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Pharmacology and immune modulating properties of 5-androstene-3β, 7β,17β-triol, a DHEA metabolite in the human metabolome

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ABSTRACT

Androst-5-ene-3β,7β,17β-triol (βAET) is an anti-inflammatory metabolite of DHEA that is found naturally in humans, but in rodents only after exogenous DHEA administration. Unlike DHEA, C-7-oxidized DHEA metabolites cannot be metabolized into potent androgens or estrogens, and are not peroxisome proliferators in rodents. The objective of our current studies was to characterize the pharmacology of βAET to enable clinical trials in humans. The pharmacology of βAET was characterized by pharmacokinetics, drug metabolism, nuclear hormone receptor interactions, androgenicity, estrogenicity, and systemic toxicity studies. βAET's acute anti-inflammatory activity and immune modulating characteristics were measured *in vitro* in RAW264.7 cells and *in vivo* in murine models with parenteral administration. βAET was rapidly metabolized and cleared from circulation in mice and monkeys. βAET was weakly androgenic and estrogenic in immature rodents, but not bound by androgen, estrogen, progesterone, or glucocorticoid nuclear hormone receptors. βAET did not induce peroxisome proliferation, nor was it systemically toxic or trophic for sex hormone responsive tissues in mature rats and monkeys. βAET significantly attenuated acute inflammation both *in vitro* and *in vivo*, augmented immune responses in adult mice, and reversed immune senescence in aged mice. βAET may contribute to the anti-inflammatory activity in rodents attributed to DHEA. Unlike DHEA, βAET's anti-inflammatory activity cannot be ascribed to activation of PPARs, androgen, or estrogen nuclear hormone receptors. Exogenous βAET is unlikely to produce untoward toxicity or hormonal perturbations in humans.

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1. Introduction

A detailed description of the pharmacology of dehydroepiandrosterone (DHEA) is dependent on an understanding of the pharmacology of its metabolome. The objective of our research was to describe the safety and anti-inflammatory pharmacology of androst-5-ene-3β,7β,17β-triol (βAET), an anti-inflammatory metabolite of DHEA, for its potential use in humans.

DHEA together with its sulfate conjugate are among the most abundant steroids found in human circulation [1]. In humans age-related declines in plasma DHEA coincide with a heightened pro-inflammatory status [2,3]. DHEA has activity in certain *in vitro* systems [4], and is remarkably effective as a chemoprotectant and anti-inflammatory agent in rodent models [4–9]. Published activities in rodent models include general anti-inflammatory effects [10], reversal of the aging phenotype [11], maintenance of bone mineral density [12], and enhanced immune [13–15], cognitive [16] and metabolic [17] functions. DHEA potentially acts through several different mechanisms [18]. It modifies glucocorticoid receptor (GR) signaling in addition to effects on glucocorticoid activity and opposes certain deleterious immune modulating activities of endogenous cortisol by reducing 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) expression and the cortisol/cortisone ratio [19–21]. Other mechanisms, such as peroxisome proliferator

Abbreviations: βAET, androst-5-ene-3β,7β,17β-triol; GR, glucocorticoid nuclear hormone receptor; PPAR, peroxisome proliferator activated receptor; AR, androgen nuclear hormone receptor; ERα, estrogen nuclear hormone receptor alpha; ERβ, estrogen nuclear hormone receptor beta; 17β-HSD, 17β-hydroxysteroid dehydrogenase; LPS, lipopolysaccharide; PK, pharmacokinetics; LC–MS/MS, liquid chromatography–mass spectrometry; GLP, Good Laboratory Practice; SQ, subcutaneous; HBsAg, hepatitis B surface antigen; CAR, carrageenan; PLN, popliteal lymph node; TNP–OVA, trinitrophenyl–ovalbumin conjugate; ASC, antibody secreting cells.

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activated receptor alpha (PPARα) activation and up-regulation of certain P450 enzymes, which occur in rodents, do not pertain to humans [18].

Although highly active in rodents as an immune-modulating agent, human clinical trials with DHEA have not demonstrated substantial anti-inflammatory activity, and benefits of treatment, when reported, are generally subtle compared to rodents, and may be accompanied by androgenic side effects (particularly in women) [22–24]. The inability to translate the benefits of DHEA therapy from rodents into humans has been referred to as "*The DHEA conundrum*" [25]. Differential metabolism between rodents and humans may contribute significantly to the divergent effects between the rodent and non-rodent species [26]. The discovery of the anti-inflammatory activity associated with poly-hydroxylated DHEA metabolites indicates that the desirable properties of DHEA may be shared by or reside in one or more of these metabolites, which are readily formed in rodents, but to a lesser extent in humans [27–30].

