Steroids 76 (2011) 145–155

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Novel components of the human metabolome: The identification, characterization and anti-inflammatory activity of two 5-androstene tetrols

Clarence N. Ahlem[a,*], Theodore M. Page[a], Dominick L. Auci[a], Michael R. Kennedy[a], Katia Mangano[b], Ferdinando Nicoletti[b], Yu Ge[a], Yujin Huang[a], Steven K. White[a], Sonia Villegas[a], Douglas Conrad[c], Angela Wang[c], Christopher L. Reading[a], James M. Frincke[a]

[a] Harbor Biosciences, Inc., 9171 Towne Centre Drive, Suite 180, San Diego, CA 92122, United States
[b] Department of Biomedical Sciences, School of Medicine, University of Catania, Catania, Italy
[c] San Diego Veterans Administration Healthcare System, 3350 La Jolla Village Dr., San Diego, CA 92161, United States

ARTICLE INFO

Article history:
Received 27 August 2010
Received in revised form 13 October 2010
Accepted 13 October 2010
Available online 23 October 2010

Keywords:
Androstene
Tetrol
Metabolism
Inflammation
Metabolome
Human

ABSTRACT

Two natural 5-androstene steroid tetrols, androst-5-ene-3β,7β,16α,17β-tetrol (HE3177) and androst-5-ene-3α,7β,16α,17β-tetrol (HE3413), were discovered in human plasma and urine. These compounds had significant aqueous solubility, did not bind or transactivate steroid-binding nuclear hormone receptors, and were not immunosuppressive in murine mixed-lymphocyte studies. Both compounds appear to be metabolic end products, as they were resistant to primary and secondary metabolism. Both were orally bioavailable, and were very well tolerated in a two-week dose-intensive toxicity study in mice. Anti-inflammatory properties were found with exogenous administration of these compounds in rodent disease models of multiple sclerosis, lung injury, chronic prostatitis, and colitis.

1. Introduction

Dehydroepiandrosterone (DHEA) is not a significant product of the rodent adrenal gland [1–4], but exogenous DHEA exhibits anti-inflammatory activity in a wide range of metabolic or inflammatory disease models in rodents [5,6]. Unfortunately, clinical trials evaluating DHEA supplementation in patients with inflammatory conditions have reported undesired estrogenic and androgenic side effects, but not significant reductions in disease parameters [7–9]. These clinical outcomes may be related to poor oral bioavailability and rapid metabolism through the sex steroid pathways and into inactive conjugates [6,10–12].

The historical focus on androgens and estrogens has left much of the DHEA metabolome relatively unexplored. Reports describing the ability of exogenous DHEA or DHEA-sulfate (DHEA-S) to rejuvenate rodents [13] and regulate stem cells [14,15] as well as causal evidence linking adrenal dysfunction with inflammatory diseases [16] have generated a high level of academic and industrial interest, but although there are numerous publications describing the effects of DHEA, the precise role of the DHEA metabolome in biological systems remains something of an enigma.

It is well established that rodents are an endocrine species whereas humans have evolved to utilize a locally controlled paracrine–autocrine hormone system [17,18]. Accordingly, steroidogenesis is profoundly different between the two species. Exogenous DHEA in rodents, but not humans, is typically metabolized into an array of highly oxidized androstenes [19,11]. Several investigators have published evidence that many of the anti-inflammatory properties initially attributed to DHEA reside in these more highly oxidized metabolites [20–28]. These metabolites include androst-5-ene-3β,17β-diol (5-AED) and 7-hydroxy members of the series, such as androst-5-ene-3β,7β,17β-triol (βAET). Although 5-AED and βAET have substantial anti-inflammatory activity in rodents [20,29,30], these molecules do not have suitable pharmaceutical properties, e.g., they lack sufficient metabolic stability to allow for efficient oral delivery and sustained blood concentrations. We have developed synthetic versions of several of these compounds in our effort to improve their pharmaceutical properties [29,31,32].

During the course of characterizing the stability of our steroid library to microsomal metabolism, we observed several compounds that appeared to be completely resistant to metabolism. These compounds were 5-androstenetetrols, which had been prepared by chemical synthesis, and the observed stability led to their further characterization. Here we report the discovery of androst-5-ene-3β,7β,16α,17β-tetrol (HE3177) and androst-

* Corresponding author. Tel.: +1 858 320 2571; fax: +1 858 558 6470.
E-mail address: cahlem@harborbiosciences.com (C.N. Ahlem).

0039-128X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2010.10.005

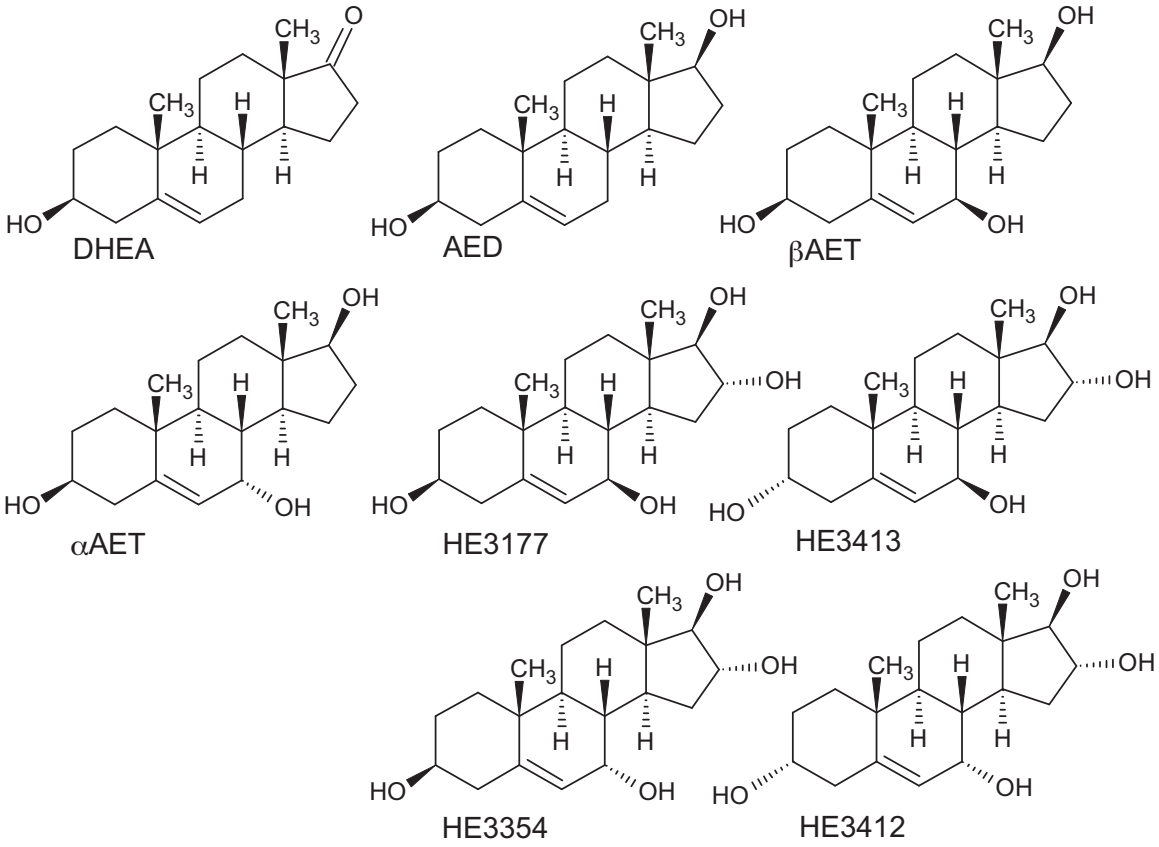

Fig. 1. Structures of selected DHEA metabolites.

5-ene-3α,7β,16α,17β-tetrol (HE3413) in human plasma and urine.

We report preliminary studies on their pharmacokinetics and toxicology, and demonstrate their oral bioavailability. These compounds appear to be end products of metabolism, as they were highly resistant to primary and secondary metabolism *in vitro* and *in vivo*. Neither compound appears to interact with steroid binding nuclear hormone receptors or peroxisome proliferator activated receptor (PPAR), and in preliminary high-dose evaluations, neither elicited acute toxicity, sex-hormone effects, or indications of immunotoxicity. Next, we tested these novel tetrols in several rodent models of chronic inflammatory disorders, and found them to possess potent anti-inflammatory activities. In addition to their potential as pharmaceuticals, since both are natural products, either may be suitable for use as a dietary supplement to delay or prevent diseases associated with chronic inflammation, including cancer, autoimmune disease and metabolic syndrome.

2. Materials and methods

All *in vivo* experiments were conducted in accordance with national and local animal welfare regulations and with Institutional Animal Care and Use Committee (IACUC) approvals.

2.1. Steroids, chemicals, and reagents

The tetrol compounds, androst-5-ene-3α,7β,16α,17β-tetrol (HE3413), androst-5-ene-3β,7β,16α,17β-tetrol (HE3177), androst-5-ene-3β,7α,16α,17β-tetrol (HE3354), androst-5-ene-3α,7α,16α,17β-tetrol (HE3412), androst-5-ene-3β,7β,17β-triol (βAET), and androst-5-ene-3β,7α,17β-triol (αAET) were synthesized at Harbor Biosciences (San Diego, CA) (see Fig. 1 for chemical structures). Tetrols were synthesized from commercially available dehydroepiandrosterone (DHEA). Reagents and anhydrous solvents were obtained from commercial sources and used without further purification. Synthetic procedures ranged from seven steps with an overall yield of ~15% (HE3177) to a 9 step process with an overall yield of ~2.5% (HE3413). NMR spectra were acquired in d_4-methanol with a Bruker 400 MHz NMR spectrometer. Purity was measured by HPLC using a 4.6 mm × 150 mm Agilent XDB-C18, 3.5 μm column (Agilent Technologies) at ambient temperature, with a mobile phase of 10–90% acetonitrile in water, 0.1% trifluoroacetic acid (TFA) (gradient completed in 10 min, 1 mL/min). Eluted compounds were monitored with a diode array detector (Agilent Technologies) and an evaporative light scattering detector (SEDEX 85 LT-ELSD, SEDERE Sas, France). The molecular mass was determined by LC–MS/MS using an Xbridge Phenyl column (2.1 mm × 150 mm, 3.5 μm, Waters, Beverly, MA) eluted with a mobile phase gradient of acetonitrile in water, 0.1% formic acid, with the column temperature maintained at 40 °C. The column eluent was subjected to positive-mode electrospray ionization (ES+), and the analytes were detected with a tandem quadrupole mass spectrometer (Waters, Beverly, MA), monitoring the M+1 (−1 or $2H_2O$) mass transitions (323.4 > 305.4 > 287.4 amu).

