An Orally Bioavailable Synthetic Analog of an Active Dehydroepiandrosterone Metabolite Reduces Established Disease in Rodent Models of Rheumatoid Arthritis

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ABSTRACT

Dehydroepiandrosterone (DHEA) treatment provides diverse anti-inflammatory benefits in rodent models of diseases, including rheumatoid arthritis (RA), but only limited benefits to patients. In rodents, DHEA is metabolized to (among others) androstene-3β,7β,17β-triol (AET), which retains potent anti-inflammatory activity. 17α-Ethynyl-5-androstene-3β,7β,17β-triol (HE3286) is a novel, metabolically stabilized, orally bioavailable derivative of AET. In the DBA mouse model of collagen-induced arthritis (CIA), once-daily oral treatments (gavage) with HE3286 (40 mg/kg), beginning at onset of disease, significantly decreased disease. Benefit was associated with reduction in joint inflammation, erosion, and synovial proliferation as measured by histological analysis and mRNA of proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-6, IL-1β, and IL-23. Significant benefit was also observed in the CIA model even when treatments were delayed until 7 days after the onset of arthritis. Furthermore, dose-dependent benefit was observed in the DBA mouse model of collagen antibody-induced arthritis, as well as reductions in IL-6 and matrix metalloproteinase-3 mRNA levels in joints at the peak of disease and at the end of the study. HE3286, in contrast to dexamethasone, was not immune-suppressive in several classic animal models of immune function. Instead, HE3286 treatment was associated with reduced nuclear factor-κB activation and in our previous studies, with increased regulatory T cells. We hypothesize that HE3286 may represent a novel, perhaps first-in-class, anti-inflammatory agent and may more fully translate the benefits of DHEA, hereetofore largely limited to rodents, into treatments for human diseases, including autoimmune disorders such as RA.
highly oxygenated metabolites were identified. We hypothesized that these oxygenated DHEA metabolites were responsible for activities attributed to DHEA in rodents. Androstene-3β,7β,17β-triol (AET) was described previously (Butenandt et al., 1938; Reynolds, 1966) as a more highly oxygenated natural DHEA metabolite found in humans (Hill et al., 2005) that provided anti-inflammatory benefit in animal models of autoimmunity and trauma (Offner et al., 2002; Auci et al., 2003; Marcu et al., 2006). However, metabolic instability and poor oral bioavailability of AET suggested limited pharmaceutical usefulness of this natural hormone (Hollis-Eden Pharmaceuticals Inc., unpublished observations). Therefore, analogs of AET were prepared and screened for improved pharmaceutical properties. The lead candidate, HE3286 was composed of the core structure AET with the addition of an ethynyl group at the 17α-position, which stabilized the molecule to metabolism. Similar modifications failed at retaining biological activity, whereas others resulted in new drugs, such as 17-ethynyl estradiol, an orally bioavailable and pharmaceutically improved version of estradiol (Ranney, 1977). No one could predict whether HE3286 would be useful in the treatment of autoimmune disease. We probed the potential of oral HE3286 for utility in the treatment of RA and found surprisingly broad-based and potent anti-inflammatory activity.

There are various models of RA in the rodent, and each embraces elements of the pathophysiology relevant to the human disease. CIA is an autoimmuned-mediated polyarthritis sharing important similarities with RA (Holmdahl et al., 2002). Mice with CIA display synovitis and erosion of cartilage and bone. The autoimmune response in the CIA model is characterized by both the stimulation of collagen-specific T cells and the production of high titers of specific antibody. The intense synovitis, easily observable histologically, corresponds precisely with the clinical onset of arthritis. Because of the pathological similarities between CIA and RA, the CIA model has been the subject of extensive investigation and is a common model in which many putative antiarthritic agents are tested. In our previous studies, we reported that HE3286-treated mice displayed a significant decrease in CIA disease score and increased regulatory T cells compared with animals treated with vehicle alone (Auci et al., 2007). In the collagen antibody-induced arthritis (CAIA) model, the typical disease course involves a short period of acute disease followed by gradual resolution in joint inflammation (Nandakumar et al., 2003). The CAIA model permits an analysis of the contribution of non-T- and B-cell compartments to the pathogenesis of experimental RA. Here, we report the results of our studies testing HE3286 in these models of RA and in classic in vitro and in vivo models of immune function. We tested the ability HE3286 to limit NF-κB activation and to provide benefit in both CIA and CAIA models. We also evaluated its effect on both cellular and antibody-mediated immune function. Clinical benefit in RA may be achieved by HE3286 with an improved safety profile.