βAET is an anti-inflammatory DHEA metabolite with multi-faceted properties that include glucocorticoid-opposing actions [19,31] and immune modulation [27,30]. βAET is metabolically derived from DHEA through reduction of the 17-ketone by 17β-hydroxysteroid dehydrogenase (17β-HSD) and oxidation at C-7, which occurs in certain tissues, such as brain, liver, gut, lymphoid organs, joints and adipose through the activity of CYP3A [26] and CYP7B [19,32,33]. Human metabolic pathways do not favor the formation of highly oxidized DHEA metabolites [26], which are thus collectively present in low nanomolar concentrations in human circulation [34,35]. Other investigators have shown that they are not metabolized into sex steroids [36] and are not ligands for the peroxisome proliferator activated receptor [18]. Studying βAET pre-clinical safety in various models and expanding the characterization of its anti-inflammatory activity will facilitate the evaluation of βAET in humans.

2. Methods

2.1. Test compound

Androst-5-ene-3β,7β,17β-triol was custom manufactured for Harbor Biosciences by Niels Clauson-Kaas Chemical Research Laboratory (Farum, Denmark). βAET was formulated for murine and monkey pharmacokinetic studies as a solution in sulfobutylether β-cyclodextrin or as an aqueous microsuspension. Micronized βAET was formulated for nonclinical toxicology studies as a suspension of 100 mg/mL βAET in 40% (v:v) PEG200, 2% (v:v) benzyl alcohol, 2% (v:v) benzyl benzoate and q.s. propylene glycol for subcutaneous injection (SQ). *In vivo* efficacy studies used βAET formulated as an aqueous microsuspension.

2.2. Nuclear hormone receptor interaction studies

Nuclear hormone receptor interactions were measured as previously described [37]. Binding interactions for androgen receptor (AR), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), glucocorticoid receptor (GR), and the progesterone receptor (PR) were quantified in homogeneous competition assays using the PolarScreen^{TM} fluorescence polarization system (Invitrogen, Carlsbad, CA). Transactivation of AR, ERα, ERβ, and GR was measured in stably transfected human cancer cell lines expressing nuclear receptor-sensitive luciferase reporter genes. AR and GR were assayed with cell line MDA-kb2 (American Type Culture Collection CRL-2713) harboring a mouse mammary tumor virus/luciferase cassette. ERα and ERβ were assayed with cell line T47D-kBluc (American Type Culture Collection 2865) stably transfected with a synthetic plasmid containing three copies of an estrogen response element fused upstream of a luciferase gene. PPARα, PPARγ, and PPARδ were assayed in UAS-bla HEK 293T cells using the fluorometric Gene Blazer assay system (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.3. Nitric oxide production by RAW cells in vitro

RAW264.7 cells were obtained from ATCC and grown in DMEM containing 10% FCS (Invitrogen Corporation, Carlsbad, CA). As in our previous studies [38], βAET was dissolved in DMSO and added at a maximum of 0.1% (v/v). Sub-confluent layers of RAW264.7 cells grown in 24-well plates in phenol red-free DMEM + 10% FBS were stimulated with 100 ng/mL LPS (*Salmonella minnesota* Re595, Calbiochem, San Diego, CA) in the presence or absence of serial dilutions of βAET. After 24 h, nitric oxide in the supernatants was determined using Griess reagent (Amresco, Solon, OH). Cell viability was assessed by exclusion of propidium iodide.

2.4. In vivo study conduct

All experiments with animals were conducted in accordance with respective institutional guidelines and regulatory agencies, and were reviewed and approved by institutional animal care and use committees. Nonclinical safety studies in rats and monkeys were conducted at Charles River Laboratories (formerly CTRB, Senneville, Canada). Androgenicity and estrogenicity studies in mice and rats were conducted at Huntington Life Sciences (East Millstone, NJ). The in-life phase of the study that measured βAET pharmacokinetics in monkeys was performed at Xenometrics, Stillwell, KS. Pleurisy studies were performed at the University of Catania, Italy. Popliteal lymph node assays were performed at IRAS, Utrecht, The Netherlands. Mouse aging studies were performed at University of Utah, Salt Lake City, UT.

2.5. Pharmacokinetics

βAET pharmacokinetics (PK) was measured in CD-1 mice and cynomolgus monkeys (*Macaca fascicularis*). βAET (40 mg/kg) was administered as a solution in cyclodextrin to 8-week old male CD-1 mice ($n = 3$ per time point) by subcutaneous injection. Blood samples were collected by cardiac puncture under CO_2 anesthesia 15, 30, 60, 120 and 240 min post administration and processed to serum. The 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 quadrupole mass spectrometer (Waters, Beverly, MA). A single dose of 10 mg/kg βAET formulated as a solution in cyclodextrin was administered by intravenous injection to four male monkeys. Plasma samples were collected prior to dosing and 0.05, 0.25, 0.50, 0.75, 1, 2, 4, 8, and 24 h after administration. Concentrations of βAET and metabolites androst-5-ene-3β,7α,17β-triol, 3β,7α-dihydroxy-androst-5-en-17-one, 3β,7β-dihydroxy-androst-5-en-17-one, and androst-5-ene-3β,7β,16α,17β-tetrol were measured by LC–MS/MS as described above. Pharmacokinetic parameters were determined with WinNonlin software (Pharsight, Mountain View, CA).

2.6. Androgenicity and estrogenicity studies

Androgenicity was assessed by the method of Hershberger et al. [39]. Briefly, vehicle, 200 mg/kg βAET microsuspension or 1.5 mg/kg testosterone (positive control) was administered to 4–5-week-old castrated CD-1 mice ($n = 10$ per group) by subcutaneous injection for 7 days. On day 8, animals were weighed and sacrificed.