Dimethylsulfoxide (DMSO), N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), methyl-*tert*-butyl ether (MTBE), human sulfotransferase, human UDP-glucuronosyltransferase, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, octanol, phosphate-buffered saline, dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol (5-AED), and dexamethasone were purchased from Sigma Chemical Company (St. Louis, MO). Androst-5-ene-3α,17β-diol (DHA, HE3209), 3β,7β-diol-androst-5-en-17-one (HE3129), 3β-ol-androst-5-ene-7,17-dione (7-keto-DHEA), androst-5-

ene-3α,16α,17β-triol (HE3411), androst-5-ene-3α,7β,17β-triol (HE3593), and 3α,7β-diol-androst-5-en-17-one (HE3768) were purchased from Steraloids (Newport, RI). β-Cyclodextrin sulfobutyl ether (Captisol®) was purchased from CyDex, Inc. (Overland Park, KS). Vehicles for oral administration of compounds were prepared by Harbor Biosciences: 300 mg/mL β-cyclodextrin sulfobutyl ether and 1 mg/mL carboxymethylcellulose (Spectrum, Gardena, CA), 9 mg/mL NaCl (Sigma, St. Louis, MO), 20 mg/mL polysorbate 80 (Spectrum, Gardena, CA), and 0.5 mg/mL phenol (Sigma, St. Louis, MO).

2.2. Analytical methods for tetrol determinations

2.2.1. Structure determination using a gas chromatography–mass spectrometry method

Human plasma and urine (1 mL) was extracted with 10 mL MTBE, dried under nitrogen, and derivatized with MSTFA. The derivative (1 μL) was analyzed on a Varian 3800 gas chromatograph coupled to a 1200 L mass spectrometer in electron impact mode (EI) with a Varian CP-Sil column (0.15 mm × 10 m) eluted with helium at 1 mL/min (Varian, Palo Alto, CA). Tetrols were identified by a combination of their retention time, mass transitions (610 > 520 @-9 V, and 520 > 415 @-13 V), and by the ratio of these two transitions, and by comparison to authentic standards, when applicable.

2.2.2. Structure determination using a liquid chromatography–mass spectrometry method

Human plasma (10 mL) was washed with 50 mL hexane. The aqueous phase was extracted with 150 mL ethyl acetate, dried under nitrogen, and derivatized with 500 μL of 50 mg/mL nicotinyl chloride in pyridine at 80 °C for 1 h. The reaction was cooled to room temperature and quenched with 1 mL 5% sodium bicarbonate. The derivatized steroids were extracted with 10 mL MTBE, dried under nitrogen, dissolved in 100 μL acetonitrile/water (45:65, v/v), and analyzed on a Varian ProStar HPLC coupled to a Varian 1200 L mass spectrometer in MS/MS mode (Varian, Palo Alto, CA), using a Phenomenex Polar RP C18 column (2 mm × 250 mm, Phenomonex, Torrance, CA) eluted with a water/acetonitrile gradient. Some analyses were performed with two columns in tandem. Steroid tetrols were identified by their 743 > 497 @-14 V mass transition and their retention time. For the determination of tetrol concentrations in biopharmaceutical assays (solubility, Caco-2 transport, *in vitro* metabolism), tetrols were extracted with the specified volume of ethyl acetate, dried under nitrogen, dissolved in 100 μL acetonitrile/water (45/65), and analyzed by LC–MS/MS as above, but without derivatization, and detected by their retention time and 287 > 269 @-7 V mass transition.

2.2.3. Pharmacokinetics determinations using a liquid chromatography–mass spectrometry method

Serum or plasma samples were extracted with MTBE. The organic phase was collected and evaporated to dryness, and dissolved in HPLC mobile phase (water/acetonitrile, 90:10, v/v). Samples were analyzed on a Waters Xbridge Phenyl column by reversed-phase high-performance liquid chromatography (Agilent, Palo Alto, CA and Leap Technologies, Carrboro, NC) coupled with a tandem mass spectrometer (Waters, Beverly, MA). Concentrations were determined from a calibration curve using Masslynx analysis software (Waters, Beverly, MA). Analytes below the quantifiable assay limit (BQL) were assigned values of zero for calculation of pharmacokinetics parameters (WinNonlin 5.2; Pharsight, Mountain View, CA).

2.3. Biopharmaceutical characterization

2.3.1. Pharmacokinetics assessments in mice

Male CD-1 mice (6–8 weeks of age) received a single oral gavage of the test compound formulated as an aqueous solution in β-cyclodextrin sulfobutyl ether. Cohorts of 3 animals were sacrificed by CO_2 asphyxiation and cardiac puncture prior to dosing ($t = 0$), and at different times after dosing up to 8 h. Serum was prepared and stored at −20 °C until analysis. Test compound concentrations in serum were measured by LC–MS/MS, and the mean concentration of the cohort was used to report PK parameters.

2.3.2. Pharmacokinetics assessments in monkeys

The absolute oral bioavailability of HE3413 was determined in cynomolgus monkeys (*Macaca fascicularis*). HE3413, 10 mg/kg, in an aqueous β-cyclodextrin sulfobutyl ether solution was administered both orally and intravenously to groups of 4 males (body weight 3.7–4.5 kg). Blood was collected serially for 24 h (0, 0.25, 0.5, 1, 2, 4, 8, and 24 h for oral and 0, 0.05, 0.25, 0.5, 0.75, 1, 2, 4, 8, and 24 h for IV) after drug administration and analyzed for by LC–MS/MS. The absolute oral bioavailability (% F) was expressed as the dose mass adjusted ratio of the integrated drug plasma concentration, $AUC_{(0-infinity)}$, for the oral relative to the IV dose.

2.3.3. Microsomal metabolism

The test compounds (10 μM) were incubated with 0.25 mg human liver microsomal extract (In Vitro Technologies), 10 mM glucose-6-phosphate, 10 mM NADP, and 5 U glucose-6-phosphate dehydrogenase in 0.5 mL PBS for 75 min at 37 °C. An equivalent amount of androst-5-ene-3β,17β-diol was incubated separately as an enzyme activity control. Steroids were extracted with 5 mL ethyl acetate and analyzed by LC–MS.

2.3.4. In vitro tetrol formation from 5-androstene precursors

Steroid procursors (10 μM) were incubated for 90 min at 37 °C with 0.25 mg liver microsomal extract with 10 mM glucose-6-phosphate, 10 mM NADP, and 10 U/mL glucose-6-phosphate dehydrogenase. The reaction mixtures were subsequently extracted with ethyl acetate, derivatized with nicotinyl chloride, and analyzed using a high sensitivity tandem column LC–MS/MS method. Peak areas were determined for responses with the appropriate retention time of androstenetetrols and 743 > 497 amu mass transition.

2.3.5. Measurement of sulfation and glucuronidation

The susceptibility of test compounds to sulfation and glucuronidation was measured *in vitro* by comparing the disappearance of test compounds in the presence and absence of activated cofactor. Test compounds (10 μM) were incubated with human liver S9 fraction (1 mg/mL) in 0.5 mL PBS containing 5 mM magnesium chloride in the presence or absence of 100 μM adenosine 3′-phosphate-5′-phosphosulfate (for sulfation assays), or 800 μM UDP-glucuronic acid (for glucuronidation assays). The assays were incubated at 37 °C for 15 min (sulfation) or 1 h (glucuronidation). 5-AED (sulfation) and DHA (glucuronidation) were incubated in parallel as controls. Following incubation, steroids were extracted with 5 mL ethyl acetate and analyzed by LC–MS.

2.3.6. Partition constant

Compound solubility in water was measured using Millipore MultiScreen HTS solubility plates (Millipore, Billerica, MA) according to the manufacturer's instructions. Compounds were dissolved in dimethyl sulfoxide, and diluted to 250 μM in water. The concentration of soluble compound in the filtrate was determined

by LC–MS, and the $\log_{10}$ of the partition constant in PBS (pH 7.4)/octanol was calculated [33].

2.3.7. Caco-2 membrane transport measurement

The apical to basolateral transport of tetrols and reference compounds was measured in Caco-2 cells (In Vitro Technologies, Baltimore, MD) as described [34]. Test compounds were dissolved in apical buffer at 10 μM, and samples of basolateral buffer were taken at 0, 20, 40, 80, and 120 min, extracted with 5 mL of ethyl acetate, and analyzed for the concentration of compound by LC/MS–MS as described above.

2.4. Nuclear hormone interactions

2.4.1. Nuclear receptor binding assessment

Assessment of binding activity for various nuclear receptors was performed by homogeneous competition assays using the PolarScreen™ fluorescence polarization system (InVitrogen, Carlsbad, CA) as previously described [32]. The highest concentration tested was 10,000 nM.

2.4.2. Nuclear receptor transactivation assays

Transactivation of androgen (AR), estrogen (ERα, and ERβ), and glucocorticoid (GR) receptors were measured as previously described [32]. Human mineralocorticoid receptor (MR) and peroxisome proliferator-activated receptor (PPAR-α, PPAR-γ and PPAR-δ) transactivation was measured with the Gene Blazer® β-lactamase assay system (InVitrogen, Carlsbad, CA) following the manufacturer's instructions. The highest concentration tested was 10,000 nM.

