TOXICOLOGICAL SCIENCES **126(2)**, 353–361 (2012)
doi:10.1093/toxsci/kfs022
Advance Access publication January 23, 2012

The Anabolic Androgenic Steroid Fluoxymesterone Inhibits 11β-Hydroxysteroid Dehydrogenase 2–Dependent Glucocorticoid Inactivation

Cornelia Fürstenberger,*,† Anna Vuorinen,‡,§ Thierry Da Cunha,*,† Denise V. Kratschmar,*,† Martial Saugy,¶,|| Daniela Schuster,‡,§ and Alex Odermatt*,†,[1]

*Swiss Center for Applied Human Toxicology and †Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland; ‡Institute of Pharmacy/Pharmaceutical Chemistry and §Center for Molecular Biosciences Innsbruck-CMBI, University of Innsbruck, A-6020 Innsbruck, Austria; ¶Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Geneva-Lausanne, Switzerland; and ||Centre Hospitalier Universitaire Vaudois, University of Lausanne, 1066 Epalinges, Switzerland

[1]To whom correspondence should be addressed at Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland. Fax: +41 61 267 1515. E-mail: alex.odermatt@unibas.ch.

Received November 16, 2011; accepted January 13, 2012

Anabolic androgenic steroids (AAS) are testosterone derivatives used either clinically, in elite sports, or for body shaping with the goal to increase muscle size and strength. Clinically developed compounds and nonclinically tested designer steroids often marketed as food supplements are widely used. Despite the considerable evidence for various adverse effects of AAS use, the underlying molecular mechanisms are insufficiently understood. Here, we investigated whether some AAS, as a result of a lack of target selectivity, might inhibit 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2)–dependent inactivation of glucocorticoids. Using recombinant human 11β-HSD2, we observed inhibitory effects for several AAS. Whereas oxymetholone, oxymesterone, danazol, and testosterone showed medium inhibitory potential, fluoxymesterone was a potent inhibitor of human 11β-HSD2 (half-maximal inhibitory concentration [IC_{50}] of 60–100nM in cell lysates; IC_{50} of 160nM in intact SW-620, and 530nM in MCF-7 cells). Measurements with rat kidney microsomes and lysates of cells expressing recombinant mouse 11β-HSD2 revealed much weaker inhibition by the AAS tested, indicating that the adverse effects of AAS-dependent 11β-HSD2 inhibition cannot be investigated in rats and mice. Furthermore, we provide evidence that fluoxymesterone is metabolized to 11-oxofluoxymesterone by human 11β-HSD2. Structural modeling revealed similar binding modes for fluoxymesterone and cortisol, supporting a competitive mode of inhibition of 11β-HSD2–dependent cortisol oxidation by this AAS. No direct modulation of mineralocorticoid receptor (MR) function was observed. Thus, 11β-HSD2 inhibition by fluoxymesterone may cause cortisol-induced MR activation, thereby leading to electrolyte disturbances and contributing to the development of hypertension and cardiovascular disease.

Key Words: **anabolic androgenic steroid; fluoxymesterone; testosterone; 11β-hydroxysteroid dehydrogenase; hypertension; glucocorticoid; cardiovascular disease.**

The misuse of anabolic androgenic steroids (AAS) represents a serious issue both in sports and in specific subsets of the population (Basaria, 2010). AAS are not only used as ergogenic drugs by athletes to enhance performance during competition but also for bodybuilding and for improving body image. Estimates suggested that the lifetime prevalence of AAS use in the United States is about 3% and that up to 6% of high school boys and 2% of girls use AAS (Basaria, 2010; Buckley *et al*., 1988; Hall, 2005; Kanayama *et al*., 2009; Yesalis *et al*., 1997). In addition to endogenously occurring steroids and synthetic AAS developed for clinical applications such as muscle wasting in human immunodeficiency virus infection and aging, an increasing number of designer steroids that have never been clinically tested often are present in dietary supplements, mostly without proper labeling (Kuhn, 2002; Parr and Schanzer, 2010).

The use of AAS has been associated with a series of adverse effects, including hepatotoxicity, psychological disturbances, virilization, suppressed spermatogenesis, gynecomastia, as well as premature mortality mainly as a result of cardiovascular complications (Handelsman, 2011; Parssinen *et al*., 2000). AAS exert direct effects on cardiomyocytes and vascular cells and indirect effects by decreasing high-density lipoprotein cholesterol and leading to early abnormalities in blood clotting factors (Ansell *et al*., 1993; Calof *et al*., 2005; Glazer, 1991). An impaired systolic and diastolic function has been directly associated with the duration and dose of AAS use in a recent study in power athletes (D'Andrea *et al*., 2007). Furthermore, AAS use has been associated with increased fluid retention and elevated blood pressure (Sullivan *et al*., 1998). Despite the recognition of the serious adverse cardiovascular effects of AAS misuse, the underlying molecular mechanisms remain largely unknown.

Impaired corticosteroid metabolism and activation of mineralocorticoid receptors (MR) have been associated with

cardiovascular disease (Briet and Schiffrin, 2010; Hadoke *et al*., 2009; Lastra *et al*., 2010). Two clinical hallmark studies supplementing the standard therapy (angiotensin-converting enzyme inhibitor, loop diuretic, and digoxin) with the MR antagonists spironolactone (Randomized Aldactone Evaluation Study, Pitt *et al*., 1999) or eplerenone (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study, Pitt *et al*., 2003b) revealed significantly reduced mortality in patients with myocardial infarction. Moreover, in patients with essential hypertension and left ventricular hypertrophy (4E-left ventricular hypertrophy study, Pitt *et al*., 2003a), the administration of the MR antagonist eplerenone lowered blood pressure and reduced left ventricular hypertrophy. Importantly, aldosterone levels were low in these patients, suggesting activation of MR by glucocorticoids, alternative ligands, or ligand-independent receptor activation (Frey *et al*., 2004; Odermatt and Atanasov, 2009).

Glucocorticoid-induced MR activation is especially relevant in situations of impaired activity of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts active 11β-hydroxyglucocorticoids (cortisol and corticosterone) into their inactive 11-keto forms (cortisone and 11-dehydrocorticosterone) (Frey *et al*., 2004; Odermatt and Kratschmar, 2012). In addition to distal colon and renal cortical collecting ducts, coexpression of 11β-HSD2 and MR has been reported in the atria and the ventricles of the heart and in endothelial cells and vascular smooth muscle cells of the aorta (Caprio *et al*., 2008; Klusonova *et al*., 2009; Naray-Fejes-Toth and Fejes-Toth, 2007). Inhibition of 11β-HSD2 is expected to cause excessive cortisol-induced MR activation in these tissues. The impact of impaired 11β-HSD2 activity has been investigated in apolipoprotein E (−/−)/11β-HSD2 (−/−) double knockout mice where the loss of 11β-HSD2 stimulates inflammation and strikingly promotes atherosclerosis in an MR-dependent manner (Deuchar *et al*., 2011). In humans, genetic defects in the gene encoding 11β-HSD2 result in cortisol-induced MR activation and severe hypertension, ultimately leading to death by cardiovascular complications (Ferrari, 2010).