The seminal vesicles and prostates were removed and the percentage increase in wet weight of the seminal vesicles and prostates was compared to control vehicle. Estrogenicity was assessed by uterine weight increases in ovariectomized rats. Four- to five-week-old ovariectomized Wistar rats ($n=10$) received four daily subcutaneous injections of vehicle, 300 mg/kg βAET or 0.01 mg/kg 17β-estradiol 3-benzoate (positive control). Approximately 24 h after the last dose, animals were weighed and sacrificed. The uteri were removed, blotted dry and weighed. The percentage increase in uterine weight was compared with vehicle control.

2.7. Nonclinical safety

βAET was evaluated for genotoxic effects in Good Laboratory Practice (GLP) assays in accordance with FDA guidelines for testing new chemical entities in support of clinical trials [40].

2.7.1. Genotoxicity

Genotoxicity tests were conducted with and without metabolic activation in bacteria (Ames test, highest concentration = 5000 μg per plate) and Chinese hamster ovary cells (CHO chromosome aberration test, highest drug concentration = 3100 μg/mL) *in vitro*, and in Sprague-Dawley rats *in vivo* (bone marrow micronucleus test, high dose = 2000 mg/kg) in studies of standard design for the pharmaceutical industry.

2.7.2. Systemic toxicity studies

Twenty-eight day GLP toxicity studies were conducted in Wistar rats and cynomolgus monkeys. Rat studies: Treatment groups of 15 rats per gender received 28-daily subcutaneous injections of 0, 25, 50, and 100 mg/kg βAET, and 5 animals per group were allowed to recover for 14 days after completion of dosing. Data collection included clinical signs, dermal irritation scores at the injection site, body weight, food consumption, blood and urine chemistries, ophthalmology, gross necropsy with organ weights, and histopathology. Toxicokinetics was measured on days 1 and 28 in satellite animals. Monkey studies: Treatment groups of 3 monkeys per gender received 28 daily injections of 0, 42.5, 85, or 170 mg βAET. Data were collected as described for rats, but without recovery animals. Toxicokinetics was monitored in the main study animals.

2.8. Immune responses in young and aged βAET treated mice

Female BALB/c mice (8 per group; aged: 20 months, young: 3 months) were bred from breeding stock originally purchased from the National Cancer Institute, and housed in the University of Utah vivarium. Recombinant hepatitis B surface antigen (rHBsAg, 2 μg; Recombivax-HB, Merck and Co, Inc., Whitehouse Station, NJ) in aluminum hydroxide (14 μg) was delivered into the left thigh (primary immunization) of each animal by subcutaneous injection (50 μL). Certain animals received a booster (secondary) immunization 42 days later. Within 20 min after the primary immunization, animals received a single subcutaneous injection containing either vehicle alone (50 μL), 0.3 or 3.0 mg of test compound. On days 14, 21 and 42 after immunization, animals were sacrificed, serum obtained, and levels of HBsAg-specific antibodies and IgG1 or IgG2a isotypes evaluated by ELISA as in previous studies [41].

2.9. Carrageenan-induced pleurisy

Six- to eight-week-old CD1 male mice (Charles River, Calco, Italy) were maintained in a pathogen-free vivarium at the University of Catania, School of Medicine (Catania, Italy). The mice received sterilized food and water *ad libitum*, and were adapted to the ambient environment for at least seven days before experimentation. Mice were allocated into one of the following groups ($n=8$): (1) carrageenan (CAR) only, (2 and 3) CAR plus treatment 24 h and 1 h before CAR with either 0.3 or 3 mg subcutaneous (s.c.) βAET formulated as an aqueous suspension, (3) CAR plus s.c. vehicle 24 and 1 h before CAR, and (4) CAR plus 200 μg polyclonal rabbit anti-mouse-TNFα antibody (i.p.) 24 and 1 h before CAR (all treatments were given in a volume of 100 μL). Pleurisy was induced as previously described [38,42]. Animals were sacrificed 4 h after CAR challenge (peak of the neutrophil and exudate response). The pleural cavities were washed with 1 mL of saline; the exudate and washing solution were removed by aspiration, and the total fluid volume was measured. The exudate volume was calculated by subtracting the 1 mL injected from the total volume recovered. Leukocytes were counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