2.5. Acute toxicity in mice

Experiments to determine the acute toxicity and estrogenic potential were performed at MPI Research (Matawan, MI). Three groups of 10 female Crl:CD1®(Icr) mice, approximately 6 weeks of age, received 10 mL/kg twice daily oral gavage for 14 consecutive days of either 30% β-cyclodextrin sulfobutyl ether (vehicle control) or 20 mg/mL HE3413 or HE3177 (400 mg/kg/day) as a solution in cyclodextrin. The mice were observed twice daily for clinical signs of toxicity and weighed on days 1, 7, and 14. On the fifteenth day, the animals were sacrificed, and subjected to gross necropsy. Blood from 5 animals per group was used to measure hematological parameters, and blood from the remaining 5 per group was used to measure a limited panel of clinical chemistry parameters (emphasizing liver function tests). The liver, uterus, and adrenal glands from each animal were weighed and evaluated for histopathology.

2.6. Immune suppression assessment by mixed lymphocyte response

The classic mixed lymphocyte reaction (MLR) was used to assess immune suppressive potential essentially as previously described [35]. Peripheral blood mononuclear cells (PBMC) were isolated from three healthy donors (2 females, 1 male, between 18 and 65 years of age) after informed consent. Each of the three donors was tested as responders in an allogeneic MLR assay that used allogenic stimulator cells pretreated with mitomycin C (25 μg/mL for 30 min at 37 °C in a 5% CO_2 atmosphere and washed) in lieu of radiation. Compounds were tested at 100 and 1000 ng/mL in cultures incubated for six days. The data are expressed as mean ± SEM of the normalized (% DMSO control) quadruplicate values obtained from the three different responders.

2.7. Anti-inflammatory assays

The anti-inflammatory activity of HE3177 and HE3413 was assessed in rodent (SJL/J mice and Dark Agouti rat) models of experimental autoimmune encephalomyelitis (EAE), experimental autoimmune prostatitis (EAP), LPS induced lung injury, and dextran sodium sulfate induced colitis (DSSIC).

2.7.1. SJL/J mouse EAE

Mice, 6–8 weeks of age (Charles River Italia, Udine, Italy) were adapted (at least 7 days) and maintained in a pathogen-free vivarium (University of Catania, School of Medicine, Catania, Italy), and had access to sterilized food and water *ad libitum*. Relapsing and remitting EAE was induced with a subcutaneous 200 μL injection containing 75 μg proteolipid protein (PLP) as previously described [36]. Body weight and disease symptoms were monitored daily and scored (blinded): 0 = no illness; 1 = flaccid tail; 2 = moderate paraparesis; 3 = severe paraparesis; 4 = moribund state; 5 = death. Before immunization mice were randomized to each experimental group (n = 7–8) and treated daily (6 days/week) with either 100 μL of HE3413 (4 mg/kg), or HE3177 (4 mg/kg) by oral gavage. Treatment was initiated at disease onset (12 days after immunization, disease scores 2–3 in approximately 25% of mice) and continued for 18 days. Mice were monitored for an additional 3 weeks after treatment. A two-way ANOVA was used to determine statistically significant differences in disease severity among the treatment groups. The Mann–Whitney *U* test was used to determine the significance between the tetrol- and vehicle-treated groups.

2.7.2. Dark Agouti rat EAE

Female rats, 8–10 weeks old, 150–175 g (Scanbur AB, Sweden), maintained under standard (non-specific pathogen free) laboratory conditions (Department of Biomedical Sciences, Section of General Pathology, Catania, Italy), were adapted for at least 7 days with free access to food and water. Rats were subcutaneously immunized (day = 0) at the base of the tail with 200 μL of a homogenized emulsion containing 50 mg of whole DA rat spinal cord in Complete Freund's Adjuvant containing 10 mg/mL *Mycobacterium tuberculosis* (Difco, Detroit, USA). A progressive paralysis resulted approximately 8–9 days after immunization, initially in the tail, ascending up to the forelimbs. Body weight and disease symptoms were monitored daily beginning on day 7, and severity scored in a blinded manner according to the same scale used in mice (see above). Rats were randomized to experimental groups before immunization. When an animal's clinical EAE score ranged between 1 and 2 (days 10–12), once daily oral gavage was initiated with either 125 μL of cyclodextrin vehicle (n = 10) or HE3413 in cyclodextrin (10 mg/kg; n = 7), 6 days per week for 20 days. Animals were subsequently monitored daily until day 34. Daily severity scores from individual animals were used to determine group differences. The Mann–Whitney *U* test was used to determine the significance between the tetrol- and vehicle-treated groups.

2.7.3. Murine experimental autoimmune prostatitis

Animals were adapted at least 7 days and kept under standard (non-specific pathogen free) laboratory conditions (University of Catania, Catania, IT) with free access to food and water. Male NOD mice (n = 8 per group), 6–8 weeks old (Jackson Laboratories, ME) were immunized with a subcutaneous injection of 300 μg CD-1 mouse prostate homogenate in CFA containing 1 mg/mL *M. tuberculosis* (Difco, Detroit, MI) [37]. A total volume of 150 μL administered for immunization was divided between four sites: right and left foot pad (25 μL); tail base and shoulder (50 μL). Animals received a daily oral gavage of either 100 μL cyclodextrin vehicle or HE3413 (80 mg/kg) on days 14–30 after immunization. Mice were sacrificed 4 h after the final treatment, and prostates excised and snap-frozen

in Tissue Tek (Miles Laboratories). Fifty cryostatic sections covering the entire prostate were prepared, stained with H&E and scored by a pathologist (blinded to treatment) for organized intraprostatic lymphomononuclear cell infiltrate nodules and reported as the number of infiltrates/cross-section. The significance between groups was tested using Student's *t* test.

2.7.4. LPS induced lung injury

Female C57 black/6 mice (6–8 weeks old; Harlan, San Diego, CA) were used in these studies. Animals were acclimated for 3 days prior to the experiment in a controlled laboratory environment (San Diego Veterans Administration Healthcare System, La Jolla, CA) with free access to standard rodent chow and water. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was purchased from Sigma, St. Louis, MO. Animals received an oral gavage of 100 μL of cyclodextrin vehicle ($n = 7$), 40 mg/kg HE3413 ($n = 7$), or HE3177 ($n = 8$) formulated as a solution in cyclodextrin, 24 h and 1 h before LPS challenge. Challenge was performed by lightly anesthetizing the mice with isofluorane and then directly administering 50 μL LPS solution (1 mg/mL in sterile saline) into the trachea with mice in a vertical position using a gel loading pipette under direct observation through a medical otoscope. Forty-eight hours after the LPS challenge, the animals were sacrificed, bronchoalveolar lavage (BAL) samples were collected (BAL performed 3× using sterile PBS; 1.3 mL were typically recovered) and myeloperoxidase (MPO) levels in lungs measured as previously described [38]. TNFα and IL-6 ELISA kits were purchased from Assay Designs (Ann Arbor, MI) and used according to the manufacturer's instructions. Significance was determined using a two-sided Mann–Whitney exact test.

2.7.5. Dextran sodium sulfate (DSS)-induced colitis

Male C57BL/6 mice, 6–8 weeks old, 25–30 g (Charles River, Wilmington, MA) were maintained in a pathogen-free ambient environment with *ad libitum* sterilized food and water and adapted for at least 7 days before induction. Prior to dextran sodium sulfate (DSS) treatment, the mice were randomized into groups of five to receive either daily oral gavage of 100 μL of cyclodextrin vehicle or HE3413 (10 and 40 mg/kg) for 5 or 15 days, starting immediately prior to DSS administration. Colitis was induced with DSS as previously described [39]. Briefly, mice were administered 3% DSS *ad libitum* in drinking water for 5 days, after which, mice received DSS-free water. On days 5 and 15 the respective groups were anesthetized with isoflurane for blood (serum) collection *via* cardiac puncture, and immediately euthanized by CO_2 asphyxiation. The colons were scored visually for diarrhea (0 = normal fecal pellets, 1 = slightly loose feces, 2 = loose feces, and 3 = watery feces) and colon inflammation (0 = normal, 1 = slight inflammation, 2 = moderate inflammation and/or edema, and 3 = heavy inflammation, edema and/or ulcerations). The colon samples were fixed in buffered formalin and paraffin-embedded sections were prepared and stained with Hematoxylin and Eosin (H&E) for histological analysis of inflammation, edema, epithelial defects, gland atrophy, hyperplasia and dysplasia. Haptoglobin levels in serum samples were measured by ELISA (GenWay, San Diego, CA). Significant differences were determined using a two-sided Student's *t*-test.

3. Results

3.1. Characterization of synthesized 5-androstenetetrols

5-Androstenetetrols used as analytical standards and/or pharmacological agents were prepared by unambiguous chemical synthesis, and were greater than 98% pure using HPLC analysis with evaporative light scattering detection. The melting range (mp), proton NMR spectrum, HPLC retention time (t_R) and percent purity (ELS detector), and molecular mass (LC–MS/MS) are reported for HE3177, HE3354, HE3412, and HE3413.

Androst-5-ene-3β,7β,16α,17β-tetrol (HE3177), mp 229.1–231.4 °C. ^{1}HNMR (CD_3OD, 400 MHz): δ 5.24 (s, 1H), 4.00 (m, 1H), 3.74 (d, 1H, $J = 6.9$ Hz), 3.42 (m, 1H), 3.36 (d, 1H, $J = 6.3$ Hz), 2.28–1.06 (m, 15H), 1.07 (s, 3H), 0.77 (s, 3H). HPLC: $t_R = 4.26$ min (98.7%). LC–MS/MS: $t_R = 3.84$ min, 305.2 amu (M+1 $-H_2O$).

