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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.

Article from the Special issue on Targeted Inhibitors.

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,

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.

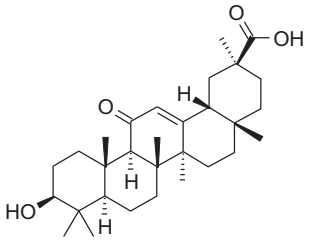
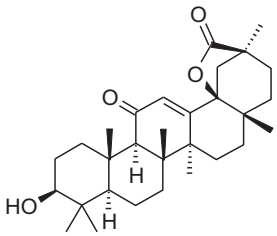
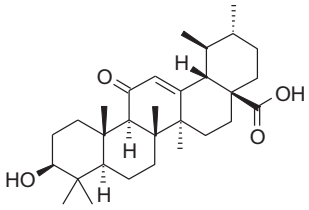
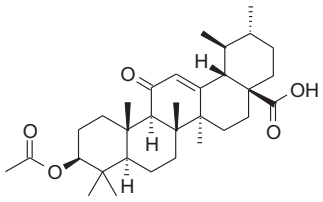
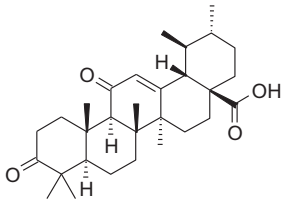
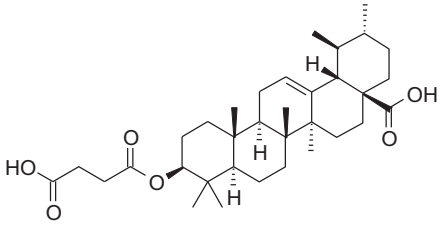
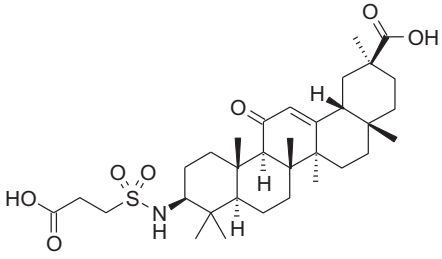
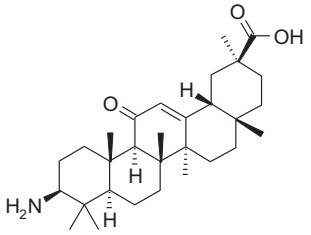
Compound [1 μM]	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
GA		778 ± 71	256 ± 33
1		49 ± 5	25,800 ± 6800
2		2100 ± 400	No inhibition
3		1360 ± 450	No inhibition
4		890 ± 240	>20,000
5		25 ± 13	3200 ± 500
6		147 ± 51	390 ± 51

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

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

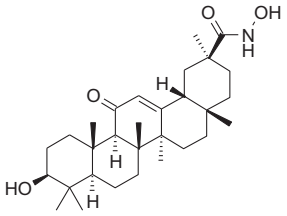
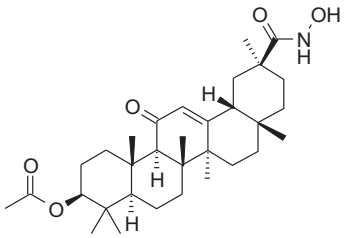
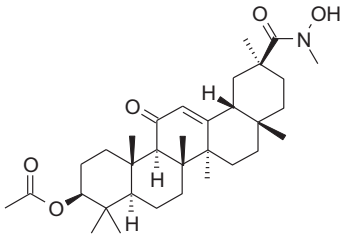
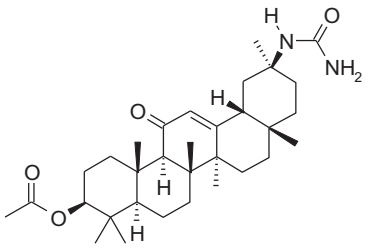
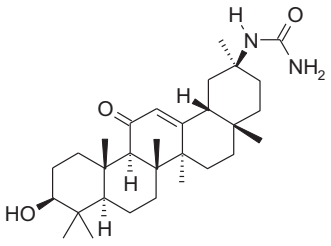
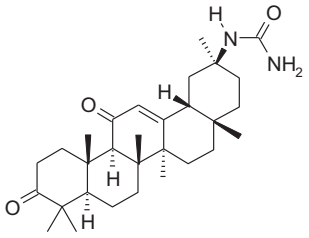
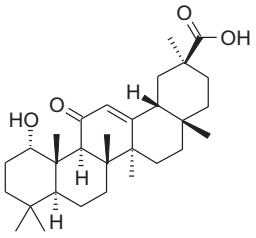
Compound	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
9		1060 ± 70	122 ± 15
10		630 ± 106	60 ± 4
11		1010 ± 140	2.9 ± 1.4
12		8300 ± 1500	104 ± 25
13		5500 ± 900	17 ± 5
14		>10,000[a]	45 ± 6
15		2000 ± 100	90 ± 13