2.10. Popliteal lymph node (PLN) assay

Non-specific pathogen-free female BALB/c mice (6–12 weeks old) were obtained from the Utrecht University breeding facility (Gemeenschappelyk Dier Laboratorium, Utrecht, The Netherlands) and randomly assigned to specific treatment groups. Animals were housed at Utrecht University animal facilities in accordance with respective institutional guidelines and requirements of the relevant regulatory agencies. PLN assay: naïve mice (3–5 mice per group) were injected s.c. into the right hind footpad with 50 μL of a freshly prepared mixture of βAET (0.3 or 3.0 mg) together with a subsensitizing dose (10 μg) of TNP–ovalbumin conjugate (TNP–OVA) prepared as previously described [42]. Seven days after injection, the popliteal lymph node (PLN) was excised and separated from adherent fatty tissue. PLNs were isolated in ice cold PBS/1% BSA and single cell suspensions were prepared, washed, ($350 \times g$ at 4 °C), resuspended in 1 mL PBS/1% BSA, counted using a Coulter counter (Coulter Electronics, Luton, UK) and adjusted to 1×10^6 cells/mL. ELISPOT and cytokine ELISA assays (IFNγ, TNFα, and IL-4) and were performed as in our previous studies [38]. Flow cytometric analysis was performed as previously described [38] with staining for CD3 CY (145-2C11), CD4 FITC (RM4-5), CD8 PE (53-6.7), and CD19 PE (1D3) (BD PharMingen, The Netherlands).

2.11. Statistical analysis

For *in vitro* studies, and *in vivo* animal studies, data were analyzed using two-tailed Student's t tests. All p values <0.05 were considered statistically significant.

3. Results

3.1. Nuclear hormone receptor interactions

βAET was not measurably bound by the ligand binding domain of any of the tested nuclear hormone receptors, although it did weakly transactivate AR ($EC_{50} \approx 8200$ nM, $n=1$), ERα ($EC_{50} \approx 6500 \pm 3000$ nM, $n=3$), and ERβ ($EC_{50} \approx 400$ nM, $n=1$). The GR receptor was not bound or transactivated at the highest concentration assayed (10,000 nM). PPARα/γ/δ was not transactivated by βAET at concentrations up to 10,000 nM.

3.2. Pharmacokinetics

The pharmacokinetics of βAET was measured in mice following subcutaneous injection. Cyclodextrin solubilized βAET was rapidly absorbed from the injection site and rapidly cleared from circulation. The maximum serum concentration (C_{max}) fell from ≈7200 ± 2300 ng/mL ($n=3$) at 0.25 h to only ≈60 ± 30 ng/mL ($n=3$)

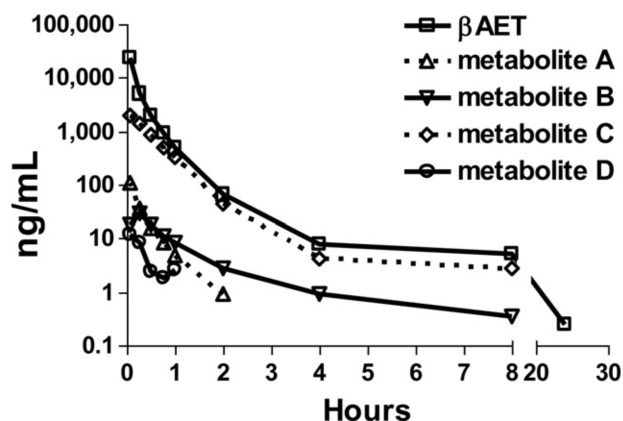

Fig. 1. βAET and metabolite PK in monkeys. βAET, 10 mg/kg, was administered to cynomolgus monkeys by IV injection as a solution in cyclodextrin. βAET and major metabolites were quantified by LC–MS/MS. βAET was rapidly cleared from circulation; major metabolites were detectable immediately after dosing, and were also rapidly cleared. βAET = androst-5-ene-3β,7β,17β-triol; Metabolite A = androst-5-ene-3β,7α,17β-triol; Metabolite B = 3β,7α-dihydroxy-androst-5-en-17-one; Metabolite C = 3β,7β-dihydroxy-androst-5-en-17-one; Metabolite D = androst-5-ene-3β,7β,16α,17β-tetrol.

at 4 h. Administered subcutaneously as a microsuspension, it was absorbed more slowly and resulted in a shallow plateau of serum drug concentrations ranging from approximately 550 to 800 ng/mL between 0.25 and 2 h, with greater than ≈400 ± 30 ng/mL ($n = 3$) remaining at 4 h. βAET was also rapidly cleared from circulation in monkeys. In monkeys IV ($n = 3$) administration of a 10 mg/kg β AET cyclodextrin solution resulted in $C_{max} \approx 24,000 \pm 6000$ ng/mL (observed at the first time point, 3 min), and cleared rapidly to ≈70 ± 40 ng/mL just 2 h after administration. The extrapolated $AUC_{(0-inf)}$ was ≈6000 ± 1000 ng h/mL, and the terminal half-life was 3.0 ± 1.9 h (Fig. 1, left). All four monitored metabolites were detected in plasma immediately after administration and were rapidly cleared from circulation (Table 1 and Fig. 1). The 3β,7β-dihydroxy-androst-5-en-17-one metabolite (produced by the action of 17β-HSD) was dominant. See Supplemental Tables 1–5 and Fig. 1 for plasma concentrations for individual animals and additional pharmacokinetic parameters for βAET and metabolites.

3.3. Androgenicity and estrogenicity

High doses of βAET (200 mg/kg) produced weak but statistically significant androgenic activity in young castrated mice. Seminal vesicle weights increased 22%, but without a significant increase in the prostate weights. In comparison, testosterone positive control administered at less than 1% of the βAET dose increased both seminal vesicle and prostate weights 135% and 92%, respectively. βAET also increased the mean absolute uterine weight in ovariectomized rats approximately 67%. Estradiol benzoate positive control (10,000-fold lower dose than βAET) increased the mean uterine weight approximately 8-fold.