In the present study, we tested the hypothesis that some widely used AAS compounds might inhibit 11β-HSD2 and/or directly modulate MR activity.

MATERIALS AND METHODS

Materials. Methenolone was purchased from Cerilliant Corporation (Round Rock, TX), formebolone from LPB Instituto Farmaceutico S.p.A. (Cinisello, Milano, Italy), and other steroids from the National Analytical Reference Laboratory (Pymble, Australia) and from Steraloids (Newport, RI). The reagents and solvents used were of analytical and high performance liquid chromatography grade. [1,2,6,7-3H]-cortisol was purchased from PerkinElmer (Waltham, MA). Cell culture media were purchased from Invitrogen (Carlsbad, CA); all other chemicals were from Fluka AG (Buchs, Switzerland) of the highest grade available.

Cell culture. HEK-293 cells stably transfected with human 11β-HSD2 (Schweizer *et al*., 2003), human SW-620 colon carcinoma cells, and human MCF-7 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 50 U/ml penicillin/streptomycin, 2mM glutamine, and 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

Determination of inhibition of human and mouse 11β-HSD2 using cell lysates. The conversion of cortisol to cortisone by 11β-HSD2 was measured as described previously (Gaware *et al*., 2011). Briefly, lysates of stably transfected HEK-293 cells were incubated for 10 min (human 11β-HSD2) or 20 min (mouse 11β-HSD2) at 37°C in a total volume of 22 µl of TS2 buffer (100mM NaCl, 1mM ethylene glycol tetraacetic acid [EGTA], 1mM EDTA, 1mM $MgCl_2$, 250mM sucrose, and 20mM Tris-HCl, pH 7.4) containing a final concentration of 200nM cortisol, of which 10% was radiolabeled, 500µM nicotinamide adenine dinucleotide (NAD^+), and vehicle or inhibitor at the desired concentration. Reactions were terminated by adding methanol containing 2mM unlabeled cortisol and cortisone, followed by separation of steroids by thin-layer chromatography (TLC) and analysis by scintillation counting. In addition, the inhibition of the conversion of cortisol to cortisone and fluoxymesterone to 11-oxofluoxymesterone by human 11β-HSD2 in cell lysates was assessed by liquid chromatography-mass spectrometry (for LC-MS method, see below). Lysates of HEK-293 cells stably expressing human 11β-HSD2 were incubated for 10 min at 37°C in a total volume of 500 µl at a final concentration of 200nM cortisol, 500µM NAD^+, and various concentrations of fluoxymesterone. To assess inhibition of fluoxymesterone oxidation, lysates were incubated for 1 h at 37°C with 500nM fluoxymesterone and various concentrations of cortisol. Reactions were stopped by shock freezing in a dry ice-ethanol bath. Stock solutions of all inhibitors were prepared in dimethylsulfoxide (DMSO) or methanol. Further dilutions were prepared prior to use in TS2 buffer to yield final concentrations between 0.6nM and 10µM.

Determination of 11β-HSD2 inhibition using rat kidney microsomes. Rat kidney microsomes were prepared basically as described earlier (Senesi *et al*., 2010). Briefly, the kidneys of male Sprague Dawley rats were homogenized, and the microsomal fraction was isolated by fractional centrifugation. The microsomes were resuspended at a protein concentration of 1 mg protein/ml of buffer containing 100mM NaCl, 1mM EGTA, 1mM EDTA, 1mM $MgCl_2$, 250mM sucrose and 20mM Tris-HCl, pH 7.4, and frozen at −70°C. For 11β-HSD2 activity measurements, microsomes at a final protein amount of 10 µg per reaction were incubated for 10 min at 37°C as described above for cell lysates.

Determination of 11β-HSD2 inhibition in intact SW-620 and MCF-7 cells. 11β-HSD2 activity in intact SW-620 cells was measured as described earlier (Gaware *et al*., 2011). Briefly, 100,000 cells per well of a 96-well plate were incubated in 50 µl steroid-free DMEM containing vehicle or inhibitor at the desired concentration, 10 nCi radiolabeled cortisol and 50nM unlabeled cortisol. SW-620 and MCF-7 cells were incubated for 2 and 4 h, respectively, at 37°C, followed by separation of steroids by TLC and scintillation counting.

Transactivation assay. HEK-293 cells (200,000 cells per 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 (0.04 µg/well), and human recombinant MR (0.35 µg/well). Cells were washed twice with DMEM 6 h posttransfection, followed by incubation for another 18 h 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 DMSO (0.05%) or aldosterone (10nM) in the presence or absence of fluoxymesterone (1µM). 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 0.5mM 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 luciferin solution (Kratschmar *et al*., 2011).

Measurement of fluoxymesterone metabolism by LC-MS. Lysates of untransfected HEK-293 cells and HEK-293 cells stably expressing human 11β-HSD2 were incubated for 1 h at 37°C in a total volume of 500 µl at a final concentration of 1µM fluoxymesterone or cortisol and 500µM NAD^+. The reactions were stopped by shock freezing using a dry ice-ethanol bath.

After thawing, samples were centrifuged at 4°C for 10 min at 500 × g and supernatants transferred onto a solid phase extraction column (Oasis HBL 1 cc [30 mg] Waters WAT094225) preconditioned with 1 ml of methanol and 1 ml of distilled water. After washing twice with 1 ml of water, steroids were eluted by twice adding 1 ml of methanol. The combined eluate was evaporated to dryness and reconstituted with 100 µl of methanol.

Steroids were resolved on an Atlantis T3 (3 µm, 2.1 × 150 mm) column (Waters, Milford, MA) at 30°C using an Agilent model 1200 Infinity Series chromatograph (Agilent Technologies, Basel, Switzerland). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). To separate fluoxymesterone and 11-oxofluoxymesterone, a linear gradient starting from 55% solvent A and 45% solvent B to 5% solvent A and 95% solvent B from 0 to 12 min was used. To separate cortisol and cortisone, a linear gradient starting from 85% solvent A and 15% solvent B to 60% solvent A and 40% solvent B from 0 to 15 min was used. The flow rate was maintained at 0.4 ml/min.