Materials and Methods

Drugs

The test compound HE3286 and the vehicle HERF202 were provided by Hollis-Eden Pharmaceuticals Inc. (San Diego, CA). HERF202 contains 30% β-cyclodextrin sulfobutyl ether sodium salt (w/v) in water. HE3286 was dissolved in vehicle and administered by oral gavage or by subcutaneous or intraperitoneal injection. Control animals were treated with an equal volume of HERF202.

Animal Care

Animals were purchased and housed in accordance with respective institutional guidelines and requirements of the relevant regulatory agencies. NF-κB and delayed type hypersensitivity (DTH) studies were performed according to regulations at the La Jolla Institute for Molecular Medicine (La Jolla, CA). CIA studies were performed by H. Offner at Oregon Health and Science University (Portland, OR), as described in a previous study (Offner et al., 2004). CAIA studies were performed by D. Boyle at University of California at San Diego (La Jolla, CA) as reported previously (Simelyte et al., 2005). Pooled lymph node (PLN) assay was performed by R. Pieters at Institute for Risk Assessment Sciences-Immunotoxicology, Utrecht University (Utrecht, The Netherlands), as reported previously (Pieters and Albers, 1999).

Measurements of NF-κB Activation in Vivo

Animals and Treatments. ICR mice (Charles River Laboratories, Inc., Wilmington, MA) were treated with HE3286 (40 mg/kg) by intraperitoneal injection or with vehicle alone 0.5 h after, or 24 h before and once again immediately before mice were challenged with 500 μg of LPS i.p. (time 0). At 0.2, 1.5, 2 and 2.5 h after the LPS challenge, animals were sacrificed, and NF-κB activation in spleen was evaluated.

Spleen Cell Lysate Preparation. Spleens were removed from each mouse and chilled in ice-cold complete Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA), cut into three pieces, and ground between glass slides. Cells were transferred to 15-ml tubes and passed through a cell strainer. Cells were spun for 5 min at 350g. Media were aspirated, and cells were resuspended in 1 ml of lysing solution to remove red blood cells (approximately 1 min at room temperature). Complete Dulbecco’s modified Eagle’s medium (12 ml) was then added, and cells were spun at 350g for 5 min. Media were aspirated, and the cells were resuspended in 10 ml of ice-cold PBS. Cells were spun again for 5 min at 350g. PBS was aspirated, and 1 ml of cold PBS was added. Cells were transferred to Eppendorf tubes on ice. Cells were spun for 10 min in a microcentrifuge, and the PBS was aspirated. Five times the pellet volume of whole cell lysis buffer (20 mM HEPES, pH 7.4, 20% glycerol, 1% Nonidet P40, 1 mM MgCl2, and 0.5 mM EDTA) was added, and then the tube was vortexed and kept on ice for 30 min. Finally, the tube was spun in a microcentrifuge for 20 min at 4°C. The supernatant fluid was transferred to Eppendorf tube and kept at ~80°C.

Evaluation of NF-κB Activation. Protein concentration for spleen cell lysates was measured by using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). To normalize the samples, 1.2 optical density750 units (1.7–3 μl) was added to each well, and NF-κB enzyme-linked immunosorbent assay was performed using the NF-κB Family Transcription Factor Assay kit (Active Motif, Carlsbad, CA). This kit is specific for detection of activated NF-κB in whole cell lysates. Experimental samples and controls were run in duplicate. Raji nuclear extract (2 μl) was used as a positive control.

Collagen-Induced Arthritis

Mice. DBA/1Lac/J male mice (12/group; The Jackson Laboratory, Bar Harbor, ME) were treated (gavage) with HE3286 or with vehicle 7 days after disease onset (50 mg/kg).

Induction and Evaluation of CIA. Bovine type-2 collagen (bCII, immunization grade) was purchased from Chondrex, Inc. (Redmond, WA). To induce CIA, 8-week-old mice were immunized with 100 μg of bCII, emulsified 1:1 with 100 μl of complete Freund’s adjuvant (Difco, Detroit, MI) intradermally at the base of the tail. Animals were monitored for onset and progression of disease beginning 4 to 7 weeks after immunization. A mouse was considered at...
onset of disease when at a score of 1. The arthritic severity of mice was evaluated using a grading system for each paw according to the following scale: 0, no redness or swelling; 1, slight swelling in ankle or redness in foot; 2, progressed swelling/inflammation and redness from ankle to mid foot; 3, swelling/inflammation of entire foot except or redness in foot; 4, swelling and inflammation of entire foot including toes; and 4, swelling and inflammation of entire foot except toes; and 4, swelling and inflammation of entire foot including toes (Offner et al., 2004).