Androst-5-ene-3β,7α,16α,17β-tetrol (HE3354), mp 202.1–204.4 °C. ^{1}H NMR peaks (CD_3OD, 400 MHz): δ 5.53 (d, 1H, $J = 3.7$ Hz), 4.03 (m, 1H), 3.73 (m, 1H), 3.47 (m, 1H), 3.42 (d, 1H, $J = 6.3$ Hz), 2.33–2.18 (m, 2H), 1.88–1.28 (m, 11H), 1.21–1.08 (m, 2H), 1.00 (s, 3H), 0.76 (s, 3H). HPLC: $t_R = 4.84$ min (100%, ELSD). LC–MS/MS: not analyzed.

Androst-5-ene-3α,7α,16α,17β-tetrol (HE3412), mp 245–248.3 °C. ^{1}H NMR peaks (CD_3OD, 400 MHz): δ 5.54 (d, 1H, $J = 5.4$ Hz), 4.02 (m, 2H), 3.70 (m, 1H), 3.43 (d, 1H, $J = 6.1$ Hz), 2.55 (m, 1H), 2.10 (m, 1H), 1.83–1.39 (m, 12H), 1.16 (m, 1H), 1.01 (s, 3H), 0.76 (s, 3H). HPLC: $t_R = 5.05$ min (100%, ELSD) LC–MS: $t_R = 6.58$ min, 305.2 amu (M+1 $-H_2O$).

Androst-5-ene-3α,7β,16α,17β-tetrol (HE3413), mp 220–222 °C. ^{1}H NMR peaks: δ 5.23 (s, 1H), 4.00 (m, 2H), 3.78 (m, 1H), 3.37 (d, 1H, $J = 6.2$ Hz), 2.53 (m, 1H), 2.09 (m, 2H), 1.83–1.39 (m, 10H), 1.16 (m, 2H), 1.08 (s, 3H), 0.77 (s, 3H). HPLC: $t_R = 4.72$ min (98.6%, ELSD) LC–MS: $t_R = 3.81$ min, 305.2 amu (M+1 $-H_2O$).

3.2. Identification of tetrols in plasma and urine

Rat liver metabolism of androst-5-ene-3β,7β,17β-triol (βAET), a natural hormone with anti-inflammatory activity [29] produced an ensemble of unknown tetrol derivatives. We hypothesized 16-hydroxylation could produce certain of these through the action of CYP2C11, and focused on four structurally related tetrol derivatives as possibilities: HE3177 (androst-5-ene-3β,7β,16α,17β-tetrol), HE3354 (androst-5-ene-3β,7α,16α,17β-tetrol), HE3412 (androst-5-ene-3α,7α,16α,17β-tetrol) and HE3413 (androst-5-ene-3α,7β,16α,17β-tetrol) (Fig. 1). These compounds were used as analytical standards to determine their natural occurrence. Our analysis revealed HE3413 and HE3177 in human plasma (Fig. 2A). The specificity of the peaks corresponding to standards was confirmed by admixing the sample with a low (2 pg/mL, Fig. 2B) and a high (5 pg/mL, Fig. 2C) concentration of the respective analytical standards. An increased spectral intensity established the identity of two naturally occurring plasma compounds, HE3413 and HE3177. The HE3354 and HE3412 tetrols were not detected. The identity of HE3413 was confirmed using a second high sensitivity method with higher chromatographic resolution (Fig. 3). The endogenous concentration was estimated to be 2 pg/mL. Additional unidentified putative tetrols were detected in human plasma sample (Fig. 4). HE3413 was also observed un-conjugated in human urine (data not shown). In a similar manner, HE3177 in human plasma was discovered and confirmed by GC-MS/MS (data not shown).

3.3. Human liver microsome studies

Androstene tetrols were found in *in vitro* metabolism studies using human liver microsomes in which common naturally occurring 5-androstenes produced tetrols (Table 1), including HE3177 and HE3413. Both the 3α- and 3β-hydroxy precursors were metabolized into both 3α- and 3β-hydroxy tetrols suggesting formation through several different steroidogenic pathways. Greater amounts of the tetrols were derived from triols, which required only a single carbon oxidative reaction for tetrol formation.

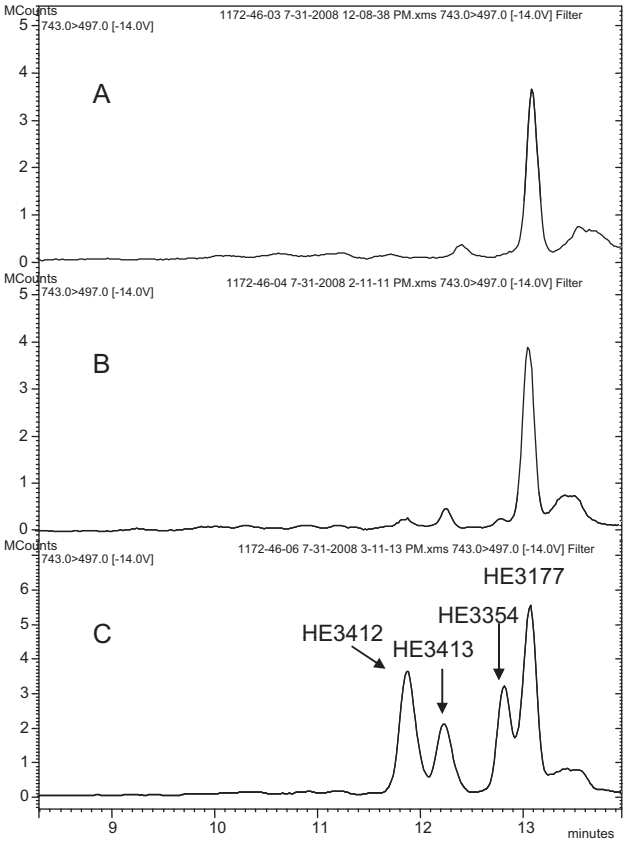

Fig. 2. Identification of androstene tetrols in human plasma using LC–MS/MS. Plasma was extracted with ethyl acetate, and steroids were derivatized with nicotinyl chloride, and identified as tetrols by LC–MS/MS. (A) LC–MS/MS chromatogram of tetrols in human plasma, (B) human plasma spiked with 2 pg/mL tetrol standards for HE3177, HE3354, HE3412, and HE3413, (C) human plasma spiked with 5 pg/mL tetrol standards of HE3177, HE3354, HE3412, and HE3413.

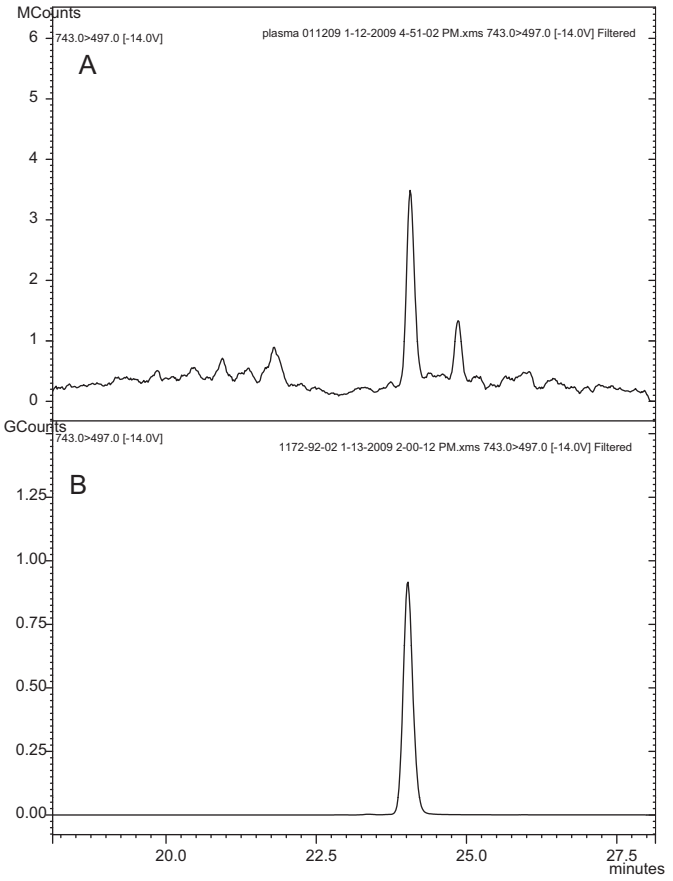

Fig. 3. Identification of androstene tetrols in human plasma using a modified high sensitivity tandem column LC–MS/MS method. (A) LC–MS/MS chromatogram of nicotinyl chloride derivatized tetrols in human plasma analyzed with a tandem column chromatographic system, (B) LC–MS/MS chromatogram of nicotinyl-HE3413 standard.

3.4. Pharmacokinetics and metabolism

3.4.1. Pharmacokinetics

The pharmacokinetics of HE3177 and HE3413 were assessed in mice following oral gavage of 40 mg/kg aqueous solutions and results were compared to 5-AED (Fig. 5). Serum levels were elevated relative to the endogenous plasma levels found in humans (~2 pg/mL) and persisted relative to 5-AED. HE3413 pharmacokinetics parameters in cynomolgus monkeys are given in Table 2. The absolute oral bioavailability (% F) was 15.8%.

3.4.2. Metabolism

HE3177 and HE3413 appear to resist primary metabolism since neither was transformed by mouse or human liver microsomes (Table 3). Similar metabolic resistance was found for HE3354 and HE3412 (data not shown). This outcome was in sharp contrast to results found with 5-AED and βAET. The *in vitro* metabolic stability of HE3177 and HE3413 was reflected in a low abundance of oxidized (hydroxy to keto) and epimerized metabolites in mouse and monkey PK studies (data not shown). HE3177 and HE3413 were not susceptible to the activity of CYP19 (aromatase) under conditions that completely convert testosterone to estradiol (data not shown).

Table 1
Metabolic conversion of androst-5-ene precursors to tetrols by human liver microsomes.