The LC was interfaced to an Agilent 6490 triple Quad mass spectrometer (MS). The MS was operated in atmospheric pressure electrospray positive ionization mode, with nebulizer pressure and nebulizer gas flow rate of 45 psi and 10 l/h, respectively, a source temperature of 350°C and capillary and cone voltage of 4 kW and 190 V, respectively. Data acquisition was performed by Mass Hunter workstation (Version B01.04). Metabolites were identified by comparing their retention time and mass to charge ratio (*m/z*) with those of authentic standards.

Qualitative determination of steroids was performed by selected positive ionization multiple reaction monitoring. Fluoxymesterone (*m/z* 337.1) and 11-oxofluoxymesterone (*m/z* 335.1) eluted at 9.1 and 9.7 min, respectively. For the quantitative determination of fluoxymesterone, prednisolone (*m/z* 361.1, retention time 7.3 min) was used as internal standard. Metabolites were quantified from calibration curves of the ratio of the peak area of the authentic fluoxymesterone standard and the internal prednisolone standard incubated with lysates of untransfected HEK-293 cells at a total protein concentration identical to that of the experimental setting and plotted against the concentration of authentic standards (normalization to the internal standard). A reference standard for 11-oxofluoxymesterone was not available and needs to be synthesized for confirmation of the structure and exact quantification. Cortisone (precursor and product ion at *m/z* 361 and 163) and cortisol (precursor and product ion at *m/z* 363 and 121) eluted at 8.5 and 8.9 min, respectively.

Determination of the binding mode of steroids in 11β-HSD2 by molecular modeling. The 2D structures of fluoxymesterone, testosterone, cortisone, and cortisol were generated using ChemBioDraw Ultra 12.0 (CambridgeSoft, U.K., 1986–2009, www.cambridgesoft.com). Two-dimensional structures were transformed into 3D using CORINA (Molecular Networks GmbH, Erlangen, Germany). These four compounds were docked into the previously generated homology model of 11β-HSD2 (Gaware *et al*., 2011) using GOLD (Jones *et al*., 1997; Verdonk *et al*., 2003). The binding site was defined by a 10 Å sphere with the hydroxyl group of Tyr232 in the center. To optimize the ligand orientation, two protein hydrogen bond constraints were defined: one to the catalytically active Tyr232 and the second one to Tyr226. During the docking, the protein was handled as rigid and ligands as flexible. The ligand flexibility was increased by allowing the ring corner to flip. GoldScore was used as a scoring function, and 10 docking solutions for each ligand were calculated. Atom types for the protein as well as for the ligands were automatically determined by the program. LigandScout (Wolber and Langer, 2005) was used for visualizing the docking results and for the analysis of the protein-ligand interactions.

RESULTS

Inhibition of 11β-HSD2 by Anabolic Androgenic Steroids Measured in Cell Lysates

In order to test the hypothesis that AAS might cause adverse effects by disrupting corticosteroid hormone action, we tested several frequently used AAS for potential inhibition of the 11β-HSD2–dependent conversion of cortisol to cortisone as well as for their impact on MR transactivation. The activity of human 11β-HSD2 was assessed using lysates of stably transfected HEK-293 cells and by determining the conversion of radio-labeled cortisol to cortisone. At a high concentration (10µM), only norbolethone and methenolone did not have any inhibitory effect (Table 1). Dehydroepiandrosterone, mesterolone, and formebolone showed weak inhibitory effects with estimated Half-maximal inhibitory concentration (IC_{50}) values of 10µM or higher. For the compounds that showed less than 40% remaining activity at this concentration, IC_{50} values were determined. This revealed potent inhibition by the 11β-hydroxylated steroid fluoxymesterone with IC_{50} of 60 ± 27nM and medium inhibitory effects for testosterone (Fig. 1A), oxymesterone, danazol, and oxymetholone. The inhibition of 11β-HSD2–mediated cortisol oxidation by fluoxymesterone was confirmed in an assay using unlabeled cortisol and quantification of the product cortisone by LC-MS. A slightly higher IC_{50} value of 100 ± 28nM was obtained (data not shown).

Adverse effects are usually studied in a next step in rodents. In preparing for animal studies, we tested whether fluoxymesterone would inhibit rat and mouse 11β-HSD2 *in vitro*. Measurements with rat kidney microsomes revealed an approximately 80-fold lower inhibitory potential with an IC_{50} of 4.9 ± 0.8µM (Fig. 1B). Similarly, an IC_{50} of 5.4 ± 0.4µM was obtained for fluoxymesterone inhibition of 11β-HSD2 using lysates of HEK-293 cells stably expressing the mouse enzyme (Fig. 1C). Testosterone was about 10-fold less potent toward the rat and the mouse enzymes with IC_{50} of 14.9 ± 4.3 and 15.3 ± 3.5µM, respectively. The weak

TABLE 1
Inhibition of 11β-HSD2 by Anabolic Androgenic Steroids

Compound	Remaining cortisol to cortisone conversion at 10µM (%)	IC_{50} (nM)
Glycyrrhetinic acid	2.4 ± 1.8	256 ± 33[a]
Testosterone	12 ± 12	1370 ± 60
Dehydroepiandrosterone	78 ± 12[b]	nd
Danazol	23 ± 17	740 ± 110
Fluoxymesterone	1.9 ± 1.5	60 ± 27
Formebolone	51 ± 12	nd
Mesterolone	74 ± 6	nd
Methenolone	90 ± 3	nd
Norbolethone	92 ± 13	nd
Oxymesterone	2.6 ± 3.5	960 ± 140
Oxymetholone	6.4 ± 4.5	710 ± 50

Notes. The 11β-HSD2–dependent conversion of cortisol (50nM) to cortisone was measured in cell lysates in the presence of 500µM of NAD^+ and various concentrations of AAS or vehicle (0.1% DMSO). Glycyrrhetinic acid was included as reference compound. Inhibitory activities represent IC_{50} ± SD from at least three independent experiments. nd, not determined.

[a]Value taken from Kratschmar *et al*. (2011).

[b]Determined at 20µM.

