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Selective inhibition of 11β-hydroxysteroid dehydrogenase 1 by 18α-glycyrrhetinic acid but not 18β-glycyrrhetinic acid

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ABSTRACT

Elevated cortisol concentrations have been associated with metabolic diseases such as diabetes type 2 and obesity. 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1, catalyzing the conversion of inactive 11-ketoglucocorticoids into their active 11β-hydroxy forms, plays an important role in the regulation of cortisol levels within specific tissues. The selective inhibition of 11β-HSD1 is currently considered as promising therapeutic strategy for the treatment of metabolic diseases. In recent years, natural compound-derived drug design has gained considerable interest. 18β-glycyrrhetinic acid (GA), a metabolite of the natural product glycyrrhizin, is not selective and inhibits both 11β-HSD1 and 11β-HSD2. Here, we compare the biological activity of 18β-GA and its diastereomer 18α-GA against the two enzymes in lysates of transfected HEK-293 cells and show that 18α-GA selectively inhibits 11β-HSD1 but not 11β-HSD2. This is in contrast to 18β-GA, which preferentially inhibits 11β-HSD2. Using a pharmacophore model based on the crystal structure of the GA-derivative carbenoxolone in complex with human 11β-HSD1, we provide an explanation for the differences in the activities of 18α-GA and 18β-GA. This model will be used to design novel selective derivatives of GA.

1. Introduction

11β-hydroxysteroid dehydrogenases (11β-HSDs) are microsomal enzymes belonging to the short-chain dehydrogenase/reductase (SDR) family. In humans and rodents, two isozymes, 11β-HSD1 and 11β-HSD2 have been identified which catalyze the interconversion of active 11β-hydroxyglucocorticoids and their inactive 11-keto counterparts [1,2]. 11β-HSD1 catalyzes the NADPH-dependent reduction (activation) of the 11-ketosteroids cortisone (human) and 11-dehydrocorticosterone (rodent) to cortisol and corticosterone, respectively. 11β-HSD1 is highly expressed in many glucocorticoid target tissues (liver, adipose tissue, skeletal muscle, macrophages). 11β-HSD2 is a NAD^+-dependent dehydrogenase and catalyzes the oxidation (inactivation) of 11β-hydroxyglucocorticoids in kidney, colon, placenta and inflamed tissue. In classical aldosterone target tissues such as renal cortical collecting ducts it protects the mineralocorticoid receptor from activation by glucocorticoids [3–6].

Active glucocorticoids play a vital role in the regulation of carbohydrate, protein, lipid, and bone metabolism, the maturation and differentiation of cells, and the modulation of inflammatory responses and stress. These activities of cortisol are mediated by glucocorticoid receptor activation and, importantly, the local concentration of active cortisol in specific tissues is tuned by pre-receptor metabolism performed by 11β-HSDs. Plasma cortisone provides an inactive pool that can be converted to active

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glucocorticoids at sites where 11β-HSD1 reductase activity is predominant.

Recent studies revealed an association of elevated 11β-HSD1-dependent glucocorticoid activation with insulin and leptin resistance, visceral obesity, dyslipidemia, type 2 diabetes, and cardiovascular complications, all features of the metabolic syndrome [7–9]. Abnormal hepatic 11β-HSD1 reductase activity seems to play an important role in syndromes of insulin resistance. Overexpression of 11β-HSD1 within adipose tissue in transgenic mice results in insulin resistance, hyperlipidemia and visceral obesity, whereas 11β-HSD1 knockout mice show decreased triglyceride and cholesterol levels and resistance to stress-induced hyperglycemia [10,11]. Therefore inhibition of hepatic 11β-HSD1 may be a promising target for the treatment of insulin resistance in metabolic diseases.