Table 2 (*Continued*)

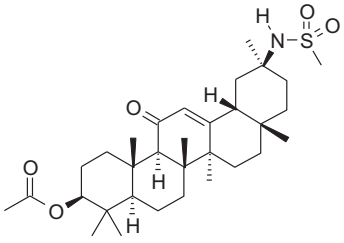
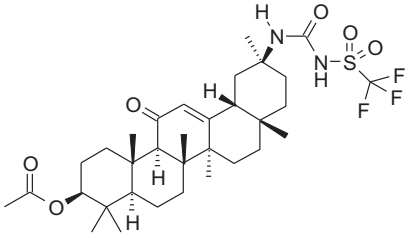
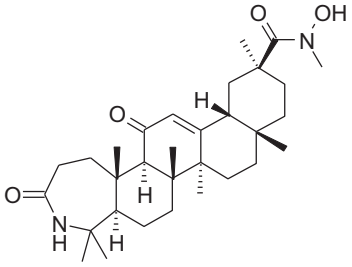
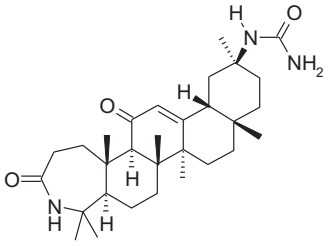
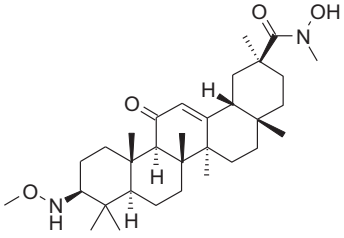
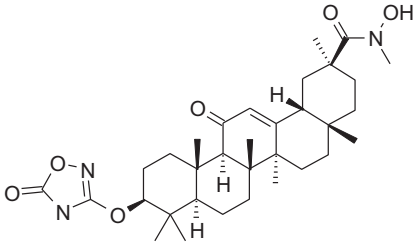
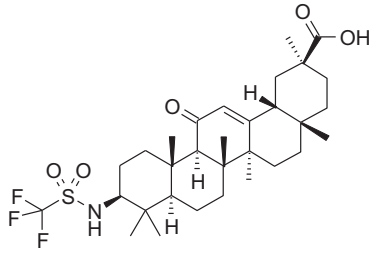
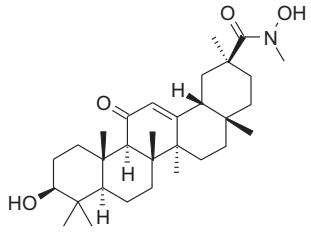
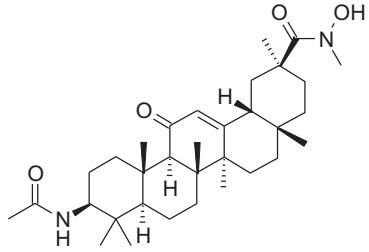
Compound	Structure	IC_{50} 11β-HSD1 [nM]	IC_{50} 11β-HSD2 [nM]
16		>40,000	6.9 ± 1.0
17		4000 ± 500	194 ± 15
18		>40,000	11 ± 2
19		>40,000	33 ± 6
20		>40,000	550 ± 90
21		500 ± 100	15 ± 2

