Metabolism and Action of Glucocorticoids and Interference with the Antioxidant Redox Pathway

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1 Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ABCC	ATP-binding cassette, sub-family C (CFTR/MRP), member
ACTH	adrenocorticotropic hormone
ADH	alcohol dehydrogenase
AME	apparent mineralocorticoid excess
ARE	antioxidant responsible element
C/EBP	CCAAT/enhancer-binding-protein
CBX	carbenoxolone
CRD	apparent cortisone reductase deficiency
CRF	corticotrophin releasing factor
CYP11B1	11β-hydroxylase
DBT	dibutyltin
GA	glycerrhetinic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
GSTA2	glutathione S-transferase alpha 2
H6PDH	hexose-6-phosphate dehydrogenase
HO-1	heme oxygenase 1
HPA	hypothalamic-pituitary-adrenal axis
HTS	high-throughput-screening
IL-6	interleukine-6
Keap1	kelch-like ECH-associated protein 1
MR	mineralocorticoid receptor

NALD	nonalcoholic liver disease
NASH	nonalcoholic steatohepatitis
NF-κB	nuclear factor-kappa B
NQO1	NAD(P)H dehydrogenase, quinone 1
Nrf2	nuclear factor (erythroid-derived 2)-like 2
S	sulforaphane
SCN	suprachiasmatic nucleus
SDR	short chain dehydrogenase/reductase
TBHQ	tertiar butyl hydroquinone
TBT	tributyltin
TNFα	tumor-necrosis-factor α

2 Summary

Disturbance of endocrine systems and signaling pathways can lead to severe disorders. Such disorders can have endogenous as well exogenous origin. The awareness of environmentally occurring xenobiotics that are able to directly interfere with and modulate the action of endogenous hormones has driven the need for mechanistic studies. Although there is a vast literature on potentially endocrine disrupting chemicals, there are only few studies investigating disturbance of glucocorticoid action by xenobiotics, despite of the importance of these hormones. In this work, the organotin dibutyltin (DBT) was identified as an endocrine disruptor of the glucocorticoid pathway. Its extensive use in plastic industry, as well as an antifouling agent explains its occurrence in water and seafood. In the present study, we were able to show that DBT disturbs GR mediated anti-inflammatory effects. Furthermore, DBT was found to potentiate NFκB mediated production of the pro-inflammatory cytokines IL-6 and TNFα in macrophages. The presented work therefore contributes to the mechanistic understanding of DBT-induced immunotoxicity.

There are several therapeutic purposes accompanied by the modulation of the endogenous hormone system. In traditional medicines natural compounds, and plant extracts are applied since centuries for different purposes, including the treatment of diseases such as diabetes and hypertension. The benefits of evidence based medicines, even if their mechanisms of action are unknown, are widely accepted. In conventional medicine the re-awareness of naturally derived compounds and their huge potential promoted the investigation of the underlying specific mechanisms of action of such compounds over the last decades. In this context, the present work investigated effects of *eriobotrya japonica*, a plant used for anti-diabetic treatment in Chinese medicine. The project aimed to identify potential constituents that are active on 11β-HSD1. Several pentacyclic triterpenes were isolated and further characterized. These compounds included potent and, compared with 11β-HSD2, selective 11β-HSD1 inhibitors such as corosolic acid and urosolic acid, as well as urosolic acid derivatives with only low inhibitory potential but considerable synergistic effects. Inhibitors for research and/or therapeutic purposes ideally display high selectivity to avoid miss-leading interpretations of their action. Furthermore, therapeutic intervention requires selective inhibitors to prevent unexpected side

effects. The most famous triterpenoid inhibiting 11β-HSD enzymes is glycyrrhetinic acid (GA), present in liquorice. GA is a potent, but non-selective inhibitor of both 11β-HSD isoformes. Recently, GA was used as a starting compound and chemical modifications of its back-bone enabled the development of potent and specific inhibitors against 11β-HSD2. The present work describes the characterization of these novel 11β-HSD2 inhibitors. The inhibitors were characterized for their inhibitory potential by determining their IC_{50} values and selectivity for 11β-HSD enzymes as well as their species specificity by using human and mouse enzymes. Moreover, the capability for the inhibition of the endogenous 11β-HSD2 enzyme in intact cells was investigated.

Selective inhibition of 11β-HSD1 was proposed over the last years as promising drug target to cope with the consequences of obesity and diabetes type II and the metabolic syndrome. The present study supports beneficial effects of 11β-HSD1 inhibition from a different point of view. Our data suggest that excessive glucocorticoid activation by 11β-HSD1 may interfere with the antioxidant redox pathway by a GR-dependent manner. The present work describes the active glucocorticoid-dependent inhibition of classic target genes of the Nrf2-Keap1 detoxification pathway on both mRNA as well as protein level. Thus, the work supports the existence of important cross-talk between GR and Nrf2. Pathologically enhanced glucocorticoid activation, as exists in patients with alcoholic liver disease (ALD), may impair the cellular detoxification capacity.

In conclusion, the presented studies highlight different aspects of the interference of small molecules with the glucocorticoid pathway, including the endocrine disruption by DBT and inhibition of 11β-HSD enzymes, by natural and synthetic compounds. The identification and characterization of specific inhibitors against 11β-HSD1 and 11β-HSD2 offers valuable mechanistic tools. Further, the work provides evidence for the interference of 11β-HSD1 action with the antioxidant redox pathway and therefore may contribute to a deeper understanding of the pathology of locally enhanced glucocorticoids.

In conclusion, the presented studies should contribute to a better understanding of glucocorticoid related pathologies and the underlying mechanisms.

3 Introduction

3.1 Glucocorticoids and mineralocorticoids: a historical overview

In 1951, the Nobel Prize in Medicine or Physiology was awarded to Tadeus Reichstein, Philip Showalter Hench and Edward Calvin Kendall for their independent work on the “discovery of hormones of the adrenal cortex, their structure and biological effects” [1]. However, glucocorticoids were already used back in 1900, when Solomon Solis-Cohen administered adrenal extracts to patients suffering from asthma [2]. He did not assign the observed beneficial effect to glucocorticoid hormones. The isolation and later the chemical synthesis of cortisone allowed to investigate the therapeutic effects of glucocorticoids in more depth, and revealed their potential in the treatment of inflammatory diseases such as rheumatoid arthritis [3, 4]. It is noteworthy, that already in those first studies, side effects such as sodium retention, hyperkalemia, psychological changes, as well as bone fractures in osteoporotic patients were recorded to accompany systemic glucocorticoid treatment [3].

Synthetic glucocorticoids are potent drugs with a wide spread use in clinics and they still represent the most abundantly used and potent anti-inflammatory therapeutic to treat infection-related inflammation as well as autoimmune driven inflammatory diseases and neuroinflammatory disorders (*e.g.* multiple sclerosis (MS)) [5-8]. Like cortisone, aldosterone was isolated from the adrenals by the group around Tadeus Reichstein and reported in 1953 as a compound called “electocortine” [9]. The new hormone was isolated, crystallized and described as a hormone “with especially high effectiveness on mineral metabolism” [9]. Shortly afterwards electrocortine was first termed aldosterone and chemical characterized ($C_{21}H_{28}O_5$) by the same group [10].

Hans Selye and co-workers discovered the link between adrenocortical hormones and both physiological and pathophysiological stress response [11]. Furthermore, it became obvious that glucocorticoids exert important impact on glucose metabolism [11]. Selye established a to date existing nomenclature, in order to distinguish between glucocorticoids (“sugar active”) and mineralocorticoids (“salt-active”) [11]. Indeed, glucocorticoids enhance hepatic gluconeogenesis, reduce glucose uptake in

peripheral tissues such as skeletal muscle and thereby retain glucose homeostasis. The terminology ”glucocorticoid”, however, does not reflect their highly versatile effects on the regulation of expression of up to 20% of the genes in the mammalian genome [12].

3.1.1 Physiological synthesis and regulation of glucocorticoids

Systematically, glucocorticoids and mineralocorticoids are synthesized from cholesterol by enzymes located in the adrenal glands. The rate limiting step of glucocorticoid biosynthesis is controlled by the steroid acute regulatory protein (StAR), which regulates uptake of cholesterol into the mitochondrial membrane (Figure 1) [13].

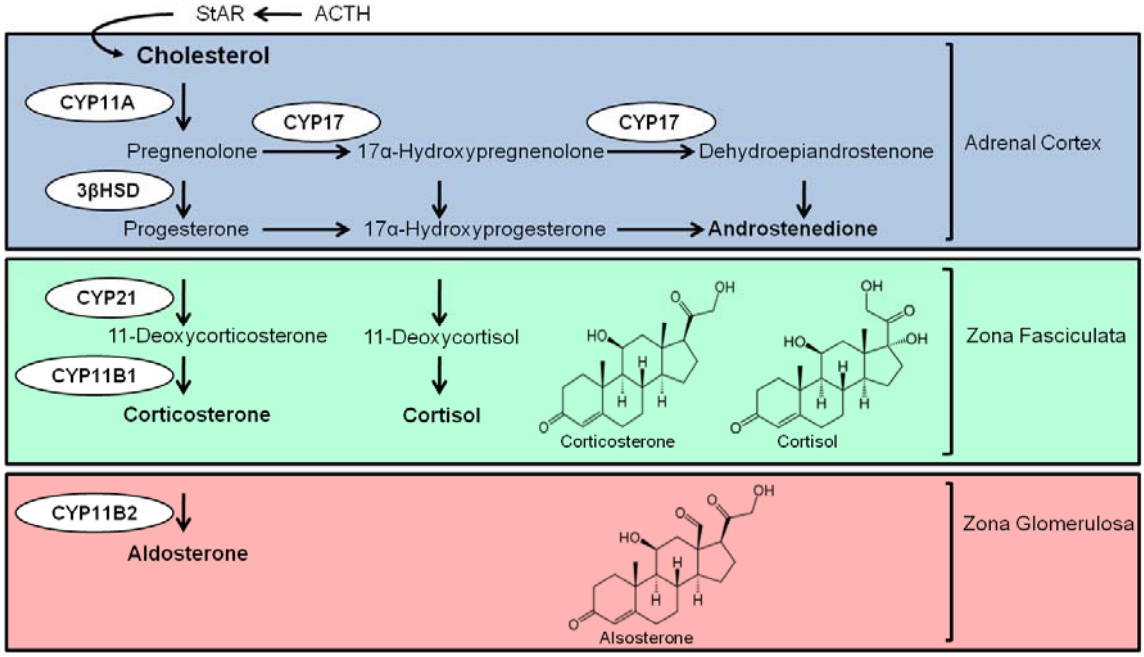


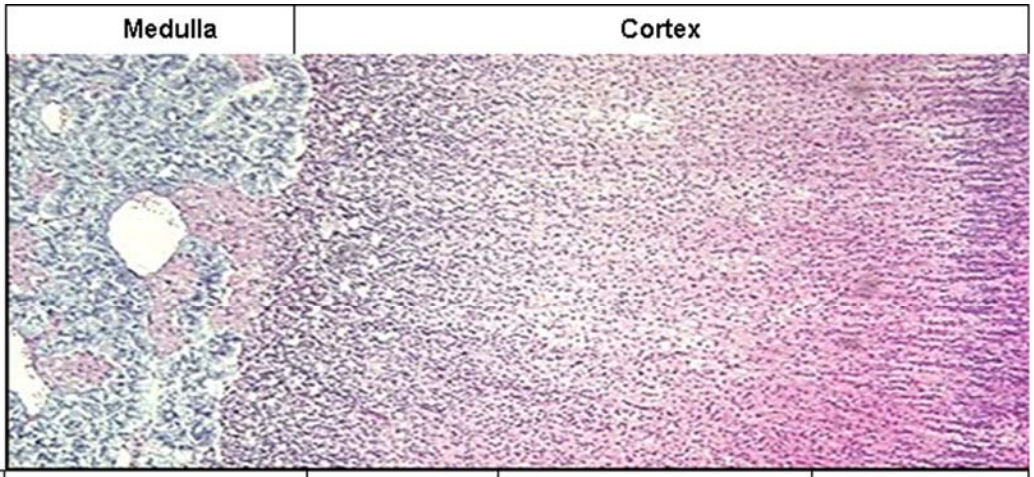
Figure 1. Biosynthesis of steroid hormones in the corresponding zone of the adrenal gland (modified from Payne *et al.* [14]).

ACTH (adenocorticotropic hormone); StAR (steroid acute regulatory protein); CYP11A (P450scc, cholesterol side-chain cleavage); CYP17 (P450c17, 17alpha-hydroxylase/17, 20 lyase); 3βHSD (3β-hydroxysteroid dehydrogenase/steroid isomerase); CYP21 (P450c21, 21 hydroxysteroid dehydrogenase type 1); CYP11B1 (11β-hydroxylase).

The final step in the glucocorticoid biosynthesis is mediated by CYP11B1, an 11β-hydroxylase (Figure 1). The produced glucocorticoids are, regarding their physico-

chemical properties, expected to pass cellular membranes and enter target cells *via* passive transport.

The characterization of the adrenal gland and the distinct distribution into three zones (Figure 2) was first described in 1886 by Arnolds *et al.* [15].



Tissue	Medulla	Zona reticularis	Zona fasciculata	Zona glomerulosa
Hormon class	Catecholamines	Sex-steroids	Glucocorticoids	Mineralocorticoids
Hormon	Epinephrine, Norepinephrine	DHEA, Estradiol	Cortisol, Corticosterone	Aldosterone

Figure 2. Histological overview of a rabbit adrenal gland, reflecting the different functional zones, as well as the adrenal medulla and their hormonal release (modified from Austgen *et al.* [16]).

The *zona glomerulosa* forms the outermost area of the adrenal cortex. Following stimulation of the renin-angiotensin-system (RAS) (Figure 3) aldosterone, with an expected half-life-time of 30 minutes, is secreted into the blood, approximately 250 µg daily [17]. In contrast, glucocorticoids (cortisol, corticosterone) are synthesised in the mid zona of the adrenal cortex, the *zona fasciculata*. Although the adrenal glands secrete certain amounts of cortisone, the majority of the cortisone in the blood is produced by the activity of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). This enzyme is mainly located in tissues involved in the maintenance of salt-water homeostasis (see below), where it catalyzes the production of cortisone from the circulating free cortisol [18].

In plasma, glucocorticoids are 100-1000 times more abundant than mineralocorticoids, with a total daily secretion of approximately 20 mg [19] and an assumed half-life time of cortisol between 60-80 minutes [20]. The predominant glucocorticoid in man is cortisol; however, also small amounts of corticosterone are

present in the human plasma. In contrast, cortisol is absent, or has very low plasma levels in rodents, with corticosterone being the major active glucocorticoid in mice and rats. Glucocorticoid release is daytime-dependent and oscillates following the circadian rhythm. The cellular source of the mammalian circadian rhythm is the suprachiasmatic nucleus (SCN) localized in the anterior hypothalamus [21]. Destruction of the SCN leads to a disturbance in the fluctuation of glucocorticoid release over daytime [22]. In human plasma, cortisol reaches the highest levels in the morning (20-400 nM) and bottom levels at night (5-100 nM) [23].

Glucocorticoid synthesis underlies a strict neuroendocrine regulation controlled *via* a three phased hormonal axis, the hypothalamic-pituitary-adrenal axis (HPA) [24]. Glucocorticoids are actively involved in their regulation by various feedback mechanisms on their own biosynthesis and secretion [24]. The HPA axis (Figure 3) is stress-controlled, thus synthesis of glucocorticoids is enhanced up to five-fold during stress [24]. Interference of the renin-angiotensin-aldosterone-system (RAAS) and the HPA axis was already shown in animal studies but has not been fully elucidated yet in humans [25].

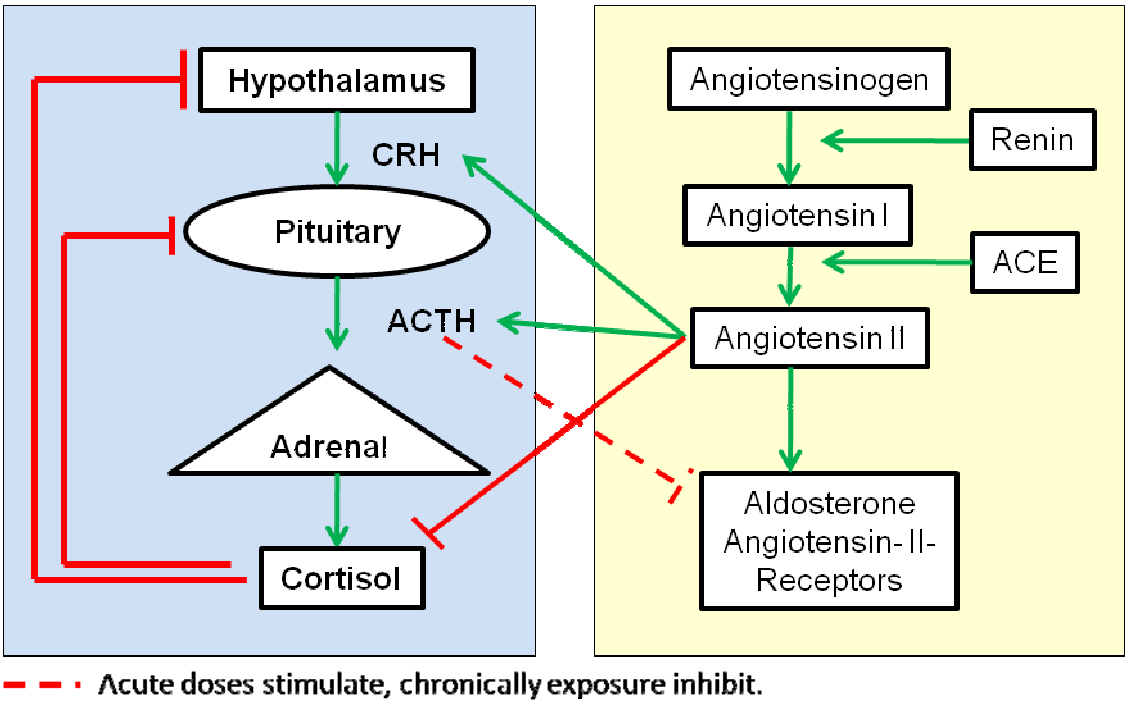


Figure 3. Overview of HPA axis (blue) and Renin-Angiotensin-System (yellow) with their possible interactions. (modified from Baghai *et al.* [25]).

CRH, corticotropin-releasing-hormone; ACTH, adrenocorticotropic hormone; ACE, angiotensin-converting enzyme.

Although free glucocorticoids are secreted into the plasma at high concentrations, 90% of the plasmatic cortisol and corticosterone is bound to corticosteroid-binding globulins (CBG) [26]. These steroid carriers belong to the clade A serine proteinase inhibitor (serpin) family, and act as substrates for the neutrophil elastase [26]. Proteolytic cleavage of CBG by neutrophil esterases irreversibly destroys glucocorticoid binding [26]. Growing evidence suggests, that CBG serves as a storage pool for glucocorticoids to enable a direct and acute release of 80% of the CBG-bound cortisol at the site of inflammation [27]. Locally released glucocorticoids thereby allow a much faster reaction against inflammatory insults. Moreover, in mouse strains with low CBG plasma levels (*e.g.* BC57BL/6) [28] as well as in CBG KO-mice [29] an enhanced susceptibility against acute inflammation was observed. The main CBG pool is produced by hepatocytes; however, CBG mRNA is also expressed in other tissues such as pancreas and kidney that may indicate a defined and tissue specific binding of locally occurring glucocorticoids [30]. Low affinity binding of cortisol also occurs to albumin in the plasma, thus final free cortisol levels are in the range of 4-10% of the total secreted cortisol [31]. Since the affinity of CBG is much lower for the inactive glucocorticoid cortisone, and given that the concentration of cortisone in the blood is five-times less than that of cortisol, the amounts of free cortisol and free cortisone are comparable [18, 32-34].

3.1.2 Pathology of impaired glucocorticoid release

The pathologies of Addison's disease and Cushing's syndrome both involve disturbances of the glucocorticoid (cortisol) content in the blood and, as a consequence, dramatically decreased or increased glucocorticoid-dependent functions. Addison's disease is characterized by reduced cortisol levels leading to impaired stress resistance, hypertrophy of the lymphoid organs, weight loss, hypoglycaemia and hypotension [35]. Causes for the disease are disruption of glucocorticoid biosynthesis as well as autoimmune driven destruction of the adrenal cortex. On the other hand, the Cushing's syndrome is characterized, among others, by central obesity, muscle atrophy, hyperglycaemia, elevated cholesterol and insulin resistance, as well as severe hypertension and immunodeficiency. This hormonal

disorder can be caused by excess of ACTH or CRH, a consequence of pituitary gland adenomas or tumors of the adrenal gland [36]. Moreover, iatrogenic causes are common after prolonged medication with glucocorticoids [37]. Regardless of the reason, the Cushing's syndrome describes the pathologically enhanced cortisol level of the blood. The therapeutic options involve glucocorticoid receptor antagonism leading to a normalization of blood pressure [38] or tumor surgery and adrenalectomy.

3.2 Pre-receptor metabolism and action of glucocorticoids

Although glucocorticoid synthesis and neuroendocrine regulation of glucocorticoid release is strictly regulated, glucocorticoid metabolism in peripheral tissues further represents a level of regulatory control contributing to the sophisticated network of glucocorticoid-mediated regulation of physiological functions.

3.2.1 11β-hydroxysteroid dehydrogenases

11β-hydroxysteroid dehydrogenases (11β-HSDs) belong to the superfamily of short chain dehydrogenases (SDR), which counts over 46'000 members, of which about 70 different genes are known in human [39]. Enzymes of the family are present in all investigated genomes and seem to be a part of the original enzyme constitution [39]. Two distinct functional 11β-HSD glucocorticoid metabolizing enzymes have extensively been investigated so far, termed 11β-HSD1 and 11β-HSD2. Both 11β-HSDs are microsomal, anchoring in the membrane of the endoplasmatic reticulum (ER) [40]. The two enzymes share only approximately 18% sequence homology, including the active motif consisting of a conserved amino acid triad of tyrosine, serine and lysine residues [41]. The catalytic domains of the type 1 and type 2 enzymes have inverted orientations [42] and opposite catalytic functions (summarized in Table 1.).

Table 1. Overview of biochemical parameters of 11β-hydroxysteroid dehydrogenases type 1 and type 2

Human	**11β-HSD1**	**11β-HSD2**
CHROMOSOME	1	16
GENE SIZE [Kb]	30	6.2
EXON/INTRON	6/5	5/4
AMINO ACIDS	292	405

PROTEIN SIZE [kDa]	34	44
TOPOLOGY	ER-membrane	ER-membrane
CATALYTIC DOMAIN	ER luminal orientation	Cytoplasmatic orientation
FUNCTION (*IN VIVO)*	Activation of glucocorticoids	Inactivation of glucocorticoids
COFACTOR	NADPH	NAD^+
FUNCTIONALITY	Bidirectional	Unidirectional
REACTION DIRECTION	Dehydrogenase and reductase	Dehydrogenase
APPARENT Km	Cortisone 300-500 nM 11β-dehydrocorticosterone 300-500 nM	Cortisol 50-100 nM Corticosterone 5-10 nM
DOMINANT EXPRESSED	Adult	Fetal/Adult
FIRST CLONING	1989 (rat liver)	1994 (human and sheep kidney)
DISTRIBUTION	Hepatocytes, adipocytes, macrophages, hippocampal neurons, kidney (proximal tubule)	Kidney (distal tubule), colon (distal), placenta

3.2.1.1 11β-hydroxysteroid dehydrogenase type 2

11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) was first cloned from human [43] and sheep [44] kidneys, and was found to be highly expressed in kidney, colon, pancreas and placenta, as well as in the ovary, prostate, and testis [43]. 11β-HSD2 is a NAD^+-dependent dehydrogenase catalyzing the conversion of 11β-hydroxy-glucocorticoids (cortisol, corticosterone) into their inactive counterpart (cortisone, 11β-dehydrocorticosterone) with high affinity and a Michaelis-Menten constant (Km) in the low nanomolar range [43].

3.2.1.2 Pathologies related to 11β-hydroxysteroid dehydrogenase type 2

Cloning of 11β-HSD2 allowed identification of loss-of-function mutations [45]. These genetic mutations in the *HSD11B2* gene were directly linked to the pathology of the syndrome of apparent mineralocorticoid excess (AME) [45, 46]. Patients suffering from AME typically present with potassium wasting (hypokalemia) and sodium retention, leading to severe hypertension. The treatment includes the administration of spironolactone, an unselective antagonist of the mineralocorticoid receptor (MR) [47]. In addition, it was shown that liquorice abuse exerts the development of symptoms resembling AME [48]. Liquorice contains 11β-glycyrrhetinic acid, an active triterpenoid and potent, but unselective inhibitor of both 11β-HSD1 and 11β-HSD2.

Mutated 11β-HSD2 raised the molecular explanation for the AME phenotype and explained the imbalance between urinary 11β-hydroxy- and 11β-ketoglucocorticoids [45, 46].

Regarding the action of 11β-HSD2 as “gate-keeper” for the MR, thus primarily regulating blood pressure, the deficiency of 11β-HSD2 activity, regardless of its reasons (genetic, food-intake/inhibition), can cause of hypertension [49, 50].

3.2.1.3 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate-dehydrogenase

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) was first cloned from the rat liver and reported in 1989 [51]. 11β-HSD1 catalyzes the interconversion of inactive (cortisone and 11β-dehydrocorticosterone) and active (cortisol and corticosterone) glucocorticoids, however, it acts predominantly as a reductase in intact cells and *in vivo,* using nicotinamideadenine dinucleotide phosphate (NADPH) as cofactor [34]. It is highly expressed in liver, gonads, adipose tissue and skeletal muscle, and lower expression levels have been found in certain regions of the brain, the lung, testis, ovary, adrenal glands and vascular cells [34].

In vitro assays using the purified protein revealed that 11β-HSD1 is a bidirectional enzyme preferably acting as dehydrogenase catalyzing the oxidation of active 11β-hydroxyglucocorticoids and using $NADP^+$ as cofactor [41, 52-54]. Initial kinetic analyses revealed an apparent Km of 1.8 μM for corticosterone and 17 μM for cortisol, respectively [34]. Later, studies with purified protein reported Km values between 300-500 nM for corticosterone and cortisol respectively suggesting a loss of enzymatic function in some older purification protocols [55]. However, as mentioned above, studies in intact cells including fibroblasts, hepatocytes, lung cells, stromal adipose cells, and hippocampal neurons revealed that 11β-HSD1 acts predominantly as a reducing enzyme generating cortisol or corticosterone and using NADPH as cofactor [34]. Data obtained from experiments using intact cells are supported by kinetic studies suggesting an apparent Km of about 0.3 μM for cortisone compared with a Km of about 2 μM for cortisol [56, 57]. Observations in isolated primary hepatocytes led to further confusion since 11β-HSD1 rapidly lost its reductase activity after a short cultivation period. In addition, 11β-HSD1 activity was shown to be influenced by the differentiation state of a cell, *e.g*. in 3T3-L1 derived mouse

adipocytes, where 11β-HSD1 reductase activity appears during differentiation of preadipocytes into adipocytes [58, 59].

Recently, the activity of the endoplasmic reticulum luminal enzyme hexose-6-phosphate dehydrogenase was identified as the determinant of the reaction direction of 11β-HSD1 [60] [61]. This enzyme generates NADPH by catalyzing the conversion of its substrate glucose-6-phosphate (G6P) to 6-phosphogluconolactone, thereby using $NADP^+$ as a cofactor (Figure 4) [60]. Recently, 11β-HSD1 and H6PDH were shown to physically interact in the inner part of the endoplasmic reticulum membrane [62].

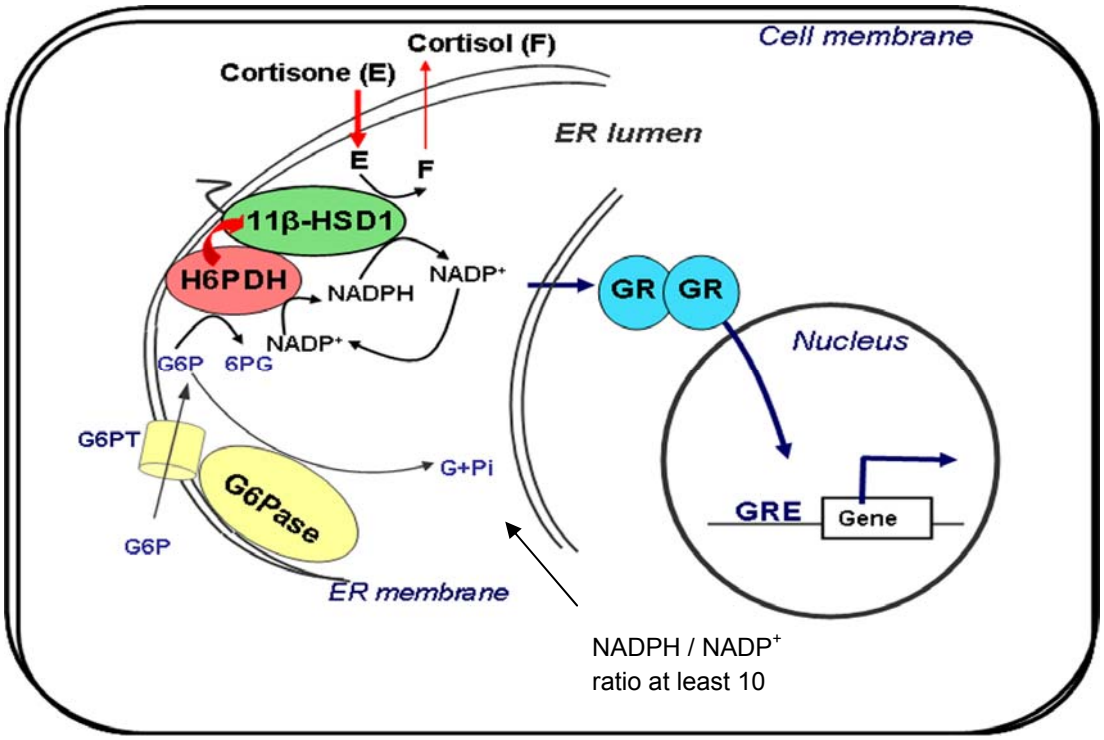


Figure 4. Physical interaction of H6PDH and 11β-HSD1 in the ER-lumen [63] [62]

Importantly, a ratio of ten to one or even higher of $NADPH/NADP^+$ is required for 11β-HSD1 to function as a reductase [54]. Besides the function of H6PDH to determine the reaction direction of 11β-HSD1, its activity can modify the effect of inhibitors of 11β-HSD1 in intact cells [53].

3.2.1.4 Pathologies related to 11β-hydroxysteroid dehydrogenase type 1

A rare syndrome called apparent cortisone reductase deficiency (CRD) has been characterized by androgen excess and decreased urinary excretion of cortisol

metabolites [64]. Homozygous mutations in the *H6PD* gene without change in the coding sequence of the *HSD11B1* [65] as well as heterozygous mutations of the *HSD11B1* gene without affected *H6PD* gene [66] were reported for the CRD phenotype.

The metabolic syndrome is characterized by a co-incidence of disturbances such as obesity, hypertension, elevated plasma triglycerides, and cardiovascular disease (Table 2). Obesity and the possible outcome, the metabolic syndrome, are consequences of inappropriate life-style leading to disturbance of multiple pathways.

Table 2. Metabolic syndrome: proposed components and associated findings (modified from Miranda *et al.* [67]).

Metabolic syndrome: proposed components and associated findings
Insulin resistance*
Hyperinsulinemia*
Obesity: visceral (central), but also generalized obesity *
Dyslipidemia: high triglycerides, low HDL, small dense LDL*
Adipocyte dysfunction
Impaired glucose tolerance or type 2 diabetes mellitus*
Fatty liver (nonalcoholic steatohepatosis, steatohepatitis)
Essential hypertension: increased systolic and diastolic blood pressure*
Endothelial dysfunction
Renal dysfunction: micro- or macroalbuminuria
Polycystic ovary syndrome
Inflammation: increased CRP and other inflammatory markers
Hypercoagulability: increased fibrinogen and PAI-1
Atherosclerosis leading to increased cardiovascular morbidity and mortality *

*Most widely incorporated into the definition of metabolic syndrome.

The prevalence of overweight and obesity has been increasing over the last decades among the Western population. Nowadays, the metabolic syndrome contributes with approximately 10% to the total mortality of developed countries, which is twice as high as the tobacco-related mortality [68] (Table 3). Thus, the metabolic syndrome has become a serious health concern and represents a cost intensive burden in developed countries.

Table 3. Contribution of the metabolic syndrome and its components compared with tobacco smoking to the total mortality of developed countries [68].

account for total mortality in middle-income countries %	
metabolic syndrome	10.00%
high blood pressure (HBP)	5.00%
high cholesterol	2.10%
obesity	2.70%
tobacco smoking	4.00%

Although similar symptoms occur in the metabolic syndrome, and in Cushing's syndrome, the metabolic syndrome is not characterized by elevated plasma glucocorticoid levels. Interestingly, glucocorticoid levels in the obese but non-diabetic condition are even reduced compared with the normal weight status [69]. Obese animal models reflect a tissue specific dysregulation of glucocorticoid activation by 11β-HSD1 in adipose tissue. The animals show normal blood corticosterone levels accompanied by increased glucocorticoid activation by 11β-HSD1 in the adipose tissue [70]. Results from these *in vivo* studies resemble those seen in humans with similar outcome. Namely, clinical studies clearly demonstrated enhanced 11β-HSD1 expression in the subcutaneous abdominal adipose tissue in the condition of obesity [71-73].

Thus, 11β-HSD1 seems to be a promising drug target, and tissue-specific inhibition of its reductase activity is expected to be beneficial in obesity and the metabolic syndrome, as well as in glaucoma and osteoporosis [34].

3.3 The nuclear receptor superfamily

The nuclear receptor superfamily comprises of 48 members in humans [74]. Most of these proteins are ligand-inducible transcription factors involved in the regulation of key physiological processes such as metabolism, development and reproduction [74]. Receptors of the family share several modulatory domains including the highly conserved zinc-finger DNA binding domain (DBD), a more variable carboxy-terminal ligand binding domain (LBD) and a highly variable amino-terminal domain [75], schematically summarized in Figure 5.

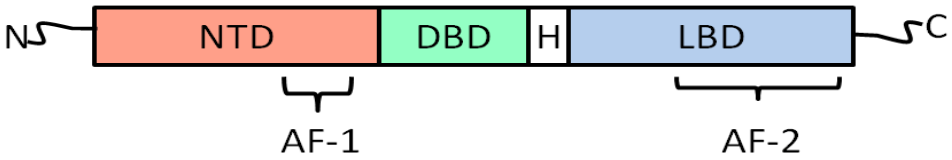


Figure 5. Basic common structural organization of the nuclear receptor superfamily (modified from Heizer *et al*. [75]).

The N-terminal region (NTD) also called A/B domain includes the conserved constitutively active transactivation region (AF-1) as well as several autonomous transactivation domains (AD). The length of the receptor N-terminus is variable and ranges from 50 up to 500 amino acids [76].

DNA binding of the receptor is realized by two conserved zinc-finger domains each consisting of four cysteines and one Zn^{2+} ion (P-box). Inside the DBD, the P-box generally recognizes the motif AGGTCA in target genes. Furthermore, the DBD is responsible for the functional dimerization of the nuclear receptors. Dimerization can occur between equal receptors (homodimerization) as well as between different nuclear receptors (heterodimerization). The nuclear fate of the receptors is dependent on nuclear localization signals (NLS). One of these short sequences is located in the hinge (H) region between DBD and LBD. The hinge region is flexible and less conserved between the family members [76]. The LBD represents the longest coherent region of the sequence. Amino acids forming the motif are less conserved; however, the secondary structure comprising 12α-helices is conserved among the members. The LBD includes the AF-2 motif responsible for the transactivation, as well as a second NLS. Many but not all functions of the LBD are ligand-induced, including activator (co-activator binding) and repressor functions (co-repressor binding) [76].

The receptors of the family are defined by a nomenclature based on sequence alignments and phylogenetic tree constructs (Table 4). Six different classes are distinguished [77]. The systematic nomenclature combines NR for Nuclear Receptor, the subfamily 0-6, the group and finally the member number.

Table 4. Members and nomenclature and of the nuclear hormone receptors (Modified from Germain et al. [77]).

Names	Nomenclature	Ligand
TRα	NR1A1	Thyroid hormones
TRβ	NR1A2	Thyroid hormones
RARα	NR1B1	Retinoic acid
RARβ	NR1B2	Retinoic acid
RARγ	NR1B3	Retinoic acid
PPARα	NR1C1	Fatty acids, leukotriene B_4, fibrates
PPARβ	NR1C2	Fatty acids
PPARγ	NR1C3	Fatty acids, prostaglandin J_2, thiazolidinediones
Rev-erbα	NR1D1	Orphan
Rev-erbβ	NR1D2	Orphan
RORα	NR1F1	Cholesterol, cholesteryl sulfate
RORβ	NR1F2	Retinoic acid
RORγ	NR1F3	Orphan
LXRα	NR1H3	Oxysterols, T0901317, GW3965
LXRβ	NR1H2	Oxysterols, T0901317, GW3965
FXRα	NR1H4	Bile acids, fexaramine
FXRβ	NR1H5	Lanosterol
VDR	NR1I1	Vitamin D, 1,25-dihydroxyvitamin D_3
PXR	NR1I2	Xenobiotics, 16α-cyanopregnenolone
CAR	NR1I3	Xenobiotics, phenobarbital
HNF4α	NR2A1	Orphan
HNF4γ	NR2A2	Orphan
RXRα	NR2B1	Retinoic acid
RXRβ	NR2B2	Retinoic acid
RXRγ	NR2B3	Retinoic acid
TR2	NR2C1	Orphan
TR4	NR2C2	Orphan
TLL	NR2E2	Orphan
PNR	NR2E3	Orphan
COUP-TFI	NR2F1	Orphan
COUP-TFII	NR2F2	Orphan
EAR2	NR2F6	Orphan
ERα	NR3A1	Estradiol-17β , tamoxifen, raloxifene
ERβ	NR3A2	Estradiol-17β , various synthetic compounds
ERRα	NR3B1	Orphan
ERRβ	NR3B2	DES, 4-OH tamoxifen
ERRγ	NR3B3	DES, 4-OH tamoxifen
GR	NR3C1	Cortisol, dexamethasone, RU486
MR	NR3C2	Aldosterone, spirolactone
PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486

AR	NR3C4	Testosterone, flutamide
NGFI-B	NR4A1	Orphan
NURR1	NR4A2	Orphan
NOR1	NR4A3	Orphan
SF1	NR5A1	Orphan
LRH-1	NR5A2	Orphan
GCNF	NR6A1	Orphan
DAX-1	NR0B1	Orphan
SHP	NR0B2	Orphan

3.3.1 Glucocorticoid and mineralocorticoid receptor

Both corticosteroid receptors, the glucocorticoid receptor (GR, NR3C1), and the mineralocorticoid receptor (MR, NR3C2), are members of the previously described nuclear hormone receptor superfamily.

In 1985, the GR of human origin was first cloned by Hollenberg and colleges [78], soon followed by the cloning of the mouse [79] and rat receptors [80]. Two years later, Arriza *et al*. used complementary DNA of the GR, under the condition of low stringency hybridization, to isolate and clone the MR [81]. MR and GR share ~90% of the amino acids forming the DNA binding domain (DBD). However, sequence homology of the ligand binding domain (LBD) shows only approximately 50% homology. GR is expressed in nearly every tissue, whereas MR expression is also frequent but more defined. MR is expressed mainly in tissues actively involved in salt-water homeostasis. Importantly, however, the receptor is also involved in the regulation of biochemical processes that are very distinct from these “classical functions”, such as in the limbic system where it regulates behavioral plasticity [82] or in the heart [83]. For MR expression and function the presence or absence of enzymes controlling the access of active ligand, thereby regulating receptor activation need to be considered. (for review see chapter 7).

The endogenous ligands of GR are cortisol (Kd 10-50 nM) and corticosterone (Kd 60nM); The widely used synthetic glucocorticoid, dexamethasone has a ten-fold higher affinity with a Kd 1-8 nM [84-86]. The MR has broader substrate specificity and it binds aldosterone, 11-deoxycorticosterone, corticosterone, cortisol and progesterone with Kd 0.5-3 nM [87, 88]. A physiological regulatory mechanism exists to protect the MR from activation by glucocorticoids: in tissues where the regulation

of the maintenance of salt-water balance takes place, such as the distal tubule and the cortical collecting duct of the kidney, distal colon and sweat-glands, the receptor is co-expressed with the glucocorticoid inactivating enzyme 11β-HSD2 (see Chapter 3.2.1 above). A well known GR antagonist is mifepristone (also known as RU-486), which was originally developed as a progesterone receptor antagonist to prevent pregnancy, but binds also GR with high affinity [89, 90]. The most abundantly used unselective MR antagonist is spironolactone, which is still used in the clinics and has found recent attention to treat essential hypertension and heart failure [91-93]. Use of spironolactone was already reported in 1960 for the treatment of patients suffering from edema, congestive cardiac failures and nephrotic syndrome [94]. Another, newer MR antagonist is eplerenon (Inspra®), which although selective, has a rather low affinity to the receptor (Kd approximately 30 μM) [95].

3.3.2 General mechanism of transactivation

The unliganded receptor is localized in the cytoplasm as part of a multiprotein complex, including molecular chaperones such as heat shock protein (HSP) 90, HSP70, HSP56, as well as other proteins such as p23 and CYP40. In the presence of active hormone ligand, the receptor undergoes conformational changes, dimerizes, discloses its NLS and releases associated proteins from the receptor complex [96, 97].

Activated GR and MR homodimers translocate to the nucleus with help of the importin system [97]. Receptor complexes then bind to glucocorticoid response elements (GRE). The GRE consensus sequence is defined as GGTACANNNTGTTCT [38]. GREs are located in the promoter region of GR and MR target genes. Binding of the active receptor complex and recruitment of the transcription machinery leads to the induction or repression of transcription [98, 99].

The activity and action of nuclear receptors is further modulated by post-translational modifications such as phosphorylation, ubiquitination, SUMOylation, methylation or acetylation. Splice variants and variants due to distinct translational initiation with different or similar activities of these described nuclear receptors are also known [100-104].

3.4 Antioxidant redox pathway

The antioxidant redox pathway is part of the cellular detoxification system. The metabolic detoxification process of cells involves detoxification of xenobiotics as well as potential endogenous toxicants with the aim to inactivate the compound and finally excrete a water soluble and harmless product.

In general, detoxification processes can be separated in three different steps, involving distinct proteins:

Phase I reactions: This step is often called functionalization, and reactive groups can be introduced in lipophilic molecules to facilitate subsequent conjungation, thereby enhancing solubility. Phase I biotransformation involves cytochrom P450 monooxygenases, monoaminooxigenases and dehydrogenases/reductases that are responsible for the oxidation, reduction and hydrolysis of compounds. The metabolic products of phase I reactions are often highly reactive metabolites that, if not efficiently removed, are able to lead to toxic insults [105, 106].

Phase II reactions: These reactions comprise conjugation, of reactive carboxyl, hydroxyl, sulfhydryl, and amino groups with glucuronicacid, sulphate or glutathione. The products of this metabolic process display higher solubility and are mostly less active or even inactive. Conjungation is mediated by a variety of enzymes including gluthation-S-transferases, sulfotrasferases, methyltransferases, UDP-glucuronosyl-transferases and N-acetyltransferases [105, 107].

Phase III reactions: These reactions involve transport proteins, such as multidrug resistance-related proteins (mdr) and ATP-binding cassette proteins (ABC-transporters) as well as other transporters and carriers [108], and they mediate excretion of the solubilized chemical.

Enzymes of the antioxidant redox pathway belong to all three phases of the detoxification process, with many of them belonging to phase II. However, these enzymes and transporters have the commonality of a specific regulatory DNA element, which mediates their induced expression after electrophile insults [109-112]. One of the first reports on an enzyme later well known as a member of the phase II detoxifying machinery was published in 1958 by Ernster *et al.* [113]. Ernster *et al.*

reported a soluble NAD(P)H: (quinone acceptor) oxidoreductase (NQO1), which was present in rat liver homogenates also known as DT-diaphorase [114]. The purified NQO1 protein was characterized as a dicoumarol sensitive enzyme, catalyzing a two electron reduction reaction [115]. Because of its characteristics, NQO1 was expected to play a role in cytoprotection against toxic chemicals [114].