Carbenoxolone, the hemisuccinate derivative of 18β-GA, is a non-selective inhibitor of both isozymes and results in increased whole body insulin sensitivity and decreased glucose production in a clinical study. Carbenoxolone has no effect on forearm insulin sensitivity in man, implicating that carbenoxolone increases hepatic insulin sensitivity and decreases glucose production [12]. Therefore, 18β-GA is a valuable starting point for the further development of more selective inhibitors.

Indeed, several 11β-HSD1 inhibitors identified in high throughput screening campaigns and lead optimization programs are currently being developed for the treatment of metabolic diseases [13–16]. Such inhibitors need to be specific and should not inhibit 11β-HSD2. The principal function of 11β-HSD2 is the inactivation of active cortisol in specific tissues such as kidney, colon and placenta in order to prevent activation of mineralocorticoid receptor from activation by cortisol. Reduced activity of 11β-HSD2 results in activation of the mineralocorticoid receptor by cortisol and leads to hypernatremia, hypokalemia and finally hypertension [17,18].

In previous work the selectivity of several 18β-GA derivatives has been studied using 11β-HSD1 and 11β-HSD2 isolated from rat liver and rat kidney, respectively [19–22]. Some of the published compounds were selective for rat 11β-HSD1 over rat 11β-HSD2 and also showed inhibitory activity against human 11β-HSD1. Other studies demonstrated significant species-specific differences in the activities of inhibitors and the conversion of substrates by 11β-HSD1, implicating that the primary biological assay should be performed with the human enzymes [23–26]. Here we describe the synthesis of 18α-GA, a diastereomeric derivative of the natural product 18β-GA (Fig. 1) as well as the activity of both, 18α-GA and 18β-GA against human recombinant 11β-HSD1 and 11β-HSD2. On the basis of the crystal structure of 11β-HSD1 in complex with the 18β-GA derivative carbenoxolone we established a pharmacophore model which provides an explanation for the differences in the activity of 18α-GA and 18β-GA against human 11β-HSD1.

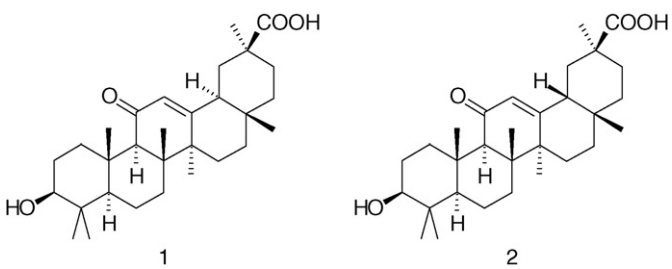

Fig. 1. Structure of 18α-GA and 18β-GA.

2. Materials and methods

2.1. Chemical synthesis of 18α-glycyrrhetinic acid (18α-GA)

18α-GA was synthesized according to method described by Ullah et al. [27]. KOH (16.0 g, 285 mmol) was added to diethylene glycol (100 mL) and heated to 100 °C. 18β-GA (40 g, 85 mmol) was added, and the solution was stirred at 220 °C for 2 h. Afterwards the mixture was cooled to room temperature. Water (500 mL) and concentrated hydrochloric acid to pH ~5 were added to the cooled solution. The solid was filtered, washed with water (200 mL), and then extracted with hot acetone (200 mL). The remaining white solid was filtered and re-crystallized from ethanol (2 × 1.5 L) to give (18.0 g, 43.7%) of 18α-GA as colorless needles.

mp: 329–330 °C (ref. [28]: 330–335 °C); HPLC purity > 99%; TLC ($CHCl_3$:MeOH, 96:4).

$[\alpha]20 = +74.6°$ ($c = 0.081$, EtOH).

^{1}H NMR (400 MHz, DMSO-d6): δ (ppm): 5.33 (s, 1H), 3.00 (dd, J = 11.3, 4.3 Hz, 1H), 2.46 (d, b, J = 13.3 Hz, 1H), 2.28–2.30 (m, 2H), 1.89 (m, 1H), 1.79 (m, 1H), 1.69–1.18 (m, 17H), 1.17 (s, 3H), 1.09 (s, 3H), 1.05 (s, 3H), 1.05–0.92 (m, 1H), 0.90 (s, 3H), 0.72–0.67 (m, 4H), 0.65 (s, 3H).