**Histology.** Fixed joints were decalcified in formic acid and processed for paraffin embedding. Tissue sections (5 μm) were stained with hematoxylin and eosin for histopathological analysis as described in our previous study (Offner et al., 2004). The histological arthritic scores were determined in an unblinded manner for proliferative and inflammatory changes and graded from 0 to 3 for each limb. For synovial proliferation, grading was as follows: grade 0, no proliferation; grade 1, mild proliferation with two to four layers of reactive synoviocytes; grade 2, moderate proliferation with four plus layers of reactive synoviocytes, increased mitotic activity, and mild or absent synovial invasion of adjacent bone and connective tissue; and grade 3, severe proliferation and characterized by invasion and effacement of joint space and adjacent cartilage, bone, and connective tissue. For articular inflammation, grading was as follows: grade 0, no inflammation; grade 1, mild inflammation with one aggregate or minimal diffuse leukocyte infiltrates; grade 2, moderate infiltration with two or more aggregates of leukocytes; and grade 3, severe inflammation with significant coalescence to diffuse infiltrates of leukocytes.

**Cytokine and mRNA Detection.** Levels of cytokines in splenocyte culture supernatants were measured as in our previous studies (Sinha et al., 2007). Total RNA was isolated from spleens using the RNeasy mini kit protocol (QIAGEN, Valencia, CA) and then converted to cDNA using oligo(dT), random hexamers and SuperScript II RT enzyme (Invitrogen). Real-time PCR was performed as described in our previous study (Polanczky et al., 2005) using QuantiTect SYBR Green PCR master mix (QIAGEN) and primers (Applied Biosystems, Foster City, CA). Reactions were conducted on the ABI Prism 7000 sequence detection system (Applied Biosystems) to detect the following genes: L32 (forward, GGA AAC CCA GAG GCA TTG AC; reverse, TCA GGA TCT GCC CCT TGA AC), IFN-γ (forward, TGC TGA TGG GAG GAG ATG TCT; reverse, TGC TGT CTG GCC TGC TGT TA), TNF-α (forward, CAG CCG ATG GGT TGT ACC TT; reverse, GCC GAG CAG TAA; reverse, GCA TGC AGA GAT TCC GAG AGA). Other groups were treated to day 14.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>NF-κB A.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE3286 (40 mg/kg)</td>
<td>2</td>
<td>0.084 ± 0.0001</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2</td>
<td>0.109 ± 0.004</td>
</tr>
</tbody>
</table>

**Induction and Evaluation of CAIA.** To induce arthritis, mice were administered 1 mg of an anti-bCII antibody cocktail (Chondrex, Inc.) intravenously on day –2. On day 0, animals were treated intraperitoneally with LPS (12.5 μg), as described in a previous study (Simelyte et al., 2005). The arthritic severity of mice was evaluated in a blinded manner using a semiquantitative clinical scoring system for each paw: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved, and 4, severe arthritis of the entire paw and digits.

**mRNA Detection in Joints.** The right ankle joint was collected, dissected to remove extra-articular tissue, and snap-frozen in liquid nitrogen. The specimens were pulverized, weighed, and stored at –80°C. Reverse transcription-PCR was performed as described in a previous study (Simelyte et al., 2005) to determine IL-6 and matrix...
Histological analysis of joint tissue from vehicle- and HE3286-treated animals

Two hind limbs from three to four mice were submitted in paraformaldehyde. Limbs were decalcified in HCl and sectioned in half longitudinally through the stifle and tibiotarsal joints. Five-micrometer sections were processed and stained with hematoxylin and eosin. The histological arthritis scores were determined in an unblinded manner for proliferative and inflammatory changes (see Materials and Methods for details). SPS, synovial proliferation score; AIS, articular inflammation score.