Precursor structure	HE3412 (3α, 7β, 16α, 17β)	HE3413 (3α, 7β, 16α, 17β)	HE3354 (3β, 7α, 16α, 17β)	HE3177 (3β, 7β, 16α, 17β)
3β-ol-androst-5-en-17-one	0	0	94	422
Androst-5-ene-3β,17β-diol	0	0	80	115
3β-ol-androst-5-ene-7,17-dione	0	0	4199	0
3β,7β-Diol-androst-5-en-17-one	0	0	88	0
Androst-5-ene-3β,7α,17β-triol	0	1090	0	192
Androst-5-ene-3β,7β,17β-triol	49	780	0	546
3α-ol-androst-5-en-17-one	43	34	47	0
Androst-5-ene-3α,17β-diol	0	51	0	0
3α,7β-Diol-androst-5-en-17-one	274	201	278	0
Androst-5-ene-3α,7β,17β-triol	2058	2167	895	0
Androst-5-ene-3α,16α,17β-triol	19	431	21	0

Steroid procursors (10 μM) were incubated for 90 min at 37 °C with 1 mg/mL human liver microsomal extract with 10 mM glucose-6-phosphate, 10 mM NADP, and 10 U/mL glucose-6-phosphate dehydrogenase. After the incubation, the reaction mixtures were extracted with ethyl acetate, derivatized with nicotinyl chloride, and analyzed using a high sensitivity tandem column LC/MS–MS method. Peak areas were determined for peaks with appropriate retention times androstenetetrols and 743 > 497 amu mass transitions; values represent the areas of each peak.

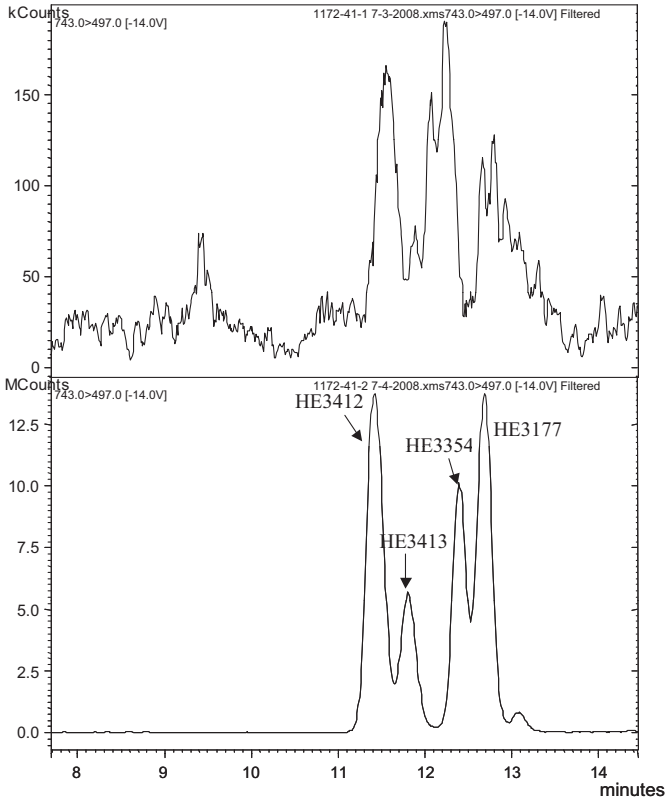

Fig. 4. Unidentified (putative) tetrols in human plasma. LC–MS/MS chromatogram of human plasma, indicating the presence of numerous unidentified androstenetetrols (top panel); LC–MS/MS chromatogram of androstenetetrol standards (bottom panel).

Tetrols appear to be very resistant to secondary metabolism, a property that is presumably related to their intrinsically high water solubility. Their metabolic stability probably contributes to their slow rate of clearance compared to the more readily conjugated di- and tri-oxygenated C-19 androstene DHEA metabolites.

3.4.3. Partition constant and Caco2 transport

The octanol:PBS partition constants (Log *D*) of HE3413 and HE3177 were similar, and considerably lower than diols and triols. The tetrols, as expected, were more water soluble relative to less oxidized members of this structure class (Table 3), but the solubility of HE3413 was surprisingly high, 28 mM. The low partition constant was apparently reflected in relatively poor transport across Caco-2 cells (Table 3).

Compound	C_{max}, ng/mL	T_{max}, hr	$AUC_{(0-8)}$, ng*hr/mL
HE3177	571	0.5	765
HE3413	1,457	0.5	2,181

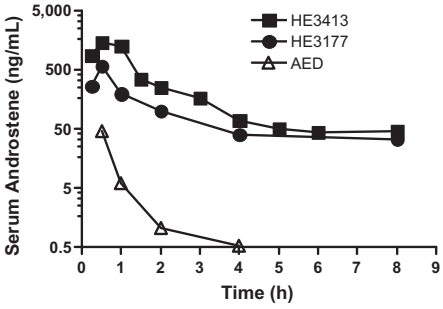

Fig. 5. Pharmacokinetics of the 5-androstenetetrols, HE3413, HE3177, and 5-AED in CD-1 mice. Male CD-1 mice received a single oral gavage of HE3413, HE3177 (aqueous solutions), or 5-AED (aqueous solution in cyclodextrin). Cohorts of 3 animals were sacrificed at each time point. Steroid concentrations in serum were measured by LC–MS/MS.

3.4.4. Nuclear hormone receptor interactions

Nuclear receptor binding experiments demonstrated that HE3413 and HE3177 do not competitively bind any of the steroid receptors, AR, ERα, ERβ, PR, or GR, and did not transactivate the nuclear hormone receptors for AR, ERα or ERβ, GR, MR, or PPAR-α (Transactivation EC_{50} values were all greater than 10,000 nM, except for HE3413 PPAR-α EC_{50} = 4000 nM). These results suggest that HE3413 and HE3177 do not exert their activity through classic steroid hormone nuclear receptor binding or through PPAR-α/γ/δ.

3.5. Systemic toxicity in mice

The systemic toxicity and estrogenicity of HE3413 and HE3177 (400 mg/kg) were assessed in CD-1 female mice. Both compounds were well tolerated. There were no clinical signs of toxicity or changes in body weight. There were no significant treatment effects on organ weights, histopathology (adrenal glands, liver, uterus), hematology parameters, and blood chemistry (data not shown). The absence of peroxisome proliferation *in vivo* was consistent with

Table 2
HE3413 pharmacokinetics parameters (SD) in cynomolgus monkeys.

Route of administration	C_{max} (ng/mL)	T_{max} (h)	$AUC_{(0-24)}$ (ng[a] h/mL)	$AUC_{(0-infinity)}$ (ng[a] h/mL)	$T_{1/2}$ (h)
IV	18,609 (4,438)	0.05 (0.0)	5,881 (454)	5,888 (452)	
Oral-gastric	292 (167)	1.5 (0.6)	877 (625)	929 (618)	7.5*

[a] Harmonic mean.

Table 3
Comparison of biopharmaceutical properties of HE3177 and HE3413 with 5-AED and βAET.

Compound	Solubility[a]	Log *D*[b]	Metabolism[c]	Sulfation[d]	Glucuronidation[e]	Caco-2[f]
5-AED	5	3.3	3.89	9.75	1.02	ND
βAET	164	1.8	0.583	ND	ND	21.5
HE3177	1,740	0.53	0	0	0	1.57
HE3413	28,900	0.43	0	0	0	2.74

ND: not determined.
[a] Solubility (μM) in H_2O at 22 °C, pH 7.4.
[b] Log of the distribution coefficient in octanol/phosphate-buffered saline pH 7.4 at 22 °C.
[c] Metabolism of compound by human liver microsomes in nanomoles compound metabolized/mg microsomal protein/h.
[d] Sulfation of compound by human liver S9 fraction expressed in nanomoles compound sulfated/mg protein/h.
[e] Glucuronidation of test compound by human liver S9 fraction expressed as nanomoles of compound glucuronidated per mg protein/h.
[f] Apical to basolateral transport by Caco-2 cells, expressed as picomoles transported/well/h.

the *in vitro* results, which again differentiates the pharmacology of these tetrols from those members of this structure class with lower oxidation states.

3.6. Immune suppression assessment by mixed lymphocyte reaction

The immunosuppressive potential of HE3177 and HE3413 was assessed using an allogeneic MLR. Blood was obtained from three individual donors (1 male, 2 females) and each of two concentrations (100 and 1000 ng/mL) tested in quadruplicate. Neither concentration of either tetrol suppressed lymphocyte proliferation (high concentration: 102% ± 14 and 116% ± 18 of vehicle control respectively). In contrast, dexamethasone (1 μM) significantly ($p = 0.0004$) suppressed (>85%) proliferation in this same human MLR assay.

3.7. Anti-inflammatory activity of HE3177 and HE3413 in mouse inflammation models

3.7.1. Experimental autoimmune encephalomyelitis (EAE)

HE3413 was assessed in both the female SJL/J mouse and the Dark Agouti (DA) rat EAE models of neuro-inflammation resembling multiple sclerosis. In both models, disease scores in tetrol-treated animals were significantly ($p < 0.0001$) reduced compared to controls (Fig. 6A). Mice received daily doses of HE3177 or HE3413 (4 mg/kg) or vehicle beginning on day 12 (first disease incidence) and continuing through day 33. Cumulative disease index (CDI) for this period was reduced in the tetrol-treated groups compared to vehicle-treated animals. ANOVA analysis showed that CDI from groups receiving HE3177 and HE3413 were significantly different from those receiving vehicle ($p < 0.005$). Benefit appeared to be sustained for greater than three weeks after cessation of dosing. In the DA rat model, where only HE3413 was tested (Fig. 6B), the compound significantly ($p = 0.018$) reduced CDI and blunted peak relapse (day 29) compared to vehicle (CDI 1.1 ± 1.1 *versus* 2.0 ± 0.0 respectively; $p = 0.02$).