The identification of additional enzymes responsible for the detoxification of xenobiotic insults such as glutathione-S-transferases (GST), promoted research on their transcriptional regulation and determining their basal and induced status, respectively [116]. Two distinct cis-acting regulatory elements, the xenobiotic response element (XRE) and the antioxidant response element (ARE) (also termed electrophile response element (EpRE) [117]), were identified and characterized in the sequence of GSTs and NQO1 [116, 118-120]. The first characterized XRE was known to be part of the regulated DNA of the cytochrome P-450 (CYP1A1) gene. However, ARE sequences share little homology with XRE motifs [118] (Table 5). Since GST as well as NQO1 are involved in the protection of the cell against oxidative stress, AREs were proposed to represent the consensus sequence for a so far unidentified transcription factor, which would be constitutively expressed and might sense oxidative stress [121]. Additional enzymes such as the billirubin converting hemeoxidase 1 (HO-1) [122], and members of the UDP-glucuronosyl transferases (UGT) [123], ferritin H, as well as glutamate cysteine ligase catalytic subunit [117] were proven to contain AREs in their promoter region. Together, these enzymes constitute the family of phase II detoxification enzymes. Enzymes responsible for the conjugation of chemicals ensure their final excretion, thereby increasing the cellular capacity to cope with endogenous and exogenous oxidants.

Table 5. Comparison of xenobiotic- (XRE) and antioxidant responsive elements (ARE) [108]

	Regulatory element	
	XRE	**ARE**
Consensus sequence	5´-C/GT-GCGTG-A/T-3´	5-gagTcACaGTgAGtCggCAaaatt-3
First identified in	CYP1A1	GST Ya
Inducers	β-naphthoflavone, tetra-chlordibenzo-*p*-dioxin (TCDD)	t-butylhydroquinone, β-naphthoflavone
Receptor	Arylhydrocarbon receptor (AhR)	nuclear factor-erythroid 2-related factor 2 (Nrf2)

Later, the nuclear factor-erythroid 2-related factor 2 (Nrf2) as well as Nrf1 were shown to be the major transcription factors acting on ARE elements, thereby inducing the expression of many phase II enzymes, including NQO1 [124] (Figure 6).

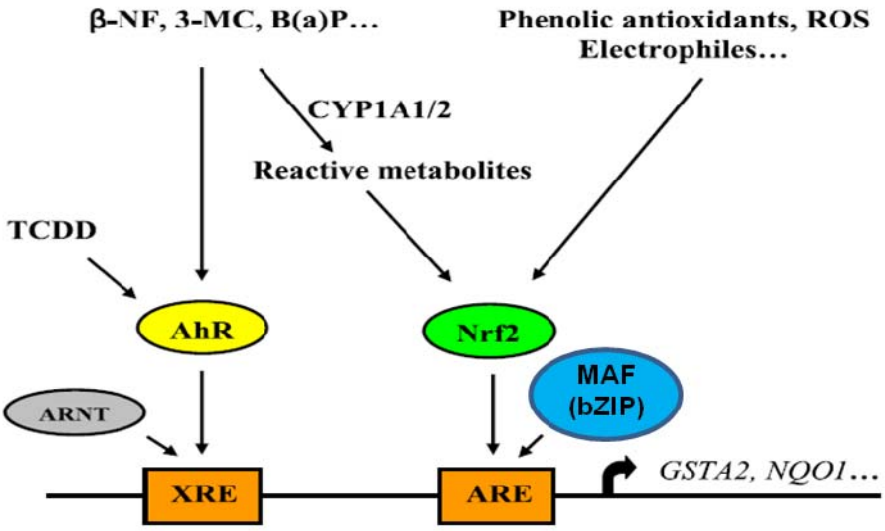


Figure 6. Regulation of NQO1 and GSTA2 by the arylhydrocarbon receptor as well as the Nrf2-Keap1 pathway reflecting two different detoxification pathways (modified from Nguyen *et al.* [125]).

β-NF, β-naphthol; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetraclorodibenzo-p-dioxin; 3-MC, 3-methylcholanthrene; ARNT, aryl-hydrocarbon receptor nuclear translocator; AhR, aryl-hydrocarbon receptor.

Nrf2 triggers the transcriptional regulation of its target genes upon the induction of a broad range of structurally highly diverse chemical classes.

Chemicals activating ARE include [126]:

- Synthetic phenolic antioxidants (butylatedhydroantisol, tert-butylhydroquinone)
- Synthetic antioxidants (ethoxyquin, pyrrolidinedithiocarbamate)
- Coumarin (hydroxycoumarin)
- Isothiocyanate (sulforaphane)
- GSH-depleting agent (diethylmaleate)
- Phorbol ester (phorbol 12-myristate 13-acetate)
- Flavonoids (β-naphthoflavone)
- 1,2-dithiole-3-thione (Oltipraz)

3.4.1 Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2)

Using the human myelogenous leukaemia cell line K562 and tandem repeats as a recognition site Moi *et al.* cloned a novel member of the NF-EF2 family (Figure 7).

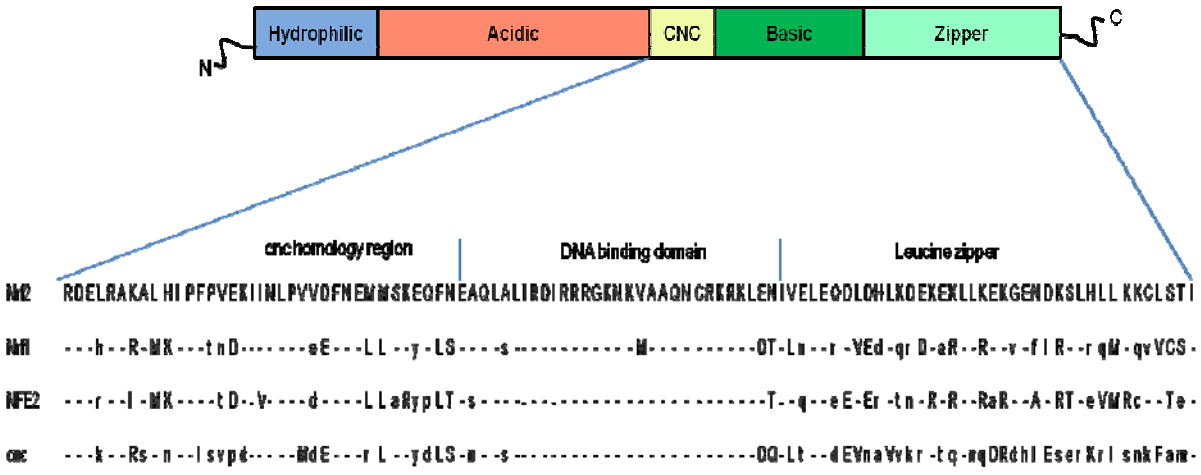


Figure 7. Amino acid sequence of Nrf2 with the already known members of the NF-E2 family (modified from Moi *et al.* [127]).

The gene was termed Nrf2 and further described as a 2.2 kb transcript, which is expressed in heart, brain, placenta, lung, liver, muscle and pancreas and encodes a predicted protein of 66 kDa [127]. Cross-species homologs of Nrf2 from chicken (ECH) [128] and mouse [129] were identified thereupon. Chromosomal localization of the human Nrf2 was defined as 17q2q31 [130]. With respect to the unusual leucine-zipper motif (hepta repeats of leucines interrupted by a polar asparagine residue) of Nrf2, Moi and colleagues postulated Nrf2 to form heterodimers, with a so far unidentified protein [127]. Indeed, the interacting protein was isolated by the same group and identified as member of the small Maf proteins [131].

Maf proteins belong to the leucine zipper-containing transcription factors, possessing with activator and repressor functions. They lack a transcriptional effector domain [132]. Members of the small Maf protein family (MafF, MafG, and MafK) form heterodimers with other transcription factors and proteins like Nrf2.

The comparison of the species homologues of the Nrf2 cDNA (Figure 7) led to the identification of six conserved regulatory domains (Neh1-6) within the protein [133] (Figure 8).

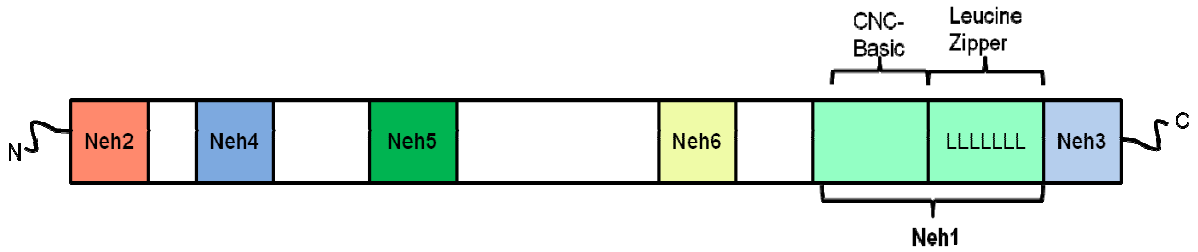


Figure 8. The schematic overview of conserved Neh-domains within the Nrf2 protein (modified from Itho *et al*. [133]).

With respect to its conserved DNA binding domain and the domain responsible for its heterodimerization with maf proteins, Nrf2 belong to the Cap n' Collar (CNC) superfamily representing a subclass of the bZIP proteins [134].

Nrf2 knock out is not lethal and the transgenes have no obvious phenotype concomitant with normal growth and development [135]. However, aged animals develop autoimmune related diseases such as multiorgan immune inflammation [136] and vacuolar leukoencephalopathy [137]. Furthermore, Nrf2 deficient animals have a vastly increased susceptibility for toxic compounds and the development of cancer [111, 138-141].

3.4.2 The Kelch-like ECH-associated protein1

The outermost amino-terminal domain (Neh2) of Nrf2 was shown to act as a negative regulatory domain [133]. In the same study, a cytoplasmic protein physically interacting with Neh2, and negatively influencing the transcriptional activity of Nrf2, was identified. The Kelch-like ECH-associated protein 1 (Keap1) [133] was named due to its similarity to an actin-binding protein found in Drosophila, and called Kelch [142]. Keap1 is able to sequester Nrf2 in the cytoplasm and repress its transcriptional activity. Electrophile compounds such as catechol and diethylmaleate abolished Keap1-mediated Nrf2 repression in a dose-dependent manner [133]. Chemicals able for the activation of the antioxidant redox response pathway (see 3.4) belong to different classes comprising highly diverse structures. However, they share the ability to modulate and interfere with the Nrf2-Keap1 complex. This action is expected to involve oxidative modifications of specific and regulatory cysteines within Keap1 resulting in a conformational change of Keap1 [143, 144]. Keap1 is a cysteinerich protein, and the 25 cysteins of murine Keap1 are conserved for rat and human [133]. The different cysteine residues are spread over the five Keap1 domains (Figure 9)

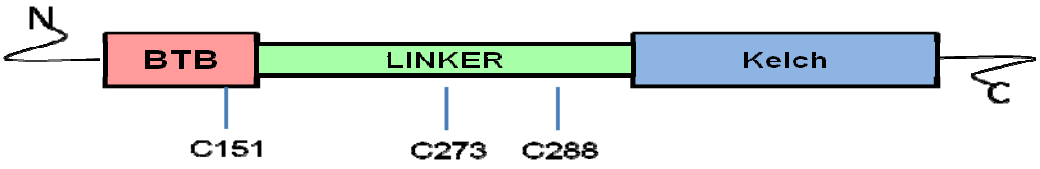


Figure 9. Schematic overview of the discrete domains within the Keap1 protein and the localization of three important regulatory cysteine residues (modified from Zhang *et al*. [145]).

N, N-terminal domain; BTB, Bric-a-brac, Tramtrack, and Broad; Linker, linker-domain; kelch, kelch repeat domain; and C, C-terminal domain.

The protein includes the N-terminal domain, the BTB domain (termed because of the similarity to the Drosophila transcription factors Bric-a-brac, Tramtrack, and Broad), the central linker domain also called intervening region (IVR), followed by the kelch domain, comprising six kelch repeats for human Keap1, and the final the C-terminal domain [145, 146]. The kelch-repeats are responsible for binding to cytoskeletal actin as well as Nrf2 [133, 147]. The highest modulatory cysteine content can be found in the central linker domain [146]. However, not all of these cysteines are regulatory [148]. Important cysteines modified by the insult of electrophile compounds are mainly located in the central linker domain (C273, C288) but also found in the BTB-domain (C151). Hereby, the modification pattern and the consequences following on the stability of Nrf2 are specific for the electrophile compound [148, 149].

Knocking-out the gene encoding Keap1 leads to postnatal lethality [150]. Lethality was related to the constitutive activity and dominant nuclear localization of Nrf2 in the transgene [150]. Keap1-KO mice suffered from excessive hyperkeratosis, mucosa detachment concomitant with inflammatory cell infiltration and, more obvious, severe growth retardation [150]. In line with these findings, transgenic mice lacking small Maf proteins rescued the lethal phenotype of the Keap1-KO demonstrating that small Maf proteins are essential for functionally active Nrf2 *in vivo* [132]. Confirmative cross-breeding with Nrf2-KO mice rescued the lethal phenotype of the Keap1-KO [150]. The expression of enzymes, known to be part of the inducible detoxification machinery regulated by Nrf2, was enhanced in the transgene animal and was further shown to be constitutively up regulated without further induction [150]. Impairment of Keap1 is further associated with different types of cancer, including lung cancer [151].

As a conclusion, both Keap1 and Nrf2 are suggested to act as intracellular sensors for oxidative stress, further leading to the transcriptional induction of genes for phase II detoxifying enzymes [133].

3.4.3 Interaction of Nrf2 and Keap1: Putative mechanism within the antioxidant redox pathway

Upon identification of the key regulators of the ARE pathway (Nrf2, Keap1, MAF) the elucidation of the mode of action and of the mechanism responsible for the regulation of gene transcription represented a major challenge. Currently, the following observations and scenario are accepted among most researchers in the field.

1. Nrf2 is a functionally active transcription factor that controls basal and inducible expression of its target genes [152].

2. Keap1 is a constitutively expressed negative regulator of Nrf2. Keap1 acts as an adaptor protein, which promotes ubiquitination of Nrf2 by the cullin-3-dependent pathway [145, 147, 152, 153].

3. Nrf2 is an unstable protein with short half-life (15-30 min), and its degradation *via* the ubiquitin-pathway is mediated by the 26S-proteasome [154].

4. Keap1 contains reactive cysteine residues, some of which were shown to be regulatory (Cys: 257, 273, 288, and 297) and therefore expected to act as redox sensors. Modification of the regulatory cysteines is electrophile-specific and can in some cases stabilize Nrf2 by preventing its degradation [148].

Recognition of Nrf2 by Keap1 is mediated by tow highly conserved motifs within the Nrf2 protein namely DLG and ETGE. In this process Keap1 is expected to bind Nrf2 in a Hinge-and-Latch fashion over a two-site-substrate recognition model [155]. The stoichiometry of the Nrf2-Keap1 complex is 1:2, while one Nrf2 molecule is bound to a homodimer of Keap1. Homodimerization of Keap1 molecules occur between the N-terminal BTB/POZ motifs. BTB/POZ motifs have been found in zinc-finger proteins, and such proteins contain, like Keap1, kelch motifs. Homodimerization instead of heterodimerisation is a common characteristic for BTB containing proteins. Furthermore, these motifs have been shown to mediate transcriptional repression as well as interaction with common co-repressors such as nuclear receptor co-repressor

1 (N-CoR) and nuclear receptor corepressor 2 (N-CoR 2 or SMRT). Keap1 acts as substrate adaptor protein for the Cul3-dependent E3 ubiquitin ligase complex [145]. Ubiquitination of proteins requires a defined lysine residue position on the substrate for transfer of the ubiquitin moiety. The DLG motive of the Nrf2 has low affinity for Keap1 and functions as latch, responsible for the functional positioning of Nrf2 molecule for ubiquitination under non stressed conditions [155]. On the other hand, the ETGE motif has high affinity and represents the hinge [155]. Within the interaction of Nrf2 and Keap1 under non-stressed conditions the lysines comprising the Nhe2 domain of the Nrf2 substrate are ubiquitinated (Figure 10).

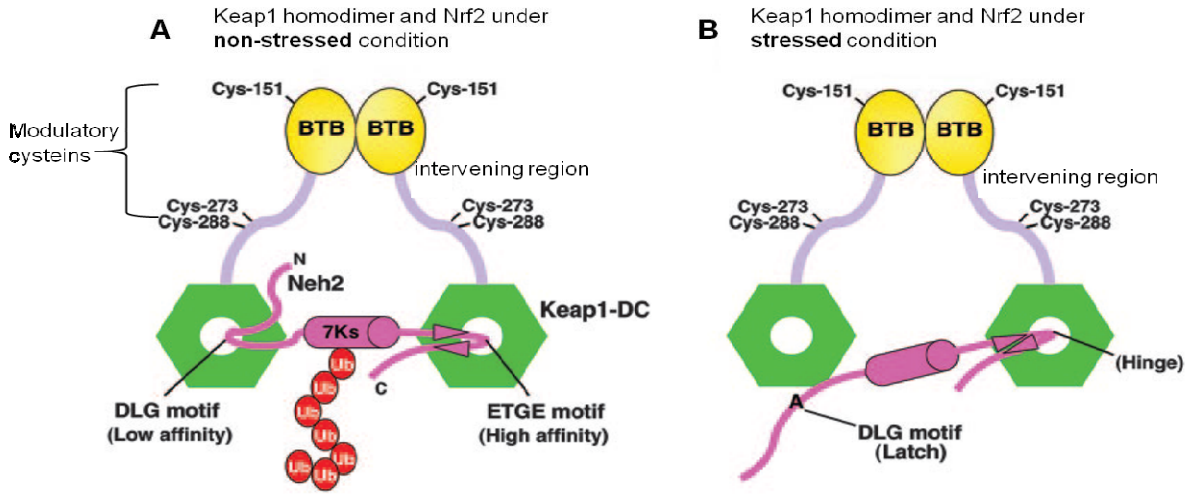


Figure 10. Interaction of Keap1 and Nrf2 over the two-site-substrate mode (modified from Tong *et al.* [155]).

Under stressed conditions, the DLG-mediated binding of Nrf2 to Keap1 is disturbed. However, Nrf2 is still bound to Keap1 over the ETGE motif, and ubiquitination of the Neh2 domain is no longer possible because of its unfavourable orientation. The aforementioned and other findings [110, 125] are also summarized in Figure 11.

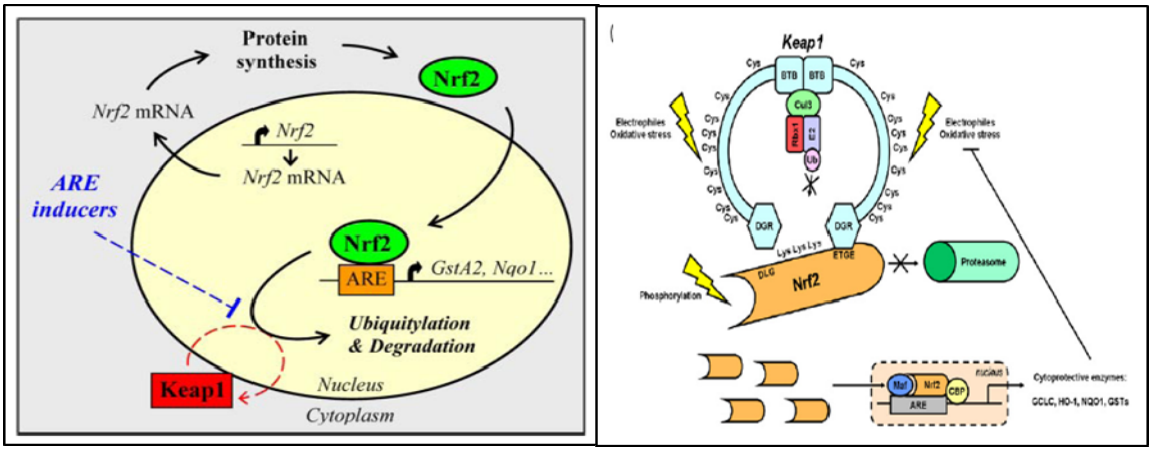


Figure 11. Putative mechanism for the suppressive action of Keap1 on Nrf2 (right; modified from [110]), and the regulation of ARE by Nrf2 (left; modified from [125]).

Nrf2 itself, without its co-regulator Keap1, is a constitutively expressed nuclear protein; however, its cellular localization remained unclear for a long time mainly due to technical difficulties [125].

After protein synthesis of Nrf2 in the cytoplasm, the functional receptor translocates into the nucleus. Inside of the nucleus, Nrf2 regulates the transcription of its target genes (GSTs, NQO1, HO-1, UGTs, etc.) [118-122]. Hereby Nrf2 forms heterodimers with its co-receptor, a member of the small Maf proteins [111, 131]. Under basal conditions, Nrf2 undergoes rapid proteosomal degradation [133, 145, 153], a process regulated by Keap1. Functional Nrf2 is translated into the cytoplasm, where it is sequestered by the constitutively expressed repressor Keap1 [144]. Modification of reactive cysteines of Keap1, due to electrophile insult of endogenous or exogenous electrophile species renders the capacity of Keap1 to promote Nrf2 proteosomal degradation [147, 148, 156]. This cytosolic fraction of Keap1 becomes saturated by Nrf2. Freshly produced Nrf2 can now enter the nucleus to regulate the transcription of target genes as already described above [110].

Whether Keap1 is able to translocate into the nucleus to remove Nrf2 by ubiquitination from these compartment, or whether Nrf2 is actively shipped from the nucleus into the cytoplasm by Keap1 for the later degradation, requires further studies [125, 147]. Furthermore, Nrf2 phosphorylation affecting its stability or transcriptional activity are described processes; however, there are still open questions that remain to be answered [143, 157].

4 DIBUTYLTIN DISRUPTS GLUCOCORTICOID RECEPTOR FUNCTION AND IMPAIRS GLUCOCORTICOID-INDUCED SUPPRESSION OF CYTOKINE PRODUCTION.

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The Study revealed the disruption of GR-dependent action in general and especially in inflammation by dibutyltin.

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Dibutyltin Disrupts Glucocorticoid Receptor Function and Impairs Glucocorticoid-Induced Suppression of Cytokine Production

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Abstract

Background: Organotins are highly toxic and widely distributed environmental chemicals. Dibutyltin (DBT) is used as stabilizer in the production of polyvinyl chloride plastics, and it is also the major metabolite formed from tributyltin (TBT) *in vivo*. DBT is immunotoxic, however, the responsible targets remain to be defined. Due to the importance of glucocorticoids in immune-modulation, we investigated whether DBT could interfere with glucocorticoid receptor (GR) function.

Methodology: We used HEK-293 cells transiently transfected with human GR as well as rat H4IIE hepatoma cells and native human macrophages and human THP-1 macrophages expressing endogenous receptor to study organotin effects on GR function. Docking of organotins was used to investigate the binding mechanism.

Principal Findings: We found that nanomolar concentrations of DBT, but not other organotins tested, inhibit ligand binding to GR and its transcriptional activity. Docking analysis indicated that DBT inhibits GR activation allosterically by inserting into a site close to the steroid-binding pocket, which disrupts a key interaction between the A-ring of the glucocorticoid and the GR. DBT inhibited glucocorticoid-induced expression of phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine-aminotransferase (TAT) and abolished the glucocorticoid-mediated transrepression of TNF-α-induced NF-κB activity. Moreover, DBT abrogated the glucocorticoid-mediated suppression of interleukin-6 (IL-6) and TNF-α production in lipopolysaccharide (LPS)-stimulated native human macrophages and human THP-1 macrophages.

Conclusions: DBT inhibits ligand binding to GR and subsequent activation of the receptor. By blocking GR activation, DBT may disturb metabolic functions and modulation of the immune system, providing an explanation for some of the toxic effects of this organotin.

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Introduction

Organotins are among the most toxic and widely distributed environmental chemicals. The most abundant organotin in the environment is tributyltin (TBT), a molluscicide and fungicide widely used as an antifouling paint for boat and fish nets and that thus is dispersed into the marine environment [1,2]. TBT interferes with reproduction in marine animals, inducing imposex (superimposition of male sexual characters in females) in gastropod molluscs, an effect used to measure TBT pollution in sea-water [3,4]. TBT concentrations ranging from 4–323 nM were measured in blood samples from healthy human subjects [5]. In mammals, organotins are hepatotoxic, neurotoxic and immunotoxic. At doses comparable with those found in human blood, TBT promotes Th2 cell polarization and exacerbates airway inflammation, providing a possible mechanism for enhanced susceptibility to allergic diseases [6]. Thus, TBT contamination represents a serious health problem and the great concern about the toxicity of TBT is underlined by recent negotiations of the United Nations' International Maritime Organization for a global ban in the use of TBT.

In vivo, TBT is mainly metabolized to DBT in the liver, involving cytochrome P450 enzymes [7,8]. DBT, itself, is used in the production of polyvinyl chloride (PVC) plastic tubes and bottles [9], and humans are exposed to DBT by direct uptake from drinking water due to leaching from PVC water distribution pipes [10]. DBT is considered to be highly neurotoxic and immunotoxic [5,11,12], and concentrations ranging from 11–401 nM were measured in human blood [5]. Hence, DBT needs to be considered as a potential toxic chemical. Both TBT and DBT cause thymus involution by inhibiting the proliferation of immature $CD4^-/CD8^+$ thymocytes, but at high concentrations, they induce thymocyte apoptosis [13]. The immunotoxic effects of DBT are more rapid and more pronounced than those of TBT, raising the possibility that some effects of TBT are primarily

caused by its metabolite DBT [14]. The target(s) for the immunotoxic actions of DBT have not been determined.

We hypothesize that DBT affects one or more glucocorticoid responses because this steroid has important actions on the immune system [15,16]. Despite the importance of glucocorticoids in the regulation of immune functions, only few studies investigated the potential interference of environmental pollutants with glucocorticoid-mediated responses [17]. None of these studies investigated DBT as a disruptor of the immune response.

With the goal of elucidating the immunotoxic mechanism of DBT and other organotins, we investigated their potential interference with glucocorticoid action using cells expressing recombinant GR, as well as macrophages expressing endogenous GR. We found that DBT, in contrast to TBT, diphenyltin (DPT) and triphenyltin (TPT), inhibits dexamethasone binding to GR and blocks receptor activation. We then used docking software [18,19] to study the binding of these chemicals to the GR and provide evidence that DBT, but not the other organotins, disrupts an essential interaction between the A-ring on dexamethasone and the GR by binding to an allosteric site.

Results

Dibutyltin, but not other organotins tested, inhibits the transcriptional activity of GR

To investigate whether organotins disrupt GR-dependent transcriptional regulation, we employed HEK-293 cells transiently expressing recombinant human GRα and a β-galactosidase reporter driven by a glucocorticoid-responsive MMTV-promoter. In the absence of organotins, 100 nM cortisol increased GR-dependent expression of galactosidase activity 185-fold. HEK-293 cells that were transfected only with the β-galactosidase reporter showed background activity because HEK-293 cells express no or very low levels of endogenous GR. Upon simultaneous incubation of cells with DBT and cortisol, a dose-dependent inhibition of GR-mediated transactivation was observed (Fig. 1A). At 1 μM DBT, the inhibition was comparable with that of 1 μM RU486. Interestingly, the repeated exposure of cells to DBT increased the inhibitory effect on GR-mediated transactivation. Upon subjecting cells to eight changes of medium containing a given DBT concentration at 15 min intervals and preincubation for another 14 h, a two- to three-fold stronger inhibition of GR transactivation was observed than in cells that were preincubated for 16 h with the same DBT concentration (data not shown). In contrast to DBT, a slight stimulation of transactivation was found for TBT, reaching a maximal effect at 250 nM (Fig. 1B). Whereas DPT had no effect on GR-mediated transactivation (Fig. 1C), an up to two-fold stimulation was observed for TPT, with a 75% increase of transactivation at 10 nM (Fig. 1D). This stimulation was reversed at 250–500 nM. At these concentrations, no cytotoxicity could be detected in the MTT assay. Dimethyltin, trimethlytin and dioctyltin, up to 1 μM, did not affect GR-mediated transactivation. Similar results were obtained in experiments using 10 nM dexamethasone instead of 100 nM

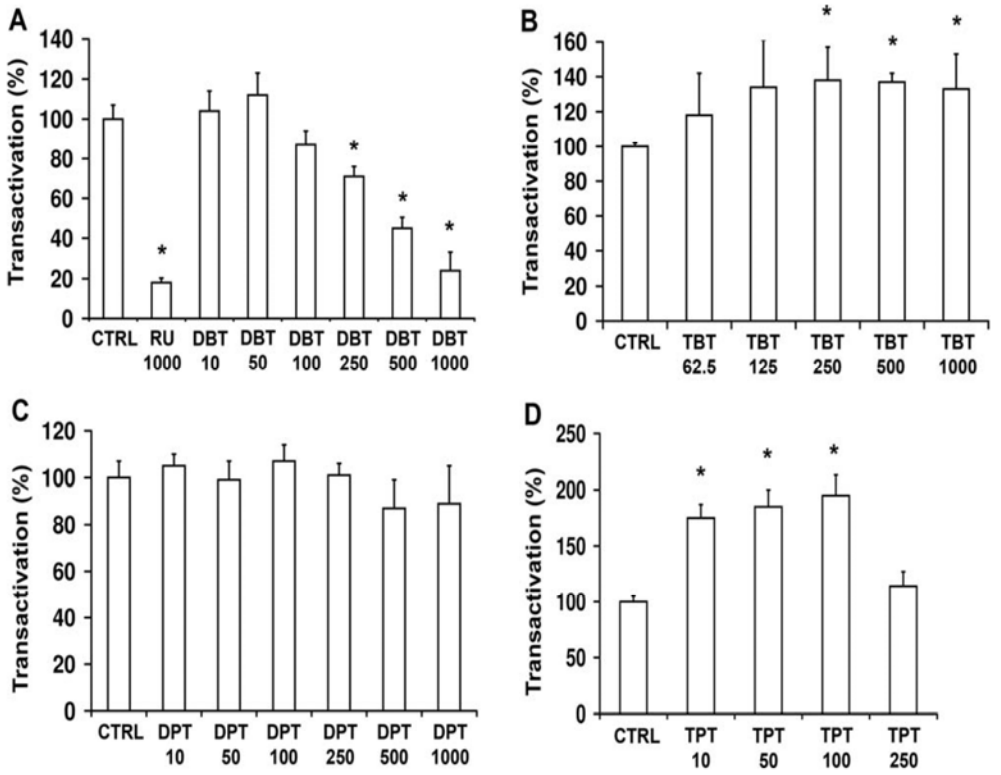

Figure 1. Inhibition of GR-mediated transactivation by organotins. HEK-293 cells transiently expressing pMMTV-LacZ, pCMV-LUC and human GR-α were simultaneously incubated with 100 nM cortisol and vehicle (CTRL), various concentrations of dibutyltin (DBT) (*A*), tributyltin (TBT) (*B*), diphenyltin (DPT) (*C*), triphenyltin (TPT) (*D*) or 1 μM of RU486 (RU). After incubation for 20 h, galactosidase reporter activity, normalized to the internal luciferase control, was determined. Data (mean±SD from four independent experiments) represent percentage relative to the control at 100 nM cortisol in the absence of inhibitors. *$p<0.05$.
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cortisol. None of the organotins tested stimulated GR-mediated transactivation in the absence of glucocorticoids (data not shown).

Dibutyltin blocks ligand binding to GR

To assess whether DBT inhibits GR function by directly interfering with ligand binding, HEK-293 cells were simultaneously incubated with radiolabeled [^{3}H]-dexamethasone and various concentrations of DBT. A dose-dependent decrease of dexamethasone binding to GR was observed, with approximately 50% inhibition of binding upon incubation with 150 nM DBT (Fig. 2A). Addition of unlabeled dexamethasone revealed a typical displacement pattern of tritiated dexamethasone in the presence or absence of DBT (Fig. 2B,C). A more pronounced inhibitory effect by DBT on dexamethasone binding to GR was obtained after preincubating cells overnight with DBT-containing medium, with an IC_{50} of about 50 nM (Fig. 2C). To determine if the reduced dexamethasone binding to GR upon preincubation with DBT was due to decreased GR protein expression, we performed Western blotting of extracts from cells expressing GR and preincubated with either vehicle or DBT. Incubation with DBT for either 3 h or 16 h did not alter GR expression relative to the actin control (Fig. 2D), suggesting that DBT did not affect the number of receptor complexes but decreased glucocorticoid binding affinity. Thus, inhibition of glucocorticoid binding to GR in the presence of DBT provides an explanation for the reduced transactivation of the β-galactosidase reporter.

DBT disrupts glucocorticoid-dependent target gene regulation in liver cells

The observation that DBT blocked GR activation in an overexpression cell system led us to test whether the disruption of GR activation could be observed in cells expressing endogenous receptor. Incubation of rat H4IIE hepatoma cells, a widely used liver cell model, with 10 nM dexamethasone for 24 h resulted in elevated PEPCK mRNA expression approximately 6-fold (Fig. 3A). This glucocorticoid-induced stimulation of PEPCK mRNA expression was diminished in a dose-dependent manner when cells were preincubated for 16 h with DBT. The effect of glucocorticoids was almost completely abolished in the presence of 500 nM DBT.

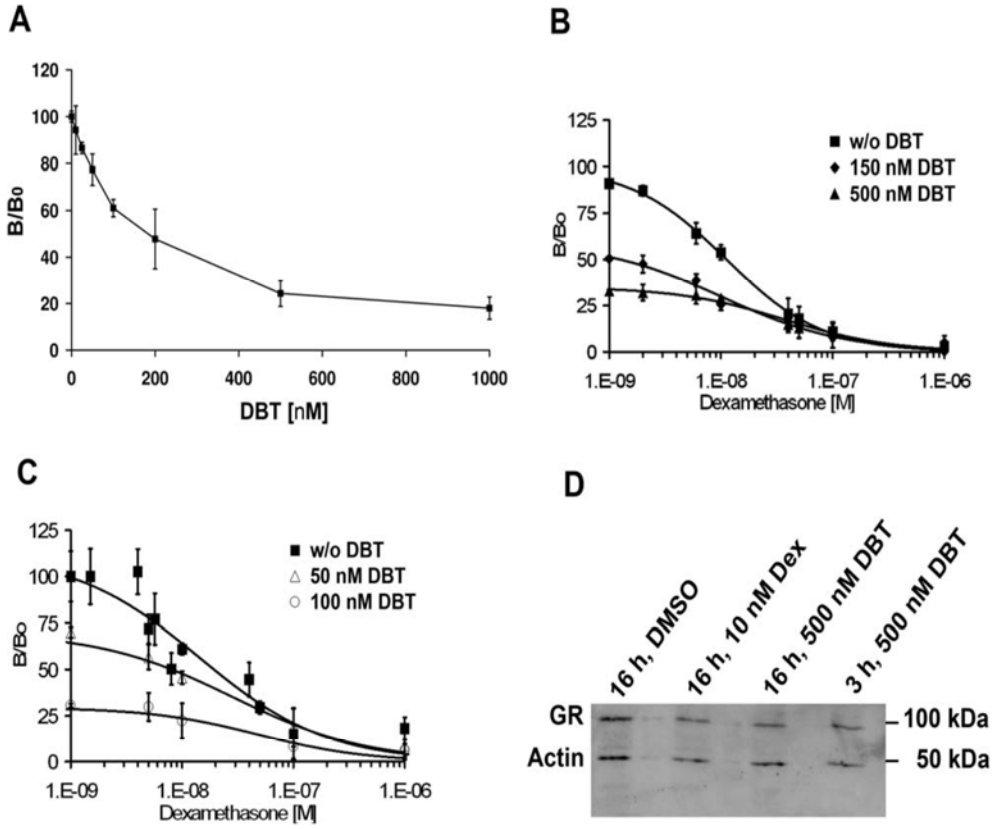

Figure 2. Inhibition of ligand binding to GR by dibutyltin. HEK-293 cells were transfected with a plasmid for human GR-α, followed by overnight incubation in serum-free medium containing vehicle (0.1% DMSO)(*A*,*B*) or in medium containing either vehicle or 50 nM or 100 nM DBT (*C*). Cells were then incubated for 3 h with 10 nM radiolabeled dexamethasone and various concentrations of DBT (*A*) or with 10 nM radiolabeled dexamethasone, various concentrations of unlabeled dexamethasone and various concentrations of DBT (*B*,*C*) prior to removal of unbound steroids and determination of bound dexamethasone by scintillation counting (*A*). Data represent mean±SD from three independent experiments. *D*, cells transiently expressing GR-α were preincubated for 16 h in serum-free medium containing vehicle (0.1% DMSO), 10 nM dexamethasone (Dex) or 500 nM DBT, followed by incubation for another 3 h with vehicle, 10 nM dexamethasone or 500 nM DBT and 10 nM dexamethasone, respectively (lane 1–3). Alternatively, cells preincubated for 16 h in serum-free medium were incubated for 3 h in medium containing 500 nM DBT and 10 nM dexamethasone (lane 4). Cells were lysed and the expression of GR protein and actin control was analyzed by Western blotting.
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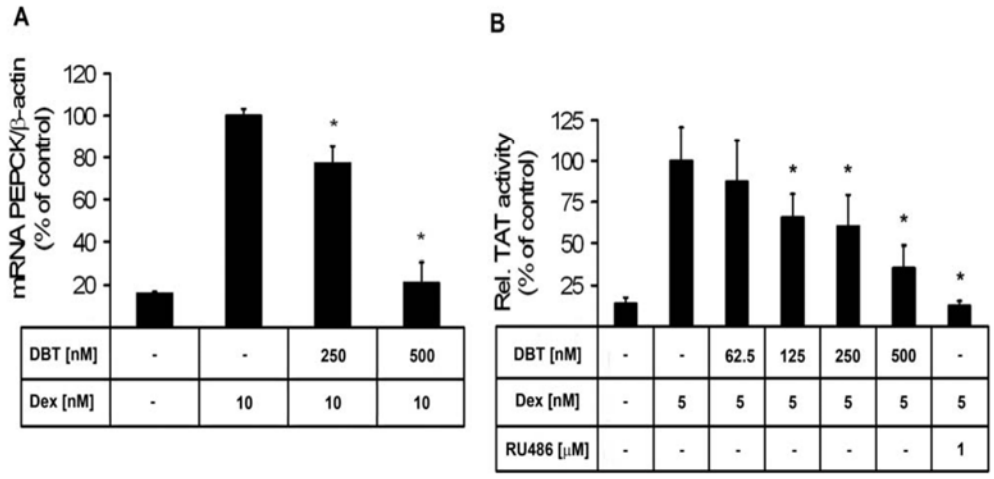

Figure 3. Inhibition of glucocorticoid-mediated expression of hepatic target genes by dibutyltin. *A*, DBT prevents glucocorticoid-induced stimulation of PEPCK mRNA expression. H4IIE hepatoma cells were preincubated for 16 h in the absence or presence of DBT, followed by adding 10 nM dexamethasone and incubation for another 24 h at 37°C. mRNA levels were determined by real-time RT-PCR using TaqMan technology. Data (mean±S.D. of triplicates from four independent experiments) are relative to the ratio of PEPCK mRNA to β-actin control mRNA from cells treated with dexamethasone in the absence of DBT. *B*, DBT inhibits glucocorticoid-induced stimulation of TAT activity. H4IIE cells were preincubated for 16 h in the absence or presence of DBT, followed by adding 5 nM dexamethasone and incubation for another 20 h. TAT activity, determined in cell lysates and normalized to protein content, is given as mean±S.D. of triplicates from four independent experiments and normalized to the positive control in the presence of dexamethasone. * $p<0.05$.
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As a second marker of GR activation, we measured the effect of DBT on TAT activity. Incubation with 5 nM dexamethasone increased TAT activity approximately 6-fold in H4IIE hepatoma cells. (Fig. 3B). Treatment of cells with DBT dose-dependently diminished the dexamethasone-induced stimulation of TAT activity. GR antagonist RU486 (1 μM) was used as a control. DBT had no effect on basal TAT activity in the absence of glucocorticoids (data not shown).

DBT impairs glucocorticoid-mediated suppression of cytokine production in macrophages

Exposure of macrophages to LPS strongly induces production of various pro-inflammatory cytokines. Among them, TNF-α and IL-6 play a pivotal role in the inflammatory response. The anti-inflammatory effect of glucocorticoids is at least in part due to the modulation of cytokine-production in a GR-dependent manner. To assess potential interference of DBT with glucocorticoid-dependent suppression of pro-inflammatory cytokines, native human monocytes were differentiated into macrophages. Macrophages were then preincubated for 16 h with vehicle or DBT, followed by simultaneous addition of LPS and 10 nM dexamethasone and incubation for another 20 h. Detection by ELISA revealed a DBT dose-dependent abrogation of the dexamethasone-mediated suppression of TNF-α and IL-6 production after LPS-induced stimulation (Fig. 4A). At 500 nM the effect of DBT was comparable with that of 1 μM RU486. DBT alone had no effect on IL-6 or TNF-α production (not shown). Similar results were observed in experiments with THP-1 cells that were differentiated into macrophages by treatment with PMA (Fig. 4B).

Dibutyltin inhibits GR-mediated trans-repression of NF-κB

Trans-repression of NF-κB activity by GR is important for the anti-inflammatory action of glucocorticoids. Therefore, we tested whether DBT might reverse glucocorticoid-dependent inhibition of NF-κB activation upon incubation with TNF-α. TNF-α strongly stimulated the expression of a luciferase reporter gene driven by the MHC promoter containing three NF-κB binding sites, an effect which was suppressed by dexamethasone (Fig. 5). As for the cytokine production, the glucocorticoid-mediated suppression of NF-κB activation was inhibited in a dose-dependent manner by DBT or by RU486.

Docking of organotins into human GR

To study the mechanism by which DBT inhibits dexamethasone binding to GR, we used Autodock 4 [18,19] to screen the GR 3D-structure [21] for binding sites for DBT and other organotins. We identified two binding sites for DBT, DPT and TPT and one site for TBT (Fig. 6). These are the steroid-binding site and a site that is adjacent to the steroid-binding site [21]. Differences in the relative occupancy for each organotin in the GR are summarized in Table 1. The dexamethasone binding site was the primary binding site in the GR for all organotins. Occupancy of the steroid-binding site varied from 51% for TPT to 100% for TBT.

In docking earch organotin into the GR, Autodock provides a summation of the van der Waals, hydrogen bond and desolvation energies (summation binding energy) and a separate calculation of the electrostatic binding energy. For organotins, the summation binding energy consists mainly of hydrophobic interactions. In general, the electrostatic binding energy is about 20% to 25% of the summation binding energy for the binding of the organotins into the GR. Thus for DBT, TBT, DPT and TPT in the steroid-binding site, the summation binding energies are −20.1 kJ/mol, −26.1 kJ/mol, −22.4 kJ/mol and −26.7 kJ/mol, respectively. DBT, which is the smallest organotin has the weakest summation binding energy. For DBT, TBT, DPT and TPT the electrostatic binding energies are −3.6 kJ/mol, −2.5 kJ/mol, −1.5 kJ/mol and −0.3 kJ/mol, respectively. Interestingly, DBT has the strongest electrostatic binding energy and TPT has the lowest electrostatic binding energy in the steroid-binding site.