^{13}C NMR (400 MHz, DMSO-d6): δ (ppm): 199.2, 179.9, 166.5, 123.5, 77.0, 60.4, 54.6, 45.1, 43.7, 42.0, 40.6, 39.3, 38.9, 37.1, 36.8, 35.7, 35.5, 33.6, 31.8, 28.8, 28.6, 27.4, 26.7, 21.0, 20.8, 18.7, 17.7, 16.9, 16.5, 16.1.

IR (KBr): ν = 3496 (w), 2969 (m), ν = 2926 (m), 2867 (w), 1704 (s), 1661 (s), 1297 (w), 1200 (w), 1112 (w), 1028 (w), 989 (w), 658 (w) cm^{-1}

2.2. Molecular modeling

The 3D pharmacophore model for 11β-HSD1 inhibitors was generated using LigandScout 2.02 (LigandScout 2.02, Inte:Ligand GmbH, Vienna, Austria, www.inteligand.com). 18α-GA and 18β-GA were constructed in Catalyst Vers. 4.11 (Catalyst 4.11, Accelrys Inc., San Diego, CA, USA, www.accelrys.com). Both structures were energetically minimized and submitted to conformational model generation using Catalyst's conFirm module in "best" mode. This algorithm allows all internal coordinates to vary and energetically analyzes all rings and chains. All generated conformations were exported from Catalyst, imported into LigandScout 2.02, and subsequently aligned to the pharmacophore model developed in LigandScout using its accurate pattern-matching 3D alignment algorithm [29,30]. The PharmScore, a geometric scoring function that corresponds to the degree of chemical feature overlap between the found conformation and the pharmacophore model, was computed under consideration of all features and exclusion volume spheres and used for the selection of compounds. The MMFF94-based minimization of 18α- and 18β-GA was performed within LigandScout. The minimization was carried out until a local energy minimum was reached.

2.3. 11β-HSD activity assays

HEK-293 cells, devoid of endogenous expression of 11β-HSD1 and 11β-HSD2 [31], were transfected with pcDNA3 plasmids containing either human 11β-HSD1 or 11β-HSD2 with a C-terminal FLAG epitope. The FLAG epitope did not affect enzymatic activities. HEK-293 cell clones stably expressing 11β-HSD1 (referred to as AT6) or 11β-HSD2 (referred to as AT8) were selected as described earlier [32]. Cells were grown in 10 cm dishes to confluence, washed with steroid-free medium, suspended and aliquots centrifuged at 150 × g for 3 min. Cell pellets were flash-frozen in a dry ice ethanol bath and stored at −70 °C until further use. Upon thawing, activities in cell lysates were immediately determined as described earlier

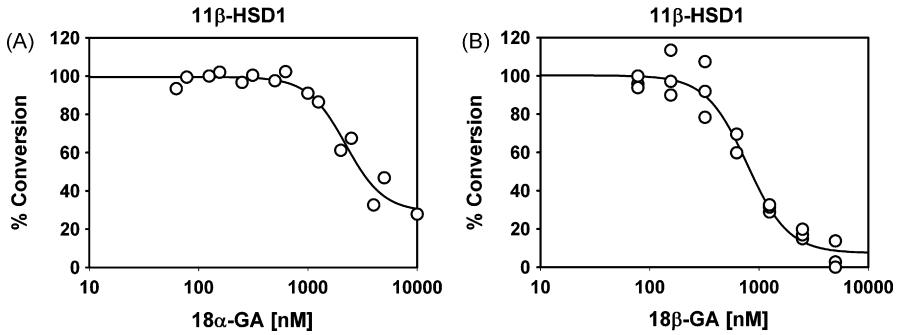

Fig. 2. Inhibition of 11β-HSD1 by 18α-GA and 18β-GA: the conversion of cortisone to cortisol was measured in lysates of HEK-293 cells transfected with human 11β-HSD1 as described in Section 2. Inhibition of 11β-HSD1 dependent reduction of cortisone to cortisol by various concentrations of 18α-GA (A) and 18β-GA (B).