3.7.2. Experimental autoimmune prostatitis (EAP)

NOD mice develop a dramatic inflammatory, non-infectious, prostatitis upon autoimmunization with gland extracts [40]. This EAP model has been used to test agents for potential activity in human chronic prostatitis [37]. Daily treatment (days 14–30) with HE3413 (80 mg/kg) provided clinical benefit in this model as determined by a significant ($p = 0.034$) decrease in the number of infiltrating lymphocyte nodules per prostate tissue section (2.23 ± 0.65 in the vehicle group *versus* 1.50 ± 0.58 in the HE3413-treated group) on day 30 (data not shown).

3.7.3. LPS-induced lung injury

Neutrophil mediated inflammation is implicated in the tissue damage and fibrosis associated with several forms of chronic obstructive pulmonary disease, and has been suggested as a potential target for therapeutic action [41]. The murine model of LPS-induced lung injury has been proposed to model neutrophil-mediated lung inflammation and tissue damage [42]. Treatment with either 40 mg/kg HE3413 or HE3177 significantly decreased the myeloperoxidase activity in BAL fluid compared to the vehicle control group (44%, $p = 0.038$ and 51%, $p = 0.014$ respectively). The levels of TNFα and IL-6 in BAL fluid were also decreased approximately 50%, but did not achieve statistical significance (data not shown).

3.7.4. DSS-induced colitis

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease have been associated with unresolved infection, chronic non-productive inflammation and a pro-inflammatory cycle that resists resolution [43]. HE3413 was evaluated in the DSS-induced colitis mouse model. The published literature indicates that in this model, five days of DSS exposure typically produces a colitis that continues well beyond 30 days [39], and in our experiments colitis was readily observed in colon samples assessed for diarrhea and inflammation on days 5 and 15 (Fig. 7). While no significant treatment effects were observed on day 5, HE3413 (10 and 40 mg/kg) significantly suppressed DSS-induced diarrhea ($p = 0.03$) and colon inflammation ($p = 0.04$) on day 15 in a dose-dependent manner. Histological analysis of the H&E stained colon segments demonstrated that HE3413 suppressed edema, leukocytic infiltration, epithelial defects, and glandular atrophy (Fig. 7D). In addition, a dose-dependent suppression of serum haptoglobin, an inflammatory acute phase protein associated with tissue deterioration in this condition [39], was observed on day 15 (Fig. 7C).

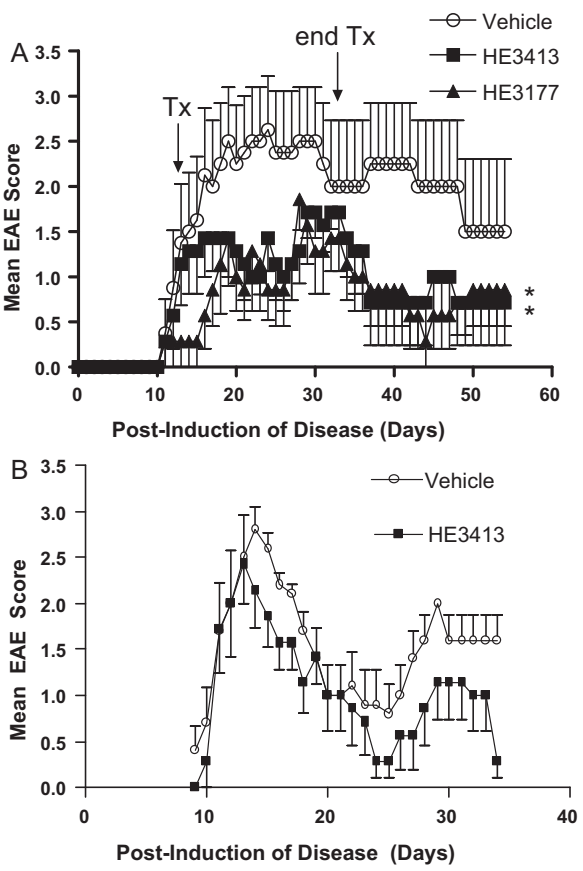

Fig. 6. Efficacy of the 5-androstenetetrols HE3413 and HE3177 in experimental autoimmune encephalomyelitis (EAE). (A) EAE was induced in female SJL/J mice on day 0 as in our previous studies [44]. Mice received daily oral gavage of vehicle, HE3413 (4 mg/kg), or HE3177 (4 mg/kg) administered at the first disease incidence (day 12). Tx indicates the beginning and end of therapy. *Significantly different from vehicle-treated group (Mann–Whitney test; $p < 0.0001$). (B) Female DA rats received s.c. injections of 0.2 mL (50 mg) of homogenized emulsion of whole DA rat spinal cord in complete Freund's Adjuvant on day 1. Rats received daily oral gavage of vehicle (0.125 mL; $n = 10$) or HE3413 (10 mg/kg; $n = 7$) (6 days per week) beginning when animals developed a clinical score of 1–2 (~days 10–12). +Significant difference between HE3413- and vehicle-treated groups total daily disease scores of individual animals (Mann–Whitney test; $p < 0.02$).

4. Discussion

A survey of human plasma revealed an ensemble of unidentified tetrols, which we investigated starting with the synthesis of four structurally related derivatives of the 7-hydroxy 5-

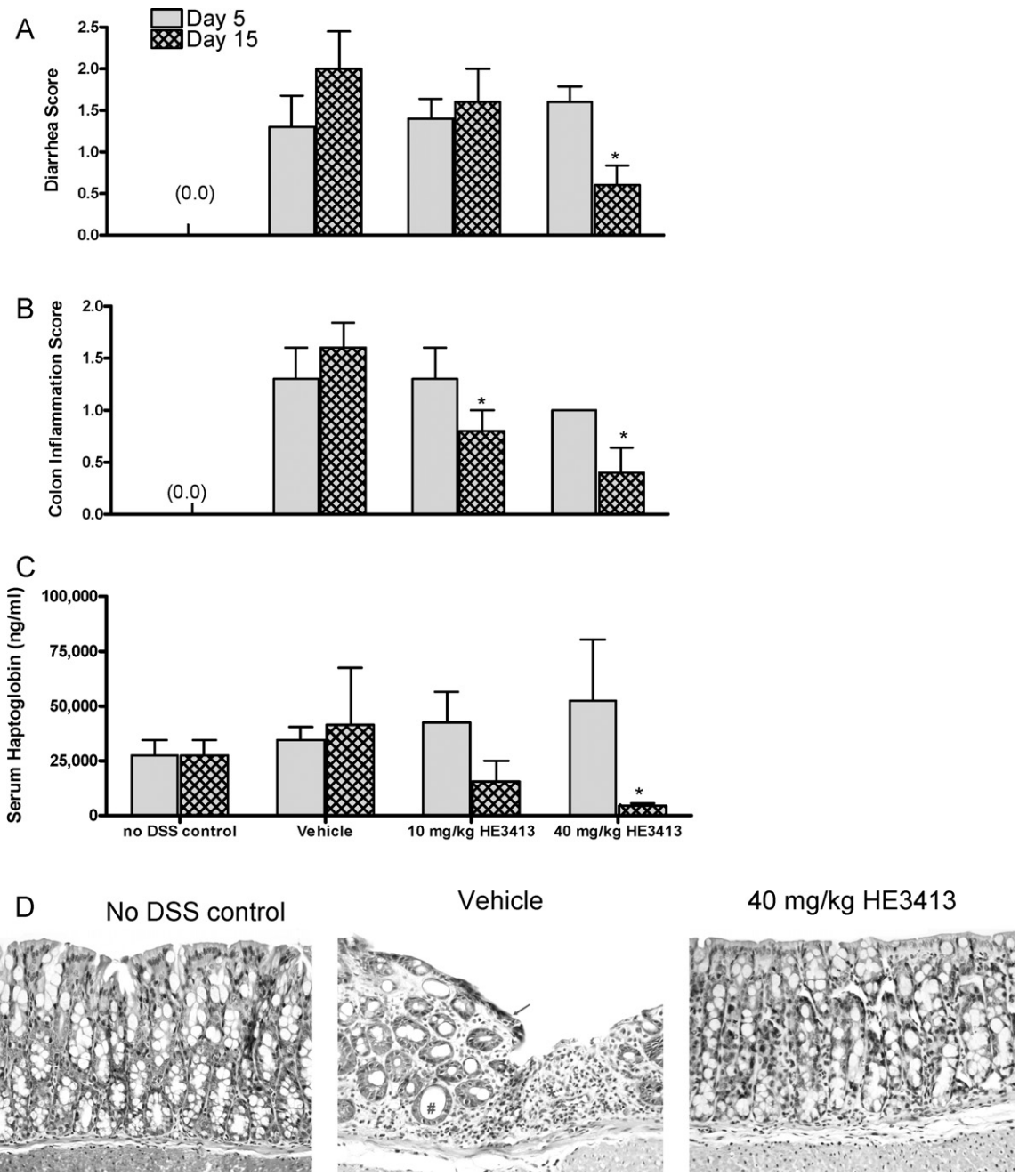

Fig. 7. Activity of HE3413, in the mouse DSS-induced colitis model of IBD. Colitis was induced in C57BL/6 male mice with 3% (w/v) dextran sodium sulfate (DSS) in drinking water initiated on days 1–5. The mice received daily oral gavage of vehicle or HE3413 (10 and 40 mg/kg) beginning on day 1. Groups of mice ($n = 5$) were sacrificed on day 5 and 15. Colons were removed and visually scored for the degree of (A) diarrhea and (B) colon inflammation. (C) Haptoglobin levels were measured in serum by ELISA. *Statistically different from vehicle control mean ($p < 0.05$; Student's t test). (D) Histological analysis of one representative colon sample (H&E staining) from each treatment group. Vehicle sample shows moderate colitis with epithelial attenuation (arrow), mucosal inflammation and glandular loss (*), and ectasia (#). All results are representative of two experiments that had similar results.

