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A novel 18β-glycyrrhetinic acid analogue as a potent and selective inhibitor of 11β-hydroxysteroid dehydrogenase 2

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Abstract—Using 18β-glycyrrhetinic acid as a template, the synthesis of a series of secondary amides at the 30-position is described and the effects of these modifications on the SAR of the 11β-hydroxysteroid dehydrogenase isozymes type 1 and 2 from the rat are investigated. An isoform selective inhibitor has been discovered and compound **5**, *N*-(2-hydroxyethyl)-3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid amide, is highlighted as a very potent and selective inhibitor of 11β-hydroxysteroid dehydrogenase 2 with an $IC_{50} = 4$ pM.

1. Introduction

The short-chain dehydrogenases/reductases (SDR) are a large well-established family of functionally heterogeneous enzymes present in all forms of life.[1] The 11β-hydroxysteroid dehydrogenases (11β-HSDs) are members of this family and are microsomal enzymes catalysing the conversion of active glucocorticoids to their 11-dehydro products and vice versa.[2] The 11β-hydroxysteroid dehydrogenase isozymes type 1 and 2 are responsible for the interconversion of cortisone (E) and cortisol (F) as shown in Figure 1.

The 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme in the rat is a 32.4 kDa protein that converts cortisone to the active glucocorticoid cortisol and is present in liver, muscle and adipose tissue.[3] Cortisol has a central role in regulating carbohydrate metabolism and its main action in this respect is to oppose the action of insulin, and 11β-HSD1 has been shown to mediate glucocorticoid action and insulin release in pancreatic islets.[4] Gluconeogenesis in the liver is reduced in knockout mice without the 11β-HSD1 gene resulting in lower blood sugar levels.[5] Recently, it has been shown that selective inhibition of 11β-HSD1 decreases blood glucose concentrations in hyperglycaemic mice.[6] The effects of the non-selective 11β-HSD inhibitor carbenoxolone (CBX **2**) on insulin sensitivity in non-obese men with type 2 diabetes have recently been shown to lower glucose levels.[7] Selective 11β-HSD1 inhibitors have the potential for treatment of metabolic disorders such as insulin resistance, obesity, hyperlipidemia and arterial hypertension.[4,8–11] Although it has been known for some time that 18β-glycyrrhetinic acid (18β-GA **1**), a principal active ingredient of liquorice root, and its hemisuccinate derivative CBX **2** (Fig. 2) are potent non-selective inhibitors of both 11β-HSD1 and 11β-HSD2,[12] only recently have selective inhibitors of 11β-HSD1 with nanomolar potency been reported.[8]

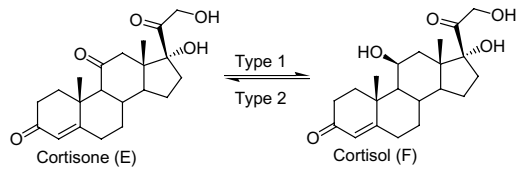

Figure 1. Interconversion of cortisone (E) and cortisol (F) by 11β-HSD type 1 and 2.

The 11β-HSD type 2 isoform in the rat is a 43.7 kDa protein that inactivates the transformation of cortisol to cortisone and is a unidirectional enzyme and exclusively

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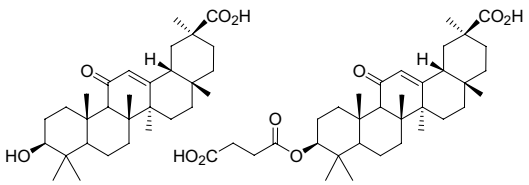


Figure 2. Structures of 18β-glycyrrhetinic acid and carbenoxolone.