[32]. 11β-HSD1 dependent reduction of [1,2-^{3}H]-labeled cortisone (American Radiolabeled Chemicals, St. Louis, MO) to cortisol was measured for 10 min at 37 °C in a volume of 22 μl containing a final concentration of 200 nM cortisone and 500 μM NADPH. 11β-HSD2 dependent oxidation of cortisol to cortisone was measured similarly using [1,2,6,7-^{3}H]-cortisol (Amersham Pharmacia, Piscataway, NJ, USA) at a final concentration of 50 nM and NAD^+ (500 μM). 18α-GA and 18β-GA at final concentrations between 50 nM and 20 μM were diluted from stock solutions in dimethylsulfoxide and immediately used for activity assays. The solvent concentration did not exceed 0.1% 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 and 95% confidence intervals (CI)) were obtained from three independent experiments.

3. Results and discussion

In this study, both diastereomers, 18α-GA and 18β-GA, were evaluated for their inhibitory activity against recombinant human 11β-HSD1 and 11β-HSD2. Previous studies analyzed the activity of 18β-GA on 11β-HSD1 and 11β-HSD2 using microsomal fractions prepared from rat liver (for 11β-HSD1) and rat kidney (which expresses mainly 11β-HSD2), respectively. For the rat enzymes 85% inhibition of 11β-HSD1 and 101% inhibition of 11β-HSD2 at compound concentrations of 10 μM have been reported [19–22]. Our measurements of the inhibitory effect of 18β-GA on human 11β-HSD1 and 11β-HSD2 resulted in IC_{50} values of 779 nM (95% CI: 632–960 nM) and 257 nM (95% CI: 186–354 nM) (Fig. 2). Statistical analysis using the extra sum-of-squares *F*-test at $p = 0.05$ revealed a significant preferential inhibition of human 11β-HSD2 over 11β-HSD1 and correspond well with previously reported data [19–22,25]. In contrast, the diastereomer 18α-GA did not inhibit 11β-HSD2 at concentrations up to 20 μM but instead dose-dependently inhibited 11β-HSD1 with an IC_{50} of 2214 nM (95% CI: 1654–2963 nM). Both 18α-GA and 18β-GA seem to act as competitive inhibitors of the 11β-HSD1 dependent conversion of cortisone to cortisol at saturating concentrations of NADPH (data not shown).

The crystal structure of human 11β-HSD1 in complex with the GA derivative carbenoxolone (2bel) from the Protein Data Bank was used for a structure-based molecular modeling approach in order to identify novel structural characteristics in the binding of GA derivatives to 11β-HSD1 [33,34]. A structure-based pharmacophore model based on the interactions of carbenoxolone with 11β-HSD1 was built using the software LigandScout and used for the identification and visualization of protein–ligand interactions. Crucial interactions included hydrogen bonds between carbenoxolone and the catalytically active amino acid residues Ser172 and Tyr183, as well as with Leu217 and between carbenoxolone and the cofactor NADPH (Fig. 3). Six hydrophobic features represented the vast hydrophobic contacts that carbenoxolone established with the 11β-HSD1 active site (Fig. 3). In addition, 23 exclusion volume spheres, representing areas where the protein lines the binding pocket, were present in the model (not shown).

The previously generated conformations of 18α-GA and 18β-GA were imported into LigandScout and automatically aligned to the pharmacophore model. The PharmScore was calculated separately for each conformation. For further analyses, the conformation with the highest PharmScore was used. For a more detailed analysis, the best fitting conformations of 18α-GA and 18β-GA were minimized in the binding site using a MMFF94 force field using LigandScout. The minimized conformations of 18α-GA and 18β-GA were used to analyze the pharmacophoric interactions of these compounds with the binding site of 11β-HSD1.