**TABLE 2**

Histological analysis of joint tissue from vehicle- and HE3286-treated animals

<table>
<thead>
<tr>
<th>Dose</th>
<th>SPS</th>
<th>AIS</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mg/kg</td>
<td>0</td>
<td>0</td>
<td>Very few mononuclear cells</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>0</td>
<td>0</td>
<td>Very few mononuclear cells</td>
</tr>
<tr>
<td>0 mg/kg</td>
<td>3</td>
<td>2</td>
<td>Marked synovial proliferation and growth into adjacent bone and cartilage. Erosion and loss of articular cartilage are present. Scattered mononuclear and neutrophilic inflammatory cells are present within the synovium and adjacent stroma.</td>
</tr>
<tr>
<td>0 mg/kg</td>
<td>2</td>
<td>1</td>
<td>Moderate synovial proliferation is present and scattered mononuclear cells in stroma and synovial space contains a focal area of neutrophils.</td>
</tr>
<tr>
<td>0 mg/kg</td>
<td>3</td>
<td>2</td>
<td>Marked synovial proliferation and growth into adjacent bone and cartilage. Erosion and loss of articular cartilage are present. Scattered mononuclear and neutrophilic inflammatory cells are present within the synovium and adjacent stroma.</td>
</tr>
<tr>
<td>0 mg/kg</td>
<td>3</td>
<td>2</td>
<td>Marked synovial proliferation and growth into adjacent bone and cartilage. Erosion and loss of articular cartilage are present. Scattered mononuclear and neutrophilic inflammatory cells are present within the synovium and adjacent stroma.</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Processing artifact prevented adequate evaluation.</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>3</td>
<td>3</td>
<td>Marked synovial proliferation and in growth into adjacent bone and cartilage. Erosion and loss of articular cartilage are present. Diffuse mononuclear and neutrophilic inflammatory cells are present within the synovium and adjacent stroma. The articular spaces contain large numbers of neutrophils and fibrin.</td>
</tr>
</tbody>
</table>

**SPS**, synovial proliferation; **AIS**, articular inflammation. N.D., not done.

**Fig. 2.** CIA joint histology in DBA mice after treatment with HE3286. Fixed joints were decalcified in formic acid and processed for paraffin embedding. Tissue sections (5 μm) were stained with hematoxylin and eosin for histopathological analysis. The histological arthritis scores were determined in an unblinded manner for proliferative and inflammatory changes (see Materials and Methods for details). SPS, synovial proliferation score; AIS, articular inflammation score.

Metalloproteinase (MMP)-3 mRNA expression. Predeveloped sequence detection reagents (Applied Biosystems) specific for IL-6 and MMP-3 were used at 0.8 μl per 20 μl of reaction mixture. Each PCR reaction mixture also included 1× TaqMan Universal Master Mix (Boyle et al., 2003).

**DTH Responses.** CD1 mice (Charles River Laboratories, Inc.) were used for these studies. On the day before the start of the experiment, male mice (eight/group) were shaved on their right flank. On day 0, mice were painted (sensitized) on the shaved flank with 50 μl of 2.5% oxazolone solution in 95% ethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands). Mice were then, beginning on the same day, treated with dexamethasone (DEX; 3 mg/kg i.p.), HE3286 (40 mg/kg, by gavage), or with vehicle (HERF202, by gavage) alone, once daily for 4 days. Twenty-four hours after the final treatment, mice were challenged on the right dorsal ear surface with 25 μl of 0.25% oxazolone solution. Both the right and left pinnae thickness were measured at the indicated time points using a micrometer caliper. Data are expressed as left ear minus right ear thickness.

**Reporter Antigen Popliteal Lymph Node Assay**

**Mice.** Nonspecific 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.

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Chemicals and Reagents. Chemicals were obtained from Sigma-Aldrich unless stated otherwise. Saline (0.9%; B. Braun Mel- sungen, Melsungen, Germany) and citrate buffer (0.1 M citric acid and 0.1 M disodium hydrogen phosphate, pH 6.0) were used to dilute test chemicals. Trinitrophenol-ovalbumin (TNP-OVA) was prepared as described previously (Pieters and Albers, 1999).

Assay. Naive mice (five/group) were injected subcutaneously into the right hind footpad with 50 μl of a freshly prepared sensitizing dose (50 μg) of TNP-OVA. Mice were treated (daily for 5 days) with DEX (5 μg i.p.), with HE3286 (4 or 40 mg/kg given by oral gavage) in HERF202 (100 μl) vehicle, or with vehicle alone, or with both HE3286 and DEX. The 5-μg dose of DEX was chosen, based on

Fig. 3. Proinflammatory cytokine mRNA detected in splenocytes taken from HE3286-treated CIA mice at the end of study (day 22 after onset). Splenocytes were pooled, frozen, subsequently thawed, and evaluated for expression of cytokine mRNA by the RNase protection assay. Data are expressed as relative units on the y-axis and either drug or vehicle on the x-axis. Bottom and top boxes indicate the first and third quartiles, respectively. The line connects the medians of the two samples. For IL-17 measurements, splenocytes (4 × 10⁶) were stimulated with bCII (25 μg/ml) for 48 h. IL-17 levels in supernatants were determined using the 10-Plex Luminex kit (Bio-Rad Laboratories).

