt Amrein of Hoffmann LaRoche, Basel, for providing dog liver cDNA and for helpful discussion and support. We thank Heidi Jamin for excellent technical support and Dr. Bernhard Dick for advice with GC/MS analyses. A O is a Cloëtta Research Fellow. This work was supported by grants from the Swiss National Science Foundation no. 3100A0–100060 and National Research Program No. 50 'Endocrine Disruptors' no. 4050–066575. T L and D S were supported by Inte:Ligand GmbH. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 7 April 2005
Accepted 22 April 2005
Made available online as an Accepted Preprint 12 May 2005