Table 1
Summary of βAET and metabolite exposure parameters in monkeys ($n = 4$).

βAET or derivative	C_{max}, ng/mL	T_{max}, h	$AUC_{(0-24)}$, ng h/mL
βAET	24,000 ± 6000	0.05	6000 ± 1000
7α-Hydroxy[a]	110 ± 30	0.05	30 ± 10
7α-Hydroxy-17-keto[b]	30 ± 20	0.20	30 ± 10
7β-Hydroxy-17-keto[c]	2000 ± 700	0.05	1200 ± 300
16α-Hydroxy[d]	10 ± 10	0.1	10 ± 10

[a] Androst-5-ene-3β,7α,17β-triol.
[b] 3β,7α-Dihydroxy-androst-5-en-17-one.
[c] 3β,7β-Dihydroxy-androst-5-en-17-one.
[d] Androst-5-ene-3β,7β,16α,17β-tetrol.

3.4. Safety and toxicology

There were no mortalities or toxicologically significant changes in clinical pathology in rats that received 0, 25, 50, or 100 mg/kg βAET microsuspension by subcutaneous injection for 28 days. Injection site erythema was observed in all βAET treated groups. βAET increased the absolute and relative liver weights of all animals and was associated with minimal diffuse hepatocellular hypertrophy (diffuse enlargement of the hepatocellular cytoplasm often associated with increased eosinophils), but not peroxisome proliferation. All findings were generally resolved by the end of the 14-day recovery period. βAET was not genotoxic in the Ames test, chromosome aberration test, or bone marrow micronucleus test.

There was no evidence of systemic toxicity in monkeys that received subcutaneous injections of βAET microsuspension for 28 days in the clinical pathology or histopathology, although drug administration produced dose-dependent injection site irritation. The pharmacokinetics in both genders combined gave an AUC for the highest dose (170 mg) of ≈1500 ± 500 ng h/mL on day 1 and 7600 ± 1300 ng h/mL on day 28, with C_{max} of ≈300 and 600 ng/mL respectively.

3.5. βAET inhibition of NO production in LPS-stimulated RAW264.7 cells

βAET (1–10,000 nM) was tested for anti-inflammatory effects in LPS-induced RAW264.7 cells *in vitro*. A meta-analysis revealed that only 10 μM βAET significantly inhibited NO production (approximately 50%, $p < 0.05$, data not shown).

3.6. Effect of βAET on immune responses in aged mice

The capacity for βAET to resolve immune senescence was measured in healthy aged (20 months) or young (3 months) mice vaccinated with HBsAg. A single subcutaneous administration of βAET given contemporaneously with vaccination resulted in significantly higher anti-HBsAg IgG titers than in animals receiving only the vaccine alone (Fig. 2, left). The titer increase was greater in animals that received high dose βAET (120 mg/kg) and comparable to the effects of DHEA control at low dose (12 mg). Anti-HBsAg IgG titers and IgG1/IgG2a ratios in aged animals were similar to DHEA at high dose and young untreated controls (Fig. 2, right). Quantitatively similar results were obtained with transmucosal administration of βAET (data not shown). Forty-two days after vaccination, serum levels of anti-HBsAg IgG were low or undetectable (Table 2). Memory response effects were assessed by booster administration on day 45, and anti-HBsAg IgG was redetermined on days 52 and 59. In young mice a marked increase in specific antibody was seen. In the aged mice with no βAET, only 4 of 8 animals responded to booster immunization and antibody titers were low. In contrast, increased anti-HBsAg IgG titers were seen in all βAET treated aged animals (data not shown).

3.7. βAET inhibition of carrageenan-induced pleurisy

Carrageenan induced cellular exudates and edema in footpads, air pouches and pleural spaces of rodents are classical models of acute inflammation [43]. In agreement with previous reports [44], we found the peak neutrophil and turbid exudate response between 4 and 5 h after administration of CAR. Relative to the two groups of CAR control mice, treatment with 120 but not 12 mg/kg βAET significantly reduced both the degree of cell infiltration and the volume of pleural exudates (Fig. 3). The effects achieved by high-dose βAET were comparable to those observed with mouse