Fig. 4 shows the pharmacophoric interactions of 18α-GA (A) and 18β-GA (B) with the binding site derived from the crystal structure of 11β-HSD1 in complex with carbenoxolone. The interaction pattern with 18β-GA contains three hydrogen bonds and six hydrophobic features and has a score of 85.98. The pharmacophore with 18β-GA shows that this isomer can optimally establish hydro-

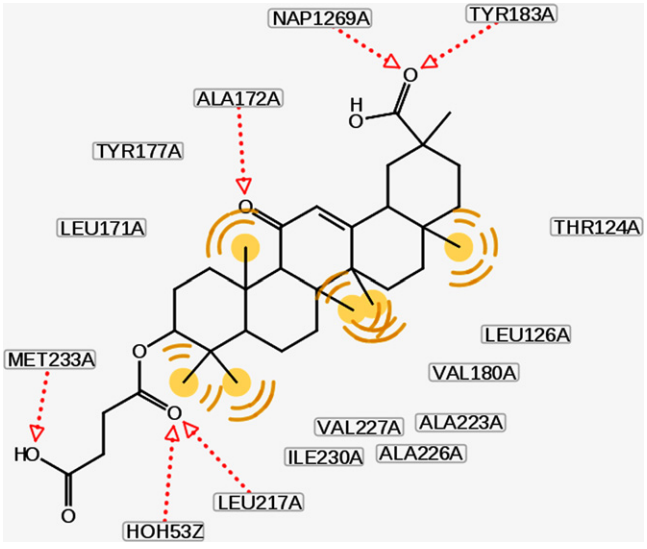

Fig. 3. 2D depiction of the carbenoxolone-derived pharmacophore model for 11β-HSD1 inhibitors. The model comprised hydrogen bonds with the cofactor NADP, the catalytically active amino acid residues Ser172 and Tyr183, as well as with Leu217. Four hydrophobic features represented the vast hydrophobic contacts that carbenoxolone establishes with the 11β-HSD1 active site. Chemical features are color-coded: red, hydrogen bond acceptors; yellow, hydrophobic features. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