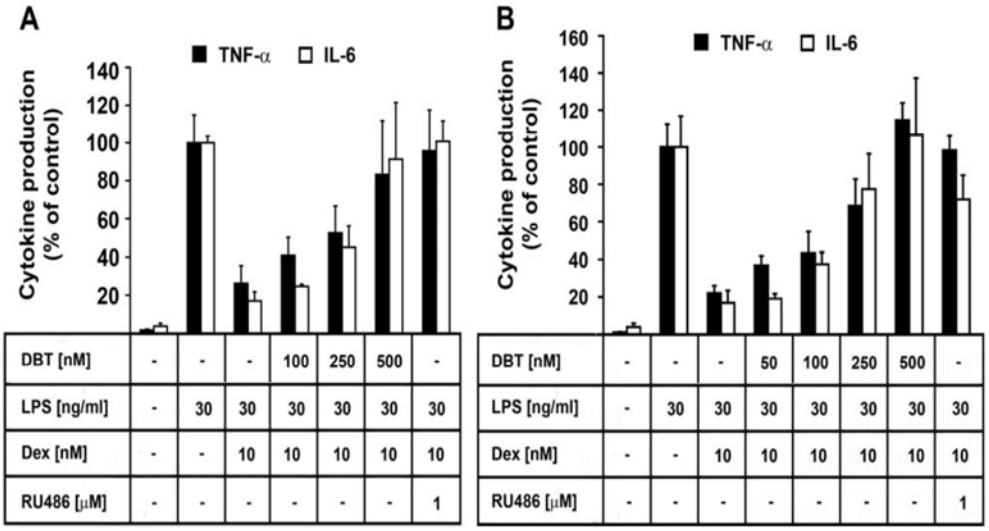

Figure 4. Dibutyltin abrogates GR-mediated suppression of cytokine production. Native human monocytes (*A*) or THP-1 cells (*B*) were differentiated into macrophages, incubated for 16 h with various concentrations of DBT prior to the addition of LPS and dexamethasone. Cells were incubated for another 20 h, and the levels of TNF-α (filled bars) and IL-6 (open bars) were determined by ELISA. Data represent mean±SD from three independent experiments, normalized to the LPS-stimulated control.
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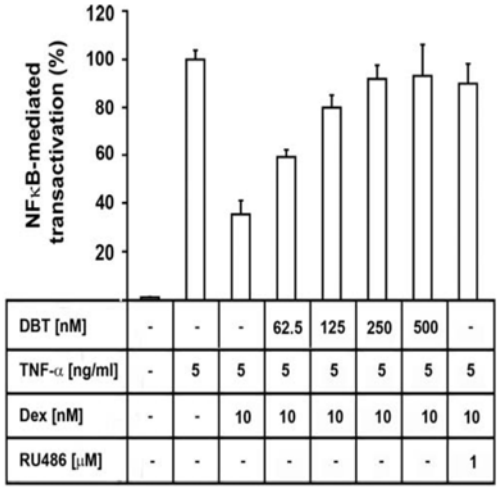

Figure 5. Inhibition of GR-mediated transrepression and potentiation of TNF-α-induced activation of NF-κB. HEK-293 cells were transfected with a plasmid for a luciferase reporter gene under the control of a promoter containing three NF-κB binding sites together with a plasmid for human GR. Cells were preincubated for 16 h with various concentrations of DBT prior to stimulation with TNF-α in the presence of glucocorticoids. Data represent mean±S.D. from three independent experiments, normalized to the TNF-α -stimulated control.
doi:10.1371/journal.pone.0003545.g005

For DBT, DPT and TPT in the allosteric site, the summation binding energies are −18.2 kJ/mol, −22.1 kJ/mol, −28.2 kcal/mol, respectively. For DBT, DPT and TPT, the electrostatic binding energies are −5.1 kJ/mol, −4.8 kJ/mol and −5.0 kJ/mol, respectively.

Organotin docking to the dexamethasone-binding site

Figure 7A shows DBT, TBT, DPT and TPT in the dexamethasone-binding site of the GR derived from the structure determined by Bledsoe *et al.* [21]. DBT and TBT overlap the region where the D-ring and C17-side chain of dexamethasone insert into the GR. Both DBT and TBT are much smaller than dexamethasone, which reduces interactions of these organotins with the GR. In contrast, DPT and TPT overlap much of the dexamethasone-binding site in the GR. In Figure 7A, we show four residues that are important in stabilizing binding of dexamethasone to the GR [21].

Organotin docking to an allosteric site

DBT, DPT and TPT docked into the GR at an allosteric site, which is close to the A-ring of dexamethasone (Fig. 7B). Of the four organotins that we studied, only DBT inhibits transcriptional activity of GR in the presence of 100 nM cortisol, which indicates that DBT exerts its inhibitory action by binding to the allosteric site. To investigate further the interaction of GR with DBT in this secondary site, we analyzed the interaction of DBT with GR under two conditions. First, we analyzed DBT in the apo-GR to simulate DBT binding in the absence of glucocorticoids. Second, we analyzed DBT in the GR-dexamethasone complex.

For the first analysis, we docked DBT into the GR and minimized the DBT-GR complex with Discover 3, to allow all of the amino acids in the GR to relax, so as to diminish unfavorable steric interactions and optimize favorable interactions between GR and DBT. Then, we inserted dexamethasone into the minimized structure and analyzed the interactions between DBT and dexamethasone with the GR. In this analysis, we focus on the effect of DBT on the interactions between the C3-ketone on dexamethasone and Arg^{611} and Gln^{570} in the GR because these amino acids have been shown to be important in stabilizing the A-ring of dexamethasone [21]. We also examined the interaction of Gln^{642} and Asn^{564} with substituents on the D ring of dexamethasone.

Analysis of DBT in the allosteric site of the GR reveals van der Waals contacts between DBT and Tyr^{660}, Tyr^{663}, Val^{543}, Leu^{544}

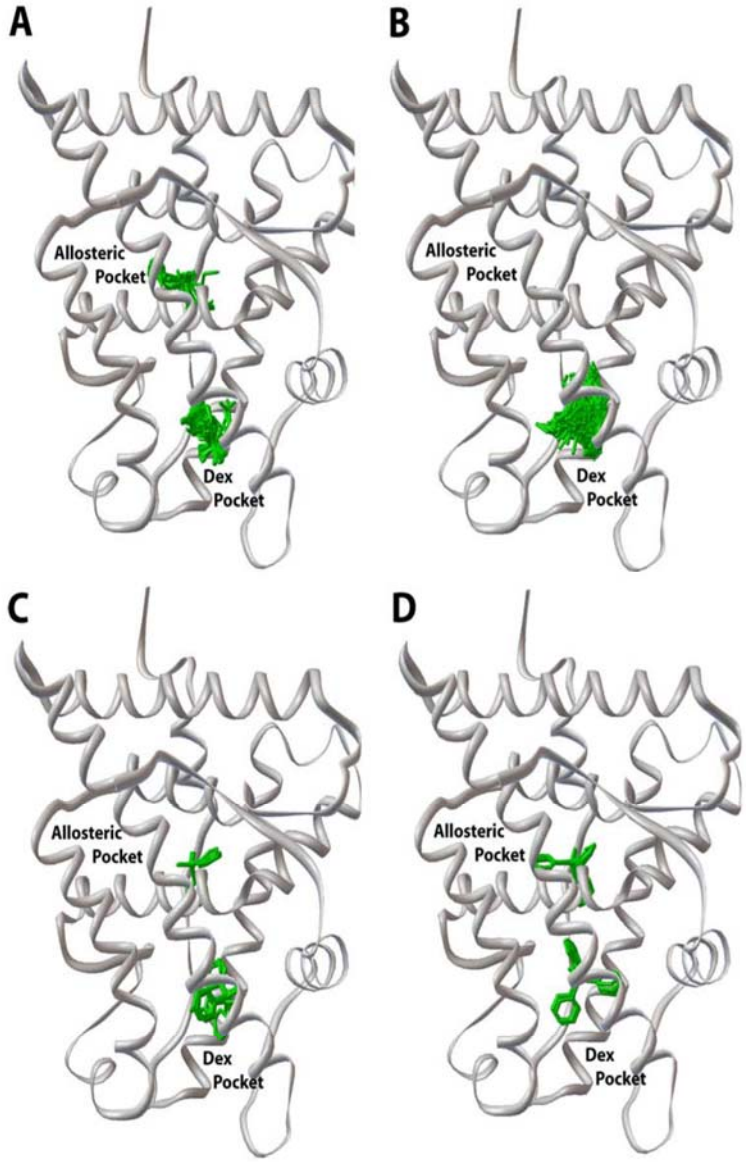

Figure 6. Docking sites for organotins in the GR. Autodock 4 was used to dock organotins (green) into the GR (1M2Z.pdb) (grey) without dexamethasone. DBT (*A*) docked into two sites on GR, with the highest percentage in the dexamethasone-binding site (Table 1). The second site for DBT was an adjacent allosteric pocket. Docking of TBT (*B*), DPT (*C*) and TPT (*D*) into the GR.
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and Arg^{611}. There also is a coulombic interaction between Glu^{540} and tin. Importantly, as shown in Figure 8A, there are two critical steric clashes between dexamethasone and the GR in the minimized GR-DBT complex. First, Gln^{570} is 1.4 Å from the C3-ketone, and second, Gln^{642} is 1.1 Å from the C17 hydroxyl group. Interestingly, other key amino acids, such as Asn^{564}, Arg^{611} and Phe^{623} [21], retain their favorable binding to dexamethasone. Thus, the 3D-models indicate that binding of DBT to an allosteric site in unliganded GR selectively distorts the ligand-binding domain and alters the interaction with dexamethasone.

To determine if the ternary DBT-GR-dexamethasone complex could relax into a conformation that would be favorable for dexamethasone binding, we minimized this complex using Discover 3. Analysis of this complex (Fig. 8B) reveals that after energy minimization the steric clashes between dexamethasone and Gln^{570} and Gln^{642} have been removed. After minimization, Gln^{642} moved to have a strong hydrogen bond with the C17 hydroxyl group, which also is present in the GR crystal structure [21]. Gln^{570} is more distant from the C3 ketone than in the crystal structure (Fig 7A). This change may be due to a van der Waals contact between Gln^{570} and DBT. Figure 8B also shows that there are changes in other interactions between DBT and residues in the allosteric pocket of the GR.

An important change, which has profound functional implications for steroid activation of the GR, is the loss of the contact

Table 1. Relative calculated occupancy by organotins in the GR.

Organotin	Dexamethasone pocket	Allosteric pocket
Dibutyltin	82%	18%
Tributyltin	100%	
Diphenyltin	87%	13%
Triphenyltin	56%	44%

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between Asn^{564} and the C11 hydroxyl on dexamethasone. Asn^{564} and corresponding asparagines on the progesterone receptor (PR) and mineralocorticoid receptor (MR) are important in stabilizing binding and transcriptional activity of cognate steroids to these receptors [22]. Asn^{564} and Gln^{570} are on α-helix 3 on the GR, PR and MR. Arg^{611} is on α-helix 5. The distance between α-helix 3 and α-helix 5 is crucial for steroid activation of the GR, PR [22] and MR [23]. Our analysis indicates that DBT will alter the conformation of α-helix 3 in the GR-dexamethasone complex.

Discussion

Despite the fact that organotins are well known and relevant environmental pollutants, we are only beginning to understand their mechanisms of toxicity. An important advance was the recent observation that the triorganotins TBT and TPT are potent activators of the nuclear hormone receptors RXR and PPARγ and that they promote adipocyte differentiation, suggesting that these organotins might contribute to the development of metabolic diseases [24–27]. Interestingly, the diorganotins DBT and DPT were found to be inactive toward RXR and PPARγ in these studies.

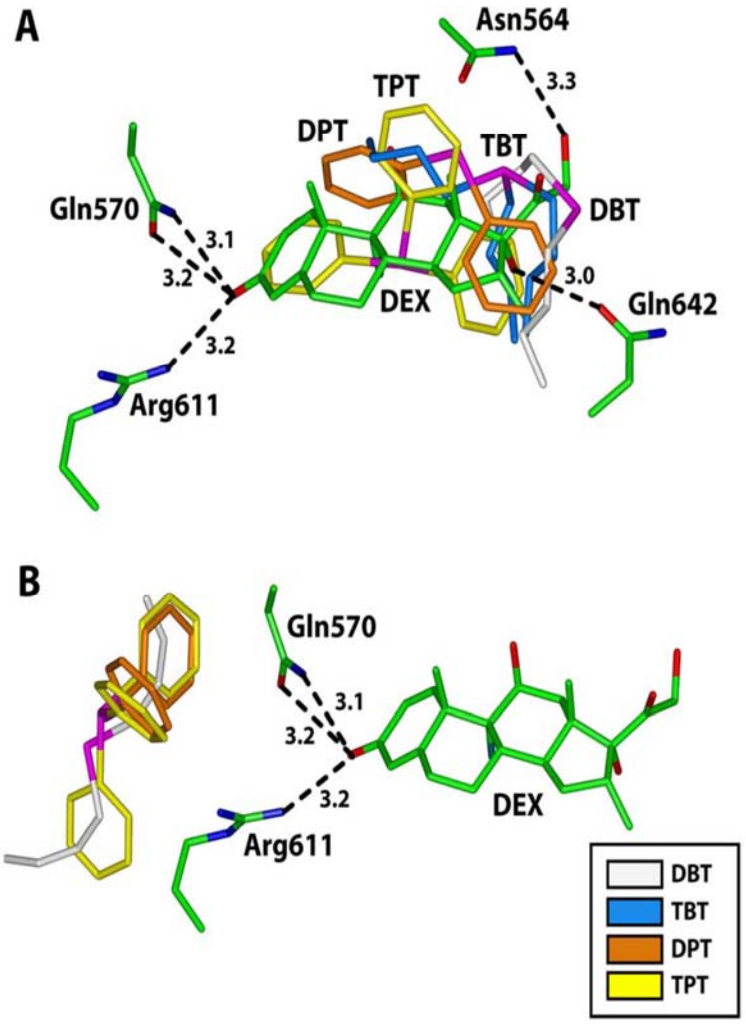

Figure 7. Docking of organotins into the dexamethasone-binding site and at an allosteric site in the GR. *A*, DBT (white), TBT (blue), DPT (orange) and TPT (yellow) fit into the dexamethasone-binding site of the GR. In these unminimized complexes with the GR, DBT and TBT overlap the D-ring and DPT and TPT most of dexamethasone. Distances between dexamethasone and Gln^{570}, Arg^{611}, Asn^{564} and Gln^{642} are taken from the crystal structure [21]. *B*, organotins docked into the GR at an allosteric site close to Glu^{570} and Arg^{611}.
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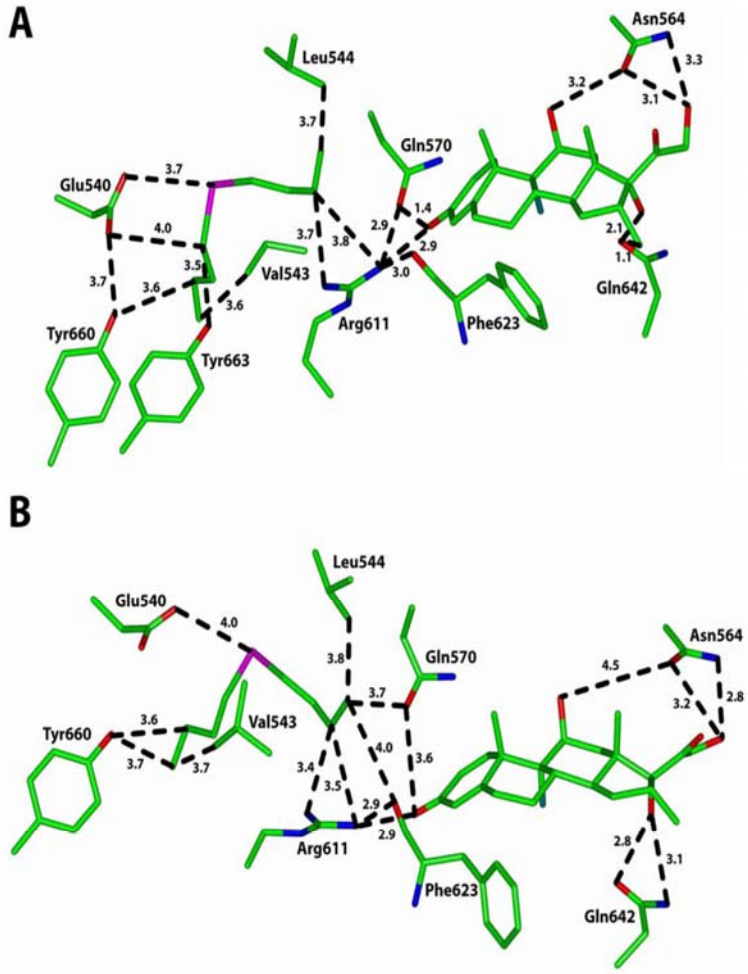

Figure 8. Interactions of dibutyltin with the GR in an allosteric site close to the dexamethasone-binding site. *A*, After minimization of the 3D-model of the DBT-GR complex, dexamethasone was inserted into the complex. There are steric clashes between dexamethasone and Gln^{570} and Gln^{642}, which are absent in the GR crystal structure [21] (Fig. 7A). The distances of Asn^{564}, Arg^{611} and Phe^{623} to dexamethasone are similar to that in the GR crystal structure. *B*, Interaction of dexamethasone with the GR after energy minimization of the DBT-GR-dexamethasone complex in (*A*) with Discover 3. Gln^{570} and Gln^{642} have moved and no longer have steric clashes with dexamethasone. Asn^{564}, however, has moved to 4.5 Å from the C11 hydroxyl on dexamethasone.
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The pathophysiological effects of DBT have been less well studied and understood. Here, we demonstrate for the first time that DBT at nanomolar concentrations, but not other organotins investigated, disrupts GR function. In a recent study we showed that the organotins DBT, TBT, DPT and TPT inhibited the enzyme 11β-HSD2, which catalyzes the conversion of active 11β-hydroxyglucocorticoids into their inactive 11-keto derivatives [28]. IC_{50} values of 11β-HSD2 inhibition between 1 μM for TPT and 5 μM for DBT were obtained. Thus, while some organotins such as TPT may enhance local glucocorticoid effects by inhibiting 11β-HSD2-dependent glucocorticoid inactivation, DBT blocks GR activation at concentrations that are ten-fold lower than those effective on 11β-HSD2. Importantly, 11β-HSD2 is not expressed in HEK-293 and H4EII cells as well as THP-1 and native human macrophages that are used in the present study, and the observed effects of DBT are not caused by altered intracellular glucocorticoid metabolism. These observations suggest that different organotins cause distinct disturbances of corticosteroid hormone action *in vivo*.

The ability of DBT to inhibit GR function is relevant for human toxicity from two ways. First, humans are exposed to DBT through its use as a heat stabilizer in the production of PVC plastic materials (water distribution pipes, tubings and bottles) from which it can leach into drinking water [9]. And second, DBT is likely to be responsible for some of the toxicity of TBT in the environment, because earlier *in vivo* studies indicated that trialkylated organotins are mainly metabolized to their dialkylated forms by hepatic cytochrome P450 enzymes through hydroxylation and dealkylation [29]. Ueno *et al.* showed that inhibition of hepatic cytochrome P450 enzymes prevented TBT-dependent hepatotoxicity [8], suggesting that hepatotoxicity is caused by its metabolites. DBT was hardly degraded in the liver of treated mice, rats and guinea pigs, indicating that the relatively inert DBT may be responsible for some of the TBT toxicity observed *in vivo*.

Glucocorticoids play a crucial role in the regulation of many physiological processes including the control of energy metabolism and the modulation of the immune system. Here, we demonstrate that DBT blocks the glucocorticoid-induced expression of hepatic PEPCK and TAT, two enzymes with a key role in energy metabolism. Furthermore, our results from experiments with LPS-stimulated native human macrophages and human THP-1 macrophages show that DBT is able to abolish the suppressive effect of glucocorticoids on the synthesis of the pro-inflammatory cytokines TNF-α and IL-6. By reducing the GR-dependent trans-repression of NF-κB activation, DBT disrupts the anti-inflammatory effect of glucocorticoids, which plays an important role for the resolution of inflammatory reactions.

Our results indicate that DBT disrupts GR-mediated regulation of gene transcription at the initial step of receptor activation by abolishing ligand binding to the receptor. The effects of DBT on glucocorticoid-induced gene expression in liver cells and in macrophages resembled those of RU486, supporting the evidence that DBT directly inhibits GR activity. The concentrations of DBT that affected GR-mediated gene expression in our experiments were close to those found in tissue and blood samples from human and wildlife in the range of 3–300 nM [5,30–32]. Moreover, we observed a more pronounced effect upon preincubation and after repeated exposure of cells to medium containing DBT. The lipophilic organotin molecules have been shown to accumulate near the lipid-water interface of cellular membranes [33]. Thus, organotins may reach higher concentrations in tissues, especially in those with high lipid content, than in the circulation. A cellular accumulation is also consistent with studies demonstrating up to 70,000 times higher concentrations of organotins in plankton and other organisms compared with sea water [34].

The GR-dependent effects of DBT on the LPS-induced synthesis of TNF-α and IL-6 and the subsequent activation of NF-κB found in the present study are in line with pro-inflammatory effects of DBT and TBT previously reported [35–37]. DBT as well as TBT which is metabolized *in vivo* to DBT, induced inflammation of the bile duct associated with hepatic lesions [35] and acute interstitial pancreatitis [36,37]. The present study suggests that the exposure to DBT can interfere with glucocorticoid-mediated modulation of the immune system and may contribute to inflammatory diseases. DBT and other chemicals disrupting glucocorticoid effects might contribute, among other factors, to the high incidence of allergies and asthma in developed countries [38].

Docking analysis of organotins into the GR 3D-structure revealed that DBT, TBT DPT and TPT dock nicely into a site overlapping the dexamethasone binding site, which would be expected because these organotins are smaller than dexamethasone and should fit into this site. However, DBT and TBT have fewer interactions with the GR than dexamethasone, DPT and TBT. TBT, DPT and TPT did not inhibit GR-mediated transactivation, suggesting that the inhibitory actions of DBT are due to binding to an allosteric site. This hypothesis is supported by DBT inhibition of GR-mediated transcriptional activity in the presence of 100 nM cortisol, which is over ten-fold higher than the K_d of cortisol for GR. At this concentration, the steroid binding site on the GR will be occupied by cortisol.

Docking analysis identified a binding site for DBT close to Gln^{570} on α-helix 3 and Arg^{611} on α-helix 5. DBT has several van der Waals contacts with residues in this allosteric site on the GR. Analysis of DBT in this site in the apo-GR and holo-GR reveals that DBT alters the interaction of dexamethasone with the GR. Analysis of the GR with DBT in the apo-GR revealed a conformational change in the steroid binding pocket which results in steric clashes between dexamethasone and Gln^{570} and Gln^{642} (Fig. 8A). Energy minimization of DBT in the holo-GR complex removed these steric clashes. However, after energy minization, Asn^{564} moved and lost its stabilizing contact with the C11 hydroxyl on dexamethasone (Fig 8B). The interaction between α-helix 3 and α-helix 5 is important in transcriptional activity of steroids for the GR [22]. Alteration of α-helix 3 by DBT occupying the allosteric site on the GR may explain how DBT inhibits dexamethasone binding to GR and its subsequent transcriptional activation.

Although neither TPT nor TBT altered GR-mediated transcriptional activity in the absence of cortisol, both of these organotins stimulated the activity of the GR-cortisol complex. Binding of TPT and TBT to other proteins of the transcriptional complex or effects on post-translational modifications of the receptor or its associated proteins could be important in TBT- and TPT-induced stimulation of GR-mediated transcription in the presence of cortisol.

In conclusion, the present study demonstrates that DBT, but not TBT, DPT or TPT, inhibits ligand binding to GR and subsequent stimulation of its transcriptional activity. Molecular modeling analyses indicate that binding to an allosteric site by DBT, but not by the other organotins, alters the orientation of key residues in the ligand binding pocket of the GR. Disruption of GR activation by DBT can disturb essential physiological processes such as the immune system, as shown by inhibition of glucocorticoid-mediated suppression of pro-inflammatory cytokine production in macrophages. Thus, by interfering with GR function, DBT may contribute to immune diseases.

Materials and Methods

Analysis of transcriptional activation of reporter genes

HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, 4.5 g/L glucose, 50 U/mL penicillin/streptomycin, 2 mM glutamine, and 1 mM HEPES, pH 7.4. 200,000 cells/well were seeded in poly-L-lysine coated 24-well plates, incubated for 16 h and transfected by the calcium-phosphate method with plasmid for human GR-α (0.1 µg/well), MMTV-lacZ β-galactosidase reporter (0.15 µg/well) and pCMV-LUC luciferase transfection control (0.05 µg/well). Cells were washed twice with serum- and steroid-free DMEM 6 h later, followed by incubation for 16 h with organotins. Cortisol (100 nM) was added and cells were incubated for another 20 h. Cells were washed with PBS and lysed with 50 µl lysis buffer (Tropix, Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiothreitol. 20 µl of lysate were analyzed for β-galactosidase activity using the Tropix kit and luciferase activity using a home-made luciferine-solution [39].

To measure NF-κB-dependent transcriptional activity, HEK-293 cells were transfected with plasmid 3xMHCLUC (provided by Dr. J. Cidlowski, [40]), containing three binding sites for NF-κB in the promoter preceding a luciferase gene, and a cytomegalovirus (CMV)-driven galactosidase control plasmid to adjust for transfection efficiency. The medium was exchanged by serum- and steroid-free DMEM 6 h post-transfection, and cells were incubated overnight with DBT. Cells were stimulated by adding 5 nM TNF-α with or without glucocorticoids or RU486, followed by incubation for another 24 h. Thereafter, luciferase and galactosidase activities were measured as described above.

Glucocorticoid receptor binding assay

HEK-293 cells (400,000 cells/well in 12-well plates) were transfected with 0.2 µg/well GR plasmid, washed twice 6 h later

with serum- and steroid-free DMEM and incubated in this medium overnight, followed by addition of vehicle (0.1% of methanol or dimethylsulfoxide) or DBT, dexamethasone and 10 nM [^{3}H]-dexamethasone (specific activity 70 Ci/mmol). Alternatively, cells were preincubated overnight with vehicle or DBT prior to incubation with dexamethasone. After incubation with dexamethasone for 3 h, cells were washed three times with PBS, lysed with 300 µl of 0.4 N NaOH, and lysates were subjected to scintillation counting. Non-specific binding was determined in cells transfected with pcDNA3 control plasmid or in the presence of 1 µM RU486 and subtracted from the values obtained in absence of antagonist.

Glucocorticoid receptor expression analysis

HEK-293 cells transfected with GR plasmid were washed 6 h later with serum- and steroid-free medium and incubated in this medium for 16 h with vehicle control (0.1% DMSO), 10 nM dexamethasone or 500 nM DBT. After preincubation, cells were incubated for another 3 h in the presence of vehicle, 10 nM dexamethasone or 500 nM DBT and 10 nM dexamethasone. In a another sample, cells were preincubated overnight in serum-free medium, followed by incubation for 3 h with 500 nM DBT and 10 nM dexamethasone. These conditions were chosen according to the procedure used for GR ligand binding. GR protein was detected by rabbit polyclonal anti-human GR antibody at a working dilution of 1:500 (Santa Cruz, antibody sc-1030). Actin served as a control to adjust for the amount of protein loaded on the SDS-gel and was detected with a rabbit polyclonal antibody. A horse-radish peroxidase conjugated mouse anti-rabbit antibody was used as secondary antibody for detection.

Analysis of phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels

Rat H4EII hepatoma cells were cultured in MEM medium containing 1 mM sodium pyruvate and supplemented with 10% fetal calf serum, 4.5 g/L glucose, 50 U/mL penicillin/streptomycin, 2 mM glutamine, and 1 mM HEPES, pH 7.4. H4EII cells were preincubated for 16 h in serum-free MEM in the presence of various concentrations of DBT or vehicle (dimethylsulfoxide), followed by adding 10 nM dexamethasone and incubation for another 24 h at 37°C. PEPCK mRNA expression was determined by real-time RT-PCR as described earlier [41]. Briefly, total mRNA was extracted from H4EII cells (500'000 cells per well) using an mRNA isolation kit, and 100 ng of mRNA was reverse transcribed using SuperscriptII reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad CA). Relative quantification of PEPCK mRNA expression levels was performed on an ABI7600 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reactions were performed in 96 well plates using 25 ng of cDNA, TaqMan Universal PCR Master Mix and assay on demand primers and probes from Applied Biosystems (Mm01247057_g1) following the instructions from the manufacturer. The relative expression of each gene compared to the internal control β-actin was determined using the 2-ΔCT method.

Determination of tyrosine aminotransferase (TAT) activity

H4IIE cells (300,000 cells/well in 6-wells plate) were allowed to adhere overnight, and then washed twice with serum- and steroid-free MEM followed by 16 h incubation with DBT. Dexamethasone (5 nM) was added, and cells were incubated for another 20 h, washed once with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.2% Nonidet-P40) supplemented with Complete Mini Protease Inhibitor cocktail at a working dilution of 1 tablet in 25 ml (Roche Applied Science, Rotkreuz, Switzerland). After centrifugation (10 min, 10'000×g, 4°C), cleared lysates were used to measure TAT activity. 50 µl of lysate (diluted in lysis-buffer) was added to 465 µl of reaction mix (90 mM potassium-phosphate buffer, pH 7.4, 25 mM L-tyrosine disodium, 90 mM α-oxoglutarate, pH 7.0, 0.45 mM pyridoxal-5'-phosphate, pH 6.5) and incubated for 30 min at 37°C. The reaction was stopped by adding 35 µl 10 N KOH, and after 30 min incubation at room-temperature, the absorbance at 331 nm against a blank (lysis buffer treated identically) was determined. The activity was normalized to protein content in cell lysates, as determined by BCA-solution (Pierce, Soccochim, Lausanne, Switzerland).

Analysis of cytokine production

THP-1 cells and native human monocytes (50,000 cells/well, 24-well plates) were grown in RPMI-1640 medium supplemented with 10% FCS, 50 U/ml penicillin/streptomycin, 2 mM glutamine and 25 mM HEPES, pH 7.4. Peripheral blood mononuclear cells were purified from blood of non-smoking healthy male volunteers of age 25–40 [42]. Venous EDTA-blood (180 ml) was collected and left to sediment in 6% dextran. Leukocytes were enriched for monocytes using a two-step discontinuous Percoll gradient (1.0791 and 1.0695 g/ml). The purity of isolated monocytes was 70–80%. Native human monocytes were differentiated into macrophages by incubation for 7 days in culture medium without medium change, whereas THP-1 cells were differentiated by incubation with 5 ng/ml PMA for 72 h. The medium was replaced by fresh RPMI-1640 containing DBT, and cells were incubated for 16 h. Then, 10 nM dexamethasone and 30 ng/ml LPS were added and cells incubated for another 20 h before harvesting the medium and analysis of IL-6 and TNF-α by ELISA (BD Biosciences, Allschwil, Switzerland).

Docking of organotins within human GR

The PDB files for DBT, TBT, DPT and TPT were obtained from CambridgeSoft's Chemfinder chemical compound database. Human GR (PDB:1M2Z) [21] was extracted from the PDB [43] and converted into an apo-monomer using a text editor. Then, organotins were docked into human GR using Autodock 4 [18,19]. The well depth and equilibrium separation for tin in DBT and other organotins were set to 0.63 kJ/mol and 4.40 Å. Initially, the grid size was set to cover the entire receptor. Lamarckian Genetic Algorithm (LGA) docking was run for 100 trials of 25 million energy evaluations each and a histogram of the binding energies for the various conformations was calculated [19]. This analysis identified two interior sites in the GR. One site overlapped the steroid-binding site. The other site was close to the A ring of dexamethasone. Several non-specific sites for TPT and other organotins were found on the surface of the GR. To analyze docking of organotins to sites with a specific mode of binding, we adjusted the grid to cover the interior of the GR and redid the docking as described above.

The organotin-GR complex calculated by Autodock was refined with Discover 3 software in Insight II, allowing the GR and the organotin to move to an energy minimum. Discover 3 was used with the ESSF force field and a distant dependent dielectric constant of 2 for 10,000 iterations in each energy minimization.

Statistical analysis

Values are expressed as means±SD. Data were analyzed (and significance assigned) using the ratio *t*-test in the GraphPad Prism 4 software. Data were also subjected to one- or two-way ANOVA using SigmaStat software (Jandel Scientific, San Rafael, CA).

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Author Contributions

Conceived and designed the experiments: CG MEB AO. Performed the experiments: CG CC AAD DVK. Analyzed the data: CG CC AAD DVK MEB AO. Contributed reagents/materials/analysis tools: AO. Wrote the paper: CG MEB AO.

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5 11β-HYDROXYSTEROID DEHYDROGENASE 1 INHIBITING CONSTITUENTS FROM ERIOBOTRYA JAPONICA REVEALED BY BIOACTIVITY-GUIDED ISOLATION AND COMPUTATIONAL APPROACHES.

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In this publication, we used a pharmacophore-based virtual screening approach to reveal selective 11β-HSD1 inhibitors from the leaves of loquat (*eriobotrya japonica*) a known anti diabetic in Chinese medicine; the most promising hits were evaluated using *in vitro* assays.

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11β-Hydroxysteroid dehydrogenase 1 inhibiting constituents from *Eriobotrya japonica* revealed by bioactivity-guided isolation and computational approaches

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ABSTRACT

The inhibition of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), which catalyzes the conversion of inactive 11-ketoglucocorticoids to active 11β-hydroxyglucocorticoids, emerged as promising strategy to treat symptoms of the metabolic syndrome, including obesity and type 2 diabetes. In this study the leaves of the anti-diabetic medicinal plant loquat (*Eriobotrya japonica*) were phytochemically investigated following hints from a pharmacophore-based virtual screening and a bioactivity-guided approach. Determination of the 11β-HSD1 and 11β-HSD2 inhibitory activities in cell lysates revealed triterpenes from the ursane type as selective, low micro-molar inhibitors of 11β-HSD1, that is, corosolic acid (**1**), 3-epicorosolic acid methyl ester (**4**), 2-α hydroxy-3-oxo urs-12-en-28-oic acid (**6**), tormentic acid methyl ester (**8**), and ursolic acid (**9**). Importantly, a mixture of loquat constituents with moderate activities displayed a pronounced additive effect. By means of molecular modeling studies and the identification of the 11β-HSD1-inhibiting 11-keto-ursolic acid (**17**) and 3-acetyl-11-keto-ursolic acid (**18**) a structure–activity relationship was deduced for this group of pentacyclic triterpenes. The mechanism of action elucidated in the present work together with the previously determined pharmacological activities provides these natural products with an astonishing multi-targeted anti-diabetic profile.

1. Introduction

Obesity and its related metabolic diseases, including type 2 diabetes, dyslipidemia, hypertension, and cardiovascular complications, represent a major health problem in the industrialized world. There is a great need for novel, improved therapeutic strategies to combat the consequences of these diseases.

Glucocorticoids play a central role in the modulation of carbohydrate and lipid metabolism, and the prolonged exposure to elevated glucocorticoids has been associated with metabolic disturbances such as visceral obesity, insulin and leptin resistance, hyperglycemia, elevated triglyceride and cholesterol levels, and elevated blood pressure. In recent years, it became evident that inappropriately elevated local glucocorticoid activity rather than systemic levels contribute to the adverse metabolic effects observed in obese individuals.[1] On a tissue-specific level, the enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) catalyzes the conversion of inactive 11keto-glucocorticoids (cortisone in human, 11-dehydrocorticosterone in rodents) to active 11β-hydroxyglucocorticoids (cortisol in human, corticosterone in rodents). This enzyme is highly expressed in metabolically relevant tissues such as liver, adipose and skeletal muscles. A second enzyme, 11β-HSD2, is expressed in kidney cortical collecting ducts, distal colon and placenta, and catalyzes the reverse reaction.

The consequences of elevated glucocorticoid activation by 11β-HSD1 have been well demonstrated in transgenic mice overexpressing the enzyme in adipose tissue.[2,3] These mice present typical features of metabolic syndrome. In addition, several clinical studies described the negative impact of elevated 11β-HSD1 activity on various metabolic functions.[4] Based on these observations, inhibition of 11β-HSD1 is considered a promising strategy to treat metabolic syndrome, and many potent synthetic inhibitors have been described recently.[5,6] In contrast, only few natural compounds that inhibit 11β-HSD1 have been identified at present. Because 11β-HSD1, in addition to its role in glucocorticoid metabolism, has other functions such as metabolism of 7-oxysterols, 7-oxy-neurosteroids and xenobiotics,[7] full inhibition of this

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enzyme may interfere with these alternative functions, and the use of moderate inhibitors may be a preferred strategy.

Therefore, we recently initiated a search for natural compounds inhibiting 11β-HSD1. We scrutinized extracts of six medicinal plants used as traditional anti-diabetic medicines for their potential to inhibit 11β-HSD1 activity and glucocorticoid receptor activation in transfected HEK-cells.[8] In addition, the selectivity for 11β-HSD1 was determined by measuring the effects on the 11β-HSD2-dependent oxidation of cortisol to cortisone, since inhibition of this enzyme induces a cortisol-dependent activation of the mineralocorticoid receptor and results in increased blood pressure.[1]

Among the tested extracts *Eriobotrya japonica* (Thunb.) Lindl. showed promising effects. Loquat or *E. japonica* (Thunb.) Lindl. from the Rosaceae family is not only famous for its delicious fruits, but also the leaves are well known in traditional Chinese medicine for their beneficial effect in the treatment of diabetic patients.[9] A number of chemical and pharmacological studies using animal tests confirmed the hypoglycaemic action of Folium Eriobotryae.[10–15] However, there is a lack of information in respect to the mechanism/s and site/s of action of the investigated extracts, fractions and individual loquat constituents. In our recent study we found that the methanol and the dichloromethane (DCM) leaf extracts of *E. japonica* showed both a dose-dependent inhibition of 11β-HSD1, and a preferential inhibition of 11β-HSD1 versus 11β-HSD2. In cell lysates, the DCM extract exerted an IC_{50} of 24 ± 3 μg/ml against 11β-HSD1 activity and an approximately threefold weaker inhibition towards 11β-HSD2. The effects measured in cell lysates could be confirmed by the results obtained from intact cells, that is, stably transfected HEK-293 cells.[8] Following these results and virtual hits from a previously established pharmacophore model,[16] the aim of this study was to identify those secondary metabolites responsible for the inhibitory effects of the 11β-HSD1 activity observed for the extracts of the medicinal plant *E. japonica*.

Twelve constituents from the chemical class of triterpenes have been isolated and identified. Together with further naturally derived triterpenic acids the isolated constituents have been tested for their potential to inhibit the activity of 11β-HSD1 and 11β-HSD2. The results of the compounds tested in this study were used for computational analysis (i) to rationalize the binding interactions in the 11β-HSD1 binding site and (2) to derive a structure–activity relationship for this class of compounds.

2. Results and discussion

2.1. In silico screening of a natural products database

In a previous study, we have generated a ligand-based pharmacophore model for 11β-HSD1 inhibitors.[16] Using this model as query, our 3D-multiconformational molecular database DIOS consisting of approximately 10,000 reported constituents from medicinal plants described in Dioscorides' *De materia medica*,[17] was virtually screened returning 172 hits. With 28 members, the chemical class of triterpenoids was one of the chemical scaffolds dominating this virtual hit list. Among the highest scored triterpenes was corosolic acid (**1**; Fig. 1).

This natural product is a constituent of different herbal remedies and plant derived nutritionals, particularly from the Rosaceae family; for example, it is described as constituent in almond hulls,[18] blackberries,[19] and apple peels.[20] Furthermore, it is known as the prominent ingredient from the leaf extract of *E. japonica*,[21,22] which in our previous study showed a distinct potential to inhibit the activity of 11β-HSD1.[8]

Commercially available corosolic acid (**1**) was therefore tested for its potential to inhibit recombinant human 11β-HSD1 and

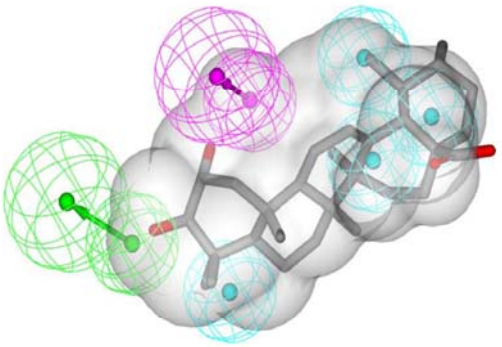


Figure 1. Corosolic acid (**1**) mapped into the pharmacophore model for 11β-HSD1 inhibitors. Chemical features are color-coded: magenta—hydrogen bond donor, green—hydrogen bond acceptor, cyan—hydrophobic, grey—shape (size constraint).

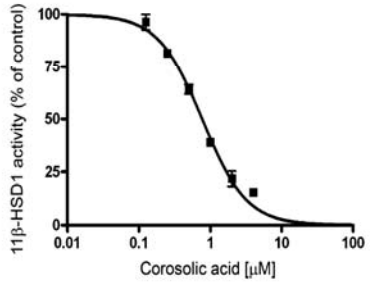


Figure 2. Concentration-dependent 11β-HSD1 inhibitory activity of corosolic acid (**1**) measured in lysates of cells expressing recombinant human 11β-HSD1. Data were normalized to control, that is, in the presence of 0.1% DMSO, and represent mean ± S.D. from three independent experiments.

11β-HSD2 activities in lysates of stably transfected HEK-293 cells. As shown in Figure 2, **1** exhibited a concentration-dependent 11β-HSD1 inhibitory activity with an IC_{50} of 0.81 ± 0.06 μM. The effect of **1** was selective as no activity against 11β-HSD2 was detected at concentrations up to 20 μM.

2.2. Phytochemical studies and bioassay-guided isolation

Using LC–MS and in comparison with commercial corosolic acid (**1**), this bioactive compound was confirmed as prominent constituent in the methanol leaf extract of *E. japonica*. This is in accordance with the recently determined quantification of triterpene acids from this plant material.[21] However, in the 11β-HSD1 inhibiting DCM extract, compound **1** could only be detected in traces. In order to identify the further secondary metabolite/s, responsible for the previously measured effect, bioassay-guided phytochemical investigations were performed with the DCM leaf extract from *E. japonica*.

Fractionation of 10.2 g of the DCM extract by silica gel column chromatography resulted in 13 fractions (A1–A13), which were tested in cell lysates on their inhibitory activity on 11β-HSD1 (Fig. 3A) and 11β-HSD2 (Fig. 3B).

At concentrations of 25 μg/ml, fractions A11–A13 diminished the activity of 11β-HSD1 to below 35% (Fig. 3A), whereas the activity of 11β-HSD2 was scarcely influenced by these fractions (Fig. 3B). Thus, A11–A13 were analysed using TLC, DAD-ELSD-HPLC and LC–MS. Repeated chromatographic separation and purification steps afforded 12 triterpenoid constituents. By using mass spectrometry, extensive 1D and 2D NMR experiments, optical rotation

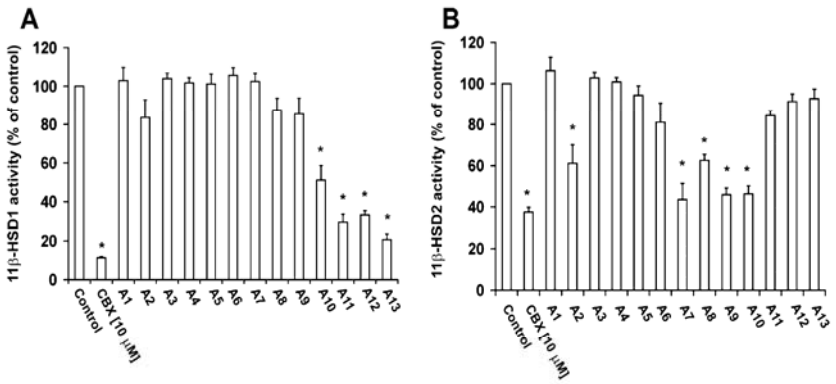


Figure 3. Inhibition of 11β-HSD1-dependent reduction of cortisone to cortisol (A) and 11β-HSD2-dependent oxidation of cortisol to cortisone (B) by fractions A1 to A13 of the DCM extract of *E. japonica* (c = 25 μg/ml) measured in lysates of HEK-293 cells stably expressing recombinant human 11β-HSD1 (A) or 11β-HSD2 (B). The non-selective inhibitor carbenoxolone (CBX) was used as a positive control. Data were normalized to control, that is, in the presence of 0.1% DMSO, and represent mean ± S.D. from three independent experiments. $^{*}p$ <0.01.

and comparison with data from the literature, the isolates were identified as ursolic acid methyl ester (**2**),[23] β-sitosterol (**3**),[24] 3-epicorosolic acid methyl ester (**4**),[25] uvaol (**5**),[26] 2-α hydroxy-3-oxo urs-12-en-28-oic acid (**6**),[27] corosolic acid methyl ester (**7**),[28] tormentic acid methyl ester (**8**),[29] ursolic acid (**9**),[22] maslinic acid methyl ester (**10**),[30] 3-*O*-*trans*-*p*-coumaroyltormentic acid (**11**), 3-*O*-*cis*-*p*-coumaroyltormentic acid (**12**), and tormentic acid (**13**; Scheme 1).[31] All the isolated triterpenes are known natural compounds; **1**–**3** and **9**–**13** have already been described as secondary metabolites from *E. japonica*; **4**, **7** and **8** were isolated as methyl esters of the known loquat constituents 3-epicorosolic acid,[23] corosolic acid,[21,22] and tormentic acid,[11] respectively. The pentacyclic triterpenoids **5** and **6** belonging to the ursane type have not been previously reported from this natural source.