acts as a NAD^+ dependent dehydrogenase of adrenal glucocorticoids. This isoform is found primarily in mineralocorticoid (MC) tissues such as the kidney, colon, salivary glands and the sodium-transporting epithelia of the lung, a non-classical MC target organ.[13] Inhibition of 11β-HSD2 could be used to potentiate the anti-inflammatory effects of glucocorticoids, leading to a reduction in the amount of glucocorticoid required for anti-inflammatory therapy and in the severity of very significant side-effects that can result from this type of medication. Inhibition of 11β-HSD2 with liquorice derivatives results in cortisol-dependent mineralocorticoid excess with hypertension and hypokalemic alkalosis.[14,15] Disruption or mutation in the 11β-HSD2 gene results in sodium retention, hypokalemia and hypertension because of inappropriate glucocorticoid occupation of the mineralocorticoid receptor in the kidney, where 11β-HSD2 is principally expressed in the distal nephron.[5,16] A recent review on the 11β-HSDs highlighted growing awareness of the link between 11β-HSD2 and cancer.[17] Adrenal cortical adenomas and carcinomas synthesise 11β-HSD2,[18] and 11β-HSD2 levels are high in pituitary tumours.[19,20] Ductal and lobular breast epithelial cells have also been shown to synthesise 11β-HSD2, with increased synthesis of 11β-HSD2 seen in invasive carcinomas.[21] Transfection experiments to study the role of 11β-HSDs showed that in stably transfected cells overexpressing *HSD11B2* cell proliferation was increased.[22] In addition, the observation that 11β-HSD2 potentiates the antiproliferative effects of glucocorticoids in endometrial and some breast cancer cell lines highlights a putative role for 11β-HSD2 activity in tumourigenesis.[23] These observations validate the need for selective inhibitors of 11β-HSD1 and 11β-HSD2 as therapeutic agents or tools for further mechanistic studies.

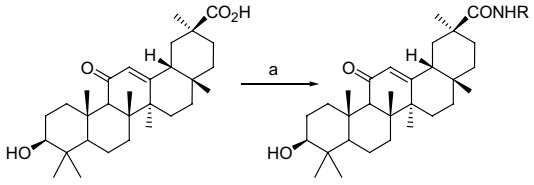
A number of natural products have been reported to inhibit the 11β-HSDs and these include xenobiotics, flavonoids, polyphenolic compounds from tea, gossypol and the constituents of Saiboku-To, a Chinese herbal remedy.[24,25] In general, these compounds are non-selective inhibitors of the 11β-HSDs and are much less potent than 18β-GA **1**. Some selectivity for 11β-HSD1 has been reported for weakly inhibiting synthetic steroids and chenodeoxycholic acid; the diuretic furosemide has also been reported as a non-selective inhibitor.[24,25]

As 18β-GA **1** and CBX **2** are potent inhibitors of 11β-HSD lacking selectivity, a programme of work was initiated to evaluate the effects on SAR by modifying the 18β-GA template on 11β-HSD activity and selectivity. The use of a number of 18β-GA derivatives was the topic of a recent patent by our group.[26] Here we introduce a range of secondary amides at the 30-position of the 18β-GA template and report the effects of the resulting novel compounds on the SAR of 11β-HSD1 and 11β-HSD2 enzymes from the rat.

2. Chemistry

The synthesis of a set of amides by modification of the carboxylic acid of the 18β-GA template **1** was targeted as a key area of the molecule, where functionality is present for rapid modification.

A set of amides **3**–**9** were synthesised by activation of the carboxylic acid moiety at the 30-position followed by reaction with a range of primary amines. Syntheses were performed in parallel using the water soluble EDCI and HOBt to activate the carboxylic acid and treatment with the requisite amine in the presence of DMAP gave the secondary amides **3**–**9** (Scheme 1).[27] The compounds listed in Table 1 were synthesised by this method.



Scheme 1. Synthesis of 18β-glycyrrhetinic acid amides. Reagents and conditions: (a) EDCI, RNH_2, CH_2Cl_2, DMAP, HOBt, rt.

2.1. General synthetic procedure for the synthesis of 18-GA 30-amides

To a solution of 18β-GA (**1**) (0.5 mmol) in DCM (15 mL) the amine (1.0 mmol), HOBt (0.26 mmol), EDCI (0.55 mmol), DMAP (0.55 mmol) and triethylamine (0.55 mmol) were added. The mixture was stirred under nitrogen at room temperature for 16–24 h. When TLC showed the completion of the reaction, the reaction mixture was poured into water and extracted with DCM. The organic phase was washed with 2% HCl and water and dried over $MgSO_4$. Evaporation of the solvent gave a residue that was purified by flash chromatography (SiO_2, ethyl acetate–hexane, gradient elution) to give the amide product. Yields of purified compounds were between 43% and 75%. The properties of a typical example and the most potent 11β-HSD2 inhibitor are shown below for compound **5**.