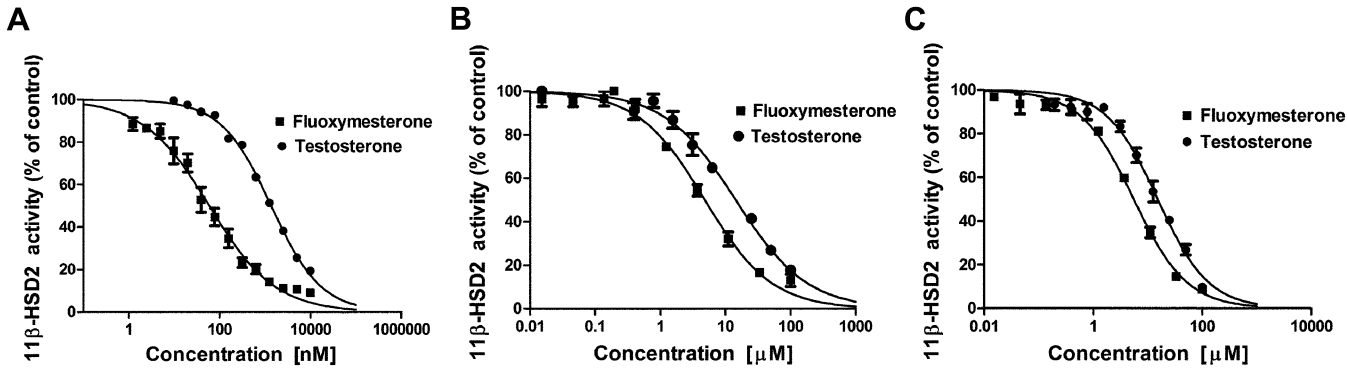

FIG. 1. Inhibition of 11β-HSD2 activity by fluoxymesterone measured in cell lysates. (A) Lysates of HEK-293 cells stably expressing recombinant human 11β-HSD2 were incubated for 10 min at 37°C with 50nM of radiolabeled cortisol and increasing concentrations of fluoxymesterone (squares) or testosterone (circles), followed by determination of cortisone formation. Similarly, inhibition by fluoxymesterone and testosterone was measured using rat kidney microsomes (B) and lysates of HEK-293 cells stably expressing mouse 11β-HSD2 (C). Values were normalized to the activity observed in the presence of vehicle (0.1% DMSO). Data represent mean ± SD from three independent experiments.

inhibitory effects of fluoxymesterone and testosterone on the rodent 11β-HSD2 enzymes make it unlikely to study the adverse effects in these species, and animal experiments were not performed.

Inhibition of 11β-HSD2 in Intact SW-620 and MCF-7 Cells

The effect of fluoxymesterone on 11β-HSD2 activity was next determined in intact cells. Two distinct cell models, human colon carcinoma SW-620 cells and human breast cancer MCF-7 cells with relatively high endogenous expression of the enzyme were applied. Cell models with lower expression may not be suitable to determine direct effects on enzyme activity due to interference with altered gene expression. Fluoxymesterone potently inhibited 11β-HSD2 in a concentration-dependent manner with IC_{50} of 160 ± 10nM in SW-620 cells and IC_{50} of 530 ± 70nM in MCF-7 cells (Fig. 2). Although the known reference compound glycyrrhetinic acid also showed potent inhibition, testosterone did not significantly inhibit 11β-HSD2 at concentrations up to 10μM in intact SW-620 or MCF-7 cells.

Fluoxymesterone Does Not Directly Modulate MR Activation

To see whether fluoxymesterone might directly modulate MR activity, transactivation assays in HEK-293 cells transiently expressing MR and a galactosidase reporter under the control of the mouse mammary tumor virus promoter were performed. Cells were incubated with 1μM of fluoxymesterone alone or together with 10nM aldosterone. The reporter gene response was indistinguishable if cells were treated with either aldosterone alone or in combination with fluoxymesterone (Fig. 3). Furthermore, basal receptor activity measured by DMSO treatment (0.05%) was not changed by fluoxymesterone. Similarly, the other tested AAS (see Table 1) did not activate or antagonize MR function, indicating that these steroids have no direct effect on the receptor.

Fluoxymesterone Is Metabolized by 11β-HSD2

Because fluoxymesterone possesses an 11β-hydroxyl group, we hypothesized that this steroid may be a substrate and therefore competitively inhibit the enzyme. Therefore, we established an LC-MS–based method using the multiple

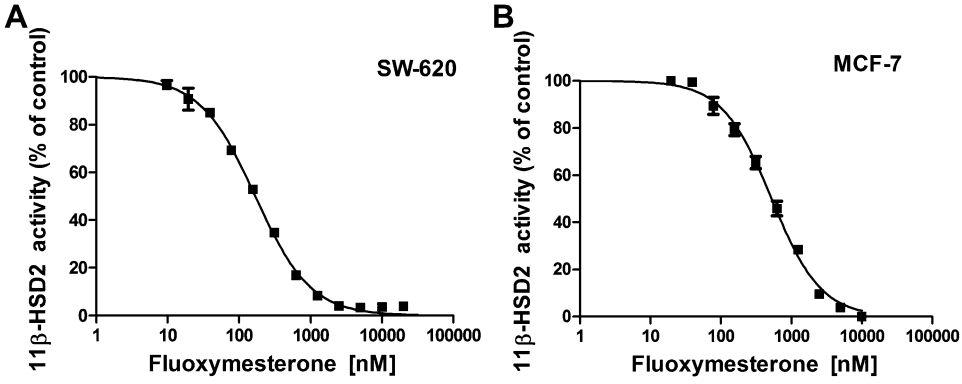

FIG. 2. Inhibition of 11β-HSD2 activity by fluoxymesterone in intact SW-620 and MCF-7 cells. Intact SW-620 and MCF-7 cells, with endogenous expression of 11β-HSD2, were incubated for 2 and 4 h, respectively, with 50nM of radiolabeled cortisol and increasing concentrations of fluoxymesterone. Values were normalized to the activity observed in the presence of vehicle (0.1% DMSO). Data represent mean ± SD from three independent experiments.

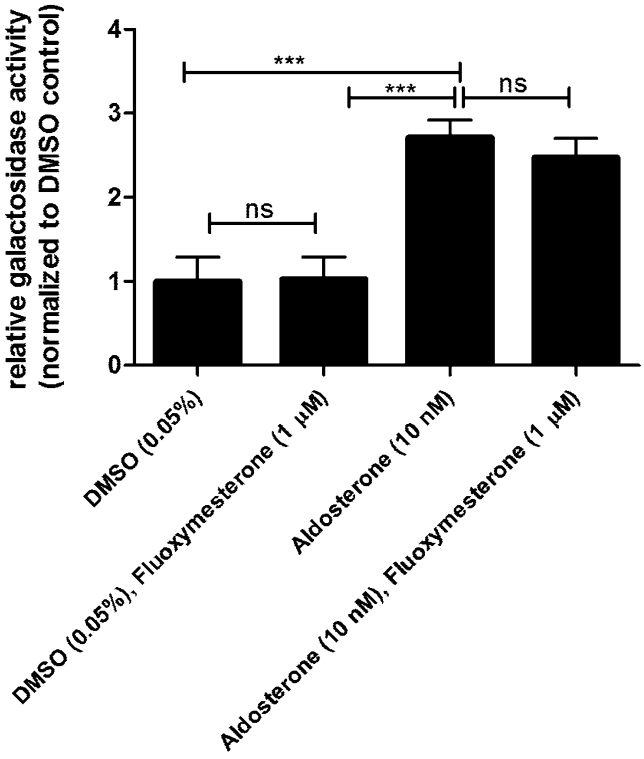

FIG. 3. Effect of fluoxymesterone on MR transactivation. HEK-293 cells were transfected with pMMTV-LacZ reporter, pCMV-LUC control plasmid, and human MR expression plasmid. Cells were incubated for 24 h in the presence or absence of 10nM aldosterone with or without 1μM fluoxymesterone, followed by determination of galactosidase and luciferase activities. Galactosidase reporter activity was normalized to the internal luciferase control. Data were normalized to vehicle control (0.05% DMSO) and represent mean ± SD from a representative experiment performed in triplicate.