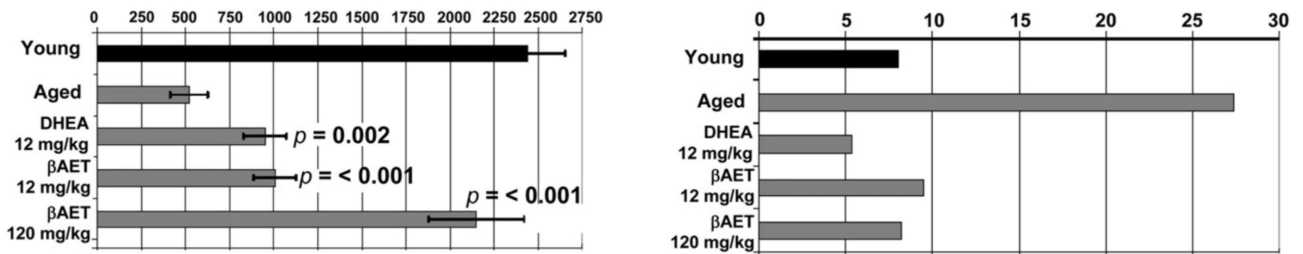

Fig. 2. Effect of βAET and DHEA on day 21 anti-HBsAg IgG titers. Healthy aged (20 months) or young (3 months) mice ($n = 8$ per group) were vaccinated with *Recombivax-HB* in alum adjuvant. Immediately following vaccination, animals received a single SQ injection of βAET (12 or 120 mg/kg), DHEA (12 mg/kg) or vehicle alone (100 L). Twenty-one days later, serum levels of HBsAg specific total IgG as well as IgG1 and IgG2a subclasses were determined by ELISA. Left panel, IgG ng/mL ± standard deviation. Right panel, ratio of IgG1 to IgG2a

Table 2
Anti-HBsAg status in vaccinated mice at various times after exposure to HBsAg.

Age category	βAET mg/kg	Number of anti-HBsAg positive mice[a]			Mean IgG anti-HBsAg titer in responding mice (ng/mL)[b]			p value[d]
		Day 42 (−3 d)[c]	Day 52 (+7 d)	Day 59 (+14 d)	Day 42 (−3 d)	Day 52 (+7 d)	Day 59 (+14 d)	
Young	0	8	8	8	46	1076	3666	–[e]
	0	0	3	4	UD[f]	36	312	–[g]
Aged	12	0	6	6	UD[f]	268	940	0.001
	120	4	8	8	18	318	1122	0.045

[a] Number of mice per group with anti-HBsAg titers ≥3 ng/mL.
[b] Averages computed for samples from mice in which anti-HBsAg was detectable.
[c] Values are the time in days (d) before (−) or after (+) secondary HBsAg vaccination on day 45.
[d] Upaired *t*-test *p* values for 14 d anti-HBsAg IgG1 compared with untreated, aged mice.
[e] Not analyzed for statistical significance.
[f] Undetectable.
[g] Comparator group for 12 and 120 mg/kg treated aged mice.

polyclonal anti-TNFα antibody (positive control). The figure represents combined data from two separate studies.

3.8. βAET enhancement of innate immunity in the PLN assay

The PLN assay, originally developed to estimate potential immune toxicities of xenobotic agents, was used to evaluate the immune modulating potential of βAET.

3.8.1. Effects on T and B cells

βAET (12 and 120 mg/kg) significantly increased lymphocyte numbers in the PLN seven days after the footpad was injected with the compound and a non-sensitizing dose of TNP–OVA (Fig. 4A). The increase in all cell types was dose dependent. Both $CD4^+$ and $CD8^+$ T cell numbers were increased and $CD4^+/CD8^+$ cell ratios decreased in a dose-dependent fashion compared to vehicle levels (2.2 and 2.6 *versus* 2.9, respectively). There was no other consistent change in cellular ratios or proportions.