*N-(2-Hydroxyethyl)-3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid amide (**5**)*. White crystalline solid (110 mg, 43%); mp 160–163 °C; HPLC purity 96% (t_R 6.2 min in 55% water–methanol); ^1H NMR (400 MHz, $CDCl_3$): δ 6.06 (t, $J = 5.9$ Hz, 1H, NH), 5.61 (s, 1H, 12-H), 3.67 (t, $J = 5.1$ Hz, 2H, CH_2), 3.39 (m, 2H, –NHCH_2–), 3.16

(m, 1H, 3-H), 2.72 (dt, $J = 13.6$, 3.6 Hz, 1H, 1β-H), 2.27 (s, 1H, 9α-H), 2.12 (t, $J = 8.6$ Hz, 1H, 18β-H), 1.30 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.74 (s, 3H, CH_3); FAB-MS: m/z 542 (100, MH^+); FAB-HRMS calcd for $C_{35}H_{52}NO_5$ (MH^+) 542.3845, found 542.3853.

Extensive further modifications to the GA template have been performed and will be published elsewhere. The most potent and selective 11β-HSD2 inhibitor from all modifications was identified from the series synthesised as described above and is reported herein.

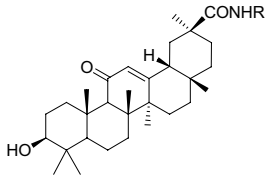
3. Biology

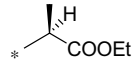
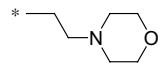
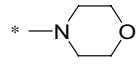
Assays were performed essentially as described previously.[26] Kidneys and livers from normal Wistar rats were homogenised on ice in phosphated-buffered saline (PBS)–sucrose buffer (1 g/10 mL) using an Ultra-Turrax. After the livers and kidneys were homogenised, the homogenate was centrifuged for 5 min at 4000 rpm. The supernatant obtained was removed and stored in glass vials at −20 °C. The amount of protein per microlitre of rat liver (11β-HSD1) and kidney cytosol (11β-HSD2) was determined using the Bradford method.[28] For the assay of 11β-HSD type 1 activity rat cytosol (76 μg protein) was incubated for 90 min with [^{3}H]cortisone and adjusted to a final substrate concentration of 0.5 μM with cortisone. The assay was carried out in PBS with NADPH (1 mM) as the co-factor. At the end of the incubation period, [4-^{14}C]cortisol was added to monitor procedural losses together with 50 μg of unlabelled cortisol to assist in the location of product steroid. Using this assay, conversion of cortisone to cortisol was linear with respect to time (0–120 min) and protein concentration (10–100 μg).

The assay for 11β-HSD type 2 activity employed rat kidney cytosol (136 μg protein) and [^{3}H]cortisol adjusted to a final substrate concentration of 0.5 μM with cortisol. PBS–sucrose was used as the buffer with NADP (1 mM) as the co-factor. [4-^{14}C]Cortisone was added at the end of the incubation period (60 min) to monitor procedural losses with 50 μg unlabelled cortisone being added to assist product location.

At the end of the incubation period and addition of [4-^{14}C] and unlabelled product steroids, precursor and product steroids were extracted with diethyl ether (4 mL). The organic phase was removed and taken to dryness under air. The resulting residue was dissolved in diethyl ether (50–100 μL), transferred to a thin-layer chromatography plate, which contained a fluorescent dye, and developed using the system chloroform–methanol (9:1 v/v). Product steroids were visualised under UV light. In this system, the R_f values for cortisol and cortisone were 0.5 and 0.8, respectively. The areas containing cortisol or cortisone were cut from the TLC plate and placed in a 20 mL scintillation vials. Steroids were eluted from the plate by the addition of methanol (0.5 mL) before the addition of scintillation fluid (10 mL) and determining the radioactivity using scintillation spectrometry. Enzyme activity was determined as the amount of product formed/mg protein/h after correction for procedural losses. Assays were carried out in the absence or presence of inhibitors. Initially, all compounds were tested at 10 μM.

Table 1. Secondary amide derivatives of 18β-GA (**1**) at the 30-position and their inhibition of 11β-HSD1 and 11β-HSD2



Compound	R	% Inhibition of 11β-HSD1 at 10 μM[a]	% Inhibition of 11β-HSD2 at 10 μM[a]
1		85	101
3		90	103
4		25	35
5	CH_2CH_2OH	36	92
6	$CH_2(CH_2)_4OH$	88	98
7		71	25
8		19	66
9		34	20

* Indicates point of attachment.