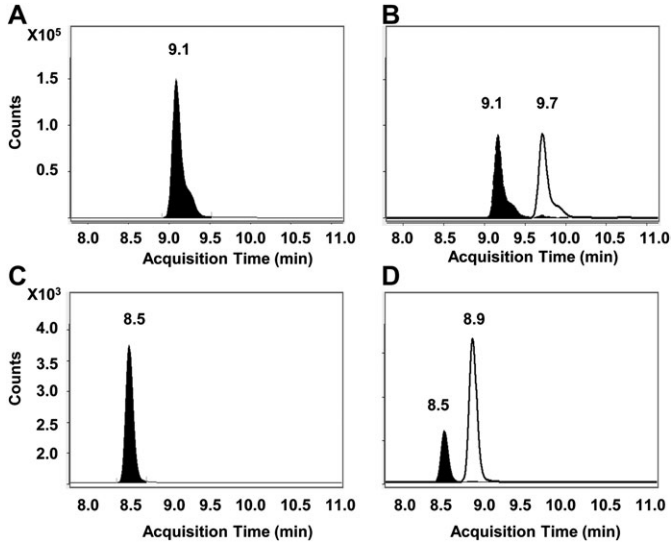

FIG. 4. Representative LC-MS chromatogram showing the metabolism of fluoxymesterone and cortisol by 11β-HSD2. Lysates of untransfected HEK-293 cells (A and C) and lysates of 11β-HSD2–expressing HEK-293 cells (B and D) were incubated for 2 h with 1μM of fluoxymesterone (A and B) or cortisol (C and D), followed by analysis of steroid metabolites by LC-MS. The area under the peak of the substrate is indicated in black and that of the product in white color. Fluoxymesterone (*m/z* 337) eluted at 9.1 min and 11-oxofluoxymesterone (*m/z* 335) at 9.7 min. Cortisol (*m/z* 363, 121) eluted at 8.5 min and cortisone (*m/z* 361) at 8.9 min.

reaction monitoring mode for the qualitative and quantitative determination of fluoxymesterone (*m/z* 337.1) and its metabolite 11-oxofluoxymesterone (*m/z* 335.1) (see Materials and Methods section). Fluoxymesterone and 11-oxofluoxymesterone were clearly separated with retention times of 9.1 and 9.7 min, respectively (Fig. 4). Similarly, conditions were established to separate and quantify cortisol (*m/z* 363, retention time 8.5 min) and cortisone (*m/z* 361, retention time 8.9 min). Upon incubation for 1 h with lysates of HEK-293 cells stably expressing 11β-HSD2, approximately 50% of the supplied fluoxymesterone (1μM) was converted. In comparison, under the same conditions, approximately 80% of cortisol was converted. No conversion was observed when lysates of untransfected HEK-293 cells were used. Thus, although both steroids are substrates of 11β-HSD2 (Fig. 5), the enzyme more efficiently oxidizes cortisol.

To further support the hypothesis that fluoxymesterone and cortisol bind to and are metabolized by 11β-HSD2, we studied the impact of cortisol on the conversion of fluoxymesterone (at a final concentration of 500nM) to 11-oxofluoxymesterone in cell lysates. A concentration-dependent inhibition with an IC_{50} of 730 ± 190nM was obtained (data not shown).

Comparison of the Binding Mode of Steroids in 11β-HSD2

All four compounds were docked into the 11β-HSD2 active site. They were all positioned in the vicinity of the catalytic residue Tyr232, close to the catalytic triad Ser219-Tyr232-Lys236 (Fig. 6A). The binding interactions of each compound were investigated in detail to explain the difference in their activities. The automatically created pharmacophoric interaction pattern showed different hydrogen-bonding patterns for each compound (Figs. 6B–E).

The natural ligand cortisol was predicted to have favorable binding interactions. The 11β-hydroxy group interacts with the

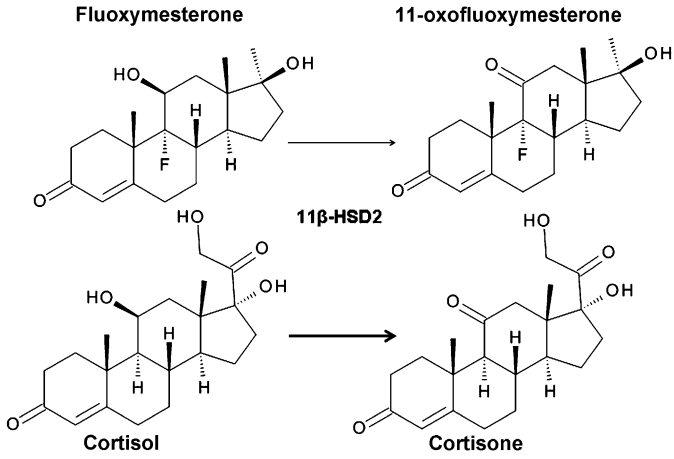

FIG. 5. Structures of 11β-HSD2 substrates and products.