Fig. 4. Effect of HE3286 treatment on well established CIA. DBA male mice (12/group) were immunized with 100 μg of bCII/100 μg of complete Freund’s adjuvant (100 μl) on day 0. Mice were then treated (by gavage) with HE3286 (50 mg/kg) daily, beginning 7 days after disease onset (day 1). Data are expressed as median CIA disease score per paw. The median duration of treatment in the vehicle-treated group was 22.0 days compared with 20.5 days in the HE3286-treated group. Bars represent interquartile ranges. Solid line connects medians. p < 0.001 versus vehicle on day 28. Arrow indicates start of treatment.
previous studies in this assay system that defined the minimally effective immune suppressive dose. Five days later, mice were killed by cervical dislocation, and the 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 g at 4°C), resuspended in 1 ml of PBS/1% BSA, and counted using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA).

**Proliferation Studies**

Spleens were obtained from C57BL/6 mice by aseptic techniques. The spleens were teased and the suspension filtered to obtain single cells. Cells (10^6 mononuclear cells/ml RPMI 1640 medium, 10% fetal bovine serum, and 50 μM 2-mercaptoethanol) were incubated in 96-well plates, 0.2 ml per well, for 3 days with no stimulus, or with approximately 5 μg/ml concanavalin A, 2 μg/ml phytohemagglutinin, or 1:100 dilution of pokeweed mitogen (Invitrogen). Parallel cultures contained HE3286 at 1000, 100, and 10 nM. Compound was dissolved in dimethyl sulfoxide at 10 mg/ml and then diluted to the desired concentration with culture medium, leading to a maximal final solution containing 0.01% dimethyl sulfoxide. DEX at 40 nM served as the positive control. Cells were incubated overnight with approximately 1 μCi/ml [3H]thymidine, and proliferation was determined by incorporation of label into cell DNA after filtration, washing, and liquid scintillography. Five individual mice were used, with each spleen cell preparation incubated in triplicate for each condition.

**Statistical Analysis**

For NF-κB profiles, overall profile homogeneity was assessed by means of the Wilcoxon-Mann-Whitney test stratified by time. The level of significance is set to α = 0.05, two-sided. Resulting p values were adjusted for multiple comparisons (Westfall et al., 1999; Cytel Software Corporation, 2005). CIA scores and cytokine levels were modeled via the linear mixed model, with treatment, time from CIA onset, and the interaction between time from onset and treatment as independent variables. To facilitate direct interpretation, CIA scores were analyzed as measured, in a scale of 1 to 4. Dose-response trends at each time point were tested for the significance of the slope through a simple linear regression through the means model and formally by use of the exact nonparametric Jonckheere-Terpstra test (Cytel Software Corporation, 2005). Statistical analysis was performed on the SAS system, version 9.1 (SAS Institute, Cary, NC).

Cumulative disease index scores were calculated as the sum of the daily arthritic severity scores ± S.D. Differences were evaluated by Student’s t test.

**Results**

HE3286 seemed well tolerated at these doses, with respect to frank toxicity and body weight.

**Effect of HE3286 on NF-κB Activation in Vivo.** When mice were pretreated (−24 and 0 h) with HE3286 (40 mg/kg i.p.) and challenged (intraperitoneally) with 500 μg of LPS, levels of activated NF-κB observed in spleens at all time points (0.2, 1.5, 2, and 2.5 h) after challenge were reduced compared with spleens from animals treated with vehicle alone (Fig. 1). The overall profiles of these groups, in terms of activated NF-κB, were significantly different (p < 0.001). Similar results at the 1.5-h time point were obtained with protocols using a single dose (40 mg/kg) of HE3286 given to animals at 0.5 h after LPS challenge (Table 1).

**Effect of HE3286 in CIA When Treatment Begins at Disease Onset.** In our previous studies, we found that when treatment with HE3286 began at disease onset, within 13 days of treatment, HE3286-treated mice displayed a significant (p < 0.04) decrease in CIA score compared with animals treated with vehicle alone from approximately 13 days after onset to approximately 18 days after onset (Auci et al., 2007).