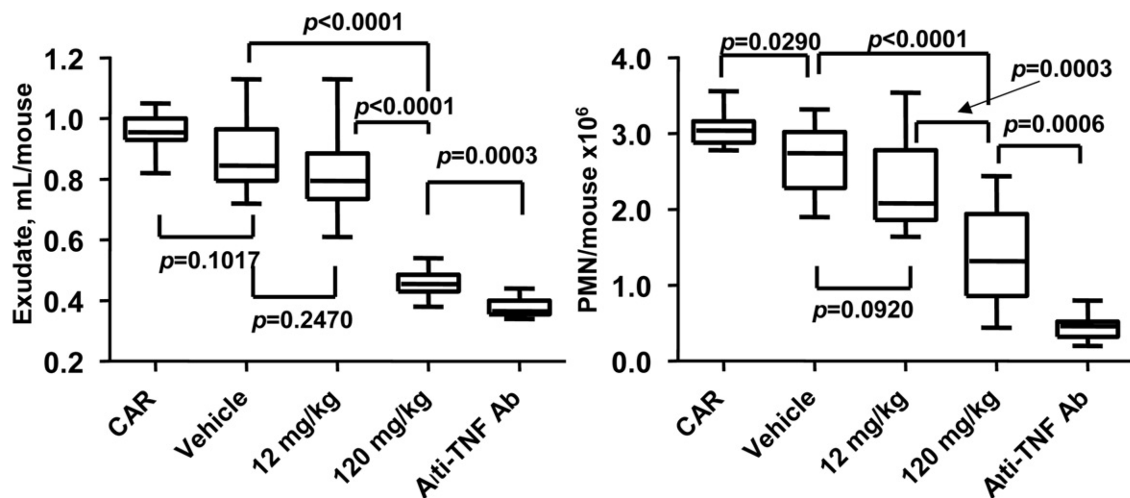

Fig. 3. Effect of βAET on PMN cell numbers and exudate volumes in the murine carrageenan (CAR)-induced pleurisy model. Male CD-1 mice ($n = 8$ per group) were anesthetized and 0.1 mL of either saline alone or saline containing 2% lambda-carrageenan (CAR) was injected into the pleural cavity. Mice were treated s.c. with βAET (12 or 120 mg/kg), vehicle (0.1 mL), or i.p. monoclonal antibody against mouse TNFα 24 h and 1 h before CAR challenge. Four hours after the CAR injection, the pleural cavities were washed with 1 mL of saline; the exudate and washing solution were removed by aspiration, and the total fluid volume was measured. The exudate volume was calculated by subtracting the 1 mL injected from the total volume recovered. The polymorphonuclear leukocytes (PMN) in the exudate were counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining. Box plots are shown for exudate volume (left) and PMN (right). Boxes depict median values with lower and upper quartiles and whiskers indicating lower and upper range of values. Statistical significance (Student's *t*-test) between treatment groups is shown with bridges between compared groups.

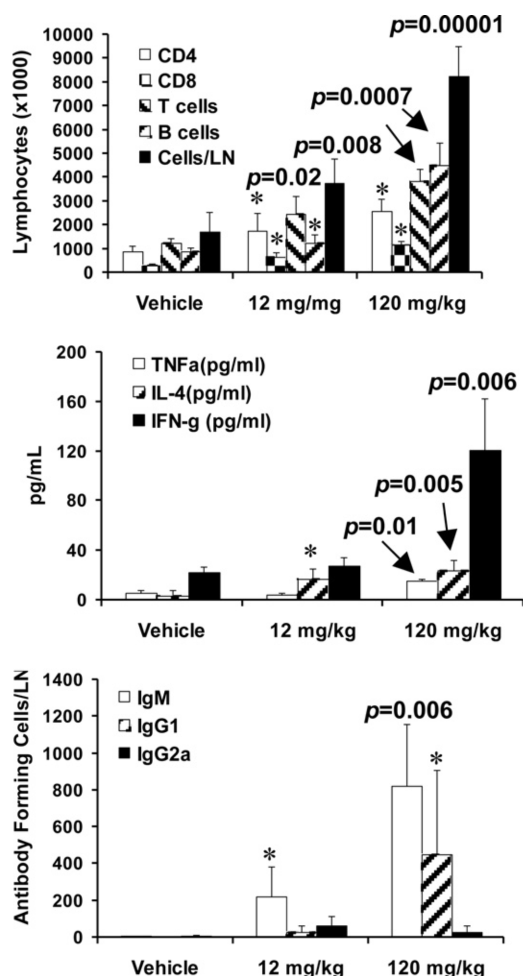

Fig. 4. The immune effects of βAET in the mouse PLN assay. βAET (12 or 120 mg/kg) or vehicle (0.1 mL) was injected into the right footpad along with a sub-sensitizing dose of TNP–OVA conjugate (10 μ in a total volume of 50 μL). Seven days later, the PLN were harvested. Total $CD4^+$, $CD8^+$ T or B lymphocytes per lymph node as determined by flow cytometry (upper panel); IL-4, TNFα, or IFNγ pg/mL determined by ELISA (middle panel); TNP-specific IgM, IgG1 or IgG2a antibody forming cells per lymph node determined by ELISPOT (lower panel). Each bar represents mean values ± standard deviation (five mice per group). *Significant ($p < 0.05$) difference when compared to control (vehicle) values.

3.8.2. Effects on cytokine production (IL-4, TNFα and IFNγ)

PLN cells from treated mice were Con A stimulated in culture overnight prior to IL-4 and IFNγ measurements. Treatment with 12 mg/kg βAET significantly increased only IL-4, whereas 120 mg/kg increased IL-4, TNFα, and IFNγ levels (Fig. 4B).

3.8.3. Effects on T cell-dependent (IgG1) and -independent (IgM) antibody-secreting cells (ASC)

The formation of TNP-specific IgM and IgG1 ASC in the PLN assay is a measure of the capacity to respectively induce T cell-independent and/or T cell-dependent stimulation. βAET induced the formation of IgM ASC in a dose dependent fashion, while significant IgG1 induction was only observed at the 120 mg/kg dose (Fig. 4C). Significant effects on IgG2a were not observed.

4. Discussion

Although DHEA has been widely studied, there are few publications concerning the pharmacology of its metabolome. βAET is one of several DHEA metabolites with anti-inflammatory activity [29,45], noting that some of these metabolites are also susceptible to metabolism, and therefore may be further transformed *in vivo* into the active entities. Recent studies suggest natural roles for βAET in preserving bone mineral content [46] and preventing the development of metabolic syndrome [35]. In a placebo controlled clinical trial it was shown to lower cholesterol in normal human subjects [47] consistent with DHEA activity in dogs reported by MacEwen and Kurzman [48].