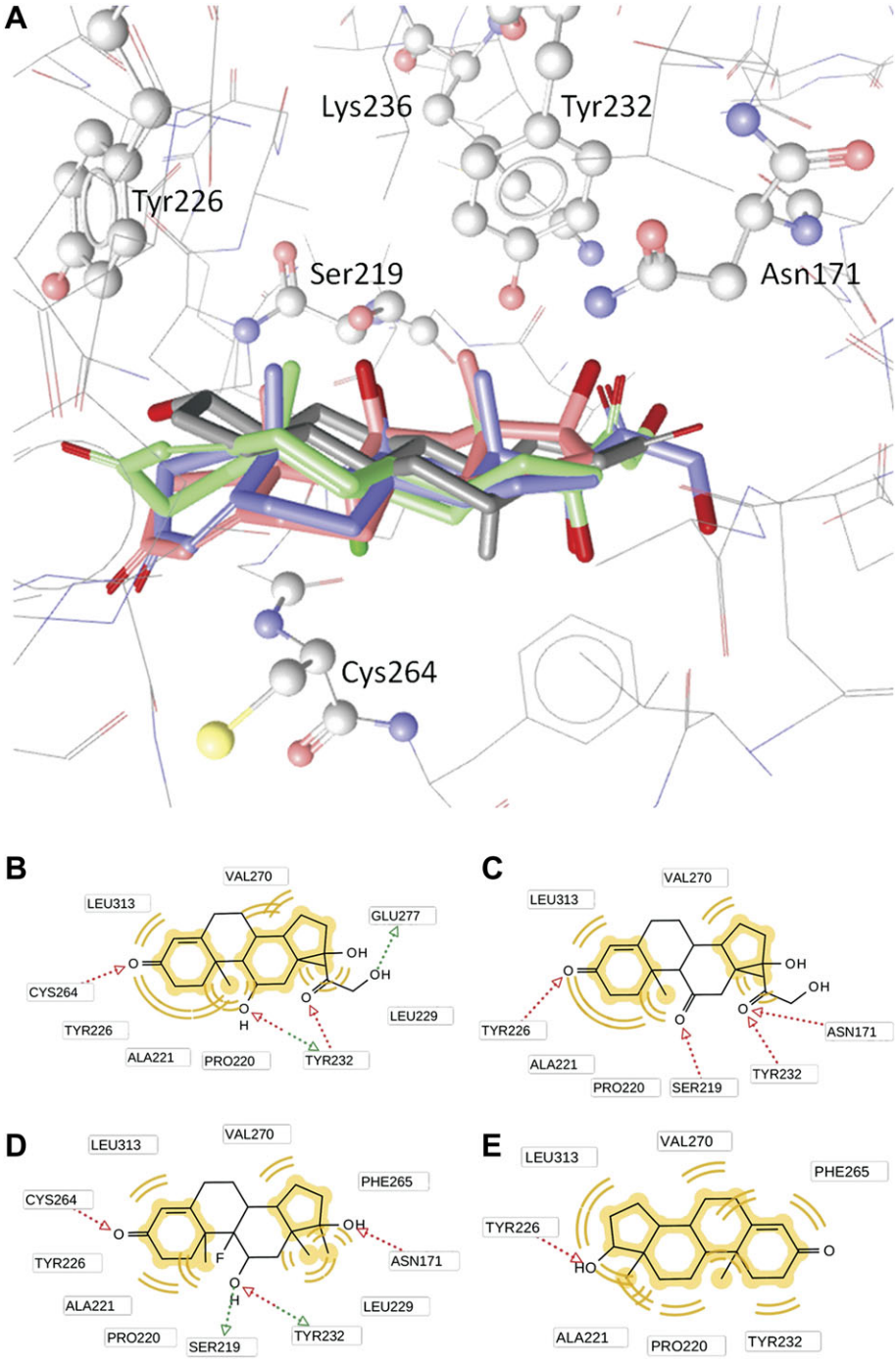

FIG. 6. Modeling of the binding of steroids to 11β-HSD2. (A) Cortisol (blue), cortisone (green), fluoxymesterone (red), and testosterone (gray) docked into the ligand-binding pocket of 11β-HSD2. The catalytic triad as well as other amino acid residues with which common interactions were observed are depicted in ball and stick style. (B–D) Two-dimensional representations of the binding interactions of steroids with 11β-HSD2. Binding interactions of cortisol (B), cortisone (C), fluoxymesterone (D), and testosterone (E) with 11β-HSD2 are shown. The hydrophobic interactions are represented in yellow, hydrogen bond acceptors in red, and hydrogen bond donors in green.

catalytic residue Tyr232, and the ligand was anchored to the binding pocket with three other hydrogen bonds. The interaction pattern of cortisone, the end product of the reaction catalyzed by 11β-HSD2, differed to the one of cortisol in terms that the product moved the 11-keto group away from the catalytically active residues. The most active inhibitor, fluoxymesterone, was observed to be anchored to the binding pocket with four hydrogen bonds. The hydrogen bonds between Tyr232, Ser219,

and the 11-hydroxy group of fluoxymesterone indicated that this molecule can be transformed into its 11-keto form by the enzyme. In fact, biological data showed that fluoxymesterone is a substrate of 11β-HSD2, but with a lower conversion rate than cortisol. This lower conversion rate leads to a competitive inhibition of 11β-HSD2 by fluoxymesterone.

Interestingly, testosterone was predicted to adapt a different binding mode compared with the other three compounds. It was located in the same area as the others; however, it had a flipped binding mode. Whereas cortisol, cortisone, and fluoxymesterone had their 3-keto groups pointing toward Tyr226, the 3-keto group of testosterone superimposed onto position 17 of the other steroids. No other hydrogen bond between testosterone and the protein was observed. This kind of binding mode as well as fewer hydrogen bonds and hydrophobic interactions with the protein could be an explanation for the weaker 11β-HSD2 inhibition of testosterone.

DISCUSSION

In this study, we analyzed several well-known and widely used AAS for their potential to inhibit 11β-HSD2 and identified fluoxymesterone as a potent inhibitor. The IC_{50} values of 60–100nM in cell lysates and 160 and 530nM measured in intact SW-620 and MCF-7 cells, respectively, are in a comparable range with the inhibitory potential of glycyrrhetinic acid, which has been demonstrated in both animals and humans to inhibit 11β-HSD2 and cause glucocorticoid-mediated MR activation, potassium excretion, sodium and water retention, and increased blood pressure (Ferrari, 2010; Ferrari *et al*., 2001; Serra *et al*., 2002). As shown for fluoxymesterone in the present study, glycyrrhetinic acid also does not directly modulate MR function but competitively inhibits 11β-HSD2, thereby leading to glucocorticoid-mediated MR activation.

Oral fluoxymesterone, which is marketed as halotestin in the United States for the treatment of hypogonadism in men and of certain hormone-dependent mammary tumors in women, can exacerbate health states where edema plays important roles, such as congestive heart failure (as described in the package leaflet, http://www.pfizer.com/files/products/uspi_halotestin.pdf). A possible mechanistic explanation for these warnings concerning fluid retention states includes the involvement of MR activation. Following oral administration, fluoxymesterone is rapidly metabolized in the liver. The adverse effects described above can be expected to be much more pronounced upon intravenous application of the drug, which is the preferred route of administration in sports and bodybuilding.