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

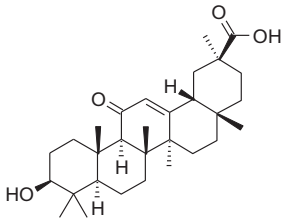
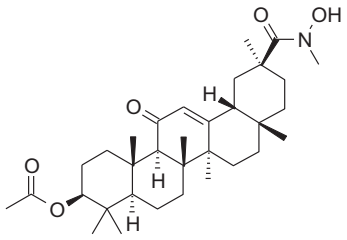
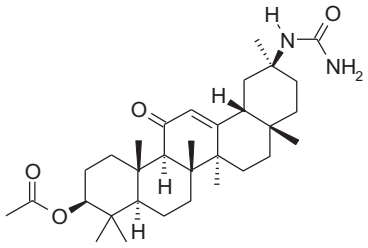
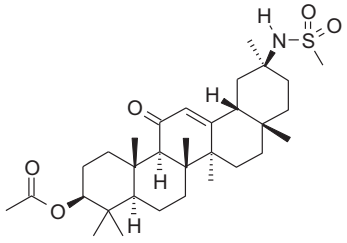
In previous studies we aimed at the synthesis of derivatives selectively inhibiting 11β-HSD1 or 11β-HSD2 with equal or higher activity than the parental compound GA [33,35,36,39]. In the present study, we characterized inhibitory activities, binding mode and impact to modulate GR activation of the most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

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

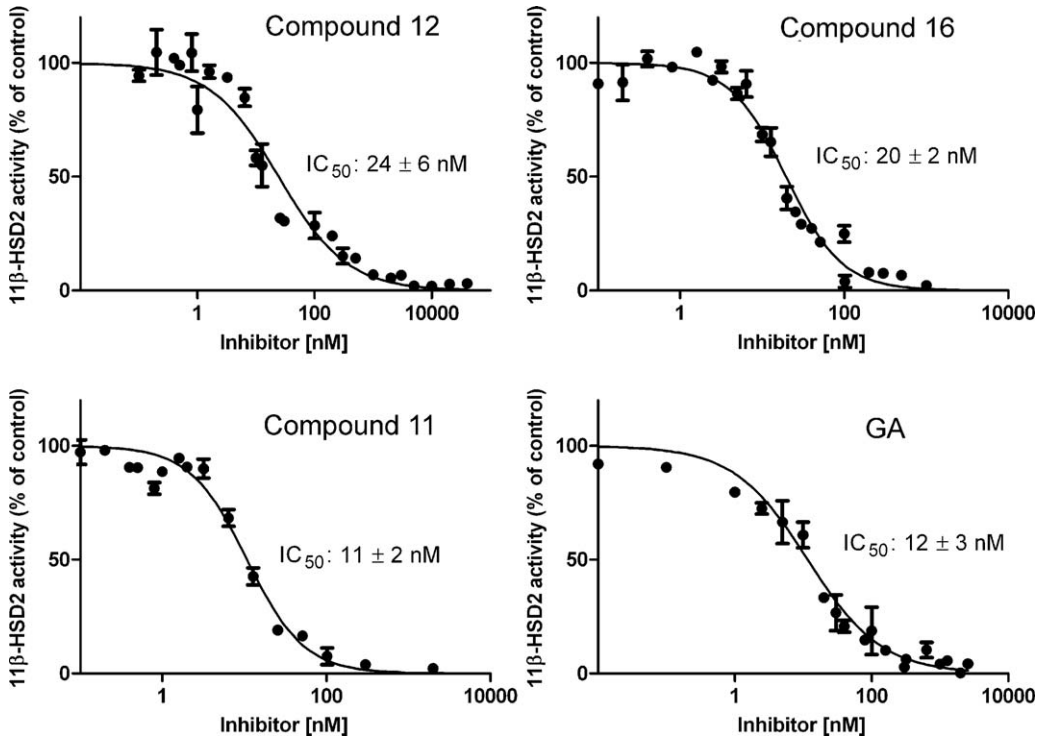


Fig. 1. Inhibition of 11β-HSD2 in intact human SW-620 colon cells. Cells were incubated for 4 h at 37 °C in DMEMsf in the presence of 50 nM cortisol and inhibitors at concentrations from 0 to 2.5 μM, followed by determination of the amount of cortisone generated. Results (mean ± SD) are from three independent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