[a] Mean of at least two measurements with typically a SD ±5% variation.

4. Results and discussion

On the rat 11β-HSD type 1 and 2 isozymes 18β-GA **1** shows potent and non-selective inhibition at 10 μM. Although being non-selective, 18β-GA **1** inhibits the type 2 enzyme to a higher degree as evidenced by the greater inhibition of 11β-HSD2 over 11β-HSD1 at different concentrations.[26] As **1** shows maximal inhibition of 11β-HSD2 at 10 μM, compounds synthesised by template modifications were screened at 10 μM against both isozymes. The secondary amide modifications at the 30-position (Table 1) gave a wide range of activities on both 11β-HSD1 and 11β-HSD2.

In general terms compounds have been classed as showing some selectivity if greater than 2.5-fold difference in % inhibition was observed provided at 10 μM >50% inhibition was seen with one isozyme. Most

compounds showed a greater inhibition of 11β-HSD2 over 11β-HSD1 and were non-selective. The cyclopropyl analogue **3** was synthesised to examine the effect of a small hydrophobic group in this region and compound **3** was a potent non-selective inhibitor of both 11β-HSD1 and 11β-HSD2. The amide from alanine ethyl ester **4** further probes for a hydrophobic interaction with some hydrogen bond acceptor and directional electronic capability in the side chain. Weak activity on both isozymes was observed with **4** and a similar cyclic compound **7** was also weakly active on the type 2 enzyme but showed moderate inhibition of 11β-HSD1 and some selectivity for this enzyme. The *N*-hydroxyethyl amide **5** was the most potent compound for 11β-HSD2 showing the greatest selectivity over 11β-HSD1. This may highlight the importance of the hydroxyl group in this region to form hydrogen bond donor/acceptor interactions with the 11β-HSD2 enzyme. The position of the hydroxyl group in **5** is important for selectivity as the 5-hydroxypentyl analogue **6** is potent on both 11β-HSD1 and 11β-HSD2. The *N*-ethylmorpholino derivative **8** shows moderate activity for the type 2 enzyme with ~3.5-fold selectivity. Interestingly, for compound **9**, where the morpholino moiety is directly attached to the amide, activity is greatly reduced and selectivity is abolished. This is an area of the template for further exploration of the effects of basic and hydrophilic groups. The only compound in this series which showed >2.5-fold selectivity as measured by % inhibition at 10 μM and an inhibition >90% at 10 μM for 11β-HSD2 was compound **5**. The 2-hydroxyethyl analogue **5** was designed to mimic the C-17 side chain of cortisol/cortisone and probe the hydrogen bonding characteristics in this region. Although being longer than the side chain in cortisone, the amide carbonyl in **5** is isosteric with the ketone at the 20-position of cortisone and the hydroxyl is in a similar spatial region to the C-21 hydroxyl in cortisone. This compound has an IC_{50} of 4 pM against 11β-HSD2 (Table 2) with only 36% inhibition against 11β-HSD1 at 10 μM and is the most potent and selective 11β-HSD2 inhibitor reported against the rat 11β-HSD2 enzyme to date.

Table 2. IC_{50} value for selective inhibitor of 11β-HSD2

Compound	IC_{50} (nM) on 11β-HSD2[a]
5	0.004

[a] Mean of at least two measurements with typically ±5% variation.

5. Conclusions

A novel highly potent and selective inhibitor of rat 11β-HSD2 has been identified and the knowledge of the preliminary SAR of glycyrrhetinic acid analogues with modifications at the 30-position as inhibitors of the 11β-hydroxysteroid dehydrogenases has been extended. Compound **5** is the most potent selective 11β-HSD2 inhibitor reported to date with an $IC_{50} = 4$ pM on rat 11β-HSD2. Compounds such as **5** should prove useful as mechanistic tools for in vivo studies to evaluate the biological effects of potent and selective 11β-HSD2 inhibitors in rodents and as templates for further design of non-steroidal 11β-HSD2 inhibitors for potential applications in oncology. The recent publication of a homology model of 11β-HSD2 should accelerate this process.[29]

Acknowledgements

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