2.3. Determination of 11β-HSD1 and 11β-HSD2 inhibitory activities

The isolated triterpene metabolites (**2**–**13**) were screened for their inhibitory activity on 11β-HSD1 and 11β-HSD2, respectively, at 20 μM. At this concentration, none of the tested compounds reduced the 11β-HSD2 activity; however, **4**, **6**, **8**, and **9** distinctly inhibited the activity of 11β-HSD1. Their IC_{50} values against 11β-HSD1 were determined to be 5.2 ± 0.9 μM (**4**), 17 ± 4 μM (**6**), 9.4 ± 0.8 μM (**8**), and 1.90 ± 0.25 μM (**9**; Fig. 4).

In order to evaluate potentially additive effects of active metabolites from *E. japonica*, compounds **4**, **6**, **8** and **9** were tested for their individual 11β-HSD1 activities using concentrations of 2, 3, 4 and 1.5 μM, respectively, which result in only partial inhibition. The obtained effects were compared with that achieved in a mixture containing the same concentration of each compound (Fig. 5). Importantly, whereas each individual compound inhibited 11β-HSD1 only partially, the mixture showed a significantly increased inhibitory potential (Fig. 5).

2.4. Evaluation of further triterpenic acids

Based on the findings from the loquat constituents, further triterpenic acids well known as plant constituents were selected to evaluate their effects on 11β-HSD1 and 11β-HSD2 (**14**–**18**; Scheme 2). Ganoderic acid A (**14**) was chosen as representative of a lanostane type triterpene from the famous TCM fungus *Ganoderma lucidum* P. Karst.[32] The pentacyclic triterpene acids 11-keto-β-boswellic acid (**15**) and 3-acetyl-11-keto-β-boswellic acid (**16**) are prominent ingredients from incense, that is, the resin of *Boswellia serrata* Roxb.[33] Guided by the 11β-HSD1 inhibiting activity of ursolic acid (**9**). its derivatives 11-keto-ursolic acid (**17**) and 3-acetyl-11-keto-ursolic acid (**18**) were also selected for a screening on 11β-HSD1 and 11β-HSD2. They were reported as constituents from Indian linaloe, that is, the resin from *Bursera delpechiana* Poiss.[34]

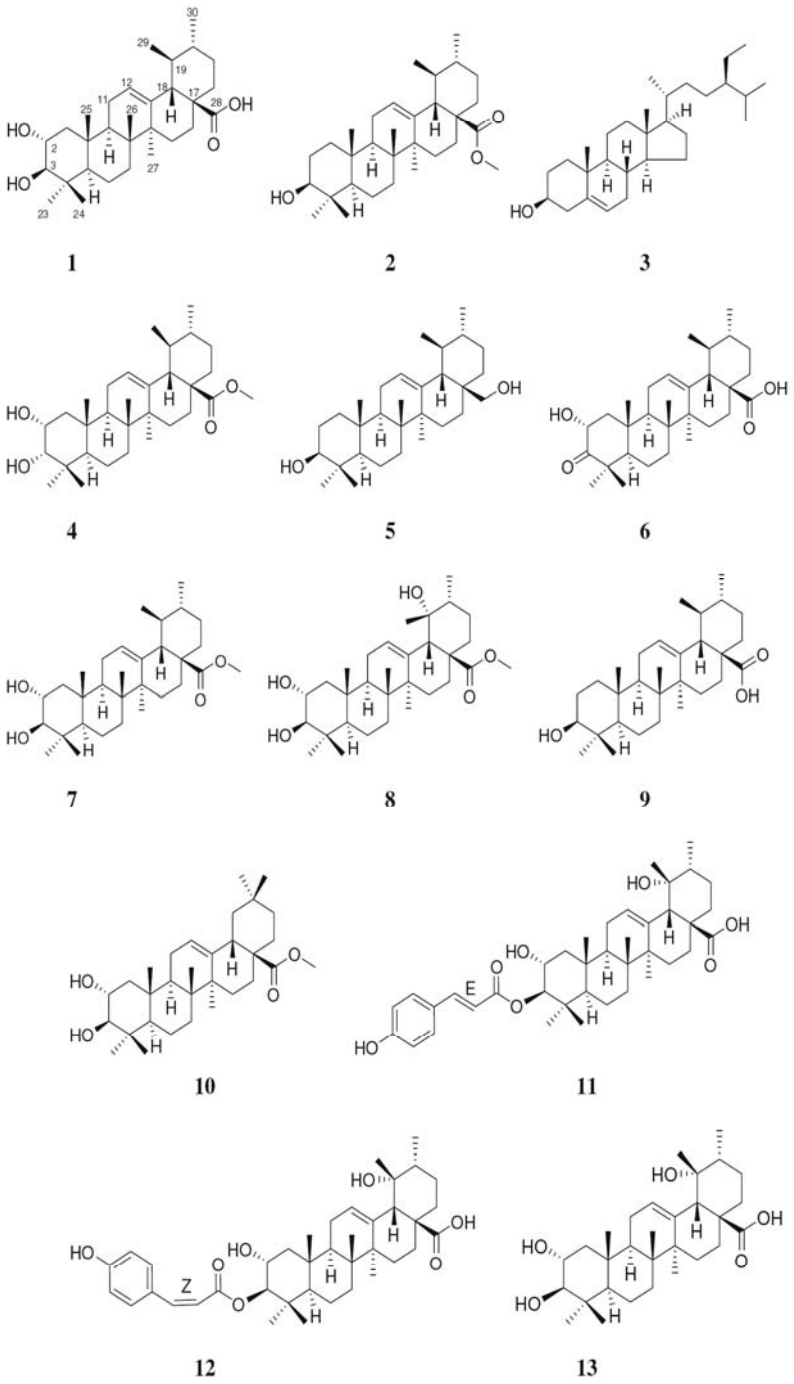
At 20 μM the triterpene acids **14**, **15**, and **16** moderately inhibited the activities of 11β-HSD1 by 46%, 52% and 63%, and that of 11β-HSD2 by 76%, 68% and 52%, respectively. Due to their unfavorable missing selectivity towards 11β-HSD1, they were not further investigated. However, the ursolic acid derivatives **17** and **18** exerted a significant and selective 11β-HSD1 inhibitory activity with IC_{50} values of 2.06 ± 0.44 μM and 1.35 ± 0.52 μM, respectively.

2.5. Structure–activity relationship (SAR) of triterpene inhibitors of 11β-HSD1

When analyzing our results, the crucial role of the carboxylic group position and derivatization became obvious. While compounds with 4-subsituted carboxylic acids were inactive, carboxylic groups in position 17 seemed advantageous for ligand binding. In some cases, methylation led to a complete loss of activity (compare **1**/**7** and **9**/**2**). However, other methyl esters still showed activity. In addition, stereochemistry of the hydroxyl group at position 3 seemed important (compare **4**/**7**). For a deeper understanding of these observations, we docked all compounds into the active site of 11β-HSD1 (PDB[35] entry 2bel chain A).

When analyzing the docking poses of corosolic acid (**1**) (Fig. 6A and B), a possible interaction of the 28-carboxylic group with the Tyr177 hydroxyl group was observed. It seems that ester formation at this position rather leads to unfavorable steric clashes with the protein than to disrupt this interaction. Carboxylic acid substituents on other positions, for example, position 4 in 11-keto-boswellic acid (**15**), were not observed to form interactions with the protein. The hydroxyl groups at positions 2 and 3 form hydrogen bonds with the backbone oxo group of Thr124. The stereochemistry and presence or absence of hydroxyl groups at these positions alters the ligands' interaction with Thr124 and thereby the anchoring of the whole molecule in the ligand binding site. From the data obtained so far, the 2*S* and 3*R* configuration leads to an optimal interaction geometry.

Several X-ray crystal structures of 11β-HSD1 in complex with potent inhibitors are reported in the literature and available in the PDB (e.g., 2irw, 2rbe, 3byz, or 3fco). With no exception, they are observed to form hydrogen bonds with the catalytically ac-



Scheme 1. Constituents from the leaves of *E. japonica*.

tive amino acid residues Ser170 and Tyr183. Although the triterpenoid carbenoxolone is also anchored with its 11-keto group between those residues in the crystal structure (PDB code 2bel), mutational analysis studies by Kim et al. show that a different amino acid—Tyr177—is essential for binding of the highly potent triterpenoid inhibitor glycyrrhetinic acid—but not the substrate cortisone.[36] This points towards a different binding mode for the triterpenoid compound class in comparison to the published synthetic inhibitors. Therefore, interactions with the catalytically active residues may not play an important role in triterpenoid inhibitor binding. The observation that ursolic acid (**9**) and 11-keto ursolic acid (**17**) do not show significant differences in IC_{50} values for 11β-HSD1 inhibition underlines this assumption.

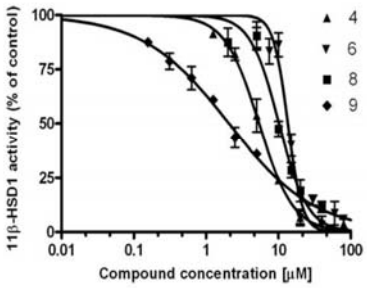


Figure 4. Concentration-dependent 11β-HSD1 inhibitory activities of compounds **4**, **6**, **8**, and **9** measured in lysates of HEK-293 cells stably expressing recombinant human 11β-HSD1. Data were normalized to control, that is, in the presence of 0.1% DMSO, and represent mean ± S.D. from three independent experiments.

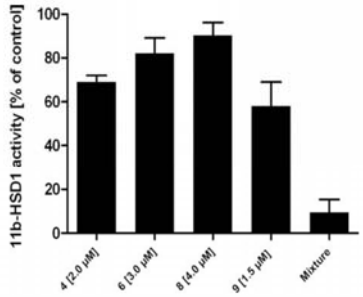


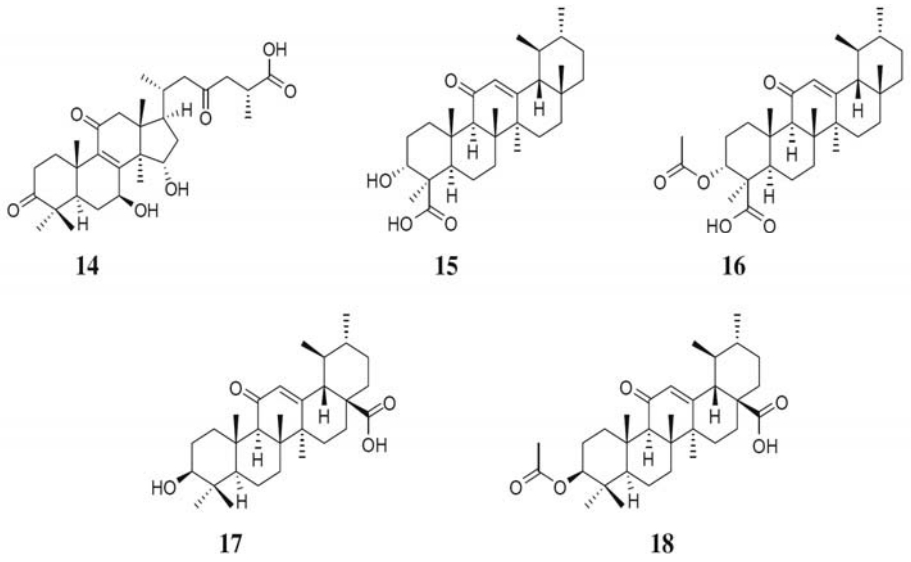
Figure 5. Inhibition of 11β-HSD1 activity by compound **4** (2 μM), **6** (3 μM), **8** (4 μM), **9** (1.5 μM), and a mixture containing the respective concentration of each compound. Data were normalized to control, that is, in the presence of 0.1% DMSO, and represent mean ± S.D. from three independent experiments.

In order to further explore the properties that are favorable for 11β-HSD1 inhibition, the four most active compounds **1**, **9**, **17**, and **18** were flexibly aligned by pharmacophoric points using LigandScout 3.0.[37] From this overlay a ligand-based 3D pharmacophore model consisting of the chemical feature set shared by all four compounds was calculated. The resulting model contains 11 hydrophobic features, representing the triterpene core structure, a negatively ionizable feature and two hydrogen bond acceptors placed on the carboxylic acid structure on C28, and one hydrogen bond acceptor on the 3*R* position (Fig. 7).

The elucidation of these important substructures was in line with the observations from the docking experiments. We additionally investigated the discriminatory power of this model by screening a database consisting of all 18 compounds using LigandScout. The model retrieved 13 compounds as hits. The ranking based on the geometric fit showed a clear enrichment of active compounds among the highly-ranked hits (Table 1).

3. Conclusion

Growing evidence suggests that selective inhibition of 11β-HSD1 lowers blood glucose concentrations, counteracts the accumulation of visceral fat and ameliorates related metabolic abnormalities in type 2 diabetes.[38] In the search for natural compounds selectively inhibiting 11β-HSD1, the traditionally used anti-diabetic medicinal plant *E. japonica* emerged as promising starting material.[8] By using this knowledge and a previously established pharmacophore model, we identified the loquat ingredients corosolic acid (**1**) and ursolic acid (**9**) as well as the ursolic acid derivatives **17** and **18**, as plant constituents able to selectively inhibit 11β-HSD1 with IC_{50} values between 0.8 and 2 μM. Moreover, several additional compounds with moderate activity and IC_{50} values in the low micro-molar range were isolated from the loquat DCM leaf extract and their structures determined. Importantly, a pronounced additive effect was observed in a mixture of constituents with moderate activity. In a recently published study, Li et al. performed an HPLC-UV quantification of Eriobotrya constituents from 11 leaf samples collected from different regions in China. Therein, pentacyclic triterpene acids revealed as the most considerable percentage of secondary metabolites in the samples.[21] It could be shown that the seven major triterpene constituents account for 10–16 mg per g crude drug. Among them, the most abundant metabolites were assigned to corosolic acid (**1**) and ursolic acid (**9**) which amount to more than 50% of the quantified triterpene fraction. This fact combined with our findings that several triterpene acids of the ursane type from loquat are endowed with a



Scheme 2. Further natural triterpenic acids.

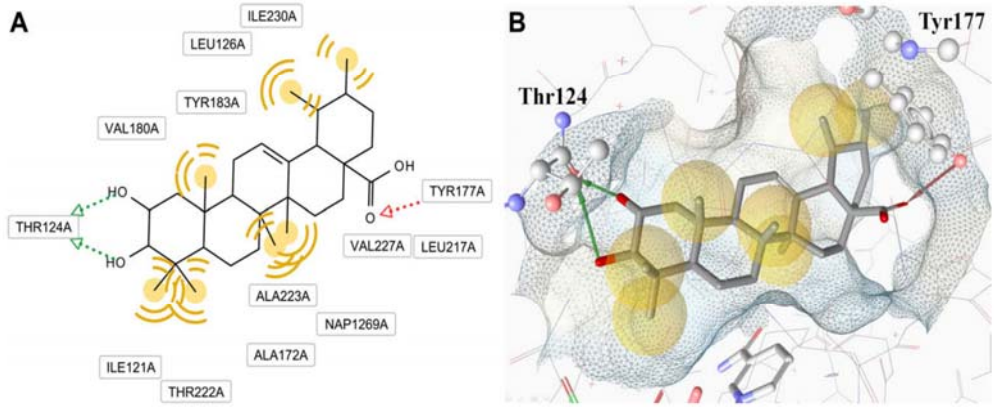


Figure 6. Predicted binding orientation of corosolic acid (**1**) in the 11β-HSD1 active site. Chemical features are color-coded: yellow—hydrophobic, green—hydrogen bond donor, red—hydrogen bond acceptor. (A) 2D view of chemical interactions observed between **1** and the protein. (B) Docked binding pose and interaction pattern of **1** bound to 11β-HSD1. At the bottom of the binding site, the cofactor molecule NADPH is located.

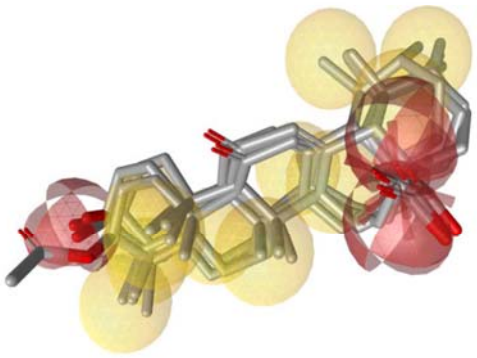


Figure 7. Merged features pharmacophore model from LigandScout derived from compounds **1**, **9**, **17**, and **18**. Chemical features are color-coded: hydrophobic—yellow; hydrogen bond acceptors—red.

Table 1
Pharmacophore-based ranking of triterpene 11β-HSD1 inhibitors

Compd	Hit list rank	In vitro IC_{50} [μM]
1	1	0.81
13	2	Inactive
4	3	5.2
9	4	1.9
17	5	2.06
18	6	1.35
8	7	9.4
5	8	Inactive
7	9	Inactive
11	10	Inactive
8	11	Inactive
10	12	Inactive
12	13	Inactive

low micro-molar inhibitory potential on 11β-HSD1 might explain the activity of the whole extract measured not only in cell lysates, but also in intact, stably transfected HEK-cells.[8]

Pentacyclic triterpenes represent a promising class of multi-target agents.[39] Corosolic acid (**1**), which widely exists in traditionally used medicinal herbs, has attracted much attention as anti-diabetic agent with hypoglycemic effects being proved on animal experiments[40] and in clinical trials.[41] A number of mechanisms have already been attested to this anti-diabetic compound.[42] Corosolic acid was identified as the most active phosphorylase A inhibiting constituent from *Lagerstroemia speciosa* leaf extract,[43,44] was able to stimulate glucose uptake by enhancing the insulin receptor phosphorylation,[45] was reported as low micro-molar inhibitor of protein tyrosine phosphatase 1B[46] and as an inhibitor of the hydrolysis of sucrose.[47] Also, ursolic acid (**9**) was shown recently to exhibit potential anti-diabetic and immunomodulatory properties in type 1 diabetic mice fed a high-fat diet.[48] The underlying mechanisms refer as well to tyrosine phosphatase 1B[46] and phosphorylase A.[49] Additionally, **9** was identified as one of the constituents from the hexane extract of *Phyllanthus amarus* causing a significant inhibition of the α-amylase, suggesting to contribute at least partly by this mechanism to the anti-diabetic effect of the investigated medicinal plant extract.[50]

Recently, a high hypoglycemic and hypolipidemic potential on normal, alloxan-diabetic, and streptozotocin-induced diabetic mice has been attested to the triterpene acid fraction yielded from the leaves of *E. japonica*.[13] In our study, among the constituents isolated from the *E. japonica* leaf extracts, we identified some potent inhibitors of 11β-HSD1 belonging to pentacyclic triterpenes of the ursane type. Intriguingly, these compounds, unlike glycyrrhetinic acid, did not inhibit 11β-HSD2, which is associated with cortisol-induced mineralocorticoid receptor activation and an elevation of blood pressure. Such inhibitors do have a considerable potential as drugs directed against glucocorticoid-related metabolic disorders. The mechanism of action elucidated in the present work together with the previously determined pharmacological activities provides these natural products with an astonishing multi-targeted anti-diabetic profile. Furthermore, clinical trials with corosolic acid (**1**),[41] and a long-term proven efficacy of Folium Eriobotryae as famous traditional Chinese medicine with hypoglycemic effect[10–15] contribute to a safe and well-approved herbal remedy.

4. Methods

4.1. Virtual screening of a natural products database

Pharmacophore models represent the three-dimensional (3D) arrangement of chemical functionalities that are essential for the interaction of a ligand with a specific pharmacological target structure (proteins, RNA, or DNA).[51] These models can be used to virtually screen multiconformational 3D databases of compounds in

order to find other molecules that fulfil the requirements for binding to the respective target. In this study, the natural products database DIOS was screened, which consists of 9676 reported constituents from medicinal plants described in Dioscorides' *De materia medica*.[17] A previously reported pharmacophore model for 11β-HSD1 inhibitors was used as search query.[16] The screening was performed within Catalyst 4.11 (Accelrys Software Inc., San Diego, CA) using the best flexible search algorithm.

4.2. Structure–activity relationship (SAR) of triterpene inhibitors of 11β-HSD1

For a better understanding of the differences in compound activity, all molecules discussed in this study were fitted into the 11β-HSD1 ligand binding site using docking. Generally, docking methods intend to predict the 3D structure of small molecules interacting with a protein binding site. Docking studies on all reported compounds evaluated in this study were carried out using GOLD SUITE (version 1.0.1; Cambridge Crystallographic Data Centre, Cambridge, UK). This program employs a genetic algorithm for finding accurate docking solutions. Furthermore, the program allows for full ligand flexibility, partial protein flexibility, and a distinct treatment of water molecules that are present in the ligand binding domain. Overall, the default parameters of the program were used. Protein and ligand atom types were determined by GOLD. Cocrystallized water from the ligand binding site was included in the docking process by setting those molecules to 'toggle' and 'spin'. The 'toggle' option lets the program decide whether the water should be present or absent (i.e., displaced by the ligand) during docking. Allowing the water molecules to 'spin' allows for the automatic optimization of the orientation of the hydrogen atoms.

Chemical interactions between the docked binding poses of the ligands and the 11β-HSD1 active site were analyzed using LIGANDSCOUT 3.0. This program automatically interprets chemical interactions observed between a ligand and a protein, based on the chemical functionalities, the geometric distances and angles between neighboring structures.[52]

The model for investigating structure–activity relationships of the discussed compounds was generated using the ligand-based modeling tool 'Espresso', a module in the program LIGANDSCOUT 3.0. This algorithm ranks all compounds according to their flexibility, and subsequently creates cascading pairwise alignments of all conformations producing a flexible overlay of the underlying molecules. This alignment set contains 3D overlays of those conformations that maximize geometric chemical feature overlap. From this set of solutions, the best flexible alignments can either be prioritized (i.e., scored) by steric overlap or pharmacophore feature overlap. For this study 'atom sphere overlap' was used as a scoring function and the highest ranked model was used for the predictions. For all other settings default values were used. Each compound was represented by a comprehensive set of conformers (conformer generation using OpenEye's program OMEGA2 (OpenEye Scientific Software, Santa Fe, NM) using default settings).

4.3. General experimental procedures

Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Optical rotation was measured on a Perkin–Elmer 341 polarimeter (Wellesley, MA) at 25 °C. FTIR spectra were recorded on a Bruker IFS 25 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) in transmission mode (4000–600 cm^{-1}) using ZnSe disks of 2 mm thickness. NMR spectra were recorded on a Bruker-DRX300 (Bruker Biospin, Rheinstetten, Germany) at 300 K in $CDCl_3$ or methanol and calibrated to the residual non-deuterated solvent signals. Upon request, NMR spectra can be obtained from the corresponding author. Column chromatography was performed under TLC monitoring using silica gel flash CC (Silica Gel 60, 40–63 μm; VWR, Darmstadt, Germany) and Sephadex® LH-20 (20–100 μm, Pharmacia Biotech, Uppsala, Sweden). TLC was performed on Silica Gel 60 F_{254} plates (0.25 mm; VWR, Darmstadt, Germany), mobile phase: chloroform/methanol/formic acid, 10:0,5:0,25 (v/v/v), and detected with vanillin/H_2SO_4 (1% w/v and 5% v/v methanolic solutions, respectively). HPLC-data were obtained on a Hewlett–Packard-(HP)-1100 system (Agilent, Waldbronn, Germany), equipped with a photodiode array detector (DAD), column thermostat and auto sampler. The LC was fitted with a Phenomenex Synergi 4 μ Max-RP 80 A column (Torrance, CA; 150 × 4.6 mm id, 4 μm) and a Merck LiChroCART 4–4 guard column with LiChrospher 100 RP18 (5 μm) packing (VWR, Darmstadt, Germany) at a column temperature of 40 °C, flow rate 1.0 ml/min, injection volume 10 μl, using DAD (205, 254, 280) and evaporative light scattering detection Alltech ELSD 2000 (Alltech, Düsseldorf, Germany); tube temperature 97 °C; gas flow 2.5 bar (impactor off). The mobile phases consisted of A: 0.02% TFA (Merck 8.08260.0100) in bidistilled water (v/v), B: methanol (Merck 1.06007.2500); linear gradient: 0 min 60% B; 18 min 98% B; 28 min 98% B. For LC–ESIMS experiments the HPLC was coupled to a Bruker Esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) replacing solvent A with a solution of 0.1% formic acid in bidistilled water (v/v). MS-parameters: split 1:5; ESI positive mode; spray voltage: 4.5 kV; the sheath gas: N_2, 30 psi, the dry gas: N_2, 6 l min^{-1}, 350 °C; scanning range: 50–1000 *m/z*.

All chemicals were analytical grade. Solvents were either analytical grade or puriss. grade and distilled before use.

4.4. Material

Authenticated leaves of *E. japonica* (Thunb.) Lindl. were collected in the Botanical Garden of Innsbruck. A voucher specimen (JR-20061023-A1) was deposited in the Herbarium of the Institute of Pharmacy/Pharmacognosy, Leopold-Franzens University of Innsbruck, Austria.

Corosolic acid (**1**) and ganoderic acid A (**14**) were purchased from Chromadex Inc. (Santa Ana, CA) with a HPLC-purity of ⩾95% and >98%, respectively. 11-keto-β-boswellic acid (**15**), 3-acetyl-11-keto-β-boswellic acid (**16**), 11-keto-ursolic acid (**17**), and 3-acetyl-11-keto-ursolic acid (**18**) were obtained from Phytoplan, Heidelberg, Germany, at a HPLC-purity of >98%.

4.5. Extraction and isolation

Five hundred and fifty grams of the air-dried and milled leaves of *E. japonica* were extracted with 1600 ml DCM three times for 24 h at room temperature. The plant material was filtered off and the solvent was evaporated under reduced pressure to afford 11.9 g DCM extract. In the same way, the remaining plant material was then extracted three times with 1800 ml methanol to gain 45.1 g of the methanol crude extract. 10.2 g of the DCM extract were fractionated using a flash silica gel CC (400 g; 40 × 5.0 cm) with 500 ml step gradients from petrol ether to DCM and from DCM to methanol to yield 13 fractions (A1–A13). Fraction A11 (3880 mg) was subjected to Sephadex CC (75 × 3.5 cm) and eluted with DCM: acetone (85:15) yielding 17 fractions (B1–17). B4 (182 mg) was separated via flash silica gel CC (80 g; 42 × 2.2 cm) with 200 ml step gradients from DCM to acetone to afford nine subfractions (C1–9). C2–4 (29.6 mg) and C5–6 (28.7 mg) were purified over Sephadex CC with DCM: acetone (85:15), respectively, to afford 4.3 mg of a white, microcrystalline powder (**2**; optical rotation $[\alpha]_D^{20}$ +48.4 (methanol, *c* 0.43); MS (ESI): *m/z* 471 $[M+H]^+$, 493 $[M+Na]^+$, 963 $[2M+Na]^+$; 1D and 2D NMR data in accordance with literature)[53] and 18.7 mg of white, microcrystalline compound **3** (optical rotation $[\alpha]_D^{20}$ −24.2 ($CHCl_3$, *c* 0.89); MS

(ESI): *m/z* 393 $[M+H]^+$; 1D and 2D NMR data in accordance with literature);[54] B7 (65.1 mg) was fractionated using a Sephadex CC and eluted with DCM: acetone (85:15) to obtain fractions D1–7. Purification of D6 (23.1 mg) via methanol Sephadex CC afforded 5.4 mg of a whitish, microcrystalline powder (**4**; optical rotation $[\alpha]_D^{20}$ −24.1 (methanol, *c* 0.54); MS (ESI): *m/z* 469 $[M+H-H_2O]^+$, 487 $[M+H]^+$, 509 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature).[25] Combined fractions B9 and B10 (552 mg) were separated using a flash silica gel CC (65 g; 41 × 1.6 cm) with 100 ml step gradients from DCM to acetone to obtain eight subfractions (E1–8). Crystallization of E2 (48.0 mg) from methanol yielded whitish crystals (**5**; 34.9 mg; optical rotation $[\alpha]_D^{20}$ +62.6 ($CHCl_3$, c 0.62); MS (ESI): *m/z* 443 $[M+H]^+$; 1D and 2D NMR data in accordance with literature).[53] E4 (23.3 mg) and E5 (19.3 mg) were purified over Sephadex CC with DCM/acetone (85:15), respectively, to afford 4.8 mg of a white, crystalline powder (**6**; optical rotation $[\alpha]_D^{20}$ +42.2 (methanol, *c* 0.48); MS (ESI): *m/z* 471 $[M+H]^+$; 1D and 2D NMR data in accordance with literature);[27] and 4.7 mg of a white, microcrystalline powder (**7**; optical rotation $[\alpha]_D^{20}$ +42.0 (methanol, *c* 0.46); MS (ESI): *m/z* 469 $[M+H-H_2O]^+$, 487 $[M+H]^+$, 509 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature).[28]

E6 (10.1 mg) was purified with methanol Sephadex CC to yield 7.9 mg of white crystalline compound **8** (optical rotation $[\alpha]_D^{20}$ +31.4 (methanol, *c* 0.79); MS (ESI): *m/z* 525 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature).[29]

Combined fractions A12 and A13 (440 mg) were subjected to Sephadex CC (80 × 2.0 cm). Elution with methanol resulted in eight fractions (F1–8). Combined F5 and F6 (61.2 mg) were re-chromatographed on silica gel (15 g; 38 × 1.0 cm) with 100 ml step gradients from DCM to methanol to obtain eight subfractions (G1–8). Recrystallization of G1 and G2 from methanol yielded 2.0 mg of white, microcrystalline compound **9** (optical rotation $[\alpha]_D^{20}$ +71.4 (methanol, *c* 0.20); MS (ESI): *m/z* 479 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature)[55] and 1.7 mg of white microcrystalline compound **10** (optical rotation $[\alpha]_D^{20}$ +61.4 (methanol, *c* 0.17); MS (ESI): *m/z* 509 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature),[56] respectively.

B16 (270 mg) was subjected to silica gel flash CC (45 g; 50 × 2.0 cm) with 100 ml step gradients from petrol ether to DCM and from DCM to acetone (fractions H1–10). For purification, combined fractions H3–5 (49 mg) were re-chromatographed twice by Sephadex CC (50 × 2.0 cm) using methanol as mobile phase to afford 12.5 mg of an inseparable 2:1mixture of **11** and **12**, respectively (MS (ESI): *m/z* 635 $[M+H]^+$; 1D and 2D NMR data in accordance with literature).[31] Hydrolysis of 10 mg of this mixture was performed with 5% sodium hydroxide by incubation at room temperature for 15 h. The reaction mixture was neutralized with 10% H_2SO_4 and extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness and purified with Sephadex CC (35 × 1.0 cm) using methanol as mobile phase to gain 5.4 mg of white needles of **13** (optical rotation $[\alpha]_D^{20}$ +28.8 (methanol, *c* 0.54); MS (ESI): *m/z* 511 $[M+Na]^+$; 1D and 2D NMR data in accordance with literature).[31]

The purity of all isolated compounds was determined by HPLC to be ⩾95%.

4.6. Pharmacological testing

For measurements of 11β-HSD1 reductase activity, lysates of HEK-293 cells stably expressing human recombinant 11β-HSD1 were incubated for 10 min at 37 °C in a total volume of 22 µl containing 200 nM [1,2-^{3}H]-labelled cortisone (American Radiolabeled Chemicals, St. Louis, MO) and 500 µM NADPH. 11β-HSD2 dependent oxidation of cortisol to cortisone was measured similarly for 10 min at 37 °C in lysates of HEK-293 cells stably expressing human 11β-HSD2 using [1,2,6,7-^{3}H]-cortisol (Amersham Pharmacia, Piscataway, NJ, USA) at a final concentration of 50 nM and 500 µM NAD^+. Extracts of *E. japonica* at 25 µg/ml and pure compounds at final concentrations between 50 nM and 50 µM were diluted from stock solutions in methanol and immediately used for activity assays. The solvent concentration did not exceed 0.5% and had no effect on enzyme activities. Reactions were stopped by adding methanol containing 2 mM unlabeled cortisone and cortisol, followed by separation of steroids by TLC and scintillation counting. Enzyme kinetics was analyzed by non-linear regression using four parameter logistic curve fitting (Sigmaplot, Systat Software Inc.). Data (mean ± SD) were obtained from at least three independent experiments.

Acknowledgements

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6 CHARACTERIZATION OF ACTIVITY AND BINDING MODE OF GLYCYRRHETINIC ACID DERIVATIVES INHIBITING 11β-HYDROXYSTEROID DEHYDROGENASE TYPE 2

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In this publication, we characterized a set of novel glycyrrhetinic acid derivates for their selective inhibition potential against 11β-HSD2. Inhibitors were developed by chemical modification of the glycyrrhetinic acid backbone

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Characterization of activity and binding mode of glycyrrhetinic acid derivatives inhibiting 11β-hydroxysteroid dehydrogenase type 2☆

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ABSTRACT

Modulation of intracellular glucocorticoid availability is considered as a promising strategy to treat glucocorticoid-dependent diseases. 18β-Glycyrrhetinic acid (GA), the biologically active triterpenoid metabolite of glycyrrhizin, which is contained in the roots and rhizomes of licorice (*Glycyrrhiza* spp.), represents a well-known but non-selective inhibitor of 11β-hydroxysteroid dehydrogenases (11β-HSDs). However, to assess the physiological functions of the respective enzymes and for potential therapeutic applications selective inhibitors are needed. In the present study, we applied bioassays and 3D-structure modeling to characterize nine 11β-HSD1 and fifteen 11β-HSD2 inhibiting GA derivatives. Comparison of the GA derivatives in assays using cell lysates revealed that modifications at the 3-hydroxyl and/or the carboxyl led to highly selective and potent 11β-HSD2 inhibitors. The data generated significantly extends our knowledge on structure–activity relationship of GA derivatives as 11β-HSD inhibitors. Using recombinant enzymes we found also potent inhibition of mouse 11β-HSD2, despite significant species-specific differences. The selected GA derivatives potently inhibited 11β-HSD2 in intact SW-620 colon cancer cells, although the rank order of inhibitory potential differed from that obtained in cell lysates. The biological activity of compounds was further demonstrated in glucocorticoid receptor (GR) transactivation assays in cells coexpressing GR and 11β-HSD1 or 11β-HSD2. 3D-structure modeling provides an explanation for the differences in the selectivity and activity of the GA derivatives investigated. The most potent and selective 11β-HSD2 inhibitors should prove useful as mechanistic tools for further anti-inflammatory and anti-cancer in vitro and in vivo studies.

1. Introduction

Glucocorticoids are essential hormones that act as "global regulators" of physiological and pathological processes and modulate the expression of up to 20% of the genes in the mammalian genome [1]. They are involved in the regulation of lipid synthesis, carbohydrate metabolism and protein turnover, and they act as key regulators of stress responses, blood pressure, cell growth and differentiation, neuronal activities, and immune functions [2–6]. A sophisticated regulatory network is required for glucocorticoids to modulate such a wide range of functions in a highly tissue- and time-specific manner.

The intracellular availability of glucocorticoids and activation of glucocorticoid receptor (GR) is tightly regulated by 11β-hydroxysteroid dehydrogenases (11β-HSDs). Two distinct 11β-HSD enzymes have been identified [7–9]. 11β-HSD1 is expressed in many cell types with high levels in liver, adipose and skeletal muscles, and functions in vivo primarily as an oxoreductase to convert inactive 11-ketoglucocorticoids (cortisone in human, 11-dehydrocorticosterone in rodents) to active

Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; GA, glycyrrhetinic acid; GR, glucocorticoid receptor; H6PDH, hexose-6-phosphate dehydrogenase; MR, mineralocorticoid receptor; PDB, Protein Data Bank; PGE2, prostaglandine-E2; SDR, short-chain dehydrogenase/reductase; TLC, thin-layer chromatography.

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11β-hydroxyglucocorticoids (cortisol in human, corticosterone in rodents) using the cofactor NADPH [10]. 11β-HSD2 catalyzes the reverse reaction, uses cofactor NAD^+ and is expressed in mineralocorticoid target tissues like kidney, colon, sweat and salivary glands but also in placenta, inflamed tissue and many tumors and cancer cell lines [11–20].

Impaired local glucocorticoid metabolism has been associated with several disease states and modulation of intracellular glucocorticoid availability is considered a promising strategy to treat glucocorticoid-dependent diseases. Elevated 11β-HSD1 activity has been associated with metabolic disorders, and there are currently extensive attempts to develop selective 11β-HSD1 inhibitors for therapeutic interventions [21–23]. In contrast, inhibition of 11β-HSD2 has first become known due to the adverse effects of enhanced renal sodium retention and elevated blood pressure in patients with mutations in *HSD11B2* and in individuals ingesting high amounts of licorice, which contains the non-selective 11β-HSD inhibiting triterpenoid glycyrrhetinic acid (GA) [24]. However, recent observations provided evidence for beneficial effects of 11β-HSD2 inhibition in chronic inflammatory diseases of the colon and on colon cancer cell proliferation. Zhang et al. reported decreased cyclooxygenase-2 (COX-2)-mediated prostaglandine-E2 (PGE2) production in tumors and a prevention of adenoma formation, tumor growth, and metastasis in mice upon pharmacological inhibition of 11β-HSD2 using 18β-glycyrrhetinic acid (GA) or gene silencing [18]. Other investigators found significantly decreased 11β-HSD1 expression in pharyngeal mucosa from patients with squamous cell carcinomas of the head and neck, and reduced levels of 11β-HSD1 but elevated levels of 11β-HSD2 in pituitary tumors [14,25]. Thus, selective 11β-HSD2 inhibition may have a beneficial impact on tumor cell growth. Furthermore, a recent clinical study suggested that 11β-HSD2 inhibition promotes potassium excretion and prevents hyperkalemia in chronic hemodialysis patients [26]. 11β-HSD2 inhibitors may be useful in specific situations such as chronic hemodialysis or may find local applications where inhibition of the renal enzyme can be avoided.

In the above mentioned proof-of-concept studies, the nonselective inhibitor GA was used. GA is widely used as a sweetener in confection products. Although it is well tolerated and does not show significant adverse effects upon short-term administration, the prolonged systemic exposure to high concentrations cause hypertension as a result of vasoconstriction and excessive renal sodium retention due to 11β-HSD2 inhibition and cortisol-dependent activation of mineralocorticoid receptors (MR) and GR [24,27–29]. Because GA potently inhibits 11β-HSD1 and 11β-HSD2 the effects observed after its administration cannot be unambiguously assigned to one of these enzymes. Thus, for the use as tools to further elucidate the physiological role of these two enzymes highly selective inhibitors are required.

Recently, GA was used as a starting point for the development of selective 11β-HSD1 and 11β-HSD2 inhibitors [30–36]. The aim of the present study was the biological characterization of a set of novel and selective 11β-HSD2 inhibitors. The activities of selected inhibitors were compared in assays using cell lysates and intact cells, and their impact on 11β-HSD-dependent modulation of GR transactivation activity was determined. Possible species-specific differences were considered by comparing inhibitory activities of the compounds on human and mouse 11β-HSD2. In an attempt to understand the selectivity of the GA derivatives to inhibit 11β-HSD1 and 11β-HSD2, respectively, we constructed an 11β-HSD2 homology model based on structural information of the related 17β-HSD1 and applied our recently constructed pharmacophore models of 11β-HSD1 [37,38]. The structural analyses provide an explanation for the differences in the selectivity and activity of the GA derivatives investigated.

2. Experimental procedure

2.1. Materials

The GA derivatives used in this study (Tables 1 and 2) were synthesized as described elsewhere [35,36,39] and were of >98% purity as determined by HPLC. [1,2-^{3}H]-cortisone was purchased from American Radiolabeled Chemicals (St. Louis, MO), [1,2,6,7-^{3}H]-cortisol from Amersham Pharmacia (Piscataway, NJ, USA), 5H-1,2,4-triazolo(4,3-a)azepine,6,7,8,9-tetrahydro-3-tricyclo(3·3·1·13·7)dec-1-yl (T0504) from Enamine (Kiev, Ukraine), cell culture media from Invitrogen (Carlsbad, CA) and all other chemicals from Fluka AG (Buchs, Switzerland) of the highest grade available.

2.2. Cell culture

HEK-293 cells, transfected with human or mouse 11β-HSD1 and 11β-HSD2, respectively [40,41], and human SW620 colon cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 50 U/ml penicillin/streptomycin, 2 mM glutamine, and 1 mM HEPES, pH 7.4. Mouse 11β-HSD2 cDNA was cloned by PCR from total RNA of a kidney from a male C57BL/6J mouse. A C-terminal FLAG epitope was attached for facilitated quantification of the protein.

2.3. Transient transfection

HEK-293 cells (200,000 cells/well) were seeded in poly-L-lysine coated 24-well plates, incubated for 16 h and transfected using calcium phosphate precipitation with pMMTV-lacZ β-galactosidase reporter (0.20 μg/well), pCMV-LUC luciferase transfection control (0.05 μg/well), human recombinant GR-α (0.35 μg/well) and either 11β-HSD1, 11β-HSD2 or pcDNA3 control (0.20 μg/well) to ensure equal total DNA content.

2.4. GR transactivation assay

Cells were washed twice with DMEM 6 h post-transfection, followed by cultivation for another 18 h at 37 °C in DMEM to allow sufficient expression. Cells were then washed once with steroid- and serum-free DMEM (DMEMsf) and cultivated at least 3 h at 37 °C. The culture medium was replaced with fresh DMEMsf containing steroids (100 nM) in the presence or absence of test compounds (1 μM). After incubation for 24 h cells were washed once with PBS and lysed with 60 μl lysis buffer of the Tropix kit (Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiothreitol. Lysed samples were frozen at −80 °C for at least 20 min. Lysates (20 μl) were analyzed for β-galactosidase activity using the Tropix kit. Luciferase activity was analyzed in 20 μl samples using a homemade luciferine-solution [42].

2.5. Determination of 11β-HSD activity in cell lysates

For measurements of 11β-HSD1 reductase activity, lysates of HEK-293 cells stably expressing human recombinant 11β-HSD1 were incubated for 10 min at 37 °C in a total volume of 22 μl 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) containing 200 nM [1,2-^{3}H]-cortisone and 500 μM NADPH. 11β-HSD2 dependent oxidation of cortisol to cortisone was measured similarly for 10 min at 37 °C in lysates of HEK-293 cells stably expressing human 11β-HSD2 using [1,2,6,7-^{3}H]-cortisol at a final concentration of 50 nM and 500 μM NAD^+. Stock solutions of all inhibitors were prepared in dimethylsulfoxide (DMSO) at a final concentration of 20 mM. Inhibitors,

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Table 1
Compounds preferentially inhibiting 11β-HSD1. The 11β-HSD1-dependent reduction of cortisone (200 nM) to cortisol and the 11β-HSD2-dependent oxidation of cortisol (50 nM) to cortisone were measured in cell lysates using 500 μM of NADPH or NAD^+, respectively. Glycyrrhetinic acid (GA) was included as reference compound. Inhibitory activities represent $IC_{50} \pm SD$ from three independent experiments.

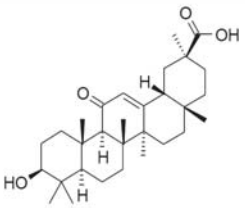
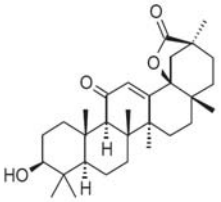
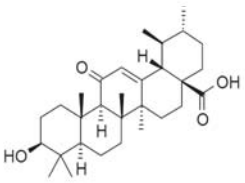
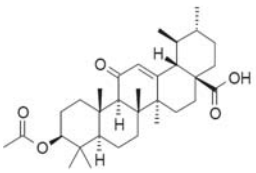
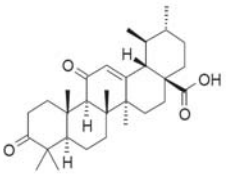
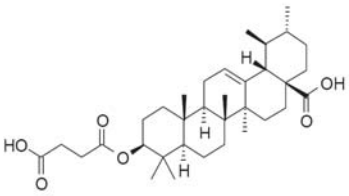
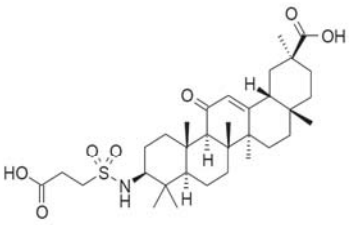
Compound [1 μM]	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
GA		778 ± 71	256 ± 33
1		49 ± 5	25,800 ± 6800
2		2100 ± 400	No inhibition
3		1360 ± 450	No inhibition
4		890 ± 240	>20,000
5		25 ± 13	3200 ± 500
6		147 ± 51	390 ± 51

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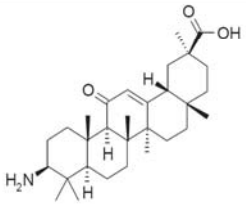
Table 1 (*Continued*)

Compound [1 μM]	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
7		79 ± 8	245 ± 11

diluted from stock solutions in TS2 buffer to yield final concentrations between 50 nM and 50 μM, were immediately used for activity measurements.