The anti-inflammatory activity of βAET in a classical model of *acute* (as opposed to chronic) inflammation has not been previously reported. Qualitatively, the activity we observed was similar to DHEA [4], 5-androstenediol [27], and 17α-alkynyl-βAET [30,49] in chronic inflammation models. βAET's activity in RAW264.7 cells was similar to DHEA [50,51], although both DHEA and βAET were active only at 10 μM, a concentration not readily achieved or sustained *in vivo*. Chronic inflammation can either be Th1 or Th2 biased and the broad, anti-inflammatory activity of βAET may reflect an immune modulating function that toggles between either cell-mediated or humoral responses. In the present studies, we reported enhanced Th1-biased responses (improved anamnestic and cell mediated response and resolution of immune senescence) in aged mice. These observations are consistent with earlier work of Padgett and colleagues who reported that exogenous βAET limits a polyclonal Th2 response in mice [52]. In contrast, the immunological effects of βAET in the PLN assay suggested Th2 promotion of antigen specific responses. These later observations are consistent with our previous reports that βAET treatment reduced disease in rodent models of experimental autoimmune encephalomyelitis [53] and TNBS-induced colitis [49]. In these models, reduced disease is often associated with an immune shift from a Th1/Th17 status to a Th2/regulatory T cell profile [54,55]. Taken together, this suggests that immune modulation (i.e., either Th1 or Th2 promoting or suppressing) is context dependent and in each case it asserts an effect toward immune homeostasis. Importantly, the effects are not immunosuppressive. The mechanism of action by which this occurs is not known, although it is reasonable to assume that βAET acts similarly to a closely related compound, 17α-alkynyl-βAET (HE3286), which has been reported to modulate the TNFα and MAP kinase signal transduction pathways [37,56].

In our toxicology evaluations, we found βAET to be very well tolerated. There was no dose limiting treatment effect in either rats or monkeys. Subcutaneous injection irritation associated with the test article microsuspension was the only untoward effect observed in rats. Although βAET treatment resulted in slightly increased liver weights, it did not induce peroxisome proliferation, as reported for other C-7 oxidized DHEA derivatives as well [18]. Of concern in steroid product development are unintended nuclear hormone receptor binding and transactivation. The *in vivo* evaluations of βAET suggested these effects were minimal, and were consistent with our *in vitro* evaluation indicating no GR interaction, and negligible AR and ERα/β transactivation at sustainable plasma drug concentrations. Although a previous report by Lardy et al. indicated that oxidation at C-7 prevents conversion of βAET to potent androgens and estrogens [36], weak androgenic and estrogenic activities were observed in immature rodents. The biochemical basis for this activity is not known, but sex hormone effects were not recapitulated in either mature rats or monkeys, perhaps indicative of the sensitivity of immature rodents to exogenous hormonal influences. Interestingly, the 17α-ethynylated synthetic analog of βAET is weakly estrogenic in rodents even at very low exposures, but not in dogs even at high exposures [57]. Although estrogenic in rodents, the anti-inflammatory activity of the ethynylated analog was not dependent on ligation of ERα [49].

βAET is formed by the actions of three enzymes on DHEA, CYP3A or CYP7B, 11β-HSD-1, and 17β-HSD type 5 (AKR1C3) [33,58–64]. Both the initial product of the CYP3A/CYP7B reaction, 7α-hydroxy-DHEA, and the subsequent product of 11β-HSD-1,

7β-hydroxy-DHEA, have immunomodulation activity. 7α-Hydroxy-DHEA and 7α-hydroxy-epiandrosterone can stimulate specific antibody responses [32], while 7β-hydroxylated forms are anti-inflammatory [65,66]. βAET, like DHEA and sex steroids, is highly susceptible to metabolism. βAET was cleared rapidly from circulation by both primary and secondary metabolism and elevated plasma drug concentrations were not sustained beyond approximately 4 h. βAET was also rapidly cleared from circulation in humans after parenteral administration [47]. However βAET concentrations were likely to be higher in lymph nodes than in circulation [67–69] thereby enabling pharmacological activity in the murine PLN assay. The major unconjugated βAET monkey metabolite, 3β,7β-dihydroxy-androst-5-ene-17-one, is a product of the oxidative forms of 17β-HSD, which are considered generally responsible for C-17 hydroxyl oxidation and inactivation of sex steroids [70]. These enzymes are present in many organs and abundant in the liver. Indeed inhibition of enzymatic oxidation of the 17β-hydroxyl function of βAET via 17α-alkynylation confers oral bioavailability and the ability to achieve and sustain pharmacologically active concentrations of the native βAET pharmacophore [57]. Accordingly findings with this pharmaceutical may define the roles of βAET, a natural component of the human metabolome. In addition to a role in stem cell fate decisions [71], it is involved in metabolic processes, and possesses immune modulation properties that were previously shown to temper autoimmune disease in murine models and shown here to inhibit the acute inflammatory response and resolve immune senescence in aged mice. However, βAET's metabolic lability and limited bioavailability preclude its use as a practical treatment for acute inflammation, whereas the pharmacology of 17α-alkynyl-βAET is suitable for with this application.

5. Conclusion

Our studies have defined the pre-clinical pharmacology, toxicology and safety that led to clinical studies of βAET [47]. In addition to an apparently benign safety profile, βAET appears to possess a broad-spectrum of activities that may have utility in elderly populations when used as a dietary supplement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.04.010.

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