To seek the minimally effective dose, the study was repeated and more extensive histological evaluation was performed. The 40-mg/kg dose group showed benefit as determined by a strong trend toward reduced cumulative disease index scores (the sum of the daily arthritic severity scores; 94.5 ± 37 in the HE3286-treated group versus 164 ± 90 for the vehicle-treated group; p = 0.06). Lower doses of HE3286 (10 or 4 mg/kg) were not effective (cumulative disease index scores of 172 ± 73 and 138 ± 59; p = 0.54 and 0.87, respectively). Joint tissue samples taken from mice at the end of the study revealed expected histopathological changes, including marked synovial proliferation, erosion, and loss of articular cartilage in joints from three of four of the vehicle-treated animals analyzed. In agreement with our previous study,
intact smooth tissue, fewer mononuclear cell infiltrates and less proliferation were observed in most joints from animals (n = 3) treated with 40 mg/kg HE3286 (Fig. 2; Table 2) (Auci et al., 2007).

Effect of HE3286 Treatment on NF-κB-Associated mRNAs and Cytokines in Spleen. Treatment with HE3286 (40 mg/kg) was also associated with changes in ex vivo cytokine mRNA expression by splenocytes taken at end of the study. Levels of mRNA for IFN-γ, IL-1β, IL-23, IL-6, and TNF-α seemed to be decreased in spleens taken and pooled at the end of the study (Fig. 3). However, the pooling of samples led to an effective sample size of three replicates and did not allow for effective statistical analysis of these observations. IL-17 levels were decreased by almost 50% in cell cultures from the animals treated with the highest dose of HE3286 compared with vehicle-treated controls [vehicle median (IQR), 17.5 (16.6, 17.7) versus HE3286 median (IQR), 9.8 (9.7, 10.2)]. Decreases were also observed in TNF-α, IL-13, IFN-γ, and IL-2. Levels of IL-5, IL-6, IL-1β, and IL-10 were unchanged (data not shown).

Effect of HE3286 in CIA When Treatment Begins in Well Established Disease. We next determined whether treatment with HE3286 could also provide benefit in this CIA model when treatments were delayed until disease was well established. CIA, as judged by disease score, was well established in mice by 7 days after onset (Fig. 4), in which the average disease score per paw was approximately 1.3 of a possible score of 4. Within 12 days of the start of treatment, mice treated with HE3286 had CIA scores that trended lower compared with vehicle-treated mice (p < 0.001). Disease progressed in severity in the vehicle-treated group reaching maximal per-paw score of 4 by 23 days after CIA onset. In contrast, disease did not seem to progress in the HE3286-treated group, in that the average disease score never rose significantly above 1.6 for the duration of the study.

Effect of HE3286 in CAIA. In CAIA, daily treatment with HE3286 produced clear benefit in a dose-dependent manner (Fig. 5). CAIA began to develop by day 3 in most of the vehicle-treated and HE3286-treated animals, in which the overall, average disease score was 0.5 to 0.75. Disease peaked in all groups on days 5 to 7. Remarkably, arthritis in the high-dose group (80 mg/kg) was virtually absent throughout the course of the study. The benefit provided by HE3286 treatment was dose-dependent, because the 40-mg/kg dose produced benefit that was greater than the 20-mg/kg dose, but less than the 80-mg/kg dose (Jonckheere-Terpstra p < 0.001). All animals lost weight as a result of disease induction. However, all HE3286-treated animals returned to baseline weight by the end of the study, in contrast to vehicle-treated animals, which did not (data not shown). In one group of animals, treatment with HE3286 (40 mg/kg) was only given on days 5–14. HE3286 treatment provided this group of animals with significant (p < 0.05) benefit beginning on day 9. HE3286 treatment was associated with dose-dependent decreases (compared with vehicle administration) in IL-6 (Fig. 6, top) and MMP-3 (Fig. 6, bottom) mRNA in joints of animals taken at peak of disease (day 7) and at the end of the study (day 14). This study was repeated using the 40-mg/kg dose of HE3286 with similar results. However, lower doses (10 and 1 mg/kg) were not effective (data not shown).

Effect of HE3286 in Classic Immune Assays, Including the Murine DTH and PLN Assays. Mice treated with either vehicle or with saline (PBS) exhibited strong DTH responses between 2 and 72 h after challenge with oxazolone solution (Fig. 7). Treatment with HE3286 (40 mg/kg) did not seem to effect the generation of immunological memory in this model, because ear swelling was similar in the HE3286-treated animals and those treated with saline or vehicle. In contrast, mice treated with DEX had greatly reduced ear swelling, compared with either the HE3286-treated, saline- or vehicle-treated groups, indicating that DEX powerfully suppressed the DTH memory response. HE3286 did not markedly interfere with DEX-mediated suppression of DTH memory responses.