2.6. Determination of 11β-HSD activity in intact cells

Enzymatic activities were determined in intact HEK-293 cells stably expressing 11β-HSD2 [41] or 11β-HSD1 and H6PDH [43] as described previously [37]. Briefly, 30,000 cells were seeded per well of poly-L-lysine coated 96-well Biocoat plates (Becton-Dickinson, Basel, Switzerland). The medium was replaced 24 h later by 40 μL fresh DMEMsf containing either vehicle or inhibitor and 10 μL medium containing either 10 nCi [1,2-^{3}H]-cortisone and 200 nM unlabeled cortisone to assess reductase activity or 10 nCi radiolabeld cortisol and 200 nM unlabeled cortisol to measure dehydrogenase activity. Cells were incubated for 1 h or 45 min, respectively, at 37 °C, reactions stopped by adding an excess (2 mM) of unlabeled cortisone and cortisol in methanol, followed by separation of steroids by thin layer chromatography (TLC) and determination of the conversion of radiolabeled substrate by scintillation counting.

For determination of 11β-HSD2 activity in intact SW620 colon cells, 100,000 cells per well were incubated in 50 μL DMEMsf containing the desired concentration of inhibitor, 10 nCi radiolabeld cortisol and 50 nM unlabeled cortisol. Cells were incubated for 4 h at 37 °C and analyzed by TLC and scintillation counting.

2.7. Calculations and statistical analysis

Enzyme kinetics was analyzed by non-linear regression using the four parameter logistic curve fitting. All data (mean ± SD) were obtained from at least three independent experiments and significance was assigned using the ratio *t*-test in the GraphPad Prism 5 software.

2.8. Homology modeling

For homology modeling template selection, a BLAST [44] protein search on the 11β-HSD2 sequence was performed. The search was limited to human proteins; for all other parameters, default settings were kept. The BLAST search returned 17β-HSD2 as most similar human protein. However, for this enzyme, no X-ray crystal structure is currently available in the Protein Data Bank (PDB) [45]. The most closely related protein with available crystal structure was 17β-HSD1 (28% identity, 41% similarity). This template selection was further confirmed by a protein sequence alignment of 11β-HSD1, 11β-HSD2, and 17β-HSD1, performed using the program ClustalW2 [46,47] with default settings. The homology model was constructed using SwissModeler, a freely available online program [48–50]. For the modeling, the alignment mode of the program was used because of its higher accuracy compared to the automated mode. 17β-HSD1 (PDB code 1iol [51], chain A) was used as a template. Visual inspection of the model was carried out using LigandScout 3.0 [52]. Superimpositions of the 11β-HSD2 homology model and human 11β-HSD1 (PDB code 3fco [53], chain A) were performed by Maestro (Schrödinger).

2.9. Docking

The program GOLD [54–56], which is based on a genetic algorithm for calculating putative binding orientations, was used for the docking studies. Early termination of docking in cases where the first docking poses were very much alike was not allowed in order to get deeper insights into possible binding modes. ChemScore was used as scoring function. The program was allowed to determine the atom types of the ligands and the protein automatically. Seven compounds, **1**, **11**, **12**, **13**, **16**, **18**, and **19**, were docked both into the homology model of 11β-HSD2 and into the crystal structure of human 11β-HSD1 (PDB code 3fco, chain A). The proteins were handled as rigid and the ligands with flexible conformations during the docking. The binding modes were visualized using LigandScout 3.0. This program automatically analyzes protein–ligand interactions and generates structure-based pharmacophore models based on the nature and geometry of these interactions. The generated pharmacophore models for each ligand were used for the binding mode analysis.

2.10. Pharmacophore modeling

The pharmacophore model was constructed using LigandScout 3.0. The program was used for predicting ligand–protein interactions and for creating ligand-based pharmacophore models [57]. Six 11β-HSD2-selective compounds were aligned by similar chemical features and translated into so-called shared feature pharmacophore models, which consisted of features present in all aligned ligands. Pharmacophoric features included hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), hydrophobic (H), positive ionizable, negative ionizable, and aromatic rings. Exclusion volume spheres, which are forbidden areas where the ligand is not allowed to map, could also be added to the model at places where the amino acid residues of the protein are located. The purpose of these exclusion volume spheres is to mimic the size and shape of the ligand binding pocket and prevent to spacious hit molecules from fitting into the model.

2.11. Database generation and virtual screening

The 3D database for virtual screening was composed of the compounds described by Beseda et al. [35] and the seven compounds which were also used for docking studies. The 3D-structures for all compounds were constructed using ChemBio3D Ultra 11.0.1 (CambridgeSoft, 2009). Conformations for the ligands were calculated employing Discovery Studio 2.5 (Accelrys Software Inc., 2009) using default settings (FAST method, maximum 255 conformations per

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Table 2
Compounds preferentially inhibiting 11β-HSD2. Inhibitory activities represent $IC_{50} \pm SD$ from at least three independent experiments.

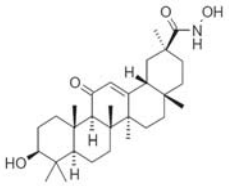
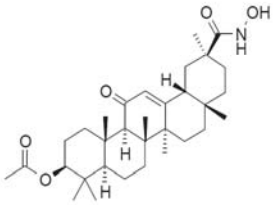
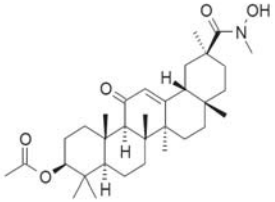
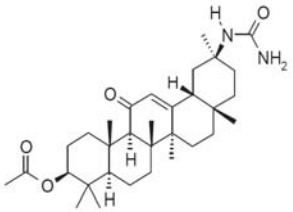
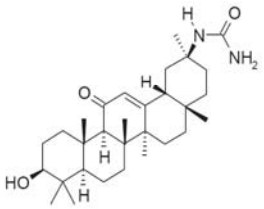
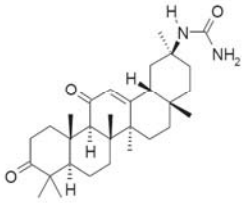
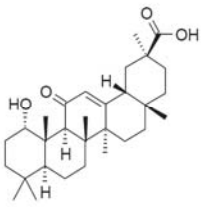
Compound	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
9		1060 ± 70	122 ± 15
10		630 ± 106	60 ± 4
11		1010 ± 140	2.9 ± 1.4
12		8300 ± 1500	104 ± 25
13		5500 ± 900	17 ± 5
14		>10,000[a]	45 ± 6
15		2000 ± 100	90 ± 13

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Table 2 (*Continued*)

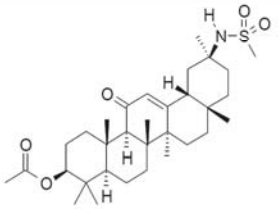
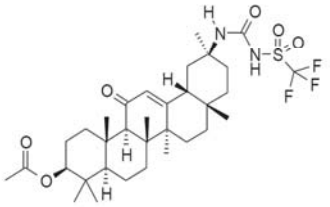
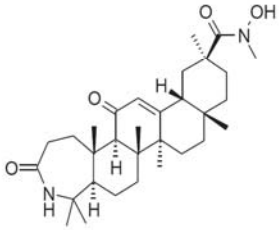
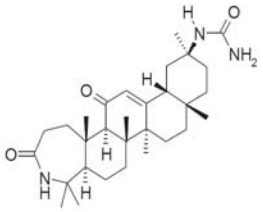
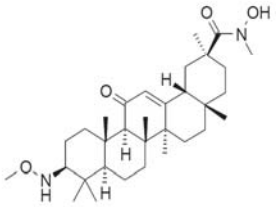
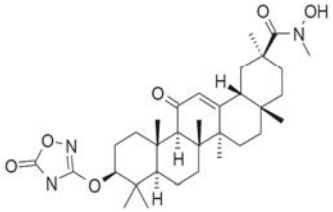
Compound	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
16		>40,000	6.9 ± 1.0
17		4000 ± 500	194 ± 15
18		>40,000	11 ± 2
19		>40,000	33 ± 6
20		>40,000	550 ± 90
21		500 ± 100	15 ± 2

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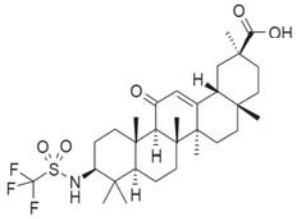
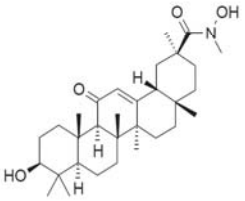
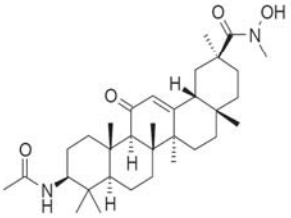
Table 2 (*Continued*)

Compound	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
22		1300 ± 200	74 ± 18
23		3000 ± 1600	16 ± 8
24		645 ± 115	1.2 ± 0.9

[a] Not tested at higher concentrations due to limited solubility.

molecule). The conformations were translated into a LigandScout database using the database generation (Idbgen) function of LigandScout 3.0. The screening was carried out employing the Iscreen tool of LigandScout 3.0.

3. Results

3.1. Analysis of GA derivatives preferentially inhibiting either 11β-HSD1 or 11β-HSD2 measured in cell lysates

In previous studies we aimed at the synthesis of derivatives selectively inhibiting 11β-HSD1 or 11β-HSD2 with equal or higher activity than the parental compound GA [33,35,36,39]. In the present study, we characterized inhibitory activities, binding mode and impact to modulate GR activation of the most selective and active compounds (Tables 1 and 2).

An initial screen for inhibitors of 11β-HSD1 and 11β-HSD2 performed in cell lysates in the presence of 1 μM of the respective compound, using GA as a positive control, revealed several preferential or selective inhibitors of 11β-HSD1 and 11β-HSD2, respectively. The reference compound GA potently inhibited both enzymes, with a slight preference to inhibit 11β-HSD2. In contrast, the 29(18)-lactone derivative **1** potently inhibited 11β-HSD1 (IC_{50} of 49 ± 5 nM), with weak effect on 11β-HSD2 (IC_{50} of 26 ± 7 μM, Table 1). Compound **1** was also tested against mouse enzymes and showed comparable inhibitory activity on 11β-HSD1, whereas concentrations as high as 20 μM did not affect 11β-HSD2 activity. Other compounds preferentially inhibiting 11β-HSD1 include the previously identified naturally occurring compounds 11-oxo-ursolic acid **2** and 3-acetyl-11-oxo-ursolic acid **3** [38], with IC_{50} values of 2.1 ± 0.4 μM and 1.4 ± 0.5 μM, respectively, without inhibiting 11β-HSD2. The related 3,11-dioxo-ursolic acid **4** and 3-succinyl-ursolic acid **5** also preferentially inhibited 11β-HSD1 (IC_{50} of 890 ± 240 nM and 25 ± 13 nM, respectively)(Table 1). The 3-sulfonamide derivative **6** and 3-amino derivative **7** were more potent inhibitors of 11β-HSD1 (IC_{50} of 147 ± 51 and 79 ± 8); however, they also lowered 11β-HSD2 activity (IC_{50} of 390 ± 45 and 245 ± 11). Among these compounds, the 29(18)-lactone derivative **1** showed high inhibitory potency and sufficient selectivity towards 11β-HSD1 and was thus selected for further biological analyses.

Since several GA derivatives and related triterpenoids, including ursolic acid and corosolic acid, that inhibit 11β-HSD1 but not 11β-HSD2 have been reported [31,32,38], our analysis primarily focused on the identification of 11β-HSD2 inhibiting compounds. Recently synthesized GA derivatives were analyzed first at 1 μM final concentrations for their selectivity to inhibit 11β-HSD2 compared with 11β-HSD1 in cell lysates, followed by determination of IC_{50} values.

As shown in Table 2, several compounds inhibited 11β-HSD2 with IC_{50} values in the nanomolar range from 1.2 to 550 nM. These compounds displayed at least ten-fold selectivity over 11β-HSD1. The 29-hydroxamic acid derivative **9** and the 3-acetyl 29-hydroxamic acid derivative **10** showed potent inhibition of 11β-HSD2 (IC_{50} 122 nM and 60 nM, respectively) but rather moderate selectivity (approximately ten-fold). Introduction of a methyl group at the nitrogen of the hydroxamic acid group in **11** resulted in an improved potency towards 11β-HSD1 (IC_{50} 2.9 nM) and higher selectivity (350-fold). Replacement of the carboxylic acid of 3-acetyl-GA by a urea group in **12** caused somewhat lower activity towards 11β-HSD1, but the high selectivity over 11β-HSD2 (80-fold) was retained. The 3-hydroxy-29-urea **13** and the 3-oxo-29-urea derivative **14** both potently inhibited 11β-HSD2 with high selectivity. Interestingly, 1α-hydroxy GA **15** retained the activity towards 11β-HSD2 and was less active against 11β-HSD1 compared with the parental compound. The most potent and selective inhibitors were the 3-acetyl-29-methylsulfonamide derivative **16**,

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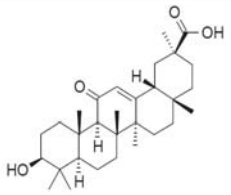
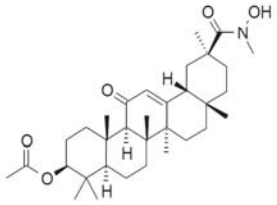
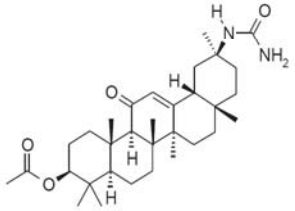
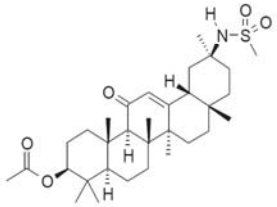
Table 3
Comparison of inhibition of human and mouse 11β-HSD2. The 11β-HSD2-dependent oxidation of cortisol (50 nM) to cortisone was measured in cell lysates using 500 μM of NAD^+. Inhibitory activities represent $IC_{50} \pm SD$ from at least three independent experiments.

Compound		IC_{50} human 11β-HSD2 [nM]	IC_{50} mouse 11β-HSD2 [nM]
GA		256 ± 33	299 ± 46
11		2.9 ± 1.4	27 ± 6
12		104 ± 25	207 ± 23
16		6.9 ± 1.0	186 ± 39

the hydoxamic acid derivative with an enlarged ring A **18**, the urea derivative with an enlarged ring A **19** and the 3-metoxylamino-29-N-methylhydroxamic acid derivative **20**. Among these compounds the hydroxamic acid derivative **11**, the urea derivative **12** and the methylsulfonamide derivative **16** of 3-acetyl GA were chosen for further evaluation.

3.2. Species-specific differences of the selected 11β-HSD2 inhibitors

Previous studies demonstrated significant species-specific differences in the potency of 11β-HSD1 inhibitors, including GA derivatives [32,40,58]; however, little information is available on species-specific differences of 11β-HSD2. Therefore, we compared the potential of GA and compounds **11**, **12** and **16** to inhibit human and mouse 11β-HSD2. Activity assays were performed in cell lysates in the presence of 50 nM cortisol and various concentrations of inhibitor. Whereas inhibition of human and mouse 11β-HSD2 by GA was comparable, approximately two-, ten- and 30-fold higher IC_{50} values were obtained with the mouse enzyme for compounds **12**, **11** and **16**, respectively (Table 3). These observations emphasize the importance of determining the potential of a compound to inhibit the enzyme of the relevant species before conducting efficacy studies or studies to assess on-target toxicity.

3.3. Inhibition of 11β-HSD2 in intact human SW-620 colon cells

Recent reports suggested that inhibition of 11β-HSD2 may be beneficial in the treatment of chronic inflammation of the colon [18]. We therefore investigated whether the selected GA derivatives are able to inhibit 11β-HSD2 in intact human SW-620 colon cells with endogenous expression of this enzyme. Cells were incubated at a final substrate concentration of 50 nM cortisol with GA or its synthetic 11β-HSD2-selective derivatives **12**, **11** and **16** followed by determination of IC_{50} values. All four compounds inhibited the conversion of cortisol to cortisone by 11β-HSD2 in a concentration-dependent manner (Fig. 1). Interestingly, the IC_{50} values obtained in intact SW-620 cells did not reflect the rank order of potency observed in the assays using lysates, where the enzyme is freely accessible by the inhibitors. Similar observations were made in intact HEK-293 cells (data not shown). Although **11** was about 80 times more active in the lysate assay than GA, both compounds inhibited 11β-HSD2 equally well in intact colon cells. This seems to be mainly a result of a more potent effect of GA, *i.e.* an IC_{50} of 12 nM in intact SW-620 cells *versus* 256 nM in the HEK-293 lysate assay. Despite their higher inhibitory potency in the lysate assay, **12** and **16** were two-fold less potent than GA in intact SW-620 cells. The discrepancies between IC_{50} values obtained from assays using lysates and intact cells may be

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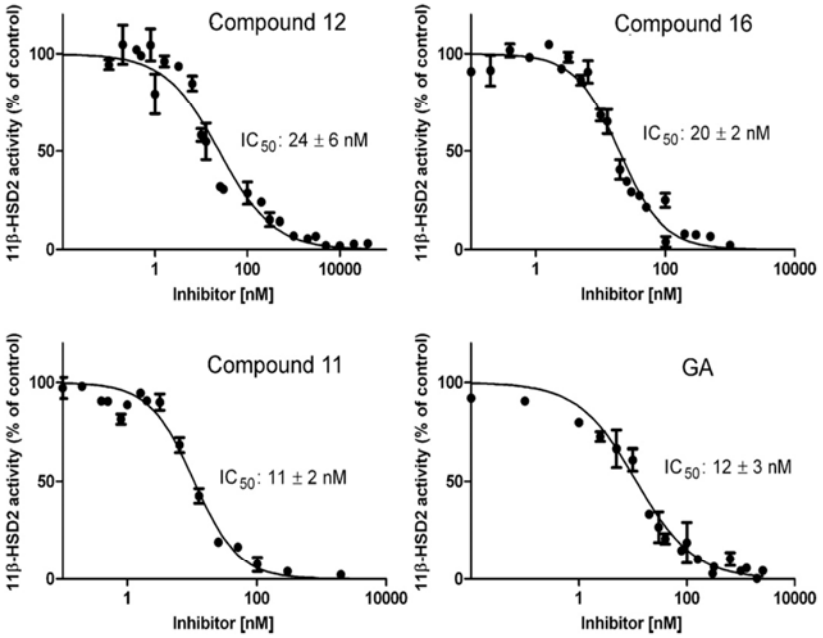

Fig. 1. Inhibition of 11β-HSD2 in intact human SW-620 colon cells. Cells were incubated for 4 h at 37 °C in DMEMsf in the presence of 50 nM cortisol and inhibitors at concentrations from 0 to 2.5 μM, followed by determination of the amount of cortisone generated. Results (mean ± SD) are from three independent experiments.

explained by differences in uptake and/or export of the compounds and the concentrations reached at the location of the enzyme in intact cells.

3.4. Modulation of GR transactivation by selective 11β-HSD inhibitors

On a tissue- and cell-specific level the transactivation capacity of the GR is tightly regulated by 11β-HSD enzymes. By converting inactive into active glucocorticoids (11β-HSD1) or the reverse reaction (11β-HSD2) these enzymes specifically regulate the access of glucocorticoids to the GR and MR. For a proof-of-concept analysis that selective 11β-HSD inhibitors can be used to modulate glucocorticoid signaling, we chose compounds **1** and **12** as selective 11β-HSD1 and 11β-HSD2 inhibitors, respectively, and compared their effects with that of the previously described selective 11β-HSD1 inhibitor T0504 and the unselective inhibitor GA on GR transactivation activities. For that purpose, HEK-293 cells transiently expressing either GR (Fig. 2A), GR and 11β-HSD1 (Fig. 2B) or GR and 11β-HSD2 (Fig. 2C) were incubated for 24 h with 1 μM of the respective inhibitor in the presence or absence of 100 nM cortisone or cortisol.

In cells expressing only GR 100 nM cortisol induced a 15-fold increase of the MMTV-LacZ reporter gene, whereas cortisone did not activate GR, as expected (Fig. 2A). In the absence of glucocorticoids 1 μM of **12**, T0504 or GA did not affect GR transactivation. Compound **1** led to a weak activation of GR (3.2-fold) in the absence of cortisol, suggesting that this compound acts as a weak GR agonist.

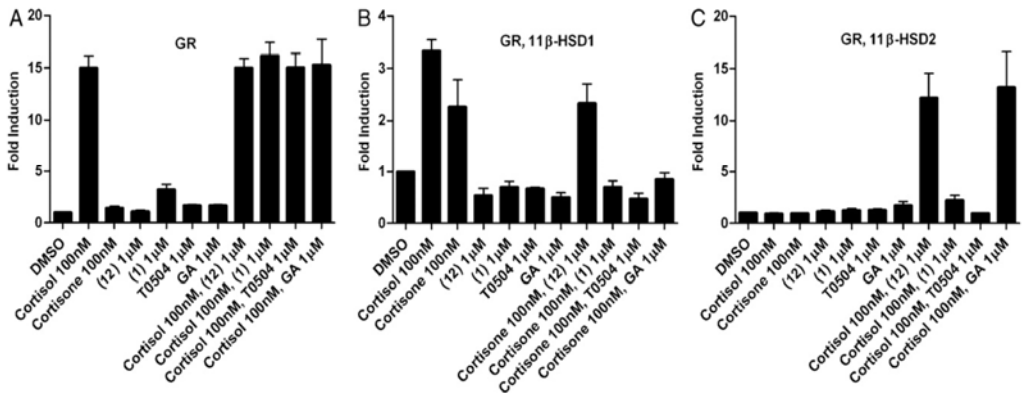

Fig. 2. Modulation of glucocorticoid-dependent GR transactivation by selected 11β-HSD inhibitors. HEK-293 cells were transfected with pMMTV-LacZ reporter, pCMV-LUC control plasmid, human GRα expression plasmid and either pcDNA3 vector to adjust total DNA in transfections (A), 11β-HSD1 expression vector (B) or 11β-HSD2 expression vector (C). Cells were incubated for 24 h in the presence of absence of 100 nM cortisone or cortisol and various inhibitors at final concentrations of 1 μM, followed by determination of galactosidase and luciferase activities. Galactosidase reporter activity was normalized to the internal luciferase control. Data were normalized to vehicle control (0.1% DMSO) and represent mean ± SD from three independent experiments.

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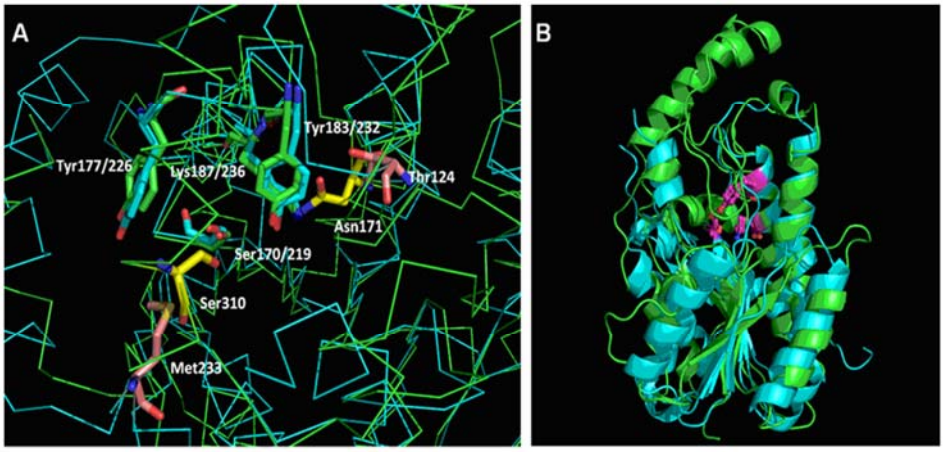

Fig. 3. Homology model of 11β-HSD2. (A) Superimposed structures of 11β-HSD1 (PDB entry 3fco) and the 11β-HSD2 model. The active site with corresponding amino acid residues Tyr 177/226, Ser170/219, and Tyr183/232 are represented in green (11β-HSD1) and blue (11β-HSD2); the main differences are highlighted in red (11β-HSD1) and yellow (11β-HSD2). (B) The protein folding of 11β-HSD1 in green and 11β-HSD2 in blue is shown, with the catalytic triad Ser170/219-Tyr-183/232-Lys187/236 highlighted in pink.

The three inhibitors did not affect the stimulation of GR transactivation by 100 nM cortisol. In cells expressing GR and 11β-HSD1 both cortisol and cortisone (100 nM) led to activation of the MMTV-LacZ reporter (3.3-fold and 2.3-fold, respectively). The less potent stimulation of GR activation by cortisone may be explained by the time required for generation of cortisol by 11β-HSD1 and the reversible activity of this enzyme in HEK-293 cells. As shown in Fig. 2B all three 11β-HSD1 inhibitors completely abolished the conversion of cortisone to cortisol and subsequent activation of GR. In contrast, the 11β-HSD2 selective compound **12** did not block GR activation by 100 nM cortisol. In HEK-293 cells transiently expressing GR and 11β-HSD2 no stimulation of GR transactivation was observed upon addition of glucocorticoids due to the efficient inactivation of cortisol by 11β-HSD2. Upon coincubation with 100 nM cortisol and 1 μM of the 11β-HSD2 inhibitor **12** and the unspecific inhibitor GA, strong stimulation of GR transactivation was observed (12.5-fold and 13.2-fold in the presence of **12** and GA, respectively). In contrast, the 11β-HSD1 selective compounds **1** and T0504 had no effect, as expected.

3.5. Generation of an 11β-HSD2 homology model

The homology model of 11β-HSD2 showed a good alignment with the template structure from 17β-HSD1, despite the presence of some long loops in the homology model. The β-sheets and α-helixes correctly followed the folding of the template, 17β-HSD1, and the overall folding pattern of SDR enzymes as described by Kavanagh et al. [59]. Parts of the C-terminal endings of the model and the template significantly differed from each other: The β-sheet from Glu356 to Arg361 in the 11β-HSD2 homology model corresponds to a loop of Asp269-Gly272 and an α-helix from Ser273 to Val283 in 17β-HSD1. The proteins differ from each other in length: The 78 amino acids longer 11β-HSD2 has two α-helixes formed by Phe265-Asn272 and Gly274-Lys280 and a loop from Gln284 to Leu287 replacing a gap in 17β-HSD1. Most importantly, the active sites and especially the conserved amino acid residues of 17β-HSD1 and 11β-HSD2 showed good superimposition. Furthermore, the conserved amino acids of 11β-HSD1 and the homology model showed good alignment (Fig. 3). The contribution of sequence differences on the ligand binding site architecture was analyzed and helped to rationalize inhibitor selectivity of the reported compounds.

3.6. Docking

Compounds **1**, **11**, **12**, **13**, **16**, **18**, and **19** were docked into the 11β-HSD1 and 11β-HSD2 ligand binding sites, respectively. An analysis of ligand binding modes revealed the differences in protein–ligand interactions between the two enzymes. All compounds were orientated in the 11β-HSD1 ligand binding domain similar to carbenoxolone in the 11β-HSD1 cocrystal structure (PDB code 2bel): The keto-oxygen in position 11 pointed towards Ser170 and/or Tyr183 and the substituents in position 20 pointed towards the cofactor binding site (Fig. 4A). This orientation allowed hydrogen bonds to Tyr177, which were observed among the docking poses of two ligands. Four out of seven compounds also formed hydrogen bonds with the backbone nitrogen of Leu217 and compound **16** forms an additional hydrogen bond to Thr124. In most of the cases, no further hydrogen bonds were observed. Among the docking solutions of **13**, **18**, and **19**, a 180° flipped binding mode in 11β-HSD1 was observed (Fig. 4B). In the best ranked, flipped docking solution for compound **18** only hydrophobic interactions were observed, which could explain the low activity.

Rollinger et al. [38] suggested such a flipped binding mode for the 11β-HSD1-selective inhibitor corosolic acid based on docking studies. Although the carbenoxolone-like binding mode was observed for all of the compounds in 11β-HSD1, the flipped binding, where 11-keto-oxygen points away from catalytic residues, seems to be favored in 11β-HSD2. All of the ligands were anchored this way into the 11β-HSD2 binding site. This orientation allowed hydrogen bonds to Asn171 and Ser310 (Fig. 5), two residues that only occur in the 11β-HSD2 binding site and may therefore be important for ligand selectivity. Some of the compounds showed additional hydrogen bonds to Asn167. The corresponding amino acid for Asn167 in 11β-HSD1 is Asn119, which does not contribute to ligand binding, because it is not part of the ligand binding pocket. The only 11β-HSD1-selective compound **1** also adopted a flipped binding mode in 11β-HSD2, but the interaction pattern lacked hydrogen bonds to Asn171 and Ser310, explaining its weak inhibitory activity towards 11β-HSD2.

3.7. Pharmacophore modeling and virtual screening

For the development of a specific 11β-HSD2 inhibitor pharmacophore model, the chemical features responsible for the selectivity

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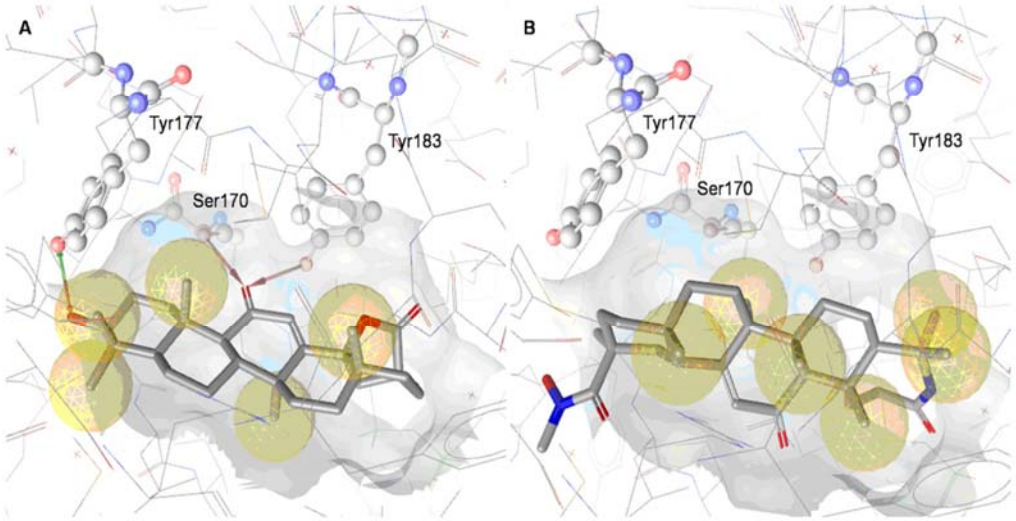

Fig. 4. Compounds **1** (A) and **18** (B) bound to 11β-HSD1. Protein–ligand interactions are color-coded: hydrogen bond acceptor – red and hydrophobic – yellow.

had to be identified. When comparing the selectivity data and structures of compounds reported by Beseda et al. [35] as well as the seven compounds presented above, the substituents in position 20 came to our attention. All 11β-HSD2-selective compounds bear substituents with both HBD and HBA functionalities in position 20, and the length of these substituents is often three atoms. Longer substituents seem to decrease the inhibitory activity or even activate the enzyme. Shorter substituents shift the inhibitors' selectivity. Without the HBD feature, the compounds seem to be unselective inhibitors of both 11β-HSDs. Furthermore, the HBA feature in position 3 seems to be important for the 11β-HSD2 inhibition.

These observations were the basis for the pharmacophore model generation. A shared feature model was derived from six 11β-HSD2-selective compounds. The automatically created model consisted of hydrophobic features placed on the methyl groups of the ligands and HBD features on positions 3, 11, and 20. The model was further modified by manually adding a HBD feature and exclusion volume spheres. In order to make the model less restrictive, some of the hydrophobic features were removed. The final model consists of six features: three HBAs, one HBD, and two hydrophobic features.

In a database search, the model retrieved six hit molecules: compounds **13**, **18**, **12**, **16**, **19**, and one unselective compound described by Beseda et al. (compound **5c**) [35] (Fig. 6A). However, the unselective compound inhibits 11β-HSD2 activity to almost 90% when tested at a concentration of 1 μM and therefore has to potently bind to 11β-HSD2. The model along with the hit molecules was injected into the binding pocket in order to analyze putative ligand–protein interactions (Fig. 6B). For most of the pharmacophore features, corresponding ligand–protein interactions were observed; however, the HBD feature at position 20 was missing an interacting amino acid residue.

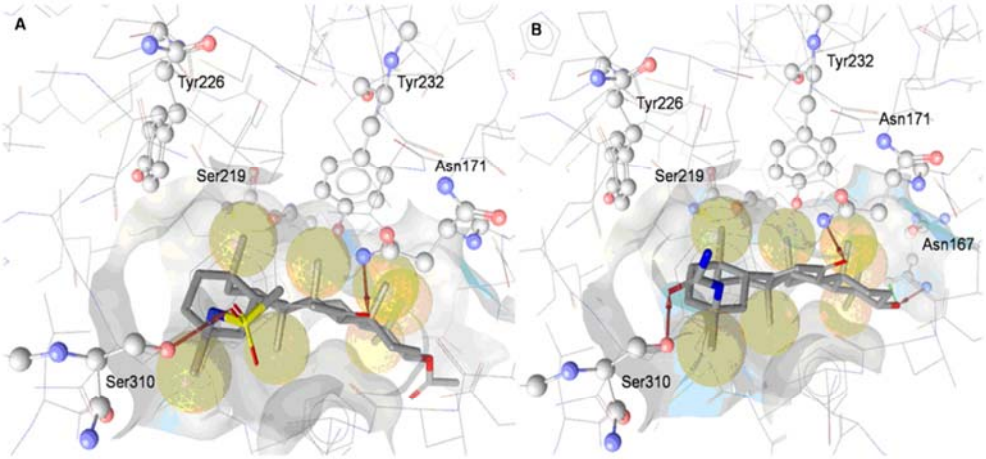

Fig. 5. Compounds **16** (A) and **13** (B) fitted into the 11β-HSD2 model. Chemical features are color-coded: hydrogen bond donor – red arrow, hydrogen bond acceptor – green arrow, and hydrophobic – yellow.

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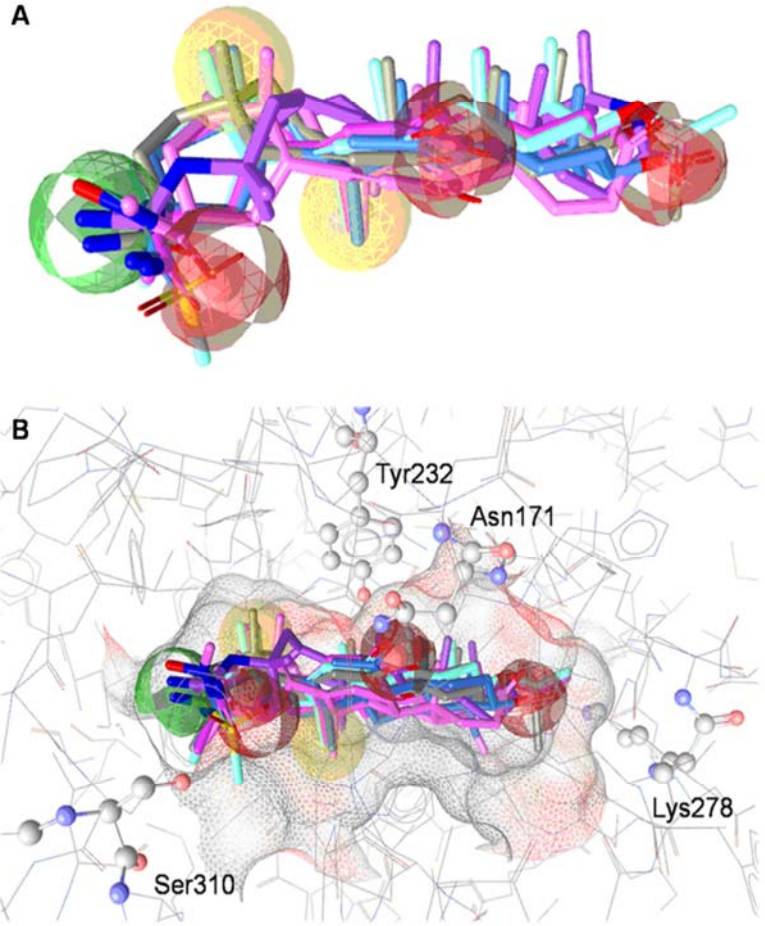

Fig. 6. Pharmacophore model of 11β-HSD2. (A) Shared feature pharmacophore model with aligned hit molecules, and (B) model and ligands fitted into the 11β-HSD2 active site. Chemical features are color-coded: HBA – red, HBD – green, and hydrophobic – yellow. The ligand binding pocket is colored by polarity.

4. Discussion

The availability of selective and potent inhibitors would greatly facilitate the elucidation of the physiological functions of 11β-HSD2. In the present study, we applied bioassays and 3D-structure modeling to characterize a series of derivatives of the well-known natural compound GA, comprising nine derivatives preferentially inhibiting 11β-HSD1 and fifteen compounds inhibiting 11β-HSD2. Comparison of the GA derivatives in assays using cell lysates revealed that specific chemical modifications of the hydroxyl and/or the carboxyl on C3 and C29, respectively, yielded potent 11β-HSD2 inhibitors with IC_{50} values in the nanomolar range and high selectivity for 11β-HSD2 over 11β-HSD1. Compounds **11**, **12** and **16** inhibited 11β-HSD2 about 40–80 times better than the parental compound GA, without significant effects on 11β-HSD1.

The docking studies, binding site analyses, and comparison of the superimposed enzymes revealed small differences between the 11β-HSD1 and 11β-HSD2 binding pockets. Most of the 11β-HSD2-selective compounds were predicted to bind to 11β-HSD2 in a flipped orientation by forming hydrogen bonds with Asn171 and Ser310. The corresponding amino acids in 11β-HSD1 are Asn123 and Met233, respectively. Asn123 does not contribute to ligand binding because it is pointing away from the ligand binding pocket, and Met233 is incapable of forming hydrogen bonds. The spatially equivalent amino acid for Asn171 in 11β-HSD1 is Thr124, which is functionally different. However, the analyses of the docked selective and unselective ligands suggest that binding to Ser310 and the HBD feature of the 11β-HSD2-selective ligands are more important to ligand selectivity than hydrogen bonds to Asn171. Furthermore, both the selective and unselective ligands have identical chemical functions in position 11 of the triterpenoid backbone, demonstrating that this position is not responsible for the observed selectivity. Based on these observations, the hypothesis of 11β-HSD2-selectivity consists of the HBA feature that could interact with Ser310 and the HBD feature, for which no direct interaction partner could be observed in the homology model. Because the created homology model did not reveal an interaction partner for the ligands' HBD feature, the possibility of a coordinated water molecule interaction was evaluated. There is enough space for a water molecule in 11β-HSD2, and water might be coordinated to the binding site in the vicinity of Pro227, Gln306, and Ser310. Thus, protein–ligand interactions of selective 11β-HSD2 inhibitors may involve a water-mediated hydrogen bond to the protein binding pocket.

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The 11β-HSD1-selective compound **1** and the 11β-HSD2-selective inhibitors **11**, **12**, and **16** were subjected to further biological analyses. All compounds tested were active in intact HEK-293 cells as well as SW-620 colon cancer cells, and no cytotoxicity was detected at the concentrations used in the experiments (data not shown). The GA derivatives **11**, **12**, and **16** potently inhibited 11β-HSD2 in intact SW-620 cells; however, the rank order of inhibitory potential was different from that seen in cell lysate assays, and the gain of potency compared with GA was no longer evident in intact SW-620 cells. These results emphasize the importance to assess inhibitory activity in relevant intact cells in order to consider cell-specific properties.

Furthermore, our analyses emphasize the importance to assess species-specific differences of inhibitors prior to conducting in vivo experiments. Using recombinant enzymes we found potent inhibition of mouse 11β-HSD2 by compounds **11**, **12**, and **16**, despite significant species-specific differences. The species effect was most pronounced for compound **16**, with approximately 30-fold weaker inhibition of mouse 11β-HSD2. This effect may be in part due to the sulfonamide group, which enhances the hydrophilicity of the molecule and may disturb hydrogen bond formation or lead to steric interference. The existence of significant species-specific differences in inhibitory potency is not surprising regarding the fact that in humans cortisol is the major substrate for 11β-HSD2, whereas it is corticosterone in rodents. Similarly, considerable species-specific effects for both substrates and inhibitors have been reported for 11β-HSD1 [10,40,58,60]. Thus, for the assessment of efficacy and potential on-target toxicity, a compound ideally should have comparable effects on the human enzyme and the enzyme of the species of interest.

The biological activity of the selective 11β-HSD1 inhibitor **1** and the 11β-HSD2 inhibitors **11**, **12**, and **16** was further demonstrated in transactivation assays in cells coexpressing GR and 11β-HSD1 or 11β-HSD2. The results underline the potential of compound **1** to study 11β-HSD1-controlled glucocorticoid-mediated gene expression, whereas compounds **11**, **12**, and **16** allow investigating 11β-HSD2-dependent modulation of GR activity. Importantly, a direct interference of the compounds with GR was excluded since the compounds were unable to activate GR in the absence of cortisol (Fig. 2), and they did not affect translocation of GR into the nucleus in response to cortisol (not shown).

Selective 11β-HSD2 inhibitors should facilitate the elucidation of the role of this enzyme in inflammatory diseases of the colon [19] as well as in colon cancer [18,61,62]. These earlier studies, using the unselective compound GA, suggested that enhanced 11β-HSD2 activity in colorectal cancer tissue leads to increased COX-2 expression, which will result in an uncontrolled production of PGE2 and promote tumor growth. Because inhibition of COX-2 by NSAIDs is accompanied with gastrointestinal side effects and selective COX-2 inhibitors may increase cardiovascular risks, their therapeutic use is restricted. Alternatively, systemic treatment with pharmacological doses of glucocorticoids results in decreased COX-2 expression and PGE2 production, thereby suppressing inflammatory response and tumor growth. However, to overcome the adverse effects of prolonged treatment with high doses of glucocorticoids, topically applied selective 11β-HSD2 inhibitors might offer an alternative strategy to modulate glucocorticoid-dependent regulation of the immune system and tumor cell growth.

The inhibitors described in the present study should facilitate proof-of-concept studies; however, their stability and tissue distribution remain to be investigated. In addition, the selectivity of the GA derivatives has to be studied by testing for effects on other members of the SDR enzyme family. Furthermore, the 11β-HSD2 pharmacophore constructed in this study offers the possibility to screen virtual compound libraries for the identification of novel classes of 11β-HSD2 inhibitors, similar to earlier approaches for 11β-HSD1 [37] and 17β-HSD3/5 [63]. Together, these attempts should promote the identification of suitable potent and selective 11β-HSD2 inhibitors for in vivo studies.

Acknowledgements

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7 TISSUE-SPECIFIC MODULATION OF MINERALOCORTICOID RECEPTOR FUNCTION BY 11β-HYDROXYSTEROID DEHYDROGENASES: AN OVERVIEW

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7.1 Abstract

In the last decade significant progress has been made in the understanding of mineralocorticoid receptor (MR) function and its implications for physiology and disease. The knowledge on the essential role of MR in the regulation of electrolyte concentrations and blood pressure has been significantly extended, and the relevance of excessive MR activation in promoting inflammation, fibrosis and heart disease as well as the adverse effects on brain function is now widely recognized. Despite this considerable progress, the mechanisms of MR function in various cell-types are still poorly understood. Key modulators of MR function include the glucocorticoid receptor (GR), which may affect MR function by formation of heterodimers and by differential genomic and non-genomic responses on gene expression, and 11β-hydroxysteroid dehydrogenases (11β-HSDs), which determine the availability of intracellular concentrations of active glucocorticoids. In this review we attempted to provide an overview of the knowledge on MR expression with regard to the presence or absence of GR, 11β-HSD2 and 11β-HSD1/hexose-6-phosphate dehydrogenase (H6PDH) in various tissues and cell types. The consequences of cell-specific differences in the coexpression of MR with these proteins need to be further investigated in order to understand the role of MR in a given tissue as well as its systemic impact.