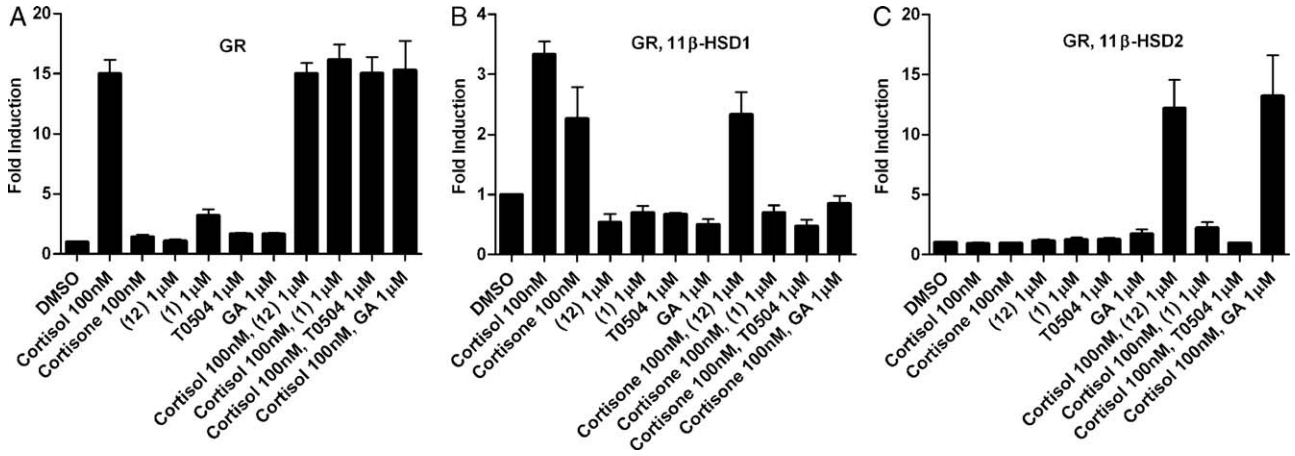


Fig. 2. Modulation of glucocorticoid-dependent GR transactivation by selected 11β-HSD inhibitors. HEK-293 cells were transfected with pMMTV-LacZ reporter, pCMV-LUC control plasmid, human GRα expression plasmid and either pcDNA3 vector to adjust total DNA in transfections (A), 11β-HSD1 expression vector (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.

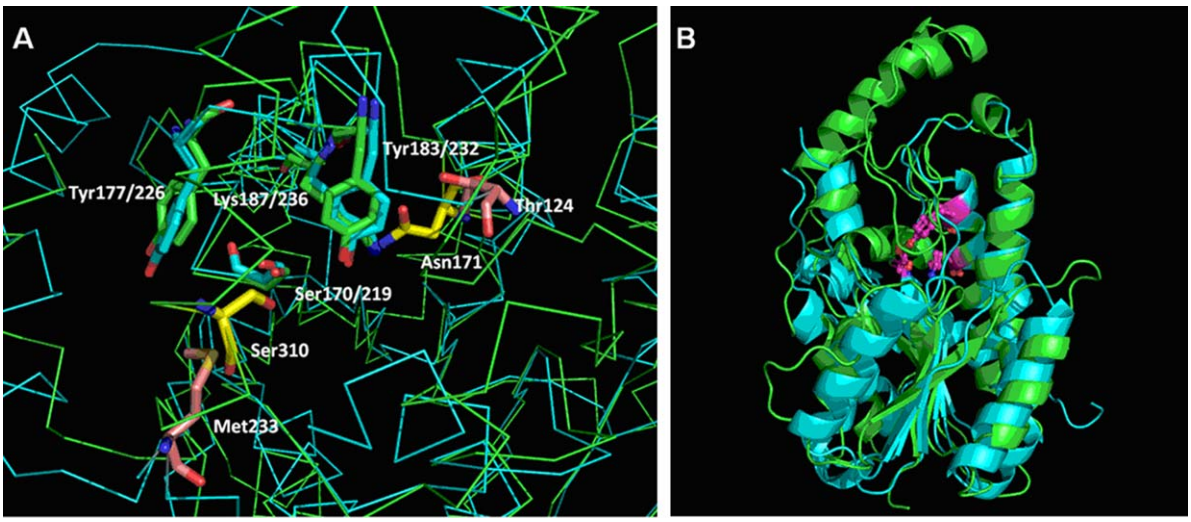


Fig. 3. Homology model of 11β-HSD2. (A) Superimposed structures of 11β-HSD1 (PDB entry 3fco) and the 11β-HSD2 model. The active site with corresponding amino acid residues Tyr 177/226, Ser170/219, and Tyr183/232 are represented in green (11β-HSD1) and blue (11β-HSD2); the main differences are highlighted 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