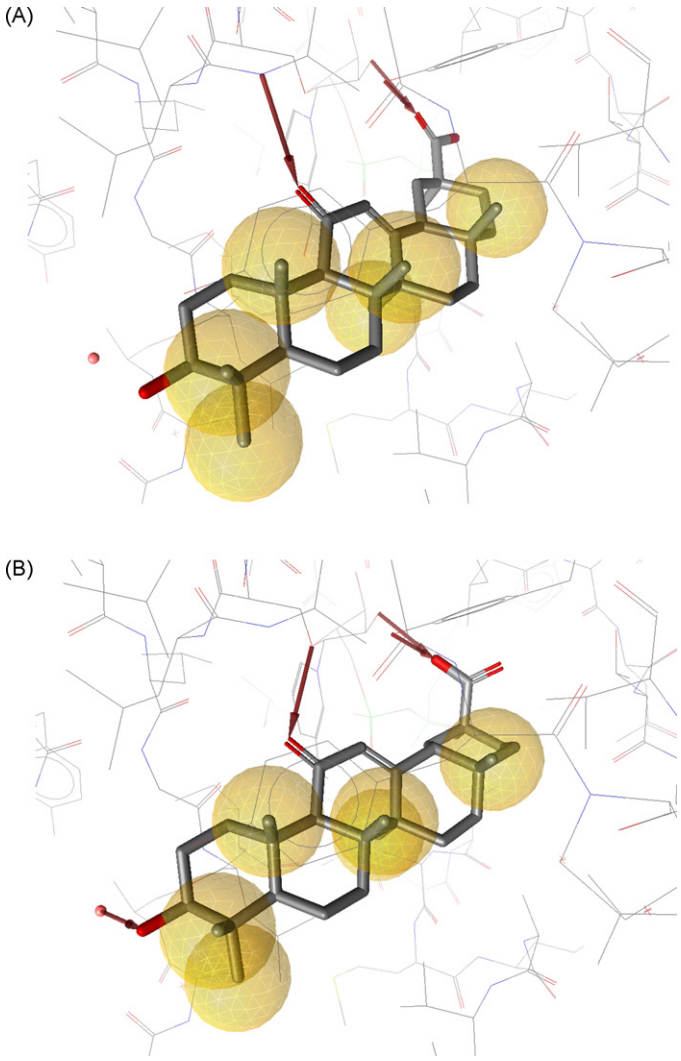

Fig. 4. Pharmacophoric interactions of 18α-GA (left) and 18β-GA (right): pharmacophoric interactions of 18α-GA (left) and 18β-GA (right) with the binding site derived from the crystal structure of 11β-HSD1 in complex with carbenoxolone are shown. Red arrows depict hydrogen bond acceptors whereas yellow spheres illustrate favorable regions for hydrophobic interactions matched by the protein and the ligand. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gen bonds with the enzymes catalytic amino acids Ser170 and Tyr183 as well as with the cofactor NADPH. The 18α-GA isomer on the contrary twists the position of the 11-oxo group in relation to the 30-carboxylic acid group. Thus it cannot ideally interact with the binding site. In addition, the hydrogen bond between the 3-hydroxy group and conserved water, which is bound to the backbone of Leu217 and the carboxylic acid group of Asp259, cannot be established by the 18α-GA derivative. The calculated score for 18α-GA (56.12) is significantly lower compared with that for 18β-GA. This observation indicates that 18α-GA and 18β-GA are not able to establish the same interactions with the 11β-HSD1 binding site. The interaction patterns as well as the calculated scores correlate with the higher activity of 18β-GA against 11β-HSD1 compared to 18α-GA.

4. Conclusions

Here, we describe the discovery that 18α-GA and 11β-GA exhibit different activities against 11β-HSD1 and 11β-HSD2 and provide an explanation for the distinct 11β-HSD1 potency of the two diastereomers. The molecular modeling studies using the pharmacophore model of the 11β-HSD1 binding pocket suggest that 18β-GA but not 18α-GA can optimally establish hydrogen bonds with the catalytic amino acids Ser170 and Tyr183 as well as with the cofactor NADPH. The lower calculated binding score (56.12) for 18α-GA compared with 18β-GA (85.98) correlates with the observed biological activities.

Both, 18α-GA and 18β-GA inhibit human 11β-HSD1 with IC_{50} values of 2214 nM (95% CI: 1654–2963 nM) and IC_{50} = 779 nM (95% CI: 632–960 nM), respectively (Fig. 2). The second isozyme 11β-HSD2 is inhibited by 18β-GA with approximately threefold higher potency (257 nM; 95% CI: 186–354 nM). 18α-GA in contrast, does not inhibit 11β-HSD2 at concentrations up to 20 μM. Although 18α-GA displays a three-fold lower inhibitory potency than 18β-GA to inhibit 11β-HSD1, it has the favorable property of not inhibiting 11β-HSD2, which is in contrast to the native 18β-GA. The selective inhibition of 11β-HSD1 is considered as promising therapeutic strategy for the treatment of different features of the metabolic syndrome like insulin and leptin resistance, visceral obesity, dyslipidemia, type 2 diabetes and cardiovascular complications [7–9]. Using a structure-based optimization process based on the information derived from the established pharmacophore model which is able to explain the differences in the activities of 18α-GA and 18β-GA novel inhibitors can be synthesized or identified among available triterpenes. 18α-GA may thus serve as a basis for the development of more active but selective natural product-derived 11β-HSD1 inhibitors.

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