7.2 Introduction

The use of complementary DNA of the glucocorticoid receptor (GR, systematic name NR3C1) and low-stringency hybridization by Arizza *et al.* led to the identification of a cDNA coding for a 107 kDa polypeptide, which was functionally characterized as mineralocorticoid receptor (MR) [1]. The MR is also known as aldosterone receptor and under the systematic name NR3C2 (Nuclear Receptor subfamily 3, group C, member 2). MR and GR share about 90% amino acid homology in their DNA binding domain (DBD) but only about 50% in their ligand binding domain (LBD). Evolutionary analyses suggested that MR and GR evolved from a common ancestor and that the MR was the first to diverge from the ancient receptor gene [2, 3]. Importantly, MR existed well before aldosterone appeared in evolution, whereas GR seems to have appeared later in evolution. This may explain the rather broad substrate specificity of MR, compared with the more selective GR. Whereas MR binds aldosterone, 11-deoxycorticosterone, corticosterone, cortisol and progesterone with similarly high affinities and Kd values between 0.5 and 3 nM, GR shows a higher selectivity to cortisol and corticosterone with Kd values of 20-70 nM [1, 4, 5].

The cloning of MR allowed its exact localization in various tissues and identification of specific cell types expressing this receptor. The subsequent cloning of 11□-hydroxysteroid dehydrogenase type 1 (11□-HSD1)[6] and 11□-HSD2 [7, 8] and determination of their tissue- and cell-specific expression patterns then allowed a comparison with the expression pattern of MR and GR. It soon became clear that MR is not only expressed in cells where 11β-HSD2 acts as a “gate-keeper” to protect MR from high concentrations of glucocorticoids and rendering specificity for aldosterone [9, 10]. As discussed below, the MR plays an important role in cells coexpressing 11□-HSD1, including macrophages, preadipocytes/adipocytes, osteoblasts/osteoclasts, and microglia cells, by modulating cell proliferation and inflammatory

response. Thus, the classic view of mineralocorticoid target tissues, where MR function is strictly regulated by aldosterone, has to be reconsidered.

7.3 Kidney

The kidney is considered as the classical mineralocorticoid target tissues. High-affinity aldosterone binding sites, corresponding to MRs, and lower affinity glucocorticoid binding sites, corresponding to GRα, have been characterized in rat kidneys almost 40 years ago [11, 12]. Aldosterone-induced renal epithelial sodium transport was found to be dependent on a nuclear transactivating receptor that was later identified as MR [1, 13]. The MR has similar high affinities to bind aldosterone, progesterone, 11-deoxycorticosterone, corticosterone and cortisol, with Kd values between 0.5 and 3 nM [1, 4], whereas the GR shows higher ligand selectivity but approximately 20-fold lower affinity for cortisol and very weak affinity for aldosterone (Kd about 500 nM). The identification of 11β-HSD2 as a "gate-keeper" to protect MR from active 11β-hydroxyglucocorticoids (cortisol in humans, corticosterone in rodents) that are present in plasma at about 1000-fold higher concentrations than aldosterone provided an explanation for the specificity of this receptor towards aldosterone [9, 10].

Investigation of the expression of MRs in human, rat and rabbit kidney revealed colocalization with 11β-HSD2 in the distal tubules and cortical collecting ducts (Table 1) [14-19]. 11β-HSD2 is an endoplasmic reticulum (ER) resident enzyme with its catalytic domain facing the cytoplasm [20-22]. Experiments with cultured cells expressing recombinant MR and 11β-HSD2 revealed a tethering of the receptor to 11β-HSD2 at the ER membrane in the absence of steroid hormones as well as in the presence of low concentrations of cortisol [23]. In contrast, low concentrations of aldosterone were efficient to induce almost complete translocation of MRs into the nucleus and to stimulate the expression of a GR/MR-dependent reporter gene (MMTV-lacZ). High concentrations of cortisol or corticosterone (>250 nM) led to the activation of MR, probably as a result of saturation of 11β-HSD2. These experiments suggested a close proximity of MR and 11β-HSD2, allowing the latter to efficiently inactivate cortisol at the site of the receptor and preventing binding of the active glucocorticoid at low concentrations, *i.e.* at nadir of circadian rhythm.

In a recent study, Ackermann *et al.* used MR- and GR-specific antibodies to determine the localization of the receptors in kidneys of rats with altered aldosterone and corticosterone levels [19]. Immunohistochemistry detected MR and GR in the nuclei of the aldosterone-sensitive distal nephron, including cells of the late distal convoluted tubule, connecting tubule and collecting duct. These cells also express high levels of 11β-HSD2 (Figure 1).

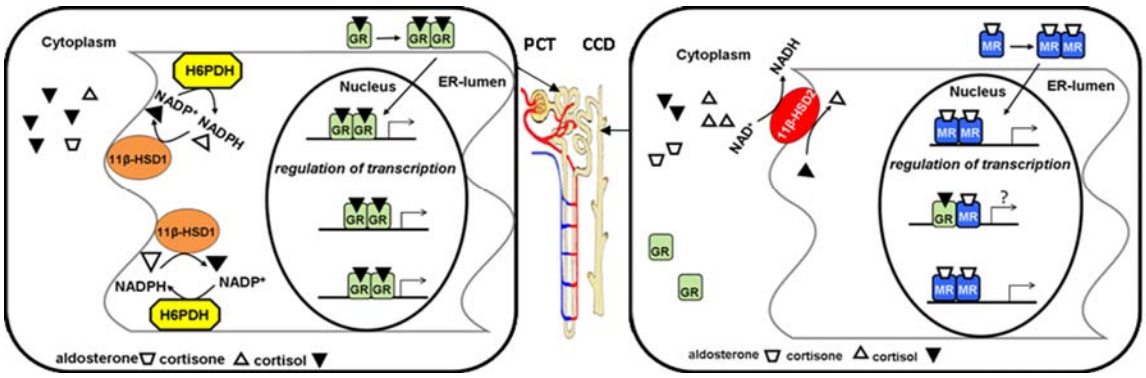

Figure 12 Schematic overview of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) regulation in with respect to the expression of 11β-hydroxysteroid dehydrogenases (11β-HSDs) and hexose-6-phosphate dehydrogenase (H6PDH) in cells of pro ximal tubules (PCT) and cortical collecting ducts (CCD).

In addition, MR and GR expression was found in the thick ascending limb and in intercalated cells, where 11β-HSD2 is absent. It was suggested that MR in intercalated cells may be involved in proton secretion, thereby playing an essential role in acid/base regulation. The role of MR in these cell types remains to be elucidated. In rats on a high-salt diet, which is known to lower plasma aldosterone, MR localization to the nuclei was unchanged, whereas GR localized to the cytoplasm in the aldosterone-sensitive distal nephron. The actual diet-induced changes in circulating aldosterone and corticosterone levels, however, have not been determined in this GR was found to be coexpressed with 11β-HSD1 and H6PDH mainly in the third segment of the proximal tubules [24-26] and probably plays a role in regulating glucose and lipid uptake and metabolism. Chronically elevated glucocorticoid activation in proximal tubules is likely to cause adverse metabolic effects and disturbances in transport processes in these cells and warrants further investigation.

Several investigators reported the expression of MR in glomerular mesangial cells and observed aldosterone-induced cell proliferation [27-32]. A recent study with cultured rat mesangial cells provided evidence for the involvement of the MR in the stimulation of mesangial cell proliferation by high glucose medium [29]. Induction of cell proliferation was prevented by incubation with an antagonist and siRNA against MR and by an inhibitor of extracellular signal-regulated kinase kinase (MEK). Furthermore, aldosterone-dependent apoptotic and mitogenic effects were demonstrated in human mesangial cells [30]. The pro-apoptotic effects of aldosterone were prevented by co-treatment with spironolactone as well as by antioxidants and free radical scavengers. Aldosterone has been shown to increase reactive oxygen species (ROS) production by a mechanism involving activation of NADPH oxidase in renal and cardiovascular tissues [33-38]. Long-term administration of aldosterone to rats caused mesangial cell proliferation and expansion of the mesangium [37]. Thus, exposure to chronically high aldosterone levels might cause mesangial cell damage, independent of its hemodynamic effects.

Glomerular mesangial cells were initially reported to express 11β-HSD1, and an upregulation of its expression was observed in the presence of the pro-inflammatory cytokines TNF-α and IL-1β [39]. However, other investigators reported expression of MR, aldosterone synthase (CYP11B2) and 11β-HSD2 in rat mesangial cells, and provided evidence for a role of mitogen-activated protein kinase 1/2, cyclin D1 and cyclin A in the aldosterone-induced mesangial cell proliferation and of a Smad2- and TGF-β1-dependent stimulation of fibronectin production [32, 40, 41]. The expression and role of the respective 11β-HSD enzyme and species differences have to be studied.

Recent evidence suggested that elevated CYP11B2 levels and MR activation in podocytes may contribute to the progression of diabetic nephropathy [42, 43]. Immortalized podocytes expressing MR, CYP11B2 and 11β-HSD2 were incubated with physiological (5.6 mM) and high (30 mM) concentrations of glucose [42]. MR and CYP11B2 expression were increased upon high glucose treatment, whereas 11β-HSD2 was not altered. Enhanced MR and CYP11B2 expression was also found in glomeruli of streptozotocin treated diabetic rats, and aldosterone levels were increased in these animals. Furthermore, treatment of type 2 diabetic Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats with the MR antagonist eplerenone enhanced the blood pressure-independent anti-proteinuric effects of angiotensinogen II type 1 receptor blocker. These observations indicate that chronically elevated MR activity may contribute to impaired glomerular function by adverse effects on podocytes.

The bidirectional enzyme 11β-HSD1 has been detected in renal medullary and interstitial cells that express GR but not MR [25, 44]. Interestingly, H6PDH seems to be absent in these cells suggesting that another enzyme might provide NADPH in the ER or that 11β-HSD1 might act as a dehydrogenase in these cells to modulate GR activity. The role of 11β-HSD1 in the modulation of GR function in these cells remains to be clarified.

study. Also, it remains unclear how altered aldosterone levels might affect GR but not MR localization. Furthermore, the time point where the samples have been taken is not indicated, and it will be important to compare the localization of MR and GR in different cell types during peak glucocorticoids and at nadir during circadian rhythm. Nevertheless, the authors observed cytoplasmic localization of both MR and GR in adrenalectomized rats, and low dose corticosterone replacement led to nuclear translocation of MR but not GR. The GR translocated to the nuclei only in cells not expressing 11β-HSD2.

Thus, there are several cell types in the kidney where the classical view of MR function does not apply. Future studies have to face the challenge to uncover the mechanisms of MR activation and its consequences in these cells as well as to elucidate the cross-talk between different renal cells, and between renal cells and cells of the vasculature, adipose tissue and immune system

Table 6 Expression and Protein appearance of MR, GR, 11β-HSD1, 11β-HSD2 and H6PDH in kidney specific cell types.

KIDNEY	MR		GR		11β-HSD1		11β-HSD2		H6PDH	
	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
Kidney					+ [25]	+ [46] [25]			+ [25]	+ [25]
Glomerulus	- [15]	- [19] [55]	+ [15] [19]	+ [19]	- [26]				+ [25]	
Aldosterone-sensitive distal nephron (ASDN)	+ [19] [56]	+ [56]	+ [19]				++ [19]		+ [25]	
Segment-specific cells of late distal convoluted tubule (DCT2)	+ [19] [15] [14]	+ [19] [55]	+ [15] +/- [19]			- [55]	++ [19] [14]	++ [14] [55]	+ [25]	
Distal convoluted tubules (DCT)	+ [15] [14] [50] [19] [57]	+ [19] [58] ++ [55]	+ [15] [19]	+ [55] [19]		- [55]	+ [53] [14] [19] [24]	- [14]	+ [25]	
Connecting tubule (CNT)	+ [19] [15] [14]	+ [19]	+ [19] [15]	+ [19]			++ [19] [53] [14]	++ [14]	+ [25]	

	[57]									
Cortex	+ [56]	+ [56] [55]	+ [19]	++ [55]			+ [19]	+ [55]	+ [59] [25]	
Interstitial cells of the medulla					+ [24] [26] [25]				-/(+) [25]	
Inner medulla		+ [55]	+ [60]	+ [55]		+ [55]			++ [59] ++ [25]	
Collecting duct (CCD).	+ [19] [14] [57] [15]	++ [19] [61] [55] [58]	+ [19]	+ [61] [55] [19]	- [25]		++ [19] [53] [14] [62] [24] [62]	++ [14]	+ [25]	
Outer medullary collecting ducts (OMCD)	+ [15]	+ [19] [55]	+ [15]	+ [19]			++ [14]	++ [14]	+ [25]	
Inner medullary collecting ducts (IMCD)	+ [15]	+ [19]	+ [15]	+ [19]			+ [14]	+ [14]	+ [25]	
Intercalated cells (IC)	- [15] [14] + [19]		+ [19]				+ [19]		+ [25]	
Proximal tubule (PT) cells	- [50] [15]		+ [50] [19]	++ [55]	+ [24]	+ [55]		- [55]	+ [25]	

Proximal convoluted (PCT)	- [15]	- [19] [55]	+ [19]	+ [19]		+ [55]		- [55]	+ [59] ++ [25]	
P1								- [55]	+/- [59] [25]	
P2								- [55]	+ [59] [25]	
P3					+ [26]			- [55]	++ [59] [25]	
Proximal straight tubules (PST)	- [15]	- [19] [55]	- [15]	+ [19]				- [55]	+ [25]	
Thick ascending limb (TAL)	+ [19]		+ [19]				- [14]	- [14]	+ [25]	
Outer medullary thick ascending limbs (OMTAL)	++ [15]	+ [19]	+ [15]	+ [19]					+ [25]	
Medullary thick ascending limbs (MTAL)	+ [15]		++ [15]						+ [25]	
Cortical thick ascending limbs (CTAL)	++ [15] [14]	+ [19]	+ [15]	+ [19]					+/- [59]	
Macula densa	+ [14]				+ [25]				+ [25]	
Henle's loop thin parts of the loop	+ [15]	+ [58]	+ [15]						+ [25]	
Interstitial cells papilla	+ [15]		+ [15]		+ [24] [26]				- [25]	

Pappilar surface epithelium	+ [15]		+ [15]						+ [25]	

7.4 Gastrointestinal tract

The MR plays an important role in the gastrointestinal tract in water and electrolyte control as well as the regulation of inflammation. In the stomach aldosterone is involved in the regulation of electrolyte transport associated with gastric acid secretion. Specific aldosterone binding sites were detected in the gastric fundic mucosa but not in antral mucosa [45]. Colocalization of MR with 11β-HSD2 could be shown on the basis of protein as well as mRNA in parietal cells of the gastric fundic mucosa. In the stomach the transport of sodium, potassium, chloride, bicarbonate and protons is mainly mediated by membrane proteins of parietal cells. The gastric fundic mucosa cells therefore resemble renal distal tubular epithelial cells as classic mineralocorticoid targets. The relevance of functional MR in the stomach was further demonstrated by the reduced gastric acid secretion after adrenalectomy [45].

Interestingly, Brereton *et al.* reported the expression of 11β-HSD1 in parietal cells of the stomach using immunohistochemistry [24]. The expression of 11β-HSD1 in stomach was verified by Moore *et al.* using RNase protection assay [46], and H6PDH expression was also reported in stomach [25]; however, in these studies the specific cell types of expression have not been determined and it needs to be clarified whether 11β-HSD1 activity indeed might play a role in parietal cells or whether it is restricted to other cell types.

The distal colon is a well accepted gastrointestinal mineralocorticoid-responsive tissue [47-49]. Specific binding of radiolabeled aldosterone provided evidence for MR expression in sigmoid, descending and transverse colon as well as epithelial cells of ascending colon, caecum and ileum in humans [49]. In contrast, Fukushima *et al.* detected MR in adult human gut cells using a polyclonal antibody [50]. They observed high expression levels in the ascending colon but weak staining in the transverse colon and no signals for goblet cells, jejunum and ileum. Hirasawa *et al.* investigated the expression of MR and 11β-HSD2 in adult and fetal tissues [51, 52]. They found coexpression of MR and 11β-HSD2 in the absorptive epithelia of duodenum, jejunum, ileum, colon, and excretory ducts of anal and esophageal glands in adult tissues [52]. High expression of MR and 11β-HSD2 was observed in colonic epithelium and weak expression in the superficial epithelium of the small intestine, suggesting relevant MR action in the upper fetal gastrointestinal tract [51]. Smith *et al.* reported immunoreactivity for 11β-HSD2 in ileal enterocytes, colonic absorptive cells and epithelial goblet cells. Lamina propria, Peyer's patch and goblet cells within the crypts of Lieberkuhn did not stain positive, while the rectum contained both negatively and positively staining cells.

The expression of 11β-HSD2 was further characterized by Naray-Fejes-Toth *et al.* in a novel transgenic mouse strain expressing a Cre recombinase under the control of the endogenous 11β-HSD2 promoter [53]. Classical mineralocorticoid target tissues as well as non-aldosterone-sensitive tissues were evaluated for galactosidase-mediated staining and results were confirmed by counterstaining with specific antibodies against 11β-HSD2. The iCre excision could be detected in colon epithelial cells, cells of the external muscular layers and for the jejunum.

11β-HSD1 expression has been found in small intestine [46]. In addition, 11β-HSD1 expressing macrophage may play a role in inflammation of the colon by producing active glucocorticoids locally at the site of inflammation. In human and rat colon samples upregulation of 11β-HSD1 and a concomitant downregulation of 11β-HSD2 was observed in colitis, indicating a role for local glucocorticoid metabolism in the regulation of colonic inflammation [54]. Future studies should address the interactions between colon epithelial cells and macrophage during inflammation.

7.5 Adrenals

The adrenal cortex can be divided into the zona glomerulosa, zona fasciculata and zona reticularis, whereby aldosterone is synthesized in the zona glomerulosa, and basal and induced glucocorticoid production occurs in the zona fasciculata. The distribution of MR and GR expression within the adrenal gland remains to be investigated. However, studies

with GR and MR knockout mice indicated important roles of these receptors for adrenal function. In fetal adrenal glands of GR knockout mice, which die immediately after birth, an extensive hypertrophy and hyperplasia of the cortical zones of the adrenal gland was observed with a disorganized and reduced medullary region and a lack of adrenalin producing cells [55]. Hubert *et al.* studied the impact of MR gene disruption on the renin angiotensin aldosterone system in 8 days old mice [56]. These mice developed pseudohypoaldosteronism type I with high plasma renin, angiotensin II and aldosterone. Histological analyses revealed a significantly enlarged zona glomerulosa, which extended more deeply toward the medullary region than in wild-type mice. The zona fasciculata was reduced and hardly detectable in MR knockout animals. Importantly, renin mRNA expression was hardly detectable in wild-type and heterozygous mice but up to ten-fold higher in the enlarged zona glomerulosa of MR knockout mice. In contrast, angiotensin receptor 1 mRNA was not changed, whereas angiotensin receptor 2 was two-fold lower in adrenals of MR knockout mice. It is not clear whether the observed changes in the adrenal glands are exclusively a result of the systemic effects of the severe sodium depletion and hypovolemia and adaptive responses or whether MR and GR in specific cells of the adrenal gland might contribute to these disturbances.

Using in situ hybridization Shimojo *et al.* found 11β-HSD1 predominantly in cells at the cortico-medullary junction within the inner cortex, where it was proposed to play a role in regulating the supply of cortex-derived corticosterone to the medullary chromaffin cells [57]. Other investigators applied immunohistochemistry and detected 11β-HSD1 in the outer layer of cells corresponding to the glomerulosa but not in the fasciculata and reticularis [24, 25]. They observed occasional spots and short streaks radiating through the zona fasciculata and reticularis and associated this expression pattern to neuronal cells and/or interstitial fibroblasts. Some staining for 11β-HSD1 was also observed in the medulla. A relatively high expression of H6PDH has been found in adrenals from rats, with highest expression in chromaffin cells [25]. Thus, H6PDH is not coexpressed with 11β-HSD1 in chromaffin cells and the role of NADPH generation in the ER of these cells remains to be determined.

11β-HSD2 mRNA was more abundant in the cortex compared with medulla and its expression was uniformly distributed over the adrenal gland [57]. In humans, 11β-HSD2 was not detected in adult adrenals but in fetal tissue [58]. Similarly, 11β-HSD2 could not be detected in adrenals from adult mice [53, 59]. In contrast, 11β-HSD2 was detected by immunohistochemistry in the fasciculata and reticularis but not in the glomerulosa and medulla. 11β-HSD2 staining was observed in cord-like structures, consistent with expression in steroid-secreting cells.

Future studies should address the role of 11β-HSDs and their corresponding receptors on adrenal function. There is limited knowledge on impaired function of these enzymes in adrenals in inflammation and metabolic diseases.

7.6 Immune system

Glucocorticoids are potent modulators of the immune system and most of their effects are mediated either directly or indirectly by GR [60, 61]. In clinics, glucocorticoids still belong to the most abundantly used and potent anti-inflammatory therapeutics. Numerous synthetic steroids are available such as dexamethasone, betamethasone, triamcinolone, budenoside, prednisolone and others. Glucocorticoids are widely used to treat acute inflammation as well as autoimmune driven chronic inflammatory diseases and neuroinflammatory disorders [62-64]. In contrast, much less is known on the role of MR in the regulation of immune functions.

Several studies showed that monocytes and macrophages coexpress MR and GR [65-71]. Interestingly, 11β-HSD1 is absent in the undifferentiated and cycling monocytes [72]. Once activated and recruited to the inflamed tissue, monocytes undergo differentiation into macrophages. During this process 11β-HSD1 expression is induced and reaches high levels in the differentiated macrophages. In addition, macrophages show high expression of H6DPH. This raises the question how MR and GR in the presence of 11β-HSD1/H6PDH can be regulated distinctly and how they are involved in the coordination of immune regulation.

Usher *et al.* generated mice specifically lacking MR in myeloid cells and showed that MR is essential for efficient macrophage activation by proinflammatory cytokines [71]. Macrophage derived from MR-deficient myeloid cells displayed an impaired activation pattern, and in mice deletion of MR in macrophages

resembled the effects of MR antagonists and protected against cardiac hypertrophy, fibrosis and vascular damage caused by treatment with angiotensin II/L-NAME. Furthermore, myeloid-derived dendritic cells express MR. Herrada *et al.* demonstrated an aldosterone-mediated increase in CD8+ T-cell activation that was dependent on dendritic cells. Aldosterone-mediated MR activation induced MAPK signaling and secretion of IL-6 and TGF-β1 by dendritic cells. Further, aldosterone induced Th17 cell-mediated immune response. The altered, aldosterone-mediated dendritic cell activity might promote inflammatory damage in the heart and other organs (see also section on heart).

MR expression was also found in neutrophils [73]. Incubation of neutrophils with aldosterone inhibited the activation of NF-κB by interleukin-8 (IL-8) and granulocyte/macrophage colony-stimulating factor. Spironolactone abolished NF-κB inhibitioin by aldosterone, indicating an MR-specific effect. Incubation with IL-8 strongly induced TNF-α mRNA expression, an effect that was prevented by aldosterone. These results suggest anti-inflammatory effects of MR in neutrophils that might be relevant when they interact with endothelial cells. Thus, MR seems to mediate pro- and anti-inflammatory effects, depending on the cell type.

7.7 Brain

Corticosteroids play a pivotal role in the control of brain activity and are involved in regulating stress response, mood, sleeping behavior, memory function and release of neuroendocrine hormones [74]. Both MR and GR are expressed in the brain, with differences in their sites of expression and functions. Immunohistochemistry, *in situ* hybridization and binding of radiolabeled aldosterone revealed high expression of MR in neurons of the hippocampus, lateral septum, medial and central amygdala, olfactory nucleus, layer II of the cortex and brain stem sensory and motor neurons [75-79]. MR is also found in the anterior hypothalamus and circumventricular tissues including chorioid plexus. The GR is widely expressed in the brain in neurons and glial cells [75, 79-81]. High expression is found in the limbic system (hippocampus, septum and amygdala), in the parvocellular neurons of the paraventricular nucleus of the hypothalamus and in the supraoptic nucleus. High expression of GR is also observed in the ascending monoaminergic neurons of the brain stems.

Importantly, in hippocampal neurons and microglia cells MR and GR are expressed in the absence of 11β-HSD2 [53, 82] but presence of 11β-HSD1 [83-85], suggesting predominant occupation of the receptors by 11β-hydroxyglucocorticoids. Low levels of glucocorticoids are expected to predominantly act through MR, thereby functioning in a proactive mode by regulating the sensitivity of neuroendocrine stress responses [76, 86]. High levels of glucocorticoids, for example during stress, lead to the occupancy of MR and GR, whereby the GR is thought to play a pivotal role in counteracting MR effects and mediating recovery from the stress response.

Confocal laser scanning microscopy revealed a specialized nuclear clustering for MR and GR in neuronal cells of the CA1 region [87]. The two receptors were found in distinct nuclear domains but also in clusters where they colocalize, indicating the formation of receptor homodimers and heterodimers. The formation of MR-GR heterodimers in rat hippocampal neurons has been demonstrated, and activation of GR was shown to inhibit MR-mediated regulation of neuronal function [88]. Using recombinant receptors Trapp *et al.* observed enhanced activation of the mouse mammary tumor virus (MMTV) promoter driven LacZ gene upon coexpressing MR and GR compared with cells transfected with one of the receptors only [89]. In contrast, Liu *et al.* coexpressed MR and GR in monkey kidney CV-1 cells and observed significantly lower activation of a TAT3-TATA-reporter construct compared to cells expressing only MR or GR [90]. These observations indicate highly cell- and promoter-specific effects by MR and GR homodimers and heterodimers.

Inflammation results in increased local and circulating levels of active glucocorticoids (for review see [63]). The inflammatory response in the brain involves a coordinated action of monocytes, macrophages, astrocytes and microglia cells. Microglia cells express both MR and GR in the presence of 11β-HSD1 [91]. Like macrophage, microglia cells belong to the specialized cells of the immune system. They express MHC II (major histocompatibility complex) and therefore act as professional antigen-presenting cells (APCs) in the brain. Microglia cells are able to produce cytokines and neurotrophic factors. High doses of the synthetic glucocorticoids methylprednisolone and dexamethasone were shown to suppress the expression of MHC II on the surface of microglia cells [92, 93]. The suppressive effect of these ligands is likely a result of their high

concentrations and GR-selectivity. In contrast, low doses of endogenous glucocorticoids that mainly act through MR may stimulate the inflammatory response.

Excess glucocorticoid action during stress or upon upregulation of 11β-HSD1 by pro-inflammatory cytokines during inflammation exerts adverse effects on hippocampal neurons and causes impaired cognitive functions. Increased glucocorticoid levels have been associated with cognitive impairments and hippocampal atrophy both in rodents and humans [94, 95]. In aging mice an increase in 11β-HSD1 levels in the CA3 hippocampus and parietal cortex correlated with impaired cognitive performance, whereby circulating glucocorticoid levels and corticosteroid receptor expression did not correlate with cognitive function [96]. Transgenic mice overexpressing 11β-HSD1 specifically in the forebrain region showed premature age-associated cognitive deficits, suggesting a causal role of elevated 11β-HSD1 expression. This is supported by the observation that mice deficient in 11β-HSD1 have lover intrahippocampal corticosterone levels and that they show a delayed decline in age-related cognitive function [84]. A reduced 11β-HSD1 expression in transgenic animals as well as inhibition of the enzyme resulted in improved memory function, suggesting that inhibition of 11β-HSD1 may show beneficial effects in treating age-related cognitive disorders [97]. In line with the adverse effects of elevated glucocorticoids in the hippocampus, transgenic expression of 11β-HSD2 in dentate gyrus granule cells reversed the adverse effects of high glucocorticoid treatment on granule cell and CA1 pyramidal cell excitability and on spatial reference memory [98].

The observation that 11β-HSD1 in neurons catalyzes the regeneration of active glucocorticoids indicates the coexpression with H6PDH. A recent analysis of H6PDH expression revealed a distinct size of the immunoreactive protein at 60 kDa in whole brain tissue compared with 90 kDa for the classical protein [25]. It remains to be clarified whether an alternatively spliced variant or a post-translationally modified H6PDH is expressed in the brain.

Colocalization of MR and 11β-HSD2, indicating aldosterone sensitivity of MR, was demonstrated in the nucleus of the solitary tract using immunofluorescence [82]. Furthermore, MR and 11β-HSD2 were both detected in amygdala, subcommissural organ, the ventromedial nucleus of the hypothalamus, and locus coerulens. The localization of 11β-HSD2 in these tissues was confirmed in transgenic mice expressing β-galactosidase under the control of the endogenous HSD11B2 promoter [53]. 11β-HSD2 expression was found in the nucleus of the solitary tract and amygdala as observed in earlier studies, in the external cuneate nuclei in the medulla, in the external granular layer in the cerebellum, in the pontine reticular formation and pontine nuclei and periaqueductal gray in the pons/midbrain region. 11β-HSD2 was also found in the hypothalamus and in several thalamic nuclei. Coexpression of MR and 11β-HSD2 in the periventricular regions render selectivity of MR to aldosterone to modulate volume regulation and sympathetic outflow as well as salt appetite.

Table 7 Expression and Protein appearance of MR, GR, 11β-HSD1, 11β-HSD2 and H6PDH in brain specific cell types.

BRAIN		**MR**		**GR**		**11β-HSD1**		**11β-HSD2**		**H6PDH**	
		Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
Brain			+ [1] [86]		+ [86] [1]	- [25]	+ [25]		- [55]	+ [25]	+ [25]
Medulla											
	Nucleus of the solitary tract	+						+++			

		[106]						[53] + [106]			
	Lateral reticular nucleus							++ [53]			
	External cuneate nucleus/ cuneate							++++ [53]			
	Spinal trigeminal nucleus							+ [53]			
Spinal Cord											
	Marginal zone		++ [61]		++ [61]						
	Substantia gelatinosa		+++ [61]		++ [61]						
	Nucleus proprrus		+++ [61]		++ [61]						
	Cochlear nucleus							++ [53]			
	Cerebral cortex				+ [55]			- [53]	- [55]		
Cerebellum	**External granular layer**						++ [107]	++++ [53]			
Pons/midbrain											
	Cochlear nucleus							++ [53]			
	Trigeminal nucleus (principal sensory)							+ [53]			
	Pontine reticular formation							++++ [53]			

	Pontine nuclei							++++ [53]			
	Pontine midline/dorsal raphe							++ [53]			
	Periaqueductal gray							+++/++++ [53]			
	Superior colliculus							+++ [53]			
Diencephalon/forebrain											
	Hypothalamic region							++/+++ [53]			
	Hypothalamic paraventricular nuclei		++ [108]		+ [108]				+ [89]		
	Medial/lateral geniculate nuclei							+ [53]	+ [89]		
	Pretectal nucleus							+ [53]			
Dentate gyrus		+ [57]	+ [108]	+ [109]	+ [55]				- [55]		
	Granular layer		++++ [61]	+++ [109-111]	++ [61] +++ [109]						
	Polymorph layer		++++ [61]		++ [61]						
	Induseum griseumlfasciola cinerea		++++ [61]		++ [61]						
Hippcampus		+ [57]									
Amygdalohippocampal area			+++		+++		+	+++	-		

			[61]		[61]		[55]	[53]	[55]		
	Cortical part		+++ [61]		+++ [61] + [55]				- [55]		
	Anterior part		+++ [61]		+++ [61]						
	Submammilothalamic nucleus			+ [109] - [110]	+ [109]			++ [53]			
	Thalamic nuclei		+ [55]		+ [55]		+ [55]	++/+++ [53]	- [55]		
	Hypothalamic preoptic nuclei			++ [109] + [110] +++ [111]	+++ [109]			++ [53]			
	ventromedial hypothalamic nucleus			+++ [109] [110] ++ [111]	+++ [109]			+ [106]			
	Subcommisural nucleus (under posterior commissure)							++ [53]	+ [89]		
	Hippocampus		+ [55, 60] [60]		+ [55, 60]		+ [55, 60]	- [53]	- [55]		
	Hippocampale pyramidale neuronen		+ [61]		++ [61]						

	CA1 (Pyrimidal Layers)	+ [61] [95] [94]	+ [108] [61]	+ [61] [95] [109] [110] [111]	+ [108] [94] [109] [55]						
	CA2			+ [109] [110] [111]	+ [55] [109]				- [55]		
	CA3 (Pyrimidal Layers)		++ [108] + [61]	+ [109] [110] [111]	+ [108] [109]						
	CA4 (Pyrimidal Layers			+ [109] [110] [111]	+ [109]						
Amygdala											
	Anterior part		+ [61]	+ [109] [110] [111]	++ [61] [109]						
	Posterodorsal part		+ [61]	+ [109-111]	++ [1] [109]						
Cerebellum											
	Deep nuclei		+++ [61]	+ [109-111]	++ [61] [109]						
	(Cortex) Purkinje	+	++	++	++						

	layer	[57]	[61]	[109] [110] [111]	[61] [109]						
	(Cortex) Granular layer		+ [61]	+++ [109-111]	++ [61] [109]						
Eye											
	Oculomotor (III)		+++ [61]	++ [109] +++ [110] [111]	++ [61]						
	Trochlear (IV)		+++ [61]		++ [61]						
	Abducens (VI)		+++ [61]	++ [109] + [110] [111]	++ [61] [109]						
Jaw											
	Motor nucleus ventral		+++ [61]		++ [61]						
	Face		+ [61]	++ [109] + [110] [111]	++ [61] [109]						
	Facial nucleus (VII)		++++ [61]		++ [61]						
Pharynx/larynx											
	Nucleus ambiguus, dorsal division		+++ [61]	++ [109,	++ [61]						

				110] [110] + [111]	[109]						
	Hypoglossal nucleus (XII)		++++ [61]	+ [109] - [110] [111]	++ [61] + [109]						
Reticular core (including central gray and raphe)											
	Periaqueductal gray **associated with PAG**		+ +(+) [61]	+ [109]	++ [61]						
	Dorsal tegmental nucleus		++ [61]	+ [109]	++ [61]						
	Locus coeruleus		+ [61]	+++ [109, 110] [111]	++ [61] +++ [109]						
Raphel	**Dorsal raphe**		+ [61]	+ [109]	++ [61] [109]						

7.8 Bone

Maintenance of bone homeostasis critically depends on the functional interactions between fibroblasts, osteoblasts and osteoclasts, and on complex interactions and feed-back regulation involving various chemokines, cytokines and hormones. Corticosteroid receptors and 11β-HSDs play an important role in the modulation of bone homeostasis and offer opportunities for therapeutic interventions in diseases including osteoporosis and rheumatoid arthritis. While physiological glucocorticoid concentrations promote osteoblast differentiation, high concentrations promote osteoblast apoptosis thereby inhibiting osteoblastogenesis.

Immunohistochemical analysis of human neonatal ribs and iliac crest biopsy specimens indicated that MR as well as GRα and GRβ are expressed in neonatal and adult human bone [99]. MR and both GR variants were found to be highly expressed in osteoblasts along bone forming surfaces in neonatal rib sections. In contrast, expression was considerably lower in multinucleated osteoclasts and GRα was absent or expressed at very low levels. Similarly, iliac crest biopsies showed expression of both GR variants in osteoblast-

like cells in cancellous surfaces, whereas very few osteocytes stained positive for GR. MR expression was found in osteoblasts and in about one-third of cancellous osteocytes. The presence of mRNA of the two GR variants and of MR was confirmed by RT-PCR in cultured primary human osteoblasts. Evidence for the lack of GRα but presence of GRβ expression in human and rat osteoclasts, and of considerably lower GR and MR in osteoclasts compared with osteoblasts, was supported by other investigators [100, 101]. Interestingly, MR was not detected in early fetal bone tissue beyond 12 weeks of gestation [102], suggesting a role of this receptor in terminal differentiation.

Early observations in patients with apparent mineralocorticoid excess (AME) provided evidence for a role of 11β-HSD2 in modulating bone homeostasis. AME patients suffered, among other complications, from retarded growth, osteopenia and minimal trauma bone fractures, effects that were ameliorated upon treatment with the MR antagonist spironolactone [103, 104]. Activation of MR by aldosterone enhanced proliferation of cultured osteoblasts from rat calvaria, an effect inhibited by specific MR antagonists [105]. Furthermore, MR antagonists inhibited the production of pro-inflammatory cytokines, including TNF-α and INF-γ, and have potential in the treatment of arthritis [106].

Using the MG-63 human osteosarcoma cell line, Cooper *et al.* reported a decreased expression of 11β-HSD2 upon treatment with TNF-α or IL-1β [107]. In contrast, primary osteoblasts express 11β-HSD1 and the levels of this enzyme were stimulated by exposure to TNF-α or IL-1β, thus leading to enhanced local concentrations of active glucocorticoids. The authors proposed that pro-inflammatory cytokines may exert some of their effects within bone, *e.g.* periarticular erosions in inflammatory arthritis, by increasing local glucocorticoid concentrations.

Jia *et al.* studied the role of glucocorticoids in transgenic mice specifically expressing 11β-HSD2 in osteoclasts [108]. Treatment of wild-type and transgenic mice with pharmacological doses of glucocorticoids enhanced apoptosis in cancellous osteoblasts and decreased osteoblast, osteoid and bone formation. Glucocorticoids stimulated the osteoclast marker calcitonin receptor on wild-type but not transgenic mice. Importantly, glucocorticoids decreased the number of cancellous osteoclasts in transgene but not wild-type mice. The observed loss of bone density in wild-type mice was prevented by 11β-HSD2 overexpression in the transgene. The authors concluded that the early, rapid loss of bone caused by glucocorticoid excess resulted from direct actions on osteoclasts.

Glucocorticoids expand the life span of osteoblasts and decrease bone density. An early increased bone resorption followed by a diminished osteoclastogenesis and a consequently decreased bone resorption was observed in a mouse model of glucocorticoid-induced osteoporosis [109]. Mice implanted with slow release prednisone pellets displayed early activation of osteoclastogenesis and adipogenesis as well as prolonged suppression of osteogenesis [110]. In this model the synthetic glucocorticoid receptor ligand prednisone required prior activation by 11β-HSD1 in the liver or locally in the osteoblast.

Buttgereit *et al.* studied the impact of osteoblast-targeted transgenic overexpression of 11β-HSD2 on joint inflammation, cartilage damage, and bone metabolism in the K/BxN mouse serum transfer model of autoimmune arthritis [111]. Wild-type and transgenic mice developed acute arthritis but in the latter arthritis and local inflammatory activity were significantly attenuated. Transgenic overexpression of 11β-HSD2 ameliorated bone resorption as well as loss of bone volume, and improved osteoblast activity, suggesting that osteoblasts modulate the immune-mediated inflammatory response in a glucocorticoid-dependent manner.

Intraarticular corticosteroid application in patients with inflammatory arthritis reduced synovial RANKL expression [112]. Glucocorticoids inhibited osteoprotegerin expression and increased receptor activator of NF-κB ligand (RANKL) synthesis by osteoblasts, thereby promoting osteoclastogenesis [112, 113]. However, following priming with TNF-α, a condition mimicking pro-inflammatory milieu of the rheumatoid joint, glucocorticoids were found to decrease RANKL expression [112]. Thus, glucocorticoids affect bone cells differently in the presence or absence of inflammatory mediators and they may have a bone conserving effect in rheumatoid arthritis despite of inducing osteoporosis in the spine.

7.9 Adipose tissue

Adipose tissue is derived from lipoblasts representing the adipocyte precursor cells. Two functionally distinct adipocytes are differentiated from these pre-adipocytes, forming either brown (BAT) or white adipose tissue (WAT). BAT in adults is derived from the abundant fat tissue of the fetal and newborn mammal organism, where it is responsible for the shiver-free heat production. The adverse metabolic effects of excessive fat accumulation have been associated mainly with WAT. Transgenic mice with disruption of uncoupling protein function and a primary deficiency of BAT were prone to obesity [114]. Functional adipose tissue includes additional cell types such as endothelial cells enabling its high vascularization, fibroblasts, and macrophages responsible for the numerous endocrine and immune functions. Adipose tissue can be broadly categorized in subcutaneous and visceral fat depots. Besides its major function as lipid storage, fat tissue performs other general functions, including protection against temperature fluctuations (subcutaneous fat) and resistance of organs like stomach, heart and kidneys against mechanical stress (visceral fat).

Adipocytes and adipose tissue infiltrating macrophages are derived from the same bone marrow stem cells, and they express MR and GR in the presence 11β-HSD1 and H6PDH. There is increasing evidence for a key role of MR in mediating adverse effects in metabolic disease. Elevated levels of MR were found in obese, diabetic mice (ob/ob and db/db), which have increased expression of pro-inflammatory and pro-fibrotic factors and reduced expression of adiponectin and PPARγ in adipose tissue [115, 116]. Treatment with the MR selective antagonist eplerenone normalized the impaired regulation of obesity-related genes, suppressed macrophage infiltration and attenuated insulin resistance [116]. Moreover, incubation of undifferentiated preadipocytes with 10 nM aldosterone for 24 h increased the expression of TNF-α and MCP1 and decreased adiponectin and PPARγ [115]. A recent study with cultured 3T3-L1 and 3T3-F442A adipocytes and human primary preadipocytes reported dose-dependent inhibition of adipose differentiation and potent anti-adipogenic effects of the MR antagonist drospirenone [117]. Further evidence for a pro-inflammatory adipogenic effect of MR was provided from a recent study with GR- and MR-deficient adipocytes [118]. Expression of the pro-inflammatory factors IL-6 and MCP1 was enhanced in GR knockout adipocytes upon treatment with corticosterone, indicating an MR-dependent stimulation of the pro-inflammatory factors. Deletion of MR resulted in a complete loss of lipid accumulation, whereas deletion of GR led to rather subtle disturbances of adipogenesis during early differentiation. These observations are in line with an earlier study using a brown adipocyte cell model [119]. Aldosterone promoted adipocyte differentiation, indicated by an accumulation of intracytoplasmic lipid droplets and a concentrations-dependent increase in intracellular triglyceride content. The aldosterone-dependent effects were not affected by the GR antagonist RU-486 but abolished by MR antagonists, indicating a key role of MR in the early phase of adipocyte differentiation.

Glucocorticoids control the terminal differentiation of adipose precursor cells and essentially modulate adipocyte function [120]. Given the more than ten-fold higher affinity of MR compared with GR for 11β-hydroxyglucocorticoids, it can be assumed that the MR is occupied by 11β-hydroxyglucocorticoids in preadipocytes and adipocytes. Depending on the available glucocorticoid concentration lower affinity GRs are activated and may counteract the effects of MR through mechanisms that need to be uncovered but likely include receptor heterodimerization. 11β-HSD1 is highly expressed in adipose tissue, although its expression is considerably lower than in liver. In contrast, 11β-HSD2 mRNA and activity was detected only at low levels in adipose stromal vascular cells [121, 122]. Comparison of the expression of 11β-HSD1 and 11β-HSD2 revealed a 22-fold and 8-fold lower expression of the latter in subcutaneous fat, respectively. Moreover, in the obese situation infiltrated macrophage expressing 11β-HSD1 further increase local cortisol production and MR activation, thus promoting inflammation [123].

In mouse preadipocytes isolated from mesenteric and subcutaneous fat 11β-HSD1 was found to function as oxoreductase, thereby activating glucocorticoids [124]. Treatment of mice with high-fat diet, leading to the stimulation of 11β-HSD1 activity, resulted in enhanced preadipocyte differentiation in wild-type but not 11β-HSD1 knockout animals. It was shown that in the widely used 3T3-L1 fibroblast-like cells 11β-HSD1 expression is absent prior to differentiation but increases with progression of differentiation and is highly expressed in the fully differentiated state [125,

126]. Importantly, H6PDH is similarly expressed before and after differentiation [125]. Furthermore, a continuous expression of H6PDH was observed during the differentiation of human adipose-derived mesenchymal stem cells, indicating that 11β-HSD1 functions as an oxoreductase in both preadipocytes and adipocytes [127]. H6PDH knockout mice, where 11β-HSD1 is thought to act as a dehydrogenase, display diminished lipogenesis, lipolysis rates and fatty acid release upon fasting, and they have significantly reduced adipose tissue mass, although average adipocyte size was not altered [128].

The association of enhanced local glucocorticoid activation by 11β-HSD1 with visceral obesity and the development of insulin resistance, type 2 diabetes and cardiovascular disease is being extensively studied (reviewed in [129, 130]). Based on the fact that the MR has more than ten-fold higher affinity than GR and that it is coexpressed with 11β-HSD1 and H6PDH in preadipocytes and adipocytes, we propose that the pro-inflammatory and pro-adipogenic effects of elevated local glucocorticoid concentrations are probably mainly mediated by activation of MR. Thus, a combination of an MR antagonist and an 11β-HSD1 inhibitor may prove beneficial in the treatment of metabolic disease.