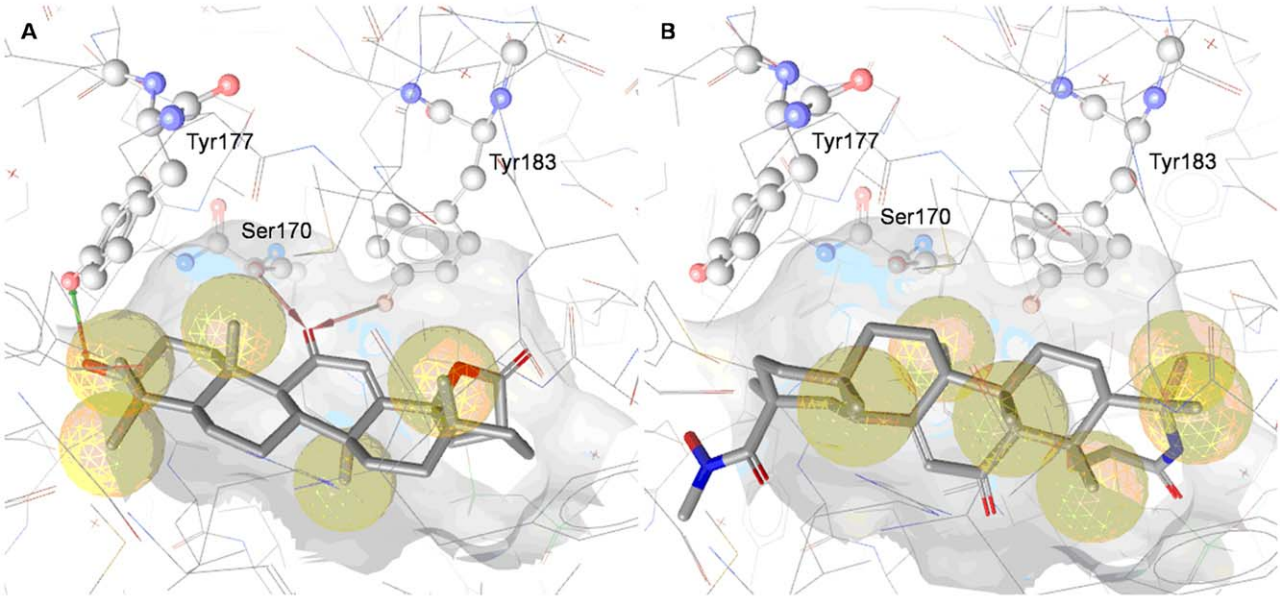


Fig. 4. Compounds **1** (A) and **18** (B) bound to 11β-HSD1. Protein–ligand interactions are color-coded: hydrogen bond acceptor – red and hydrophobic – yellow.

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.

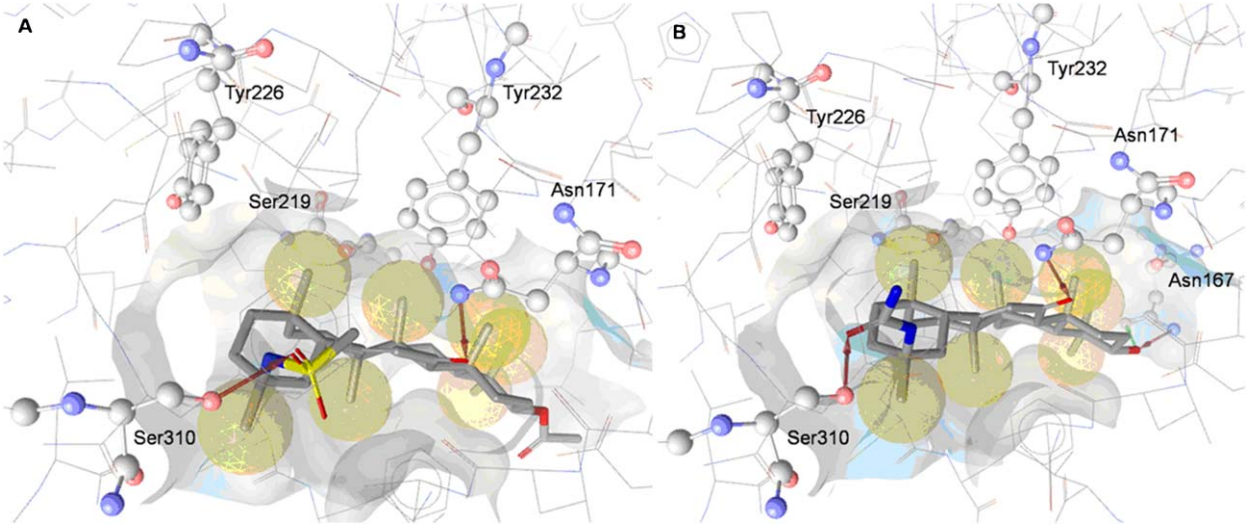


Fig. 5. Compounds **16** (A) and **13** (B) fitted into the 11β-HSD2 model. Chemical features are color-coded: hydrogen bond donor – red arrow, hydrogen bond acceptor – green arrow, and hydrophobic – yellow.