Inhibition of 11β-HSD2 in the renal distal tubules and cortical collecting ducts results in glucocorticoid-induced MR activation with subsequently increased activities of Na^+/K^+-ATPases at the basolateral membrane and of Na^+ channels (ENaC) on the apical membrane of epithelial cells, resulting in potassium excretion and sodium and water retention (Ferrari, 2010). In the vasculature, decreased 11β-HSD2 activity leads to vasoconstriction and elevated blood pressure, probably by a mechanism involving the activation of glucocorticoid receptor rather than MR (Goodwin *et al*., 2008). Furthermore, inhibition of 11β-HSD2 in vascular smooth muscle cells and endothelial cells of the heart may lead to paracrine activation of MR in cardiomyocytes. In a recent study using transgenic mice, Deuchar *et al.* (2011) showed that the ablation of 11β-HSD2 in apolipoprotein E knockout mice aggravated the progression of atherosclerosis by a mechanism involving MR activation and stimulation of inflammation in the vascular endothelium. The use of AAS has been associated with reduced levels of high-density lipoproteins and an increased ratio of low-density lipoproteins to high-density lipoproteins (Glazer, 1991; Thompson *et al*., 1989). Thus, AAS that inhibit 11β-HSD2, like fluoxymesterone, may be especially prone to cause adverse cardiovascular effects.

Regarding the inhibitory mechanism, both the 3D structural modeling and the fact that 11β-HSD2 can metabolize fluoxymesterone to 11-oxofluoxymesterone support a competitive inhibition of cortisol oxidation. The 11β-hydroxyl on fluoxymesterone has important stabilizing interactions that are absent in testosterone and other AAS. Interestingly, formebolone also has an 11β-hydroxyl but is a very weak 11β-HSD2 inhibitor, probably as a result of the methyl group in ring-A, which leads to steric hindrance and prevents optimal stabilization in the binding pocket. Although 11β-HSD2 can oxidize fluoxymesterone at position 11, this reaction represents a minor contribution to the overall metabolism of this steroid (Pozo *et al*., 2008). Compared with cortisol, fluoxymesterone was approximately three to five times less efficiently converted to its 11-oxo form, resembling the conversion of dexamethasone, which also has a fluor atom at position 9 (Rebuffat *et al*., 2004). A reference compound needs to be synthesized to test whether 11β-HSD2 might be able to reduce 11-oxofluoxymesterone as previously observed for 11-oxodexamethasone. This, together with the extensive hepatic metabolism, would explain why 11-oxo derivatives of fluoxymesterone are minor metabolites of fluoxymesterone in human urine (Pozo *et al*., 2008).

The potent inhibition of human but not rat and mouse 11β-HSD2 by fluoxymesterone emphasizes the importance to assess species-specific differences using recombinant enzymes and enzyme preparations from native tissues before conducting animal studies, both from an ethical point of view and to save resources. Clinical studies will need to investigate the potential of fluoxymesterone to inhibit 11β-HSD2 activity *in vivo*. This can be achieved by measuring its impact on the ratio of 11β-hydroxy- to 11-oxoglucocorticoids in plasma and their corresponding tetrahydro metabolites in 24-h urine samples and by examining the patients for the presence of low-renin and low-aldosterone hypertension.

FUNDING

Swiss National Science Foundation (31003A-124912) and Swiss Center for Applied Human Toxicology.

ACKNOWLEDGMENTS

A.O. has a Chair for Molecular and Systems Toxicology by the Novartis Research Foundation. D.S. and A.V. are grateful for their Young Talents Grants from the University of Innsbruck (Nachwuchsförderung).

REFERENCES

Ansell, J. E., Tiarks, C., and Fairchild, V. K. (1993). Coagulation abnormalities associated with the use of anabolic steroids. *Am. Heart J.* **125,** 367–371.

Basaria, S. (2010). Androgen abuse in athletes: Detection and consequences. *J. Clin. Endocrinol. Metab.* **95,** 1533–1543.

Briet, M., and Schiffrin, E. L. (2010). Aldosterone: Effects on the kidney and cardiovascular system. *Nat. Rev. Nephrol.* **6,** 261–273.

Buckley, W. E., Yesalis, C. E., III, Friedl, K. E., Anderson, W. A., Streit, A. L., and Wright, J. E. (1988). Estimated prevalence of anabolic steroid use among male high school seniors. *J. Am. Med. Assoc.* **260,** 3441–3445.

Calof, O. M., Singh, A. B., Lee, M. L., Kenny, A. M., Urban, R. J., Tenover, J. L., and Bhasin, S. (2005). Adverse events associated with testosterone replacement in middle-aged and older men: A meta-analysis of randomized, placebo-controlled trials. *J. Gerontol. A Biol. Sci. Med. Sci.* **60,** 1451–1457.

Caprio, M., Newfell, B. G., la Sala, A., Baur, W., Fabbri, A., Rosano, G., Mendelsohn, M. E., and Jaffe, I. Z. (2008). Functional mineralocorticoid receptors in human vascular endothelial cells regulate intercellular adhesion molecule-1 expression and promote leukocyte adhesion. *Circ. Res.* **102,** 1359–1367.

D'Andrea, A., Caso, P., Salerno, G., Scarafile, R., De Corato, G., Mita, C., Di Salvo, G., Severino, S., Cuomo, S., Liccardo, B., *et al.* (2007). Left ventricular early myocardial dysfunction after chronic misuse of anabolic androgenic steroids: A Doppler myocardial and strain imaging analysis. *Br. J. Sports Med.* **41,** 149–155.

Deuchar, G. A., McLean, D., Hadoke, P. W., Brownstein, D. G., Webb, D. J., Mullins, J. J., Chapman, K., Seckl, J. R., and Kotelevtsev, Y. V. (2011). 11beta-hydroxysteroid dehydrogenase type 2 deficiency accelerates atherogenesis and causes proinflammatory changes in the endothelium in apoe-/- mice. *Endocrinology* **152,** 236–246.

Ferrari, P. (2010). The role of 11beta-hydroxysteroid dehydrogenase type 2 in human hypertension. *Biochim. Biophys. Acta* **1802,** 1178–1187.

Ferrari, P., Sansonnens, A., Dick, B., and Frey, F. J. (2001). In vivo 11beta-HSD-2 activity: Variability, salt-sensitivity, and effect of licorice. *Hypertension* **38,** 1330–1336.

Frey, F. J., Odermatt, A., and Frey, B. M. (2004). Glucocorticoid-mediated mineralocorticoid receptor activation and hypertension. *Curr. Opin. Nephrol. Hypertens.* **13,** 451–458.

Gaware, R., Khunt, R., Czollner, L., Stanetty, C., Da Cunha, T., Kratschmar, D. V., Odermatt, A., Kosma, P., Jordis, U., and Classen-Houben, D. (2011). Synthesis of new glycyrrhetinic acid derived ring A azepanone, 29-urea and 29-hydroxamic acid derivatives as selective 11beta-hydroxysteroid dehydrogenase 2 inhibitors. *Bioorg. Med. Chem.* **19,** 1866–1880.

Glazer, G. (1991). Atherogenic effects of anabolic steroids on serum lipid levels. A literature review. *Arch. Intern. Med.* **151,** 1925–1933.

Goodwin, J. E., Zhang, J., and Geller, D. S. (2008). A critical role for vascular smooth muscle in acute glucocorticoid-induced hypertension. *J. Am. Soc. Nephrol.* **19,** 1291–1299.