7.10 Heart

In the heart MR expression has been demonstrated in cardiomyocytes and fibroblasts, cells of atria and ventricles, the aorta and pulmonary artery as well as in vascular endothelial and smooth muscle cells [131-134]. In addition, macrophage, which infiltrate the heart during inflammation, express high levels of MR.

Several clinical studies revealed an association of elevated MR activity with vascular inflammation and cardiac fibrosis, and an increased risk for congestive heart failure. Supplementation of the standard therapy of angiotensin-converting enzyme inhibitor, loop diuretic and digoxin for patients with heart failure with the MR antagonists spironolactone (RALES [135]) and eplerenone (EPHESUS [136]) demonstrated a 30% and 15% improvement in mortality. Furthermore, treatment with MR antagonists decreased blood pressure in patients with essential hypertension and left ventricular hypertrophy (4E-left ventricular hypertrophy study [137]). The MR-dependent exacerbation of tissue damage in cardiac ischemia can be ameliorated by eplerenone [138]. Thus, MR antagonists have a beneficial impact on post-myocardial infarction therapy and in treatment of patients with essential hypertension.

In line with clinical trials, animal studies addressed the mechanisms of MR activation in heart disease and provided evidence for beneficial effects of antagonists [139-146]. A causal role of MR was demonstrated in transgenic mice by conditional overexpression specifically in cardiomyocytes. These mice exhibited cardiac remodeling with severe ventricular arrhythmias and increased mortality [147]. In mice chronic severe pressure overload due to aortic constriction caused cardiac hypertrophy, followed by left ventricular dilatation and heart failure [148]. Cardiomyocyte-specific deletion of MR prevented the increase in left ventricular inner diastolic diameter and wall tension but did not prevent cardiac hypertrophy. Similarly, eplerenone did not prevent cardiac hypertrophy but delayed the transition to myocardial failure [148, 149]. Cardiac fibrosis caused by chronic pressure overload was not reduced in mice with a specific knockout of MR in fibroblasts [148]. However, deletion of MR in macrophage attenuated the production of ROS in the heart and prevented inflammation and fibrosis induced by treatment with deoxycorticosterone/salt [69] or angiotensin II [71]. These observations indicate a key role of MR in infiltrating macrophage in the progression of vascular inflammation and cardiac fibrosis.

In humans cardiomyocytes have no or very low levels of 11β-HSD1 and 11β-HSD2 [24]. Vascular smooth muscle cells do not express 11β-HSD1 but both endothelial cells and vascular smooth muscle cells were reported to express 11β-HSD2 [133, 150, 151]. In contrast, 11β-HSD1 expression was found in rodent cardiac vascular smooth muscle cells, whereby enzyme activity was higher in quiescent cells compared with proliferating cells [152]. Although some discrepancies may be due to contamination of vascular smooth muscle cell preparations with endothelial cells, species-specific differences need to be considered and care should be taken in extrapolating results from studies with rodents to the human situation.

Evidence for an important role of 11β-HSD2 in the heart was provided from observations in 11β-HSD2 knockout mice. 11β-HSD2 expression is low in fetal mouse heart,

whereas MR is highly expressed. Nevertheless, 11β-HSD2 knockout mice exhibit significantly enlarged heart size and a high mortality rate [153]. A recent study reported a highly significant negative correlation between 11β-HSD2 expression and the thickness of the left ventricular wall in sheep [154]. The chronic administration of moderate doses of cortisol during late gestation resulted in a significantly decreased 11β-HSD2 expression in the heart and caused an increase of the fetal heart weight.

Since 11β-HSD2 converts active into inactive glucocorticoids, it restores aldosterone specificity of MR and inhibits GR activation. Thus, both GR and MR might be responsible for the development of elevated heart size. Interestingly, transgenic mice with a cardiac-specific overexpression of 11β-HSD2 developed cardiac hypertrophy, which was attenuated after treatment with eplerenone. In the presence of high 11β-HSD2 activity, circulating glucocorticoids may no longer be sufficiently high to activate GR in order to counteract aldosterone-dependent MR activity. In contrast, a lack of 11β-HSD2 is expected to cause glucocorticoid-dependent MR activation and GR might no longer be able to efficiently counteract the fully activated MR, thus providing an explanation why both a lack of 11β-HSD2 and its overexpression cause cardiac hypertrophy.

Table 8 Expression and Protein appearance of MR, GR, 11β-HSD1, 11β-HSD2 and H6PDH in heart specific cell types.

Heart	MR		GR		11β-HSD1		11β-HSD2		H6PDH	
Heart/Expression	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
Whole Heart	+ [57, 168]	+ [1] [169] [168]		+ [1] [168]		+ [107] [25] [24]	++ [56] [170]		(+) [25]	+ [25]
endocardial Intestinal cells					(+) [24]					
cells surrounding cardiac vessels					(+) [24]					
VSMC	+ [144] [57, 168]				++ [24] [171]					
cardiomyocytes	+ [56] [144] [168]	+ [56] [1] [172] ++ [173] [168]	+ [168]	+ [173] [168]	- [24]					
endothelial cells	+ [144]							+ [174] - [170]		

aorta	+ [144]	+ [174] [58]		+ [174] [58]	+ [25]	+ [171] [174]	+ [170]	+ [171] [58] [170] [174]	+ [25]	+ [25]
aorta VSMC	+ [144] [175]	+ [175, 176] [176]		+ [176]		+ [174] [176]	+ [175] [170]	+ [175, 176] [176] [170]		
aorta Endothelial cells	+ [144] [175]	++ [175]				+ [177]	+ [175]	+ [174] [175] [177]		
mesenteric artery							++ [170]	+ [178]		
caudal artery							++ [170]			
atria	++ [144]						(+) [53]			
ventriculum		+ [179]		+ [179]		++ [179]	(+) [53]	+ [179]		
left ventriculum	+ [169] [56] [144]	+ [58] [169] [56]		+ [58]		++ [173]	++ [173]	+ [173]	+ [58, 173]	
right ventriculum	+ [56]	+ [56]				++ [173]	++ [173]	+ [173]	+ [173]	
small intraventricular vessels		- [56]								

7.11 Skeletal muscle

Skeletal muscles have a high demand of energy, and glucocorticoids are essentially regulating insulin-stimulated glucose uptake, glycogen storage and carbohydrate metabolism. Chronically elevated glucocorticoid levels have been associated with insulin resistance and impaired carbohydrate metabolism as well as catabolic pathways causing muscle atrophy. Recent studies provided evidence for a functional expression of MR (besides GR) in soleus muscle [155]. High aldosterone levels, in addition to their cardiotoxic effects, led to the induction of apoptosis in soleus muscle. In soleus muscle high aldosterone levels increased NADPH oxidase and the production of ROS, decreased Akt phosphorylation and GLUT4 expression, and induced insulin resistance [156]. Spironolactone ameliorated aldosterone-induced cardiac and skeletal muscle myopathy, providing evidence for an MR-dependent mechanism. As in cardiomyocytes, 11β-HSD2 expression could not be detected using immunofluorescence and ribonuclease protection assay in skeletal muscle of normal and Cre-recombinant male mice [46, 53].

11β-HSD1 is expressed in skeletal muscle with a 13-times higher level in soleus (type I-rich fibers) compared with tibialis anterior (type IIb-rich fibers)[157]. Impaired regulation of 11β-HSD1 activity in myotubes from diabetic patients may contribute to insulin resistance [158-160]. H6PDH is also expressed in soleus and tibialis anterior and plays an important role in the regulation of muscle cell differentiation and function. It can be assumed that chronic hyperglycemia leads to enhanced local glucocorticoid activation by 11β-HSD1/H6PDH, thereby activating local GR and/or MR and contributing to impaired glucose transport and insulin resistance.

The role of H6PDH has been further investigated in transgenic mice. H6PDH deficient mice developed a vacuolating myopathy, predominantly manifested in type II muscle fibers [161]. In further studies with H6PDH/11β-HSD1 double knock-out mice, this phenotype was clearly associated with H6PDH deficiency, independent of 11β-HSD1 function [157]. Fiber type differentiation can be influenced by physical activity. Marathon runners, for example, show a type I-rich fiber composition of the gastrocnemius, whereas a type IIb-rich composition is observed in sprinters. Thus, fiber type differentiation may be modulated by the level of energy demand and redox reactions in the endoplasmic reticulum. Whether and how glucocorticoids are involved in these processes remains unclear. The lack of 11β-HSD1 oxoreductase activity in knockout mice may be compensated by an activation of the HPA axis leading to higher circulating corticosterone levels [162], although strain-dependent differences need to be considered [163]. Contradicting findings were reported concerning serum corticosterone concentrations in H6PDH-deficient mice. Significantly increased adrenal weight and elevated plasma corticosterone concentrations were reported by two independent groups [164, 165]; however, two other studies reported significantly decreased plasma corticosterone levels in H6PDH-deficient animals [157, 166]. Since all of these reports are based on data from transgenic mice from the same background, *i.e.* C57BL6J strain, the reason for the observed differences remains unclear. Nevertheless, 11β-HSD1 was significantly upregulated in H6PDH knockout mice in tibialis anterior, but not in soleus or in the liver [157].

7.12 Skin

Skin consists of different layers, including the epidermis, dermis and subcutis (also named hypodermis). Innervation, vascularization, the presence of secretory glands such as sweat glands and sebaceous glands, hair folicles and pacinian corpuscles enhance the complexity of skin as an organ. Epidermis can be further divided into five distinct areas namely stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and final stratum basale. The major cell types present in the epidermis are keratinocytes, langerhans cells and “touch sensors” like merkel-ranvier cells. Keratinocytes in the four outer layers forming the epidermis are mitotically inactive and die upon their travel to the surface because of a loss of nutrition supply. During this process they undergo keratinization. In contrast to the epidermis, the dermis consists of connective tissue and contains many blood and lymphatic vessels. The subcutis is a highly immunologically active tissue and contains dendritic cells and fibroblasts as well as adipocytes.

Glucocorticoids are widely used in the treatment of inflammatory and hyperproliferative skin disorders. A major limitation, however, is the development of skin atrophy [167]. The relative contribution of GR and MR to skin atrophy remains unclear. In situ

hybridization and immunohistochemistry revealed MR expression in human keratinocytes of the epidermis, in sweat glands and sebaceous glands and in hair follicles [168]. Mice overexpressing MR specifically in keratinocytes and hair follicles exhibited developmental and post-natal impairments of the epidermis and hair follicles [169]. Transgenic mice showed premature epidermal barrier formation at embryonic day 16.5, decreased hair follicle density and epidermal atrophy and increased keratinocyte apoptosis at embryonic day 18.5 and premature eye opening when MR was overexpressed throughout gestation. When MR expression was induced after birth, mice developed alopecia and hair follicle cysts.

Important information on the role of GR in skin was provided from transgenic mice overexpressing GR specifically in keratinocytes [170]. Newborn mice exhibited severe skin lesions due to epidermal hypoplasia and underdeveloped dysplastic hair follicles. In adult mice, an impaired hyperplastic and inflammatory response to the tumor promoting agent TPA with impaired NF-κB signaling was observed.

11β-HSD2 activity was found in isolated sweat glands but was very low or absent in epidermis biopsies [168]. Whereas MR was detected in excretory ducts in the basal cell layers, 11β-HSD2 was found in the luminal cells of the ducts [52]. 11β-HSD2 was also absent in sebaceous and apocrine glands. 11β-HSD2 mRNA expression and immunoreactivity was detected in the highly vascularized dermis in arterioles [150].

The therapeutic efficacy of dermal applications of cortisone and prednisone is dependent on the oxoreductase activity of 11β-HSD1, which was found to be higher in mouse compared with human skin biopsies [171]. H6PDH was found to be highly expressed in whole human skin specimens, although no specific localization was determined. Expression of 11β-HSD1 in mouse skin was demonstrated by several groups using RT-PCR, immunodetection as well as transgenic animals [46, 53, 171]. Tiganescu *et al.* localized 11β-HSD1 expression in mice to keratinocytes in the epidermis, and to dermal standing fibroblasts and hair follicles [171]. In humans, 11β-HSD1 expression was shown to be sex-hormone dependent. Pre-menopausal women express higher 11β-HSD1 levels in epidermal keratinocytes than post-menopausal women. The opposite was found in the dermis, where 11β-HSD1 expression was higher in post-menopausal compared with pre-menopausal women.

In a recent study aldosterone was found to modulate the deposition of extracellular matrix in human skin [172]. Aldosterone stimulated the expression of collagen type I and elastin, and enhanced elastic fiber deposition in primary cultured skin fibroblasts. Interestingly, spironolactone and eplerenone stimulated the elastogenic effect of aldosterone. The authors provided evidence for a MR-independent mechanism involving the activation of insulin growth factor-I receptor and suggested the use of aldosterone in therapy for dermal lesions to prevent their recurrence after surgical excision. Although the underlying mechanism for these observations needs to be elucidated, these observations indicated that not all effects of aldosterone are mediated by MR.

7.13 Outlook

The analysis of expression patterns of MR, GR, 11β-HSD2, and 11β-HSD1/H6PDH in various organs and cell types contributed significantly to the current understanding of MR function. However, there are several key questions that need to be addressed in future studies, including the mechanism of MR activation in cells coexpressing 11β-HSD1/H6PDH, the impact of varying ligand availability during circadian rhythm, and the functional interaction of MR and GR and modulation of their activities by receptor specific associated proteins as well as post-translational modifications of the receptor and associated proteins. Further, alternative MR ligands and ligand-independent activation of MR might be relevant in some situation and likely depend on cell-specific regulation.

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8 ELEVATED 11β-HSD1-MEDIATED GLUCOCORTICOID ACTIVATION RESULTS IN IMPAIRED NRF2-DEPENDENT ANTIOXIDANT RESPONSE

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8.1 Abstract

The antioxidant redox response pathway is essential for the daily metabolic challenge of organisms. Thereby nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) and its target enzymes such as NAD(P)H dehydrogenase, quinone 1 (NQO1) and heme oxygenase 1 (HO-1) reduce oxidative stress derived from electrophilic compounds of xenobiotic and endogenous sources. The glucocorticoid receptor (GR) was shown to negatively regulate the Nrf2-dependent pathway. In this study, we focused on the impact of the 11β-hydroxysterid dehydrogenase 1 (11β-HSD1)–dependent glucocorticoid activation on the Nrf2-dependent anti-oxidant response. We show that 11β-HSD1 activity impairs Nrf2-dependent gene expression. The marker genes NQO1 and HO-1 were suppressed by 11β-HSD1 generated glucocorticoids, an effect that was reversed by inhibition of 11β-HSD1. Furthermore, our results demonstrate that elevated 11β-HSD1 expression renders cellular susceptibility against hydrogen peroxide induced cytotoxicity. The negative interference of 11β-HSD1-dependent glucocorticoid activation with the Nrf2-dependent pathway was reversed by the use of selective inhibitors. In conclusion, we show that inhibition of 11β-HSD1 can improve the cellular capacity to cope with oxidative stress and prevent susceptibility to oxidative damage.

8.1.1 Keywords

11beta-hydroxysteroid dehydrogenase, glucocorticoid receptor, metabolism, xenobiotics, redox, glucocorticoid, NQO1, Nrf2.

8.1.2 Abbreviations

11β-HSD, 11β-hydroxysteroid dehydrogenase; ER, endoplasmic reticulum; NQO1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor-erythroid 2 (NF-E2)-related factor 2; GR, glucocorticoid receptor; H6PDH, hexose-6-phosphate dehydrogenase; SDR, short-chain dehydrogenase/reductase.

8.2 Introduction

The liver is a highly metabolically active organ, regulating energy homeostasis, including carbohydrate and lipid metabolism, as well as the detoxification of

xenobiotics and many reactive endogenous chemicals. Numerous cytoprotective genes are expressed in hepatocytes, including those under the control of the transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2). Nrf2 is the key player of the antioxidant redox response pathway, representing a tightly regulated multi-diverse defense system [1]. Upon recognition of specific DNA elements on the promoters of its target genes Nrf2 regulates the basal as well as ligand-induced expression of specialized cytoprotective enzymes [2]. The consensus sequence recognized by Nrf2 5-gagTcACaGTgAGtCggCAaaatt-3 is designated as antioxidant responsive element (ARE). AREs are located in the promotor region of enzymes known to cope with oxidative and chemical stress [3]. The importance of Nrf2 is shown in knockout animals (nrf2−/−), which exhibit an enhanced susceptibility towards xenobiotic stress due to reduced basal and inducible expression of cytoprotective genes [4-7]. NAD(P)H:quinone oxidoreductases (NQO) [8], hemeoxygenase 1 (HO-1) [9] and glutathione S-transferases (GST) [10] are key phase II detoxification enzymes that are transcriptionally regulated by Nrf2 (for review see [1]). The expression of these enzymes is induced by oxidative stress caused by various mediators (xenobiotics, antioxidants, heavy metals, UV light, and ionizing radiation) [2].

A recent study reported gender divergent expression of NQO1 in two different but not all rat strains [11]. In male Sprague Dawley (SD) rats two-times lower basal hepatic NQO1 mRNA expression has been found compared with female rats. Furthermore, the induction of NQO1 expression upon treatment with known inducers (butylated hydroxyanisole, the drug oltipraz) was shown to be more pronounced in female compared with male rats. The authors concluded that females may have a greater capacity to combat oxidative stress and thus exhibit a decreased susceptibility to carcinogenes [11]. Indeed, it was shown earlier that male rats are more susceptible to carcinogenic xenobiotics [12]. Gender related differences were also found for humans [13].

Activation of the glucocorticoid receptor (GR) by dexamethasone has been shown to repress Nrf2-mediated constitutive and oltipraz- or tert-butylhydroquinone (t-BHQ)-inducible GSTA2 gene induction in the rat hepatoma cell line H4IIE [14]. Silencing mediator for retinoid and thyroid hormone receptors (SMRT), a corepressors recruited to the activated GR, was supposed to play a key role in the inhibitory

mechanism [14]. The literature on glucocorticoid-dependent transcriptional regulation is mostly based on experiments with the synthetic glucocorticoid dexamethasone that has been shown to also activate PXR at high concentrations [15-17]. Glucocorticoid-mediated regulation in the liver mainly depends on the expression and activity of 11β-hydroxysterid dehydrogenase (11β-HSD1), which converts inactive 11β-ketoglucocorticoids (cortisone, 11-dehydrocorticosterone) into their active 11β-hydroxy forms (cortisol, corticosterone) [18]. 11β-HSD1 is facing the endoplasmic reticulum (ER) and requires NADPH, which is provided by hexose-6-phosphat dehydrogenase (H6PDH) [19, 20]. By utilizing glucose-6-phosphate, H6PDH controls the NADPH/$NADP^+$ redox couple in the ER and constitutes a link between carbohydrate metabolism and hormonal regulation. The impact of 11β-HSD1 on the antioxidant redox pathway has not yet been studied. Moreover, gender differences have been observed for 11β-HSD1 in rat liver with 18-times lower expression in female rats compared with male [21, 22].

In this study we used H4IIE cells, known for their endogenous expression of functional Nrf2 [14, 23, 24] and required co-regulatory enzymes, as well as H4IIE cells transiently or stably transfected with 11β-HSD1 [25] to elucidate the impact of 11β-HSD1 on the antioxidant redox response pathway.

8.3 Experimental procedure

8.3.1 Materials

[1,2-^{3}H]-cortisone was purchased from American Radiolabeled Chemicals (St. Louis, MO), cell culture media from Invitrogen (Carlsbad, CA), Sulforaphane were obtained from Sigma-Aldrich (Buchs, Swizerland) and all other chemicals were obtained from Fluka AG (Buchs, Switzerland) of the highest purity available. 8x ARE Reporter plasmid, Nrf2 and Keap1 expression constructs have been described earlier [26].

8.3.2 Cell culture

HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 50 U/mL penicillin/streptomycin, 2 mM glutamine, and 1 mM HEPES, pH 7.4. Rat H4IIE hepatoma cells and H4IIE cells stably expressing 11β-HSD1 (clone H4H1) [25] were cultured in antibiotic free Dulbecco's modified Eagle medium (DMEM) supplemented as given above.

8.3.3 Transfection of cells

HEK-293 cells (200’000 cells/well) were seeded in poly-L-lysine coated 24-well plates, incubated for 16 h and transfected using calcium phosphate precipitation with 8 x ARE-luciferase reporter (0.20 µg/well), pCMV-LacZ galactosidase transfection control (0.03 µg/well), human recombinant GR-α (0.20 µg/well) and either human Nrf2 (0.20 µg/well).

H4IIE and H4H1 cells were transfected using electroporation (Neon™, Invitrogen) according to the protocol from the manufacturer. Briefly, cells were trypsinized, washed once with PBS, centrifuged for 2 min at 100 x g and resuspended in 288 µL resuspension buffer with the final transfection density of 1 x 10^{6} cells/mL. Cells were than subjected to a single pulse using a 100 µL gold tip at 1375 V for 30 ms, with a total amount of 2.5 µg DNA consisting of 8 x ARE-Luciferase reporter (2 µg) and pCMV-LacZ galactosidase transfection control (0,5 µg).

To assess the impact of 11β-HSD1, H4IIE cells, were transiently transfected as described above, with plasmids for 11β-HSD1 (2 µg) or pcDNA3 control (2 µg), 8 x ARE-luciferase reporter (2 µg) and pCMV-LacZ galactosidase transfection control (0,5 µg). The total amount of DNA was 6 µg.

To assess the susceptibility of H4IIE cells to hydrogen peroxide-mediated redox sensitivity, cells were transfected with either pcDNA3 (2 µg) or 11β-HSD1 (2 µg) and 4 µg of the cytosolic HyPer-plasmid [27] to yield a total amount of 8 µg of DNA. Cells (100'000 cells per well) were cultured in DMEM for 24 h at 37°C in 6-well plates containing glass coverslips. Cells were then washed once with charcoal-treated, steroid-free DMEM (DMEMct) and incubated for another 3 h. The culture medium was replaced with fresh DMEMct containing cortisone (100 nM) with or without specific 11β-HSD1 inhibitor T0504 (1 µM) [25, 28] and cells were cultured for another 24 h.

8.3.4 Detection of hydrogen peroxide sensitivity by confocal microscopy

For single cell imaging, the Leica confocal microscopy system SP5 was used. Scanning was performed at 400 Hz frequency in a 512 x 512 pixel formate. Exitation of the protonated form of HyPer [27] was performed using the 405 nm laser line. Excitation of the charged form of the chromofore was measured at 488 nm, and emission was recorded between 500 and 554 nm. Pictures were taken every 20 seconds. Ratios between 488 nm and 405 nm were recorded and calculated using the Leica confocal microscopy software.

8.3.5 Nrf2 transactivation assays

HEK-293 cells were seeded (200'000 cells/well) in 24-well plates were washed twice with DMEM 6 h post transfection, followed by cultivation for 16-24 h at 37°C in antibiotic-free DMEM to allow sufficient expression. Cells were then washed once with steroid- and serum-free DMEM (DMEMsf) and incubated for 3 h at 37°C. The culture medium was replaced with fresh DMEMsf containing sulforaphane (10 µM), T0504 (1 µM), RU-486 (1 µM), and combinations of them, in the presence or absence of steroids (100 nM). After incubation for another 24 h, cells were washed

once with PBS and lysed with 60 µl lysis buffer of the Tropix kit (Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiothreitol. Lysed samples were frozen at -80°C for at least 20 min. Lysates (20 µL) were analyzed for luciferase activity using a home-made luciferine-solution [29]. β-galactosidase activity was analyzed in 20 µL sample using the Tropix kit according to the manufacturer.

8.3.6 Determination of 11β-HSD1 activity in intact H4H1 cells

Enzymatic activities were determined in intact H4H1 cells stably expressing 11β-HSD1 as described previously [30]. Briefly, 30'000 cells were seeded per well in 96-well plates (Becton-Dickinson, Basel, Switzerland). Cells were washed once 24 h later with 50 µL DMEMsf and incubated for another 3 h at 37°C. The medium was replaced by 40 µL fresh medium containing either vehicle, sulforaphane or T0504, and 10 µL medium containing 10 nCi [1,2-^{3}H]-cortisone and 200 nM unlabeled cortisone to assess 11β-HSD1 reductase activity. Cells were incubated for 40 min at 37°C and reactions stopped by adding an excess (2 mM) of unlabeled cortisone and cortisol in methanol, followed by separation of steroids by thin layer chromatography (TLC) and determination of the conversion of radiolabeled substrate by scintillation counting.

8.3.7 Analysis of mRNA expression by real-time RT-PCR

Rat H4IIE hepatoma cells (500'000 cells per well) were cultured in 24-well plates with DMEM for 12 h at 37°C. Cells were then washed once with DMEMct and incubated for another 3 h at 37°C. The culture medium was replaced with fresh DMEMct containing sulforaphane (10 µM), T0504 (1 µM), RU-486 (1 µM) and combinations of them, in the presence or absence of steroids (100 nM), followed by incubation for another 24 h at 37°C. The expression of NQO1 mRNA was determined by real-time RT-PCR. Briefly, total mRNA was extracted from either H4IIE or H4H1 cells using the Trizol method (Invitrogen, Carlsbad, CA) according to the manufacturer. RNA concentration and purity was determined spectrophotometrically by measuring fluorescence at 260 nm, 230 nm and 280 nm. Total mRNA (2 µg) was reverse transcribed to cDNA using the Superscript-III First-Strand Synthesis System and oligodT, as recommended by the manufacturer. (Invitrogen, Carlsbad CA). Relative quantification of NQO1 mRNA expression level was performed by RT-PCR on a

RotorGene 6000 (Corbett, Australia) and SYBR-Green following the instructions from the manufacturer (KAPA SYBR® FAST qPCR Kit (Bosten, United States). The relative expression of each gene compared with the internal control GAPDH was determined using the delta-delta-CT method.

8.3.8 Calculations and statistical analysis

All data (mean ± SD) were obtained from at least three independent experiments and significance was assessed using unpaired student ***t***-test or one-way ANOVA followed by Bonferroni post tests in the GraphPad Prism 5 software.

8.4 Results

8.4.1 Glucocorticoid-mediated inhibition of Nrf2-dependent reporter gene activation in HEK-293 cells

To assess whether activation of the glucocorticoid receptor (GR) inhibits Nrf2-dependent gene regulation, we transiently expressed human Nrf2 and human GRα together with a luciferase reporter construct driven by a promotor containing an eight-times repeated antioxidant response element (ARE8L) [26] in HEK-293 cells.

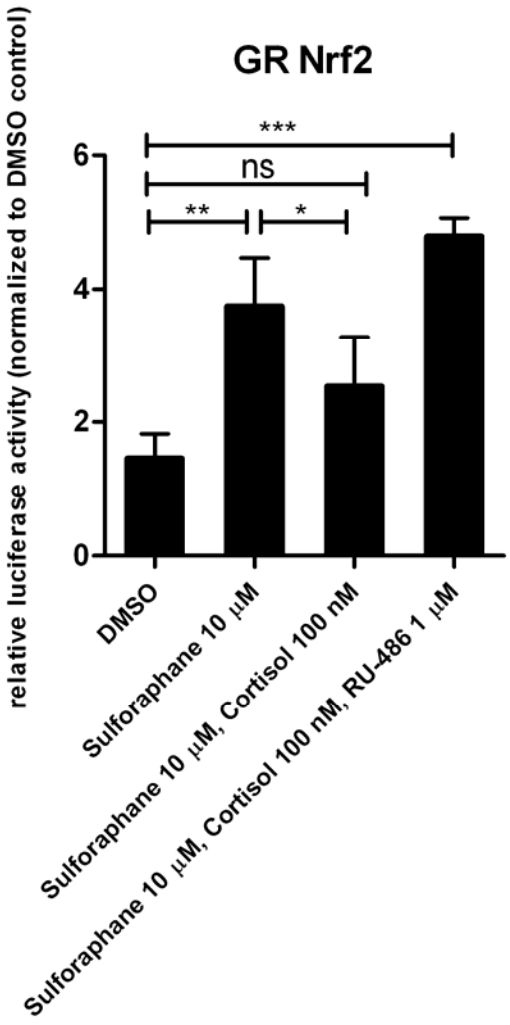

Fig. 1. Glucocorticoid receptor activation impairs Nrf2 activation in HEK-293. Nrf2 activity was measured in a luminescence based transactivation assay. HEK-293 cells transient transfected with human Nrf2, human GRα expression plasmids as well as pCMV-LacZ and ARE8L reporter plasmids cells were treated over a period of 24 h with DMSO, 10 μM sulforaphane with or without 100 nM cortisol or simultaneously with 100 nM cortisol and 1 μM RU-486. Data (obtained from a representative experiment measured in triplicate)

represent mean±SD; n =3. *, P < 0.05, **, P < 0.01 *** P < 0.001. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with control (DMSO).

Incubation of the cells with 10 μM sulforaphane led to a four-fold activation of the Nrf2-induced luciferase production. Activation of GR by 100 nM cortisol suppressed the Nrf2-dependent activation of the reporter gene by 10 μM Sulforaphane. The GR antagonist RU-486 (1 μM) was able to fully restore Nrf2-mediated activation of the ARE8L reporter.

8.4.2 Induction of the Nrf2-dependent ARE8L-reporter construct in rat H4IIE hepatoma cells

To characterize the responsiveness of the Nrf2 pathway in rat H4IIE hepatoma cells, we transiently transfected the cells with the ARE8L luciferase reporter construct. As shown in Fig. 2A, sulforaphane stimulated ARE-dependent reporter activity approximately seven-fold. Co-transfection of the cells with recombinant Nrf2 further stimulated reporter activity almost two-fold. Since Nrf2 protein has a short half-life (of about 15 min), which is significantly enhanced by proteasome inhibitors as reported earlier [31, 32], ARE-reporter activity was also assessed in cells treated with sulforaphane (10 μM) and the proteasome inhibitor MG132 (10 μM). Total luciferase reporter activity was two to three times higher in the presence of the proteasome inhibitor compared with its absence.

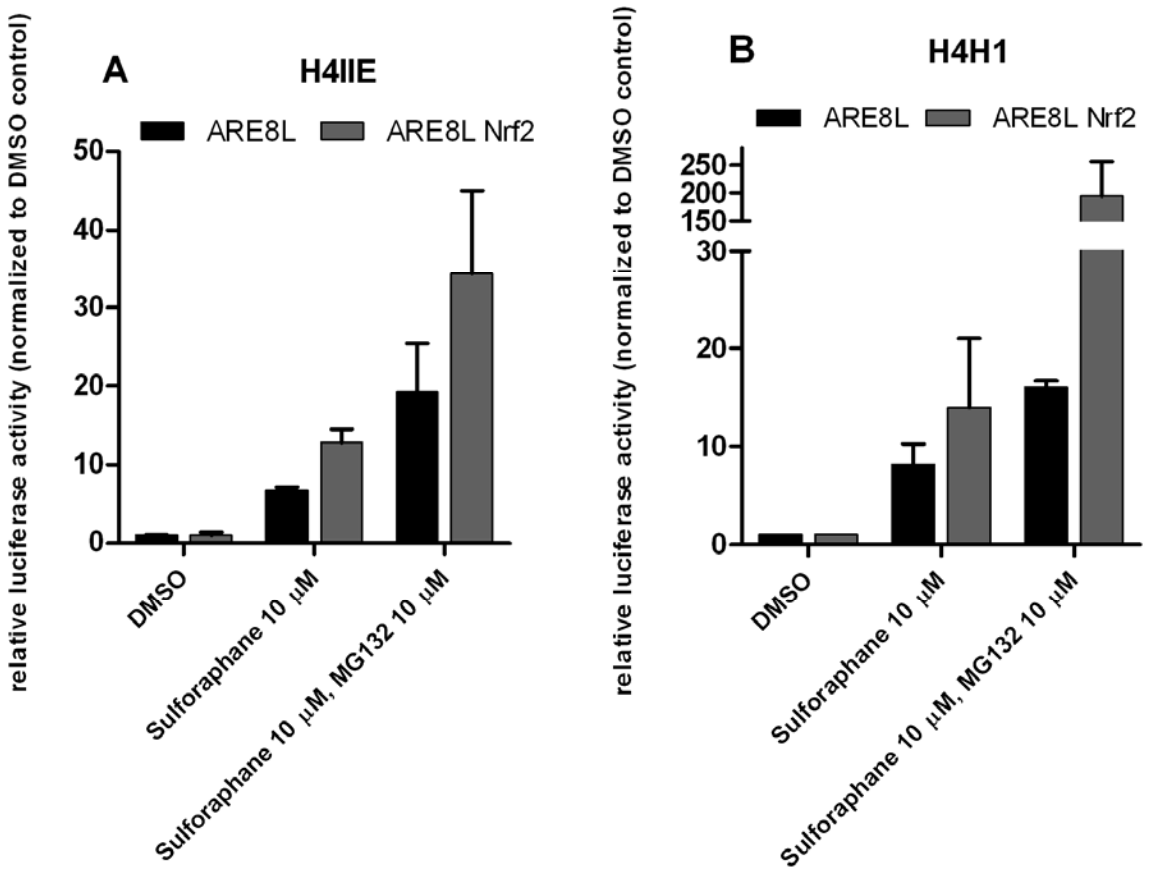

Fig. 2. Activation of the Nrf2-dependent luciferase reporter ARE8L in H4IIE cells. The activation of an Nrf2-dependent luciferase reporter driven by a promotor containing eight antioxidant response elements (ARE8L). By endogenous and over expressed Nrf2 was measured in rat H4IIE hepatoma cells (A) or in H4IIE cells stably expressing 11β-HSD1 (H4H1 clone) (B). Cells were treated with vehicle (DMSO), sulforaphane (10 μM), or sulforaphane (10 μM) and proteasome inhibitor MG132 (10 μM) for 24 h. Data represent mean ± SD from at least two independent experiments performed in triplicate.

There are currently no hepatocellular lines available that express the glucocorticoid activating enzyme 11β-HSD1. Therefore, we recently constructed a H4IIE cell clone with stable expression of 11β-HSD1 (designated as H4H1) [19]. The Nrf2-dependent ARE8L-reporter was similarly activated in this clone by sulforaphane both in the presence or absence of cotransfected recombinant Nrf2, with the exception that inhibition of the proteasome led to a much more pronounced stimulation of reporter activity upon over expression of Nrf2 (Fig. 2B).

8.4.3 11β-HSD1-mediated glucocorticoid activation suppresses Nrf2 transactivation capacity

The observation that over expression and activation of GR in HEK-293 cells inhibits the Nrf2-dependent transactivation of the ARE8L reporter gene led us to test whether suppression of Nrf2 transactivation by activated GR might be observed in cells expressing endogenous levels of these two transcription factors.

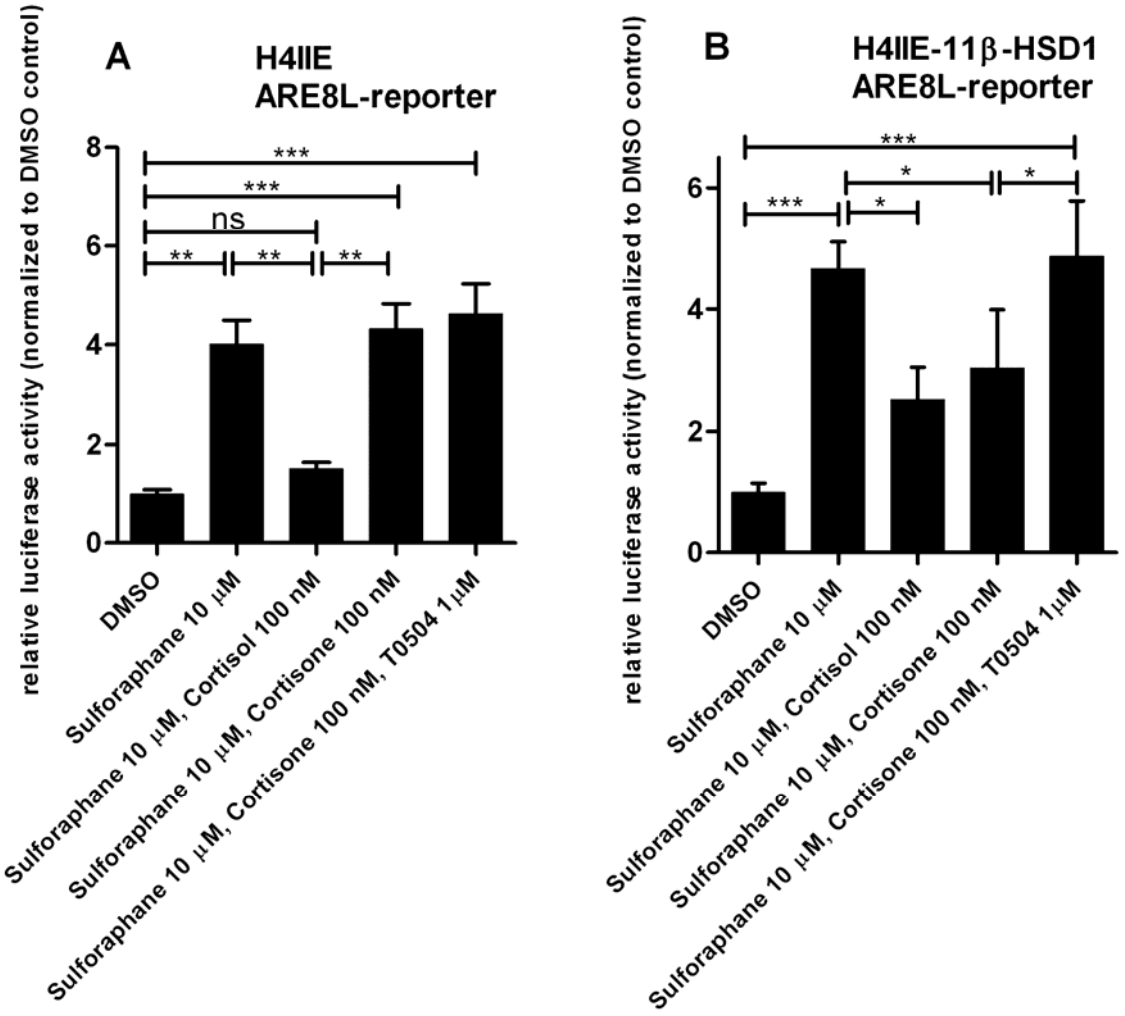

Fig. 13. Suppression of Nrf2 transactivation by glucocorticoids in H4IIE cells. H4IIE cells were transiently transfected with ARE8L-reporter plasmid and empty vector pcDNA3 (A) or with ARE8L and 11β-HSD1 expression plasmid (B). The cells were incubated with vehicle or sulforaphane, glucocorticoids and, selective 11β-HSD1 inhibitor (T0504) at the concentration indicated for 24 h followed by measurement luciferase activity. Data represent mean ± SD from at least two independent experiments measured in triplicate. *, $P < 0.05$, **, $P < 0.01$ *** $P < 0.001$. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with control (DMSO). ns, non significant.

Incubation of rat H4IIE hepatoma cells, a widely used liver cell model, with 100 nM cortisol almost completely abolished sulforaphane-induced Nrf2 activation of the ARE8L reporter gene. Neither the inactive glucocorticoid cortisone (100 nM) nor the selective 11β-HSD1 inhibitor T0504 affected Nrf2-dependent transactivation in transiently transfected H4IIE cells (Fig. 3A). H4IIE cells are devoid of endogenous 11β-HSD1 expression, as measured by real-time RT-PCR.

In H4IIE cells transiently transfected with rat 11β-HSD1 both cortisol and cortisone diminished Nrf2 activity. Impairment of Nrf2 transactivation by cortisone in 11β-HSD1 expressing H4IIE cells was fully reversed in the presence of 1 μM T0504 (Fig. 3B).

8.4.4 Sulforaphane does not affect 11β-HSD1 activity

To ensure that the observed effects are not a result of inhibition of 11β-HSD1 by sulforaphane, we measured 11β-HSD1-dependent conversion of cortisone to cortisol in H4H1 cells. H4H1 cells represent H4IIE cells stably transfected with recombinant 11β-HSD1 enzyme.

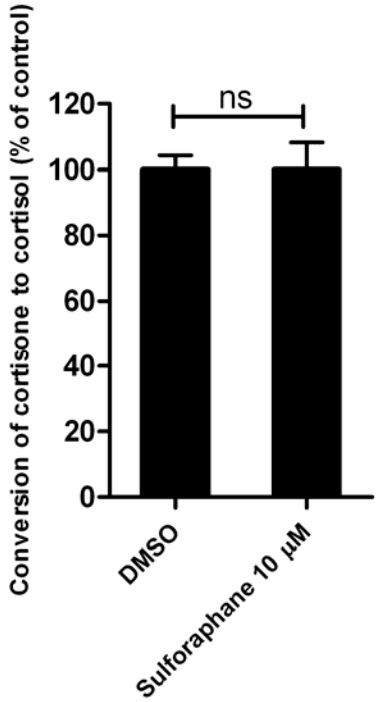

Fig. 14. Sulforaphane does not inhibit 11β-HSD1 activity. Activity was measured by the conversion of cortisone to cortisol. H4H1 cells were incubated for 24 h with 10 μM of sulforaphane or vehicle (DMSO). Data represent mean ± SD from at least three independent experiments measured in triplicate. P-value was determined using unpaired, two-tailed student t-test. ns, not significant.

Sulforaphane-treated cells showed enzymatic activity that was comparable with that of DMSO-treated cells, indicating that sulforaphane does not affect 11β-HSD1 activity.

8.4.5 11β-HSD1 inhibitors and GR antagonists improve Nrf2 transactivation capacity

To overcome experimental differences due to transfection efficacy and to further study the impact of 11β-HSD1 inhibition, on Nrf2-dependent transactivation, we applied the H4H1 cells stably transfected 11β-HSD1.

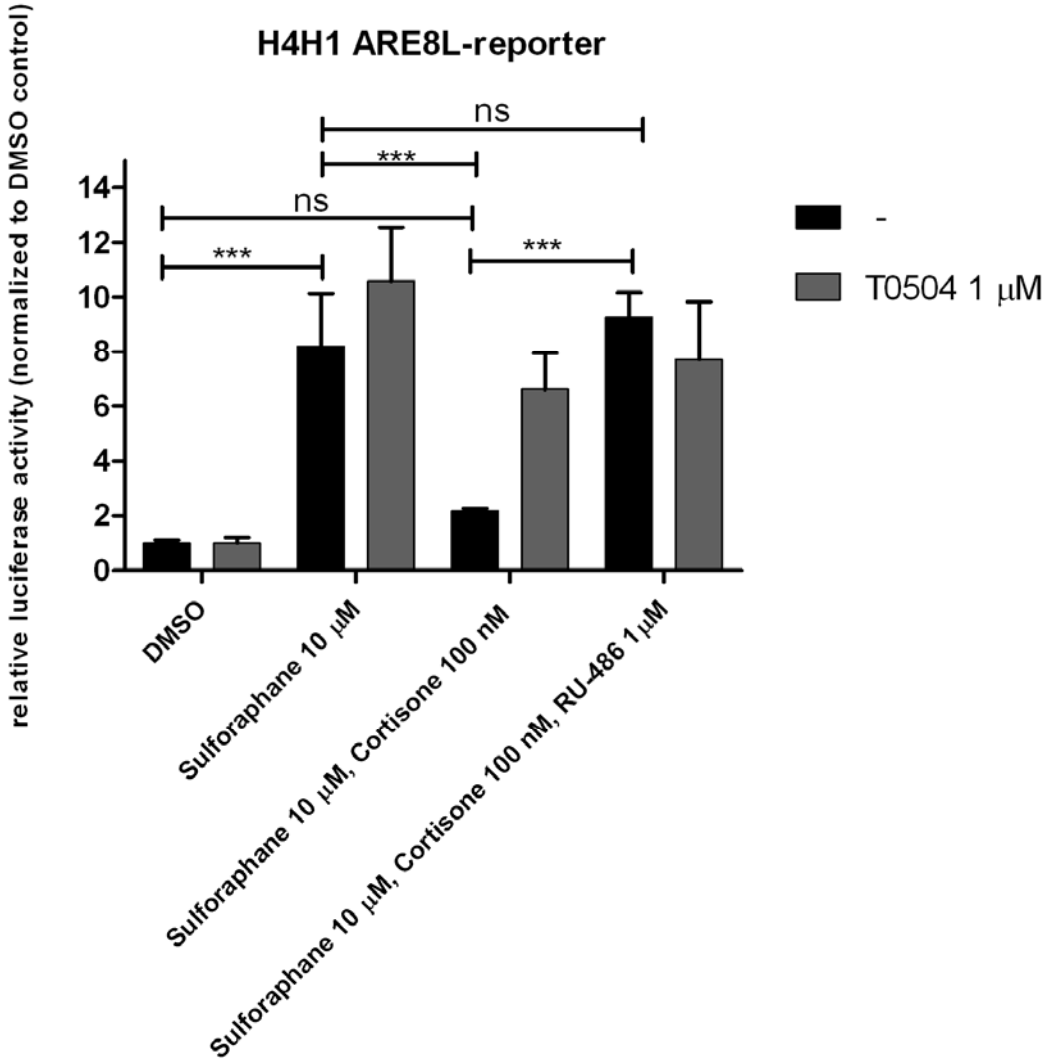

Fig. 15. 11β-HSD1 inhibitors and GR antagonists restore Nrf2-dependent transactivation H4H1 cells were transiently transfected with ARE8L and CMV-LacZ. Cells were then treated for 24 h with vehicle (DMSO), 100 nM cortisone, 10 μM sulforaphane, 1 μM of the selective 11β-HSD1 inhibitor T0504 and 1 μM of the GR antagonist RU-486 as

indicated, followed by determination of luciferase activity. Data represent two independent experiments measured in triplicate. *** P < 0.001. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with control (DMSO). ns, not significant.