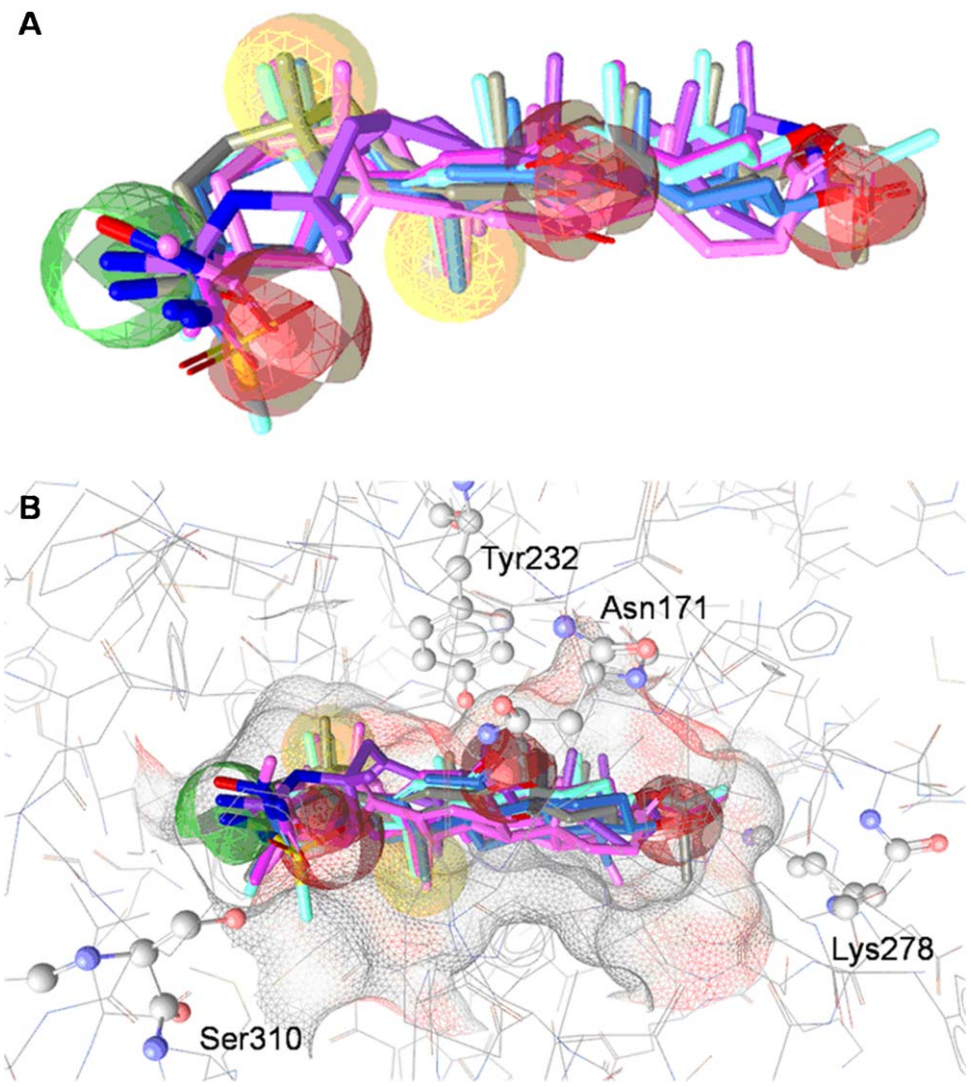


Fig. 6. Pharmacophore model of 11β-HSD2. (A) Shared feature pharmacophore model with aligned hit molecules, and (B) model and ligands fitted into the 11β-HSD2 active site. Chemical features are color-coded: HBA – red, HBD – green, and hydrophobic – yellow. The ligand binding pocket is colored by polarity.

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.

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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