Hadoke, P. W., Iqbal, J., and Walker, B. R. (2009). Therapeutic manipulation of glucocorticoid metabolism in cardiovascular disease. *Br. J. Pharmacol.* **156,** 689–712.

Hall, R. C. (2005). Abuse of supraphysiologic doses of anabolic steroids. *South. Med. J.* **98,** 550–555.

Handelsman, D. J. (2011). Androgen misuse and abuse. *Best Pract. Res. Clin. Endocrinol. Metab.* **25,** 377–389.

Jones, G., Willett, P., Glen, R. C., Leach, A. R., and Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **267,** 727–748.

Kanayama, G., Brower, K. J., Wood, R. I., Hudson, J. I., and Pope, H. G., Jr (2009). Anabolic-androgenic steroid dependence: An emerging disorder. *Addiction* **104,** 1966–1978.

Klusonova, P., Rehakova, L., Borchert, G., Vagnerova, K., Neckar, J., Ergang, P., Miksik, I., Kolar, F., and Pacha, J. (2009). Chronic intermittent hypoxia induces 11beta-hydroxysteroid dehydrogenase in rat heart. *Endocrinology* **150,** 4270–4277.

Kratschmar, D. V., Vuorinen, A., Da Cunha, T., Wolber, G., Classen-Houben, D., Doblhoff, O., Schuster, D., and Odermatt, A. (2011). Characterization of activity and binding mode of glycyrrhetinic acid derivatives inhibiting 11beta-hydroxysteroid dehydrogenase type 2. *J. Steroid Biochem. Mol. Biol.* **125,** 129–142.

Kuhn, C. M. (2002). Anabolic steroids. *Recent Prog. Horm. Res.* **57,** 411–434.

Lastra, G., Dhuper, S., Johnson, M. S., and Sowers, J. R. (2010). Salt, aldosterone, and insulin resistance: Impact on the cardiovascular system. *Nat. Rev. Nephrol.* **7,** 577–584.

Naray-Fejes-Toth, A., and Fejes-Toth, G. (2007). Novel mouse strain with Cre recombinase in 11beta-hydroxysteroid dehydrogenase-2-expressing cells. *Am. J. Physiol. Renal. Physiol.* **292,** F486–F494.

Odermatt, A., and Atanasov, A. G. (2009). Mineralocorticoid receptors: Emerging complexity and functional diversity. *Steroids* **74,** 163–171.

Odermatt, A., and Kratschmar, D. V. (2012). Tissue-specific modulation of mineralocorticoid receptor function by 11beta-hydroxysteroid dehydrogenases: An overview. *Mol. Cell. Endocrinol.* **350**, 168–186.

Parr, M. K., and Schanzer, W. (2010). Detection of the misuse of steroids in doping control. *J. Steroid Biochem. Mol. Biol.* **121,** 528–537.

Parssinen, M., Kujala, U., Vartiainen, E., Sarna, S., and Seppala, T. (2000). Increased premature mortality of competitive powerlifters suspected to have used anabolic agents. *Int. J. Sports Med.* **21,** 225–227.

Pitt, B., Reichek, N., Willenbrock, R., Zannad, F., Phillips, R. A., Roniker, B., Kleiman, J., Krause, S., Burns, D., and Williams, G. H. (2003a). Effects of eplerenone, enalapril, and eplerenone/enalapril in patients with essential hypertension and left ventricular hypertrophy: The 4E-left ventricular hypertrophy study. *Circulation* **108,** 1831–1838.

Pitt, B., Remme, W., Zannad, F., Neaton, J., Martinez, F., Roniker, B., Bittman, R., Hurley, S., Kleiman, J., and Gatlin, M. (2003b). Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N. Engl. J. Med.* **348,** 1309–1321.

Pitt, B., Zannad, F., Remme, W. J., Cody, R., Castaigne, A., Perez, A., Palensky, J., and Wittes, J. (1999). The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N. Engl. J. Med.* **341,** 709–717.

Pozo, O. J., Van Thuyne, W., Deventer, K., Van Eenoo, P., and Delbeke, F. T. (2008). Elucidation of urinary metabolites of fluoxymesterone by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. *J. Mass Spectrom.* **43,** 394–408.

Rebuffat, A. G., Tam, S., Nawrocki, A. R., Baker, M. E., Frey, B. M., Frey, F. J., and Odermatt, A. (2004). The 11-ketosteroid 11-ketodexamethasone is a glucocorticoid receptor agonist. *Mol. Cell. Endocrinol.* **214,** 27–37.

Schweizer, R. A., Atanasov, A. G., Frey, B. M., and Odermatt, A. (2003). A rapid screening assay for inhibitors of 11beta-hydroxysteroid dehydrogenases (11beta-HSD): Flavanone selectively inhibits 11beta-HSD1 reductase activity. *Mol. Cell. Endocrinol.* **212,** 41–49.

Senesi, S., Legeza, B., Balazs, Z., Csala, M., Marcolongo, P., Kereszturi, E., Szelenyi, P., Egger, C., Fulceri, R., Mandl, J., *et al.* (2010). Contribution of fructose-6-phosphate to glucocorticoid activation in the endoplasmic reticulum: Possible implication in the metabolic syndrome. *Endocrinology* **151,** 4830–4839.

Serra, A., Uehlinger, D. E., Ferrari, P., Dick, B., Frey, B. M., Frey, F. J., and Vogt, B. (2002). Glycyrrhetinic acid decreases plasma potassium concentrations in patients with anuria. *J. Am. Soc. Nephrol.* **13,** 191–196.

Sullivan, M. L., Martinez, C. M., Gennis, P., and Gallagher, E. J. (1998). The cardiac toxicity of anabolic steroids. *Prog. Cardiovasc. Dis.* **41,** 1–15.

Thompson, P. D., Cullinane, E. M., Sady, S. P., Chenevert, C., Saritelli, A. L., Sady, M. A., and Herbert, P. N. (1989). Contrasting effects of testosterone and stanozolol on serum lipoprotein levels. *J. Am. Med. Assoc.* **261,** 1165–1168.

Verdonk, M. L., Cole, J. C., Hartshorn, M. J., Murray, C. W., and Taylor, R. D. (2003). Improved protein-ligand docking using GOLD. *Proteins* **52,** 609–623.

Wolber, G., and Langer, T. (2005). LigandScout: 3-D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. *J. Chem. Inf. Model.* **45,** 160–169.

Yesalis, C. E., Barsukiewicz, C. K., Kopstein, A. N., and Bahrke, M. S. (1997). Trends in anabolic-androgenic steroid use among adolescents. *Arch. Pediatr. Adolesc. Med.* **151,** 1197–1206.