androstenetetrols (HE3413, HE3177, HE3354, and HE3412). Of these tetrols, the 7β-hydroxy epimers, HE3413 and HE3177, but not the analogous 7α-epimers, HE3354 and HE3412 were detected in human plasma. Human liver microsomes generated both the 7α- and 7β-hydroxy epimers from common 5-androstene precursors, such as DHEA, 5-AED, and DHA, which suggests that both epimers are produced, but the 7α-epimer, reported to be the metabolic precursor to the 7β-epimer, is rapidly and completely converted to the 7β form by action of 11β-hydroxysteroid dehydrogenase (11β-HSD) [44,45]. Thus, HE3413 and HE3177 were discovered and identified as new members of the human metabolome. Both are highly resistant to secondary metabolism and are orally bioavailable. Neither interacts with steroid binding nuclear hormone receptors or PPAR, nor are they metabolized into sex steroids. Although they have anti-inflammatory activity, they are not immunotoxic, and do not appear to be immunosuppressive in the manner of a glucocorticoid, although a thorough assessment of potential immunosuppressive activity was not conducted. In rodents, their potent anti-inflammatory activities are reminiscent of several other 5-androstene metabolites of DHEA [22,29]. Although poorly transported in the Caco-2 assay, in mice both HE3413 and HE3177 had greater C_{max} and AUC values following

oral administration when compared to 5-AED. This is consistent with their resistance to primary and secondary metabolism *in vitro* in contrast to the high metabolic susceptibility displayed by 5-AED and βAET. Oral HE3413 administration to cynomolgus monkeys resulted in a sustained plasma drug exposure profile.

The discovery that these novel tetrols are highly resistant to metabolism in their natural form was unexpected because natural C-19 steroids generally are highly susceptible to metabolic transformation, and have very poor oral bioavailability, properties that limit their clinical usefulness [20]. We have previously explored various strategies to overcome these limitations. For example, we have developed a depot formulation of AED (an ERβ biased agonist) that maintains serum levels of this hormone that are associated with biological activity in humans [46]. Our experience with immune regulating hormones of the 5-androstene series has been that 17α-substitution to prevent oxidation by 17β-hydroxysteroid dehydrogenase frequently results in the loss of biological activity, although 17α-ethynylation was implemented with βAET with surprisingly good results [21,29]. The resulting 17α-ethynyl-5-androsten-3β,7β,17β-triol (HE3286) retained broad-based biological activity and has not been immunosuppressive *in vivo* or *in vitro* [24,47]. In the present studies, we tested and confirmed the anti-inflammatory activity of two tetrols in rodent models of acute and chronic inflammation.

These orally bioavailable, metabolically stable, biologically active 5-androstene tetrols pose the possibility that a natural product in the human metabolome may be able to assert pharmaceutically relevant anti-inflammatory activity in target tissues.

Pharmacokinetics, drug metabolism and toxicology observations provided rationale and incentive for evaluation of these newly discovered C-19 steroid tetrols in mouse inflammation models. Anti-inflammatory activity was observed in the LPS-induced lung injury and DSS-induced colitis models, where oral administration was associated with reductions in pro-inflammatory cells and cytokines as well tissue inflammation (respectively). Anti-inflammatory activity was evidenced by reduced cellular infiltrates was also observed in experimental autoimmune prostatitis, and the symptoms of EAE were suppressed in the female SJL mouse MS model. In the later model, benefit was observed when administration was initiated after disease onset that suggests an anti-inflammatory and/or neuroprotective effect of the agent. These newly profiled androstenetetrols possess a highly potent anti-inflammatory activity that was observable at 4 mg/kg in the EAE model, compared to βAET, which required a much higher dose (120 mg/kg injected subcutaneously) to achieve a similar biological effect [24].

There are descriptions of C-18 steroid tetrols in humans in the published literature [48–53], but we could find no prior reference to C-19 tetrols in humans, perhaps because they are present at such low plasma concentrations. This suggests that the tetrols described here may be intracrine members of the human metabolome. While others have described the chemical synthesis or natural occurrence of certain C-18 tetrols in human tissues [50–54], their biological relevance was not explored. The anti-inflammatory properties of DHEA, 5-AED and βAET have been demonstrated in animal models [5,6,22,24,30]. Rodents readily metabolize exogenous androstenes into more highly oxidized compounds, and one such compound, HE3177, has been previously described in rat liver digests *in vitro* [55]. Thus, these naturally occurring androstene tetrols may contribute to biological activity observed from administration of androstenes. We have yet to explore the relationship between tetrol structure and anti-inflammatory activity.

The cellular and molecular targets relevant to the biological activity of HE3413 and HE3177 remain unknown. Since our recent studies have implicated macrophages as the target cell in the androstenetriol biological mechanism [32,47], it is tempting to speculate that they may also be the tetrol target. The murine models used in the present studies all possess a significant inflammatory macrophage component to their pathology [39,43]. However, other cell types, such as lymphocytes [56], hepatocytes [57], adipocytes [58], endothelial cells [59] may also be involved given the broad spectrum of DHEA activities. While a dedicated receptor has not been identified for the 7-hydroxy C-19 steroids, an ethynylated androstenetriol, HE3286, is reported to attenuate the NF-κB, p38, JNK, and ERK-2 pro-inflammatory pathways [32,47]. We are actively investigating the possibility that the molecularly related tetrols have a similar mode of action.

To our knowledge, this is the first report of the natural existence of the 5-androstene tetrols, HE3413 and HE3177 in human tissues. Their potent anti-inflammatory properties are described and their target tissue and mode of action inferred from that of known molecularly related agents. The discovery of these remarkably stable, anti-inflammatory components of the DHEA metabolome introduces them as naturally occurring agents that may be useful for prevention or resolution of chronic inflammatory conditions.

Disclosure statement

Authors Ahlem, Page, Acui, Kennedy, Ge, Huang, White, Villegas, Reading, and Frincke acknowledge potential conflict of interest as employees of Harbor Biosciences, Inc. Authors Mangano, Nicoletti, Conrad, and Wang declare no conflict of interest.

Role of the funding source

All experimental work reported in this article was sponsored by Harbor Biosciences, Inc. Author representatives from Harbor Biosciences participated in study design, data analysis and interpretation, report writing, and the decision to publish.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.10.005.

References

[1] Cutler Jr GB, Glenn M, Bush M, Hodgen GD, Graham CE, Loriaux DL. Adrenarche: a survey of rodents, domestic animals, and primates. Endocrinology 1978;103:2112–8.
[2] Hobe G, Hillesheim HG, Schon R, Reddersen G, Knappe R, Bannasch P, et al. Sex differences in dehydroepiandrosterone metabolism in the rat: different plasma levels following ingestion of DHEA-supplemented diet and different metabolite patterns in plasma, bile and urine. Horm Metab Res 1994;26:326–9.
[3] Wichmann U, Wichmann G, Krause W. Serum levels of testosterone precursors, testosterone and estradiol in 10 animal species. Exp Clin Endocrinol 1984;83:283–90.
[4] Belanger B, Belanger A, Labrie F, Dupont A, Cusan L, Monfette G. Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. J Steroid Biochem 1989;32:695–8.
[5] Dillon JS. Dehydroepiandrosterone, dehydroepiandrosterone sulfate and related steroids: their role in inflammatory, allergic and immunological disorders. Curr Drug Targets Inflamm Allergy 2005;4:377–85.
[6] Svec F, Porter JR. The actions of exogenous dehydroepiandrosterone in experimental animals and humans. Proc Soc Exp Biol Med 1998;218:174–91.
[7] Giltay EJ, van Schaardenburg D, Gooren LJ, von Blomberg BM, Fonk JC, Touw DJ, et al. Effects of dehydroepiandrosterone administration on disease activity in patients with rheumatoid arthritis [letter]. Br J Rheumatol 1998;37:705–6.
[8] Sirrs SM, Bebb RA. DHEA: panacea or snake oil? Can Fam Physician 1999;45:1723–8.
[9] Raven PW, Hinson JP. Dehydroepiandrosterone (DHEA) and the menopause: an update. Menopause Int 2007;13:75–8.
[10] Buster JE, Casson PR, Straughn AB, Dale D, Umstot ES, Chiamori N, et al. Postmenopausal steroid replacement with micronized dehydroepiandrosterone: preliminary oral bioavailability and dose proportionality studies. Am J Obstet Gynecol 1992;166:1163–8 [discussion 8–70].
[11] Fitzpatrick JL, Ripp SL, Smith NB, Pierce Jr WM, Prough RA. Metabolism of DHEA by cytochromes P450 in rat and human liver microsomal fractions. Arch Biochem Biophys 2001;389:278–87.