Corroborating results were obtained in other in vivo models, including the murine ovalbumin (OVA) immunization...
model and the PLN assay, an assay typically used to gauge the immune toxic potential of drugs and pollutants (Pieters, 2001; Pieters et al., 2002). In the PLN assay, mice are injected into the footpad with a sensitizing dose of hapten-carrier conjugate (TNP-OVA). HE3286 (40 mg/kg) was given orally for 5 days, and PLN was harvested. HE3286 did not decrease cell numbers or cytokine production in the PLN, in contrast to DEX treatments, which profoundly decreased all these parameters (Table 3). Studies in the PLN assay were repeated twice with similar results. In the murine OVA immunization model, where mice were immunized on days 0, 7, and 21, HE3286 was found to only slightly (≤25%) decrease serum levels of OVA-specific antibody on day 30.

HE3286 was also judged as not immune-suppressive in several in vitro models using both mouse and human cells. HE3286, at concentrations as high as 1 μM, did not exert any profound suppressive effects in the mitogen-induced lymphocyte proliferation assay (Table 4) or mixed lymphocyte response assay (data not shown). This was again in contrast to DEX, which demonstrated profound immune suppressive activity in both these models.

### TABLE 3
Effect of HE3286 in reporter antigen PLN assay

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>8.1 (7.7, 8.6)</td>
</tr>
<tr>
<td>HE3286 (4 mg/kg)</td>
<td>5</td>
<td>11.0 (9.5, 11.9)</td>
</tr>
<tr>
<td>HE3286 (40 mg/kg)</td>
<td>5</td>
<td>9.5 (8.5, 9.6)</td>
</tr>
<tr>
<td>DEX + HE3286 (4 mg/kg)</td>
<td>5</td>
<td>3.9 (3.3, 4.7)</td>
</tr>
<tr>
<td>DEX + HE3286 (40 mg/kg)</td>
<td>5</td>
<td>2.1 (2.1, 2.6)</td>
</tr>
<tr>
<td>DEX</td>
<td>5</td>
<td>2.7 (2.5, 3.2)</td>
</tr>
</tbody>
</table>

### Discussion

Oral HE3286 treatment provided significant benefit in both the CIA and CAIA models of rheumatoid arthritis, even when treatment was delayed until disease was well established. HE3286, in contrast to dexamethasone, was not profoundly suppressive in classic models of cellular or antibody-mediated immunity. Treatment was associated with reductions in activated NF-κB, proinflammatory cytokines mRNAs, and MMP-3. Across multiple studies, including those reported here and previously (Auci et al., 2007), we hypothesize a mechanism based on reduced NF-κB activation and increased regulatory T-cell levels.

In our previous studies, treatment of CIA with HE3286 was associated with significant reduction in disease score and with dramatic improvement in tissue integrity as judged by histological analysis (Auci et al., 2007). This suggested a generalized tissue-sparing activity of the drug in the joints of treated animals and correlated well with observations of reduced disease score. These observations parallel the activity reported for oral DHEA in this model (Röntsch et al., 2004), albeit at much higher doses (400 mg/kg). HE3286 provided similar benefit with a single daily administration of 1/10 the effective dose reported for DHEA and thus seems to be far more potent. The HE3286-mediated benefit we observed in CIA was also similar to that reported for anti-TNF-α treatment in this model (Joosten et al., 1999). HE2500 is another synthetic steroid that we reported previously to be effective in this same CIA model (Offner et al., 2004). HE2500 retains certain immune-regulating properties, is nontoxic, and is practically devoid of androgenic or estrogenic side effects. However, far higher doses of HE2500 were required for efficacy, both in our studies in CIA and in the rat model of adjuvant induced arthritis, as reported by others (Schwartz and Pashko, 2002). Furthermore, in contrast to HE3286, HE2500 was only effective in CIA when given by the subcutaneous route.
In the present study, we also found that treatment with HE3286 beginning at CIA onset was associated with decreases in IL-23 mRNA in spleen. IL-23 is essential for T-cell-mediated autoimmune disease and promotes inflammation via IL-17 and IL-6 (Yen et al., 2006). Furthermore, IL-23 promotes the expansion of Th17 cells (Cooke, 2006), which share reciprocal developmental pathways with regulatory T cells (Bettelli et al., 2006). Indeed, we found decreased IL-17 in splenocyte cultures from HE3286-treated animals. This suggests that the HE3286-mediated expansion of regulatory T cells we observed in our previous study (Auci et al., 2007) may relate to a suppression of IL-23 production and Th17 cells. It would be of great interest to measure regulatory T cells and/or Th17 cells in the joint tissue of treated and untreated animals during the course of CIA, and this is the subject of continued investigations in our laboratories.