The activation of Nrf2 by 10 μM Sulforaphane resulted in an eight-fold activation of the ARE8L-reporter. The activation of the ARE8L-reporter in H4H1 cells treated for 24 h with 10 μM of sulforaphane and 1 μM of T0504 was slightly higher compared with sulforaphane alone; however, the differences did not reach statistic significance. Importantly, following 24 h treatment of H4H1 cells with 100 nM cortisol and 10 μM sulforaphane significantly suppressed Nrf2-dependent transactivation of the ARE8L reporter, and reporter activation was indistinguishable to that from DMSO treated cells. The suppression of Nrf2 function due to 11β-HSD1 activity was reversed by the presence of 1 μM of the selective 11β-HSD1 inhibitor T0504 or 1 μM of the GR antagonist RU-486.

8.4.6 NQO1 expression in H4IIE cells is suppressed by cortisol but not by cortisone

To further support the suppressive effect of glucocorticoids on Nrf2 activity we determined the expression of NQO1 and GSTA2 mRNA in H4IIE cells treated with sulforaphane in the absence or presence of glucocorticoids. Sulforaphane enhanced NQO1 mRNA expression three-fold compared with control cells that were treated with DMSO. GSTA2 mRNA expression was four-fold increased by sulforaphane Co-incubation of H4IIE cells with sulforaphane compared with control cells that were treated with DMSO. Cortisol significantly suppressed NQO1 and GSTA2 mRNA expression (Fig. 6A). The inactive glucocorticoid cortisone was unable to suppress sulforaphane-induced NQO1 mRNA expression in the absence of 11β-HSD1.

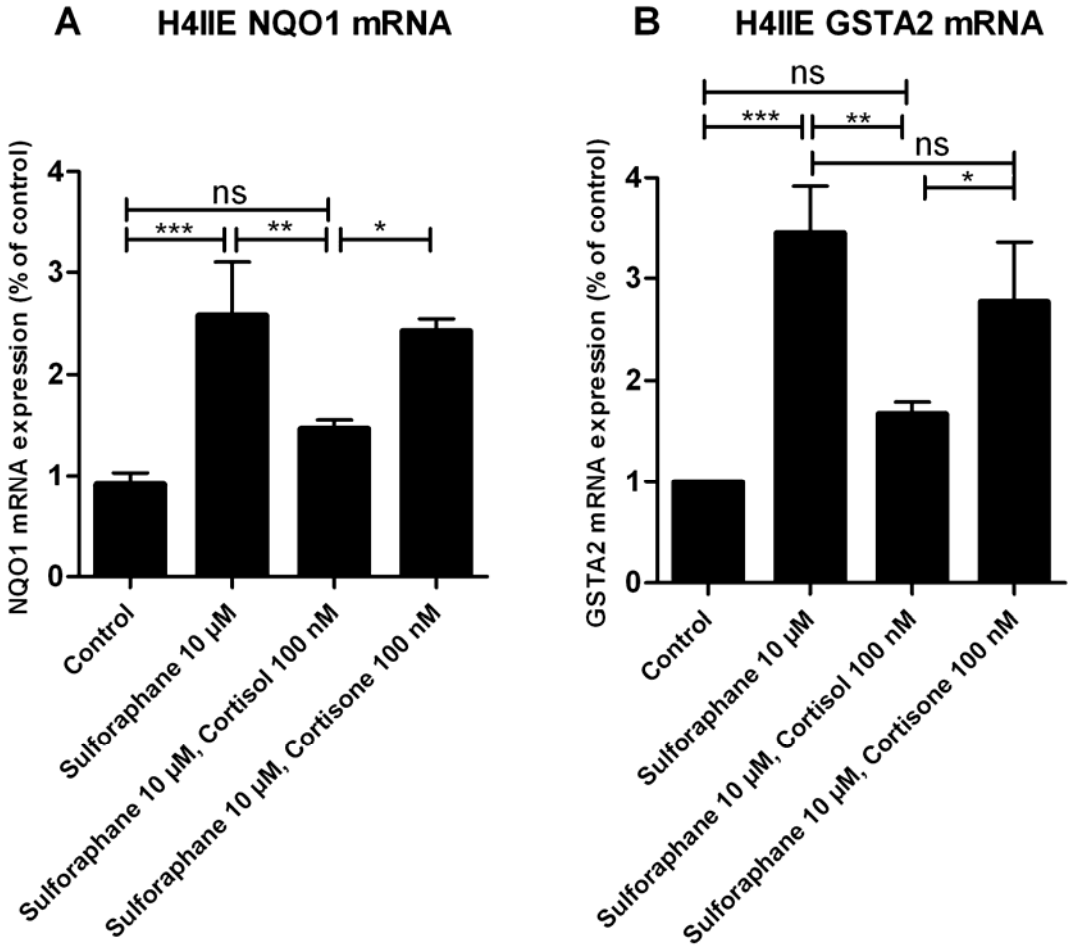

Fig. 16. Inhibition of Nrf2-mediated expression of NQO1 and GSTA2 by cortisol. H4IIE cells were incubated for 24 h at 37°C with 10 µM sulforaphane in the absence or presence of 100 nM cortisol or cortisone, respectively, followed by determination of NQO1 and GSTA2 mRNA levels by real-time RT-PCR. Data (mean ± S.D. of triplicates from three independent experiments) are relative to the ratio of target NQO1 and GSTA2 mRNA to GAPDH control mRNA from cells treated with vehicle (DMSO). *, $P < 0.05$, **, $P < 0.01$ *** $P < 0.001$. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with vehicle control (DMSO).

8.4.7 Inhibition of 11β-HSD1 restored sulforaphane-induced NQO1 mRNA expression in H4H1 cells

To investigate the impact of 11β-HSD1 on NQO1 mRNA expression we employed H4H1 cells that are stably expressing recombinant 11β-HSD1. Sulforaphane induced NQO1 mRNA expression approximately five-fold. The induction was significantly reduced upon simultaneous incubation of cells with sulforaphane and cortisone for 24

h. The 11β-HSD1 inhibitor glycyrrhetinic acid (GA) showed no significant effect on basal or sulforaphane-induced NQO1 mRNA expression of but completely reversed the suppressive effect of cortisone.

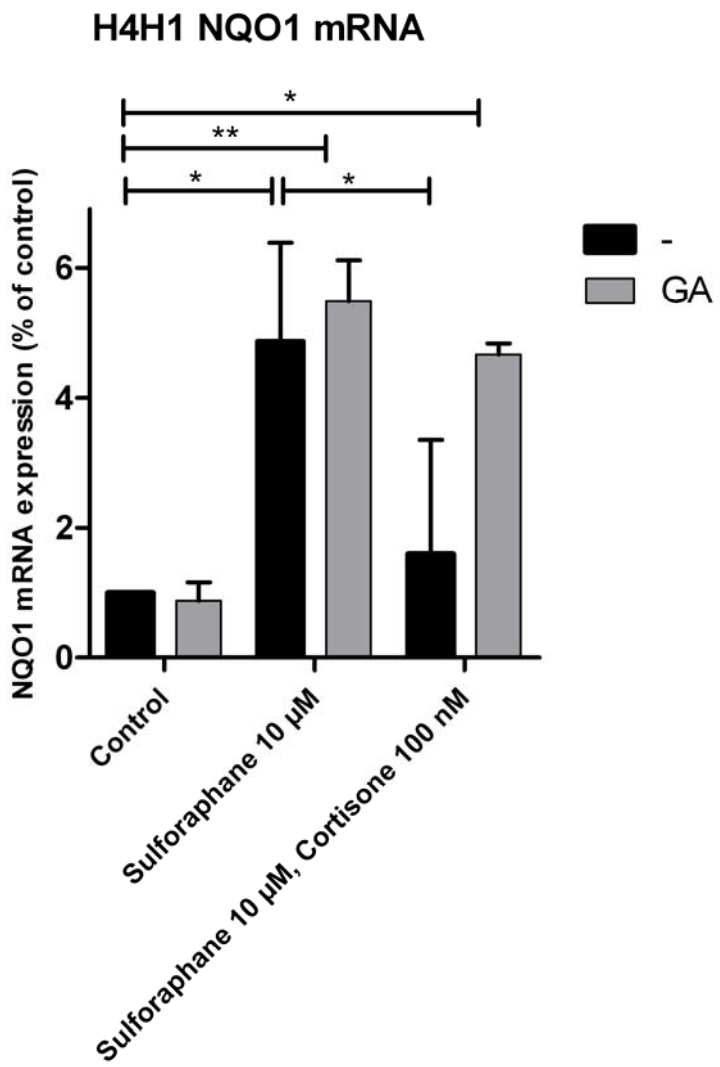

Fig. 17. Inhibition of 11β-HSD1 reversed the glucocorticoid-dependent suppression of Nrf2-mediated NQO1 mRNA expression. In 11β-HSD1 expressing H4H1 cells the addition of cortisone decreased sulforaphane-induced NQO1 mRNA expression. H4H1 cells were incubated for 24 h at 37°C with 10 µM of sulforaphane in the absence or presence of 100 nM cortisol and 1 µM glycyrrhetinic acid (GA). mRNA levels were quantified by real-time RT-PCR. Data (mean ± S.D. of triplicates from three independent experiments) are relative to the ratio NQO1 mRNA to GAPDH control mRNA from cells treated with vehicle (DMSO). *, $P < 0.05$, **, $P < 0.01$ *** $P < 0.001$. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with control (DMSO).

8.4.8 Glucocorticoid-dependent impairment of HO-1 function and susceptibility to H_2O_2

Next, we studied the impact of 11β-HSD1 activity on the Nrf2-dependent target gene HO-1. H4IIE cells were transiently transfected with a plasmid for the cytosolic hydrogen peroxide sensor HyPer and either 11β-HSD1 or pcDNA3 (empty vector). Cells were treated with 100 nM of cortisone for 24 h. The real time measurements in H4IIE cells transiently transfected with the cytosolic HyPer-senor and pcDNA3 showed a three-fold increase in the HyPer signal upon addition of 10 µM H_2O_2. The total response was reduced by about 50% after 30 min. In contrast, H4IIE transiently transfected with 11β-HSD1 showed an enhanced response to H_2O_2 with an approximately ~four-fold increased HyPer signal, indicating enhanced oxidative stress. After 30 min the HyPer signal was only slightly reduced, indicating enhanced oxidative stress, due to the impaired activity of HO-1 in 11β-HSD1 expressing H4IIE cells. In 11β-HSD1 expressing H4IIE cells simultaneously treated with 100 nM cortisone and with 1 µM T0504, the total response was slightly lower compared to cells in the absence of the inhibitor, although statistically not significant, and the cells recovered more quickly from the H_2O_2 challenge. Inhibition of 11β-HSD1 seemed to be beneficial and the total response was significantly reduced by about 30% after 30 min (Fig. 8A) compared with cells expressing 11β-HSD1 but in the absence of T0504.

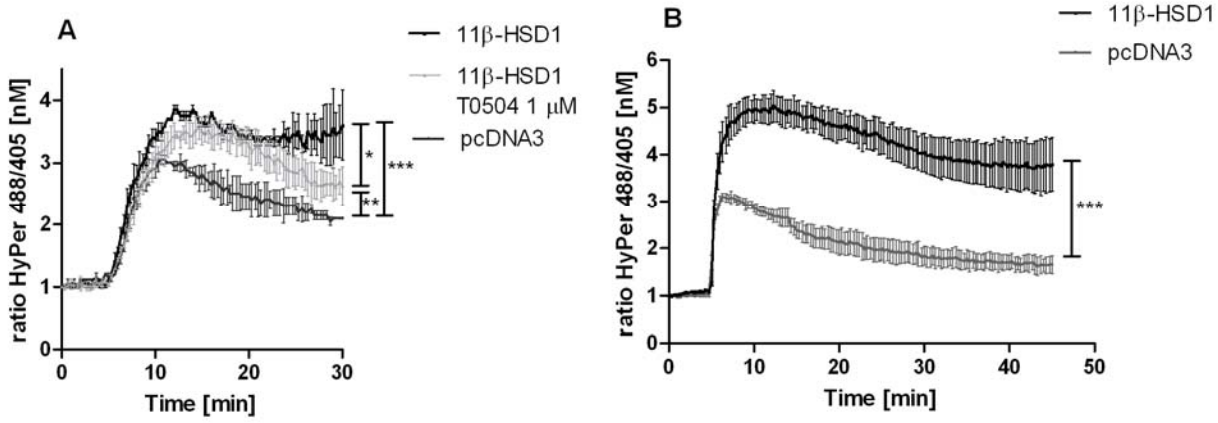

Fig. 18. 11β-HSD1 expressing cells are more susceptible to H_2O_2 induced oxidative stress H4IIE cells transiently transfected with either pcDNA3 or 11β-HSD1 expression plasmid treated for 24 h with 100 nM cortisone in the presence or absence of 11β-HSD1 inhibitor T0504. Following incubation the medium was replaced by assay buffer (HBSS)

containing 1g/L of glucose. Single cell real-time measurements were performed on a Leica confocal microscope SP5. (A) After 5 min baseline adaption, cells were exposed to a final concentration of 10 μM H_2O_2 and recovery was compared between differentially transfected cells over a period of 30 min. Data represent mean ± SEM of three different cells for each transfection. *, $P < 0.05$, **, $P < 0.01$ *** $P < 0.001$. P value was obtained using a one-way ANOVA followed by Bonferroni post tests compared with pcDNA3. (B) After 5 min baseline adaption, cells were challenged with a 100 μM H_2O_2 bolus and reduction of cytosolic oxidative stress was measured over a period of 45 min. Data represent mean+SEM of seven different cells for each transfection. *** $P < 0.001$. P value was obtained using unpaired, two-tailed student t-test.

Further, a higher concentration of H_2O_2 was used to challenge the cells (Fig. 8B) in order to address whether the observed differences were more pronounced. H4IIE cells transfected with pcDNA3 or 11β-HSD1 were treated for 24 h with 100 nM of cortisone, followed by real-time measurements and challenged with a bolus of 100 μM H_2O_2. The pcDNA3 transfected cells showed a three-fold increase in HyPer signal, followed by a rapid decline and normalization of the signal. After 45 min, the signal reached almost baseline level. On the other hand, 11β-HSD1 expressing cells also responded immediately but showed a more pronounced response to H_2O_2 (five-fold increase). In addition, the stress signal was only slightly reduced over 45 min period, indicating an impaired reduction of reactive oxygen species (ROS).

8.5 Discussion

During lifetime organisms are continuously exposed to toxicants derived from endogenous sources like reactive metabolites, reactive oxygen species (OH^-, H_2O_2, O^{2-}) as well as environmental xenobiotics. At concentrations exceeding detoxification capacities these compounds are able to disturb physiological functions, ultimately contributing to severe dysfunctions such as inflammation, cardiovascular and neurodegenerative-disorders, diabetes or cancer. To cope with the burden of reactive chemicals organisms developed a sophisticated defence mechanism. Key enzymes involved in coping with oxidative stress include those induced by the antioxidant redox response pathway under the control of Nrf2. Deficiency or impairment of Nrf2 has been closely related with many diseases such as arthritis [33, 34], diabetes [35, 36], Parkinson's [37] and various forms of cancer [38, 39]. Despite the key role of Nrf2 in redox regulation its mechanism of action is highly complex and not yet fully understood [1, 24, 40]. Only few studies address the cross-talk between Nrf2 and the essential glucocorticoid signaling pathway.

In most toxicology studies addressing the impact of glucocorticoids on detoxification reactions the potent synthetic ligand dexamethasone was used. However, dexamethasone has clearly distinct properties than cortisol the endogenous glucocorticoid. Because of its "constitutive" activity dexamethasone circumvents the important interconversion by 11β-HSD enzymes, which is in contrast to the endogenous glucocorticoids. Endogenous glucocorticoids can be metabolically inactivated by 11β-HSD2 in tissues such as the kidney and regenerated by 11β-HSD1 mainly in the liver. This is not the case for dexamethasone, since dexamethasone is not efficiently converted to 11-ketodexamethasone and because 11-ketodexamethasone is still a potent GR agonist [29]. In addition dexamethasone is approximately ten-times more potent than cortisol, and it has been shown that high concentrations of dexamethasone can activate pregnane-X receptor (PXR) [15]. PXR and its co-receptor the retinoid-X receptor (RXR) are involved in the detoxification of xenobiotics while they transcriptionally regulate the expression of phase I (CYP3A4) and phase II enzymes [15]. Some of the phase II enzymes regulated by Nrf2 (*e.g.* NQO1) contain both XRE and ARE motifs [41] in their promoter regions. Differential regulation by the use of high concentrations of dexamethasone due to PXR activation

has been reported for glutathione-S-transferase (GSTA2) [42, 43]. Therefore, it is important to distinguish between effects of synthetic and endogenous glucocorticoids.

In this study we focused on effects of endogenous glucocorticoids and on the role of glucocorticoid activation by 11β-HSD1 upstream of the GR on the Nrf2 pathway. Concentrations close to physiological levels (i.e. 100 nM) were used the present study, providing valuable information on the regulation of Nrf2 under physiological conditions.

Using H4IIE cells, we show that in the absence of 11β-HSD1 cortisol but not cortisone affects Nrf2 activity as measured in transactivation assays or in NQO1 mRNA expression (Fig.3 and Fig. 6). Thus, in tissues lacking 11β-HSD1 expression or in tissues expressing 11β-HSD2 (placenta, renal distal tubulus, distal colon) the Nrf2 pathway is modulated by extracellular availability or even insensitive to glucocorticoids, which is in clear contrast to tissues with high 11β-HSD1 expression (liver, adipose, hippocampal neurones).

A recent study reported an increased hepatic 11β-HSD1 expression in patients with alcoholic liver disease (ALD) [44]. ALD associated disorders are fatty liver, inflammation, and cirrhosis and hepatocellular carcinoma in patients with liver cirrhosis [45]. Nrf2 prevents ethanol-induced liver injury by detoxification of acetaldehyde and inhibition of metabolite accumulation. Nrf2 knockout animals ($nrf2^{-/-}$) showed dramatically increased mortality after feeding of ethanol doses which are well tolerated in wild-type mice [46].

On a basic cellular level our results indicate that 11β-HSD1 inhibition might be beneficial to restore the capacity for detoxification processes regulated by Nrf2. This was supported by transactivation assays, NQO1 mRNA expression levels (Fig. 7), and by the use of the intracellular redox-sensor HyPer reflecting the activity of HO-1 after challenge by H_2O_2 (Fig 8). In the case of ALD the authors claimed that inhibition of 11β-HSD1 could be a novel therapeutic approach to treat alcoholic pseudo-Cushing's [44]. We therefore hypothesize that inhibition of 11β-HSD1 may be further beneficial for the detoxification capacity of the liver, at least in patients with ALD.

Expression of 11β-HSD1 is increased in adipose tissue of obese humans and rodents. Transgenic mice selectively over expressing 11β-HSD1 (aP2-HSD1) in the adipose tissue develop the metabolic syndrome including visceral obesity, dyslipidemia, insulin resistance, diabetes, and hypertension [47]. In comparison to the human metabolic syndrome, plasma corticosterone levels in the aP2-HSD1 mice are unaltered while local glucocorticoid activation in adipose tissue is enhanced. In aP2-HSD1 mice hepatic 11β-HSD1 is not altered. However, corticosterone delivery to the liver is tree-times increased because of excessive activation of glucocorticoids derived from adipose tissue [47]. Obesity involves chronic inflammation and as a consequence enhanced infiltration of macrophages [48]. Macrophages express 11β-HSD1, thereby further enhancing locally active glucocorticoids. Thus, in obesity the Nrf2 pathway may be suppressed by elevated glucocorticoid levels. In contrast, 11β-HSD1 knockout animals fed with high fat diet showed reduced cytotoxic T-cell and macrophage infiltration in visceral fat compared with wild-type mice [49]. Reduced macrophage infiltration in adipose tissue observed for the 11β-HSD1 transgene further supports the assumption that 11β-HSD1 inhibition is beneficial in these situations. To study the effect of 11β-HSD1 inhibition in adipose tissue and the consequences for the Nrf2 pathway, a fatcell-specific inhibitor as developed recently [50] would provide a unique mechanistic tool. This inhibitor was successfully shown to protect against diet-induced obesity in mice [50]. In obese patients with type 2 diabetes hepatic 11β-HSD1 activity was found to be sustained, coincident with an increased whole body 11β-HSD1 activity compared with normal weight patients [51]. The transgenic ApoE-mice over expressing 11β-HSD1 specifically in the liver exhibit a phenotype resembling humans suffering under non-alcoholic fatty liver disease [52]. The phenotype includes insulin-resistance, hypertension and metabolic syndrome symptomatic concomitant with a normal weight status [53, 54].

In contrast to obesity a clinical study investigating morbidly obese patients showed a 20-fold higher 11β-HSD1 mRNA expression in liver compared with adipose tissue [56]. The study further convincingly showed that hepatic 11β-HSD1 expression in these patients is directly proportional to the body mass index (BMI). According to the outcome of the study the authors suggested that 11β-HSD1 activity in hepatic as well as visceral adipose tissue might be pathogenic in obesity [55]. Thus, enhanced 11β-HSD1 expression in liver or adipose tissue leads to an increased local activation of

glucocorticoids. 11β-hydroxygluocorticoids are then further able to repress the Nrf2 related activation of phase II detoxification enzymes including NQO1 and HO-1 (Fig. 3 and Fig. 6).

In conclusion, the present study contributes to the knowledge of the defined regulation of the antioxidant redox response pathway. It further suggests that inhibition of 11β-HSD1 may improve Nrf2-dependent cell defense, which may be beneficial for patients with ALD or obesity as well as patients with chronic inflammation such as diabetes or rheumatoid arthritis. To investigate these potentially beneficial effects *in vivo* further studies are needed.

8.6 Acknowledgements

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9 Conclusion and Outlook

11β-HSD1-mediated glucocorticoid activation is essential for the regulation of GR function. A subtle fine-tuning of 11β-HSD1 activity is critical and locally enhanced levels of cortisol (corticosterone in rodents) have been associated with metabolic disorders. This was best demonstrated, in studies with transgenic mouse models.

The present work focused in a major part on the characterization of specific inhibitors against the glucocorticoid metabolizing enzymes 11β-HSD1 and 11β-HSD2 some of the inhibitors were isolated from the natural source loquat, others were synthesized by modification of 18β-glycyrrhetinic acid, the active constituent of the roots and rhizomes of licorice (*Glycyrrhiza spp*.).

11β-HSD1 is currently considered as a promising drug target for the therapeutic intervention of obesity and its outcome, the metabolic syndrome. Results from studies with transgenic mice promoted the development of 11β-HSD1 inhibitors. 11β-HSD1 overexpression specifically in adipose tissue results in a pathologic phenotype described by accumulation of visceral fat and diabetic characteristics such as glucose intolerance, insulin- and leptin-resistance, increased free-fatty acids, as well as hypertension and chronic inflammation [158]. Furthermore, adipose specific overexpression resulted in enhanced intra-adipose glucocorticoid activation. This leads to higher adipose and portal corticosterone concentrations without affecting systemic glucocorticoid levels, measured in plasma [159]. In transgene mice with liver specific overexpression of 11β-HSD1 neither obesity nor glucose intolerance was obvious; however, the animals developed symptoms of the metabolic syndrome, including enhanced hepatic lipid synthesis, mild insulin resistance and steatosis [160]. In contrast, reduction of 11β-HSD1 activity by knock-down or knock-out protects against diet induced obesity concomitant with a lean and non-diabetic phenotype [161, 162]. Taken together, these studies among others revealed 11β-HSD1 as a potential drug target for the therapeutic intervention of metabolic diseases [159].

Inhibition of 11β-HSD1 for therapeutic purpose requires highly selective inhibitors whereby 11β-HSD2 is the first anti-target to be considered for selectivity assessment. Non-selective inhibitors such as 18β-glycyrrhetinic acid (GA) have been shown to cause hypertension as a result of potassium wasting and sodium retention due to

glucocorticoid-dependent activation of the MR [163]. Furthermore, other enzymes such as 17β-HSDs have to be considered in these approaches, to guarantee that sex hormone regulation is not affected by these inhibitors. In general, it would be more beneficial to use tissue-specific delivery of inhibitors, or pro-drugs that are activated specifically in the tissue of interest. Alternatively, topic instead of systemic applications may help to prevent unwanted side-effects as well as adverse compensatory effects. Recently, an adipose tissue-targeted 11β-HSD1 inhibitor was described, with beneficial outcome against diet induced obesity [164].

In contrast to synthetic pharmaceutical inhibitors, only few studies address the potential use of compounds from natural sources. The present work contributes to the identification of 11β-HSD1 inhibitors from natural sources. It further describes the mechanism and the site of action of the isolated compounds. Moreover, we highlighted the chemical class of pentacyclic triterpenes of the ursane type as active inhibitory compounds of 11β-HSD1.

Loquat (*eriobotrya japonica*) is used in Chinese medicine as an anti-diabetic. However, the mechanism of action was not fully elucidated. We described active plant constituents that may be responsible for some of the beneficial effects of loquat consumption in diabetes. For the discovery, a ligand-based pharmacophore model was used and selected hits were further evaluated by docking into the 11β-HSD1 binding site and the use of *in vitro* activity assays. Compounds isolated from loquat leaves were characterized as pentacyclic triterpenes. These compounds inhibited 11β-HSD1 and unlike GA they showed no or only weak effects on 11β-HSD2. The most potent compounds isolated from the extracts were corosolic acid with an IC_{50} of 0.8 µM, followed by urosolic acid with an IC_{50} of 2 µM for 11β-HSD1. From the dichlormethane extracts 12 different triterpenoids were identified, including already described constituents of *E. japonica* such as maslinic acid methyl ester, 3-O-trans-*p*-coumaroyltormentic acid, 3-O-cis-*p*-coumaroyltormentic acid and tormentic acid. However, two compounds identified as 2-α hydroxy-3-oxo urs-12-en-28-oic acid and uvaol have previously not been reported for this commonly used plant source.

Several of the identified triterpenoids exerted only weak inhibitory effect; However, a mixture of these individual compounds at concentrations were they exerted little or no effect, inhibited up to 90% of 11β-HSD1 activity, indicating a remarkable synergistic

effect. This observation is in line with the opinion that natural products might have more pronounced effects when used as whole plant extracts rather than as single isolated compounds due to synergistically active constituents. Traditional medicines are based on naturally derived products and the potential of nature to accommodate many potent and/or selective drugs seems to be huge. High throughput screening (HTS) approaches may reveal further compounds which can be used directly or serve as basis for the development of therapeutically valuable drugs. However, HTS is cost intensive; therefore, 3D-modeling accompanied with *in vitro* biological assays are an alternative to exploit the available structural knowledge of target proteins for identification of novel active entities. The predictive potential of these approaches for active drugs and/or possible adverse effects increases our understanding of the mechanisms behind drugs.

Natural compounds used in traditional medicines, and herbs and plants known as “household remedies” represent drugs that are of mechanistic value and that are broadly accepted in the population because of their natural source. The identification of substance classes, as shown in our study, therefore improves the existing knowledge on natural bioactive compounds.

With respect to its potency and selectivity for 11β-HSD1, corosolic acid may be used as a starting point for chemical modifications that might lead to more potent inhibitors with improved pharmacokinetic properties.

This approach was recently used for the development of selective 11β-HSD1 and 11β-HSD2 inhibitors using the non-selective GA as a starting compound. In the present work, we characterized nine selective 11β-HSD1 and fifteen 11β-HSD2 inhibitors for their biological activity and species specificity on 11β-HSD2. Species-specific differences of inhibitors need to considered; and have been described for 11β-HSD1 inhibition by GA-derivatives and other inhibitor classes [165, 166]. We showed that the starting compound GA was comparably active toward mouse and human 11β-HSD2. However, the chemical modification of three tested GA-derivatives enhanced the species-specificity for the human compared with the mouse enzyme. With respect to animal experiments and restriction to existing animal models for the proof-of-concept regarding subsequent clinical trials, species-specific variability represents an important aspect. The use of docking and pharmacophore

models might lead to an improved prediction of species-specific variability and potency of bioactive compounds.

11β-HSD2 is well accepted as an off-target with respect to hypertension and cardiovascular complications as a result of cortisol-dependent activation of renal MR. Recently, potential applications for the beneficial use of 11β-HSD2 inhibitors have been suggested. These approaches include end-stage renal disease patients on hemodialysis. In these patients, treatment of hyperkalemia to lower the risk of hyperkalemic arrhythmias is essential [167]. Inhibition of 11β-HSD2 by licorice consumption was able to reduce serum potassium in hemodialysis patients, probably by enhanced intestinal secretion as a result of 11β-HSD2 inhibition and MR activation in the distal colon. However, high doses of GA were used in short-time applications, and long-term studies have to address potential adverse effects. Furthermore, GA is a non-selective inhibitor of both 11β-HSD enzymes. The inhibition of 11β-HSD1 for example in liver, renal proximal tubules, in testicular leydig cells, and in the hypothalamus might cause adverse effects. Moreover, there is evidence that GA inhibits some 17β-HSD enzymes. Therefore, selective inhibitors are required for follow-up studies to confirm and clearly address the effects seen with licorice.

Hyperkalemia is also a rare but underestimated event for metastatic infiltration of the adrenals as a consequence of breast and lung cancers with the outcome of adrenal insufficiency [168]. In patients with adrenal insufficiency and for treatment of symptoms such as hypotension, hyperkalemia and hyponatremia selective 11β-HSD2 inhibitors might be beneficial. Acute intoxication with paramethoxy-metamphetamin, a frequently used party drug, can be lethal at least in part because of hyperkalemia and hypoglycemia [169]. Thus, selective inhibition of 11β-HSD2 may lead to an increase in cortisol, thereby increasing blood glucose levels and reduce hypoglycemia. Cortisol activation of MR in the intestine might be beneficial in such cases of hypokalemia as well.

In recent studies, elevated 11β-HSD2 expression was also reported for some forms of cancer and in chronic colon inflammation [170, 171]. However, most of these studies used GA. Thus, in further studies selective inhibitors against 11β-HSD2 should be used to confirm the beneficial effects of 11β-HSD2 inhibition.

Specific 11β-HSD2 inhibitors coupled with cell-permeable peptides such as oligo-arginines [172] also containing a metalloproteinase cleaving sites are conceivable. Metalloproteinases (MMP) are commonly secreted from tumors [173, 174]. Specific release of the inhibitor around the tumor tissue might be beneficial to suppress 11β-HSD2 in such situations, without affecting for example. renal 11β-HSD2 function. A possible structure for such a coupled inhibitor construct is given in Figure 14.

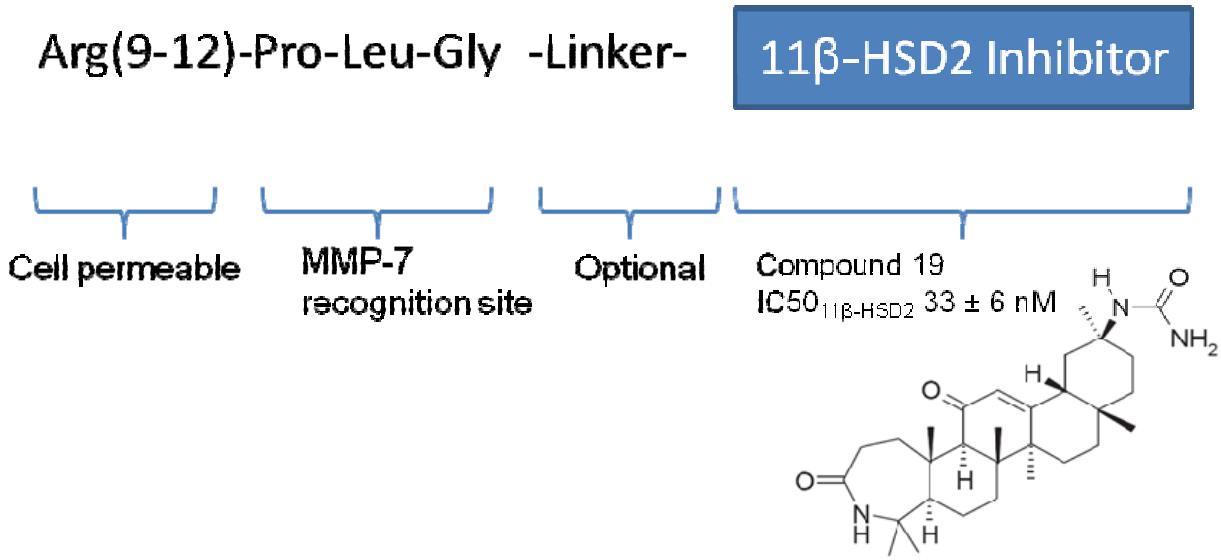

Figure 12. Schematic representation of a putative selective inhibitor coupled to a cell permeable peptide and including an MMP-7 recognition site.

Arg, Arginine; Pro-Leu-Gly, specific MMP-7 recognition site.

The cell-permeable peptide of this construct is an oligo-arginine, its beneficial length is dependent on the cell-type and varies between nine and twelve repeated arginines. The peptide is expected to enter the cell by macropinocytosis [172]. The specific recognition site Pro-Leu-Gly will be recognized by MMP-7 [175], a metalloproteinase also secreted from pancreas and colon cancer. The linker peptide may be required for functional coupling of the inhibitor and/or to protect the cleavage site from steric hindrance by the inhibitor. The last linker amino acid should contain an additional free amino group (lysine (Lys), arginine (Arg)) since this may be beneficial for chemical coupling of the inhibitor. However, in my example, I chose compound 19 (see chapter 6). This selective inhibitor of 11β-HSD2 has a free amino group, which might be easily linked to the terminal carboxy-group of glycine from the

tripeptide motif that functions as specific MMP-7 recognition site. The following aspects should be considered in general for such a hypothetic construct.

1) The total construct must be cell-permeable.
2) The cytotoxicity must be negligible for the total as well as the cleaved product(s).
3) The cleavage of the inhibitor must be possible and specific.
4) The inhibitor must be still active, even after structural changes due to cleavage from the pro-drug.
5) The construct must be soluble in a water based solution at pH 7.4.

Selective 11β-HSD2 inhibitors are highly valuable as tools for basic research. The awareness of a mosaic like expression pattern for 11β-HSD1 and 11β-HSD2 in many tissues may explain contradictive findings for the use of non-selective inhibitors. In this work, we reviewed MR expression in various organs. MR has broader substrate specificity compared with GR; therefore, MR function is tightly defined by the presence or absence of glucocorticoid metabolizing enzymes. Selective inhibition of 11β-HSD1 and 11β-HSD2 is required to clearly address mechanistic findings *in vivo* and *in vitro*.

A challenge for biologically used compounds remains cellular uptake. Therefore, the inhibitors characterized in this work were also used in intact SW-620 colon cells, known for the endogenous expression of 11β-HSD2. The tested inhibitors were found to be highly active for endogenous 11β-HSD2 inhibition in SW-620 cells. Interestingly, IC_{50} values obtained from SW-620 cells were lower compared with data obtained from HEK-293 cells or even from cell lysates. This may be explained by compound removal due to transport mechanisms in HEK-293 cells, or compound accumulation by SW-620 cells, if compared with lysates.

Compounds may act differently in intact cells compared with lysates because of active transport mechanisms that are able to remove the substance from the cell, and the specific chemical characteristics. The chemical structure *per se* might prevent cellular uptake, for example when containing highly charged groups. Further possibilities for cellular inactivity might be protein binding of the compound within the

cell, or binding to serum proteins such as albumin contained in the culture media. A general challenge is the solubility in the water-based culture media as well as the stability of the compound over the cultivation period at the temperature of 37°C and at neutral pH value. Thus, the compound must be sufficiently soluble to reach the active concentration at the site of the target enzyme. Besides, the chemical stability is essential for storage of the compound, to guarantee continuous activity without accumulation of decomposition products.

Detoxification of reactive metabolites or toxic xenobiotics is in part mediated by enzymes of the antioxidant redox response pathway. In the present study, I showed that 11β-HSD1 activity modulates the cellular capacity to cope with oxidative stress. The 11β-HSD1-dependent glucocorticoid activation suppresses the activity of Nrf2 and its target enzymes. The effect of glucocorticoids was GR-dependent [176]. Therefore, I suggest that the inhibition of 11β-HSD1 may have beneficial effects by restoring the Nrf2-dependent cell defense system. This may also contribute to the beneficial effects in obesity and the metabolic syndrome were oxidative stress-dependent damage is a major problem.

The dominant hepatic outcome of the metabolic syndrome is designated nonalcoholic fatty liver disease (NAFLD), The most severe form is nonalcoholic steatohepatitis (NASH). NASH is characterized by hepatic inflammation and fibrosis, ultimately leading to cirrhosis and hepatocellular carcinoma [177]. Both liver-specific overexpression of 11β-HSD1 in mice, and human visceral fat accumulation has been associated with the pathogenesis of NAFLD [178]. In general, fat accumulation is accompanied by enhanced expression of 11β-HSD1 and locally elevated active glucocorticoid levels. In the present work, 11β-HSD1-mediated activation of glucocorticoids was shown to suppress the Nrf2-dependent antioxidant redox response pathway. Nrf2 knock-out mice were investigated by Sugimoto *et al.* for the development of nutritional steatohepatitis [179]. They found that Nrf2 depletion leads to an increase of nutrition-mediated inflammation and fibrosis marker gene expression and the onset of NASH [139, 179]. In my experiments, I found that cortisol is able to reduce the induction of Nrf2-dependent transactivation in the rat hepatoma cell line H4IIE. Furthermore, I could show that NQO1 expression is reduced in H4H1 cells, upon treatment with cortisone. H4H1 cells stably express 11β-HSD1 and therefore efficiently convert inactive cortisone into the active glucocorticoid cortisol.

Cortisol is able to suppress Nrf2 induced expression of NQO1 and GSTA2, followed by a reduced detoxification capacity of the cell. A direct comparison of transiently 11β-HSD1 transfected H4IIE cells revealed that 11β-HSD1 expression reduces the detoxification of hydrogen peroxide (H_2O_2). H_2O_2 is removed by HO-1, another essential target enzyme of Nrf2.

These observations suggest that Nrf2-dependent detoxification pathway is at least in part suppressed by elevated glucocorticoids as a result of enhanced 11β-HSD1 expression. With respect to the data presented in this work, it might be possible that obesity, accompanied with chronically enhanced 11β-HSD1 activity and higher cortisol levels leads to a reduction of Nrf2-dependent detoxification capacity, which might further contribute to damage by increasing oxidative stress in chronically inflamed hepatic tissue. To evaluate this hypothesis, *in vivo* studies and selective 11β-HSD1 inhibition are needed. Besides, the metabolic syndrome, and its increasing importance with respect to increasing costs for treatment in industrial countries, acute and chronic liver toxicity are of interest for clinical as well as basic research. The discovery of underlying mechanisms for example of ethanol-mediated toxicity has been extensively studied; however, there are still several open questions and further research is required. Alcoholic liver disease in human is accompanied with a five-fold increase in hepatic 11β-HSD1 expression compared with healthy subjects or patients with chronic liver diseases [180]. Thus, in line with the nutrition mediated hepatic outcome also alcohol consumption and abuse is associated with fatty liver, inflammation fibrosis and cirrhosis.

Since Nrf2-induced expression is involved in ethanol detoxification, Nrf2 knock-out animals ($Nrf2^{-/-}$) are highly susceptible to ethanol doses which are well tolerated by wild-type animals (WT) [181]. Serum levels of interleukine-6 (IL-6) and tumor-necrosis-factor-α (TNFα) are significantly enhanced in transgenic mice accompanied with increased infiltration of kupffer-cells (macrophages) [181]. Macrophages express 11β-HSD1 and pro-inflammatory cytokines were shown to enhance 11β-HSD1 expression. Therefore, inflammation, regardless of its source, will further increase 11β-HSD1 expression and lead to enhanced local levels of active glucocorticoids. Locally enhanced glucocorticoid levels in inflamed tissue may be able to suppress the Nrf2-dependent detoxification machinery.

In general, inflammation is associated with increased cytokine release mediated by enhanced infiltration of macrophages. Independent of obesity, it was shown that adipose tissue inflammation is associated with hepatic fat accumulation [182]. This information suggest that in situations favoring chronic inflammation elevated 11β-HSD1 may lead to a reduced capacity of the antioxidant redox response pathway, which would lead to further oxidative damage in the inflamed tissue.

There is a clear positive correlation between tobaccos consumption and lung cancer; however, not all smokers will develop lung cancer. Gender differences in lung cancer patients have been recognized, but there are some controversies. Compared with gender-matched non-smokers male smokers have a 22-fold higher risk to develop lung cancer than non-smokers compared with a in twelve-fold higher of risk for female smokers compared with non-smokers [183]. Furthermore, the incidence for cancer development in man is higher, concomitant with a significantly lower relative survival rate over one and five years compared with women [184]. On the other hand some studies reported a higher risk for female smokers to develop lung cancer [185]. These contradictive findings may be related to age-differences of the individuals *e.g.* pre- versus post-menopausal women, to lifestyle changes, or to the investigated carcinogen causative for cancer development. 11β-HSD1 and Nrf2 are both involved in the detoxification of tobacco toxins. Furthermore, the expression of both 11β-HSD1 and the Nrf2-dependent target NQO1 were shown to be gender-dependent. 11β-HSD1 shows higher expression in male [186, 187] whereas NQO1 expression was found to be lower in male compared with female [188]. However, these studies reflect the expression in the rat and are not directly translatable to the human situation. 11β-HSD1 expression in smokers is subject to high interindividual variances with respect to protein activity and expression [183]. However, gene expression analysis between smokers with or without lung cancer revealed a significantly lower expression of 22 known Nrf2-regulated genes, including NQO1, for smokers with lung cancer [189]. Therefore, further studies for tissue specific expression of 11β-HSD1 in association with Nrf2 target genes not only in lung cancer would be desirable.

In general, imbalances in the expression or activity of any gene and protein can be associated with pathological effects. Therefore, a sophisticated regulatory network has to control key nuclear receptors such as Nrf2 to control their function. Thus, besides the beneficial effects of Nrf2 in detoxification reactions, unbalanced

activation of Nrf2 is associated with disorders, including lung cancer. Within the antioxidant redox response pathway, Keap1 is a negative regulator for Nrf2. It mediates ubiquitination of Nrf2 and thereby promotes its proteosomal removal. Keap1 gene silencing due to promotor methylation, low expression levels, as well as loss-of-function mutations are directly related to some types of lung cancer and have been shown for cancer cell lines [151, 190]. Furthermore, 11β-HSD1 may also be beneficial for the detoxification of lung cancer carcinogens. The nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) exerts carcinogenic properties after Cyp450-dependent metabolism [183]. 11β-HSD1 can catalyze the reduction of NNK to the non-carcinogenic 4-methylnitrosamino-1-(3-pyridyl)-1-butanol (NNAL), however, other enzymes belonging to the aldo-keto reductase family seem to be more important regarding NKK metabolism [183].

The action of 11β-HSD1 and Nrf2 are bivalent and interference with both underlying pathways can be beneficial as well as harmful. Well defined studies and experiments should assess potential therapeutic applications.

To further study the impact of 11β-HSD1 in the antioxidant redox response pathway, the use of liver-specific 11β-HSD1 knock-out animals would be highly useful. Elucidation of the susceptibility of the transgene compared with the wild-type animal to hepatotoxic compounds such as acetaminophen or ethanol should help to understand the role of 11β-HSD1 for liver detoxification. Moreover, H6PDH knock-out animals may be used to address beneficial effects of 11β-HSD1 inhibition since in these animals 11β-HSD1 is unable to convert cortisone to cortisol because of a depletion of its cofactor NADPH.

10 References

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