[12] Leblanc M, Labrie C, Belanger A, Candas B, Labrie F. Bioavailability and pharmacokinetics of dehydroepiandrosterone in the cynomolgus monkey. J Clin Endocrinol Metab 2003;88:4293–302.
[13] Poynter ME, Daynes RA. Restoration of immunocompetence in aging and other inflammatory disease states by dehydroepiandrosterone-3β-sulfate, an activator of the peroxisome proliferator-activated receptor alpha (PPARα). In: Kalimi MY, Regelson W, editors. Dehydroepiandrosterone (DHEA): Biochemical, Physiological and Clinical Aspects. Berlin; New York: Walter de Gruyter; 1999. p. 65–117.
[14] Suzuki M, Wright LS, Marwah P, Lardy HA, Svendsen CN. Mitotic and neurogenic effects of dehydroepiandrosterone (DHEA) on human neural stem cell cultures derived from the fetal cortex. Proc Natl Acad Sci USA 2004;101:3202–7.
[15] Risdon G, Kumar V, Bennett M. Differential effects of dehydroepiandrosterone (DHEA) on murine lymphopoiesis and myelopoiesis. Exp Hematol 1991;19:128–31.
[16] Roberge C, Carpentier AC, Langlois MF, Baillargeon JP, Ardilouze JL, Maheux P, et al. Adrenocortical dysregulation as a major player in insulin resistance and onset of obesity. Am J Physiol Endocrinol Metab 2007;293:E1465–78.
[17] Labrie F, Belanger A, Cusan L, Candas B. Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology. J Clin Endocrinol Metab 1997;82:2403–9.
[18] Labrie F, Luu-The V, Belanger A, Lin SX, Simard J, Pelletier G, et al. Is dehydroepiandrosterone a hormone? J Endocrinol 2005;187:169–96.
[19] Marwah A, Marwah P, Lardy H, Ergosteroids VI. Metabolism of dehydroepiandrosterone by rat liver in vitro: a liquid chromatographic-mass spectrometric study. J Chromatogr B Biomed Sci Appl 2002;767:285–99.
[20] Auci DL, Ahlem C, Li M, Trauger R, Dowding C, Paillard F, et al. The immunobiology and therapeutic potential of androstene hormones and their synthetic derivatives: novel anti-inflammatory and immune regulating steroid hormones. Mod Asp Immunobiol 2003;3:64–70.
[21] Auci D, Kaler L, Subramanian S, Huang Y, Frincke J, Reading C, et al. A new orally bioavailable synthetic androstene inhibits collagen-induced arthritis in the mouse. Ann N Y Acad Sci 2007;1110:630–40.
[22] Auci D, Nicoletti F, Mangano K, Pieters R, Nierkens S, Morgan L, et al. Anti-inflammatory and immune regulatory properties of 5-androsten-3{beta}, 17{beta}-diol (HE2100), and synthetic analogue HE3204: implications for treatment of autoimmune diseases. Ann N Y Acad Sci 2005;1051:730–42.
[23] Offner H, Firestein GS, Boyle DL, Pieters R, Frincke JM, Garsd A, et al. An orally bioavailable synthetic analog of an active dehydroepiandrosterone metabolite reduces established disease in rodent models of rheumatoid arthritis. J Pharmacol Exp Ther 2009;329:1100–9.
[24] Offner H, Zamora A, Drought H, Matejuk A, Auci D, Morgan E, et al. A synthetic androstene derivative and a natural androstene metabolite inhibit relapsing-remitting EAE. J Neuroimmunol 2002;130:128.
[25] Hennebert O, Pelissier MA, Le Mee S, Wulfert E, Morfin R. Anti-inflammatory effects and changes in prostaglandin patterns induced by 7beta-hydroxy-epiandrosterone in rats with colitis. J Steroid Biochem Mol Biol 2008;110:255–62.
[26] Le Mee S, Hennebert O, Ferrec C, Wulfert E, Morfin R. 7beta-Hydroxy-epiandrosterone-mediated regulation of the prostaglandin synthesis pathway in human peripheral blood monocytes. Steroids 2008;73:1148–59.
[27] Loria RM, Padgett DA. Control of the immune response by DHEA and its metabolites. Rinsho Byori 1998;46:505–17.
[28] Padgett DA, Loria RM. Endocrine regulation of murine macrophage function: effects of dehydroepiandrosterone, androstenediol, and androstenetriol. J Neuroimmunol 1998;84:61–8.
[29] Auci DL, Reading CL, Frincke JM. 7-Hydroxy androstene steroids and a novel synthetic analogue with reduced side effects as a potential agent to treat autoimmune diseases. Autoimmun Rev 2009;8:369–72.
[30] Marcu AC, Kielar ND, Paccione KE, Barbee RW, Carter H, Ivatury RR, et al. Androstenetriol improves survival in a rodent model of traumatic shock. Resuscitation 2006;71:379–86.
[31] Trauger R, Corey E, Bell D, White S, Garsd A, Stickney D, et al. Inhibition of androstenediol-dependent LNCaP tumour growth by 17alpha-ethynyl-5alpha-androstane-3alpha, 17beta-diol (HE3235). Br J Cancer 2009;100:1068–72.
[32] Wang T, Villegas S, Huang Y, White SK, Ahlem C, Lu M, et al. Amelioration of glucose intolerance by the synthetic androstene HE3286: link to inflammatory pathways. J Pharmacol Exp Ther 2010;333:70–80.
[33] Smith DA, Humphrey MJ, Charuel C. Design of toxicokinetic studies. Xenobiotica 1990;20:1187–99.
[34] van Breemen RB, Li Y. Caco-2 cell permeability assays to measure drug absorption. Expert Opin Drug Metab Toxicol 2005;1:175–85.
[35] Auci D, Mangano K, Destiche D, White SK, Haung Y, Boyle D, et al. Oral treatment with HE3286 ameliorates disease in rodent models of rheumatoid arthritis. Int J Mol Med 2010:625–33.
[36] Ahlem C, Auci D, Mangano K, Reading C, Frincke J, Stickney D, et al. HE3286: a novel synthetic steroid as an oral treatment for autoimmune disease. Ann N Y Acad Sci 2009;1173:781–90.
[37] Penna G, Amuchastegui S, Cossetti C, Aquilano F, Mariani R, Sanvito F, et al. Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. J Immunol 2006;177: 8504–11.
[38] Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. Gastroenterology 1984;87:1344–50.
[39] Melgar S, Karlsson A, Michaelsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. Am J Physiol Gastrointest Liver Physiol 2005;288:G1328–38.
[40] Rivero VE, Cailleau C, Depiante-Depaoli M, Riera CM, Carnaud C. Non-obese diabetic (NOD) mice are genetically susceptible to experimental autoimmune prostatitis (EAP). J Autoimmun 1998;11:603–10.
[41] Kim S, Nadel JA. Role of neutrophils in mucus hypersecretion in COPD and implications for therapy. Treat Respir Med 2004;3:147–59.
[42] Asti C, Ruggieri V, Porzio S, Chiusaroli R, Melillo G, Caselli GF. Lipopolysaccharide-induced lung injury in mice. I. Concomitant evaluation of inflammatory cells and haemorrhagic lung damage. Pulm Pharmacol Ther 2000;13:61–9.
[43] Torres MI, Rios A. Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy. World J Gastroenterol 2008;14:1972–80.
[44] Muller C, Pompon D, Urban P, Morfin R. Inter-conversion of 7alpha- and 7beta-hydroxy-dehydroepiandrosterone by the human 11beta-hydroxysteroid dehydrogenase type 1. J Steroid Biochem Mol Biol 2006;99:215–22.
[45] Hennebert O, Le Mee S, Pernelle C, Morfin R. 5Alpha-androstane-3beta,7alpha,17beta-triol and 5alpha-androstane-3beta,7beta,17beta-triol as substrates for the human 11beta-hydroxysteroid dehydrogenase type 1. Steroids 2007;72:855–64.
[46] Stickney DR, Dowding C, Garsd A, Ahlem C, Whitnall M, McKeon M, et al. 5-Androstenediol stimulates multilineage hematopoiesis in rhesus monkeys with radiation-induced myelosuppression. Int Immunopharmacol 2006;6:1706–13.
[47] Lu M, Patsouris D, Li P, Flores-Riveros J, Frincke JM, Watkins S, et al. A new antidiabetic compound attenuates inflammation and insulin resistance in Zucker diabetic fatty rats. Am J Physiol Endocrinol Metab 2010;298:E1036–48.
[48] Elce JS, Lai PP. Water-soluble metabolites of the estrogens. Quantitation of C-18 tetrols in rat feces. Steroids 1976;27:335–52.
[49] Badeau M, Adlercreutz H, Kaihovaara P, Tikkanen MJ. Estrogen A-ring structure and antioxidative effect on lipoproteins. J Steroid Biochem Mol Biol 2005;96:271–8.
[50] Caspi E, Njar VC. Concerning the pathway from 19-oxoandrost-4-ene-3,17-dione to estrone. Steroids 1987;50:347–62.
[51] Adlercreutz H, Jarvenpaa P. Assay of estrogens in human feces. J Steroid Biochem 1982;17:639–45.
[52] Gachancard-Bouya JL, Begue RJ. Urinary steroids from a newborn human infant. Identification of 2 alpha-hydroxy-4-pregnene-3,20-dione, 3 beta,15 beta-dihydroxy-5-pregnen-20-one and 3 beta,15 alpha-dihydroxy-5-pregnen-20-one. J Steroid Biochem Mol Biol 1994;49:213–26.
[53] Homoki J, Roitman E, Shackleton CH. Characterization of the major steroids present in amniotic fluid obtained between the 15th and 17th weeks of gestation. J Steroid Biochem 1983;19:1061–8.
[54] Numazawa M, Mutsumi A, Ogata M, Osawa Y. Synthesis of 16 alpha,19-dihydroxy-4-androstene-3,17-dione and 3 beta,16 alpha,19-trihydroxy-5-androsten-17-one and their 17 beta-hydroxy-16-keto isomers. Steroids 1987;49:247–57.
[55] Lardy H, Marwah A, Marwah P. Transformations of DHEA and its metabolites by rat liver. Lipids 2002;37:1187–91.
[56] Solano ME, Sander V, Wald MR, Motta AB. Dehydroepiandrosterone and metformin regulate proliferation of murine T lymphocytes. Clin Exp Immunol 2008;153:289–96.
[57] Peters JM, Zhou YC, Ram PA, Lee SS, Gonzalez FJ, Waxman DJ. Peroxisome proliferator-activated receptor alpha required for gene induction by dehydroepiandrosterone-3 beta-sulfate. Mol Pharmacol 1996;50:67–74.
[58] Rice SP, Zhang L, Grennan-Jones F, Agarwal N, Lewis MD, Rees DA, et al. Dehydroepiandrosterone (DHEA) treatment in vitro inhibits adipogenesis in human omental but not subcutaneous adipose tissue. Mol Cell Endocrinol 2010;320:51–7.
[59] Liu D, Iruthayanathan M, Homan LL, Wang Y, Yang L, Wang Y, et al. Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms. Endocrinology 2008;149:889–98.