HE3286 was still effective in the late-onset version of the CIA model, when treatment was delayed until disease was well established. In these experiments, disease in the HE3286-treated animals seems to have been arrested in that it failed to progress much beyond levels observed at the beginning of treatment, whereas disease continued to progress in the vehicle-treated animals. The late-onset version of the CIA model more closely resembles established RA disease as it usually presents in clinical situations.

HE3286 also provided significant benefit in both onset and late-onset versions of the CAIA model, in which the contribution of T- and B-cell compartments to the pathogenesis of experimental RA is minimized. It is interesting to note that methotrexate, a current mainstay in the treatment of RA, is not effective in this model (Lange et al., 2005). The HE3286-mediated benefit in CAIA was associated with dramatic reductions in IL-6 and MMP-3 mRNA levels in joints, measured both at the peak of disease and at the end of the study. MMP-3 is thought to be an important driver of disease in human RA (Ichikawa et al., 1998). The observation that HE3286 provides benefit in both the CAIA and CIA models probably relates to the multifaceted mechanism of action of this compound and bodes well for the potential success of HE3286 in the treatment of human RA.

We found that HE3286 treatment was associated with reductions in activation of NF-κB. Over time, evidence has accumulated implicating NF-κB as a mediator of autoimmune and a potential therapeutic target for treating autoimmune diseases (Dejardin, 2006). If an inhibition of the NF-κB pathway by HE3286 was consequential to the activity observed in models of RA, then it would be expected to have affected the expression of certain NF-κB target genes whose functions in vivo are known to play a role in the pathophysiology of inflammation. These include TNF-α, IL-1β, and IL-6. Our observations suggest that the mRNAs to these same cytokines may have been suppressed in HE3286-treated mice. Inhibition of the NF-κB pathway by HE3286 is currently under investigation in our laboratories. Although our in vivo studies on NF-κB activation suggest inhibition of the NF-κB pathway may be involved in the activity observed in models of RA, they do not address the relative potency of HE3286 with respect to other treatment approaches.

The precise mechanism(s) by which HE3286 mediates these effects remain obscure. Although HE3286, in its anti-inflammatory activity, and in its ability to expand regulatory T cells, resembles glucocorticoids, HE3286 was not found to be immune-suppressive in any of the classic models of immune function. Even with extended dosing (over 4 days) in DTH, HE3286 did not suppress generation of immunological memory. Furthermore, HE3286 does not directly interact (either via binding or transactivation) with any of the known nuclear hormone receptors, including the glucocorticoid or sex steroid receptors (T. Wang, S. Villegas, Y. Huang, S. K. White, C. Ahlem, M. Lu, J. M. Olefsky, C. Reading, J. Frincze, and J. R. Flores-Riveros, submitted for publication). Although these remarkable observations do not suggest a mechanism of action or a molecular target for HE3286, they do suggest a highly attractive safety profile. In fact, HE3286 seems to be bone-sparing in both in vitro and in vivo models (Hollis-Eden Pharmaceuticals Inc., unpublished observations). These observations do not rule out the possibility that a metabolite or metabolites of HE3286 may contribute to anti-inflammatory or other activities. With respect to potential targets, these may include surface receptors, nuclear hormone receptors, or steroidogenic enzymes such as CYP7B or CYP11B (Lathe, 2002). Such possibilities are currently under investigation in our laboratories.

HE3286 demonstrated improved metabolic stability compared with AET in studies using human microsomes. (Hollis-Eden Pharmaceuticals Inc., unpublished observations). In fact, the addition of 17α-ethyl group effectively blocks metabolism at this position. HE3286 demonstrates 25% oral bioavailability in rodents and primates. Recent observations in ongoing phase I/II trials suggest a longer half-life in humans compared with rodents (8 versus 1.5 h, respectively) and demonstrate that putative efficacious blood levels are easily attainable with chronic dosing at doses as low as 10 mg/day. This would be on the same order of potency as...
commonly prescribed glucocorticoids (Hollis-Eden Pharmaceuticals Inc., unpublished observations). Together, these observations suggest that the anti-inflammatory activity of HE3286 observed in rodents may translate to humans.

In conclusion, our series of observations suggest that HE3286 may represent a novel, first in class anti-inflammatory agent offering a superior side effect profile compared with corticosteroids for use in treatment of RA. Furthermore, given the surprising diversity of the DHEA metabolome, the development of HE3286 may also present a platform from which the enormous promise of DHEA, emanating from thousands of publications and decades of work in rodents, can at last be translated into potent pharmaceuticals able to effectively treat human disease.

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References


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