

Oral treatment with HE3286 ameliorates disease in rodent models of rheumatoid arthritis

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Abstract. HE3286 (17 α -ethynyl-5-androstene-3 β , 7 β , 17 β -triol) is an orally bio-available synthetic derivative of naturally occurring androstene-3 β , 7 β , 17 β -triol. Our present data show that oral treatment with HE3286, favourably influenced the course of arthritis in the rat model of adjuvant-induced arthritis (reduced cumulative disease scores and paw edema), and in the mouse model of collagen antibody-induced arthritis (reduced clinical paw scores). Importantly, HE3286 was not immune suppressive in human mixed lymphocyte reaction or in animals challenged with Cocksackie B3 virus. HE3286 is currently in phase I/II clinical trials in rheumatoid arthritis and ulcerative colitis and these findings further strengthen the possibility that HE3286 may represent an effective anti-inflammatory agent useful for treating chronic inflammation with a more attractive safety profile than glucocorticoids or cyclooxygenase inhibitors.

Introduction

Rheumatoid arthritis (RA) is a systemic immune mediated inflammatory disease (IMID) that possesses a multifactorial pathogenetic pathway that includes dysregulated function of T cells, B cells, dendritic cells and macrophages that may result in an unbalanced network of endogenous chemokines and cytokines such as IL-1 and TNF- α (1). This forms the basic rationale for the broad use of newly developed biologics that selectively target the action of B cells (rituzimab) or endogenous TNF- α (*infliximab*, *etanercept*, *rituximab*, *abatacept*), in this disease. While generally efficacious (2-4), these agents are costly, cannot be administered orally (5) and are ineffective or contraindicated in certain patient

populations. Similar to glucocorticoids (6), they block the inductive phase of inflammation, are powerfully immune suppressive, and prolonged usage has been associated with adverse events (7). These also include reactivation of JC virus (8), a higher risk of developing other autoimmune pathologies such as psoriasis (9), lichen planus (10), lupus (11) and demyelinating neuropathies (12). TNF- α inhibitors may also increase the risk of reactivating viral latent infection with herpes zoster (13) or Epstein-Barr virus (14) and augments the risk of tuberculosis (15) as well as the rates of serious bacterial infections (16). An association between TNF- α antagonists and development of lymphoma has been repeatedly suggested but not unequivocally proven (17).

Other classes, such as non-steroidal anti-inflammatory drugs (NSAIDs, e.g., *Celebrex* and *Vioxx*) which act to inhibit the production of prostaglandins that promote inflammation, pain, and fever (9), have also been associated with serious side effects. A new appreciation for the resolution phase of inflammation, including the discovery and characterization of resolvins, protectins, and lipoxins, may presage a new class of safer anti-inflammatory agents that promote this pathway (10). However, metabolic instability and low oral bioavailability presently jeopardizes the pharmaceutical development of these compounds (11). Thus, the search for safe, pharmaceutically relevant anti-inflammatory agents to treat IMIDs continues to command considerable attention.

The abundant adrenal steroid DHEA (dehydroepiandrosterone) provides striking therapeutic benefits across a wide array of rodent models of IMID (12-14). Carefully controlled clinical trials of DHEA in patients with IMIDs have reported low efficacy and androgenic side effects (15-18), perhaps related to low oral bioavailability and differential interspecies metabolic rates and pathways (13,19-21). Rodents rapidly metabolize exogenous DHEA into a surprisingly complex array of more highly oxidized metabolites (22), which may be responsible for the anti-inflammatory, immune regulating activity attributed to DHEA in rodents (14). Synthetic derivatives of active DHEA metabolites, with improved pharmaceutical properties and devoid of androgenic or estrogenic side effects, could potentially translate benefits observed in rodents into humans (14). The 7-hydroxylated metabolites of DHEA retain potent anti-

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inflammatory and immune modulating activity but are practically devoid of unwanted side effects (reviewed in ref. 23). Previously we showed that subcutaneous (s.c) injection of androstene-3 β , 7 β , 17 β -triol (AET) provided benefit in rodent models of ulcerative colitis (24) and experimental autoimmune encephalitis (25). By virtue of its anti-inflammatory activity, AET improved survival in a rodent model of traumatic shock (26). However, low oral bioavailability and metabolic instability of AET suggested limited pharmaceutical utility of this naturally occurring compound in humans. Therefore, analogues of AET were prepared and screened for improved pharmaceutical properties. The lead candidate, HE3286, was comprised of the core trihydroxylated androstene structure with the addition of an ethynyl group at the 17 α position, which rendered the molecule orally bioavailable and stabilized the molecule to metabolism.

We previously reported that oral treatment with HE3286 inhibited collagen-induced arthritis in the mouse (27,28). To extend and complement this finding we evaluated the effect of HE3286 in the Lewis rat model of adjuvant-induced arthritis (AIA) that represents another well known and validated preclinical model of human RA (29). We also replicated and confirmed the previously observed effects of HE3286 in the DBA mouse model of collagen antibody-induced arthritis (CAIA) (28). In addition, we demonstrated that HE3286 is devoid of immunosuppressive properties both in *in vitro* and *in vivo* experimental settings using the mixed lymphocyte reaction response and the murine model of Coxsackie B3 virus-induced myocarditis, respectively.

Materials and methods

Drugs. Test compound 17 α -ethynyl-5-androstene-3 β , 7 β , 17 β -triol (HE3286) and vehicles (HERF202 or HERF405) were provided by HollisEden Pharmaceuticals (San Diego, CA). HERF202 contains 30% β -cyclodextrin sulfobutyl ether sodium salt (w/v) in water. HERF405 contains 0.1% carboxymethylcellulose, 2% polysorbate 80 and 0.1% metabisulfite in phosphate-buffered saline pH 7.4. Control animals were treated with an equal volume of vehicle.

Animal care. Animals were purchased and housed in accordance with respective institutional guidelines and requirements of the relevant regulatory agencies. AIA rat and myocarditis mouse studies were performed by F.N. at UC. CAIA studies were performed by D.B. at UCSD as previously reported (28).

Adjuvant-induced arthritis (AIA) in the rat

Animals. A total of 40 male Lewis rats (Harlan Nossan, San Pietro al Natisone, Udine, Italy), ranging in weight from 162 to 215 grams, were used for the study. The rats were housed in a controlled environment (non-specific pathogen free) and provided with standard rodent chow and water.

Induction of arthritis and treatment. Adjuvant-induced arthritis (AIA) was induced in Lewis rats by a single s.c. injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* H37Ra (0.3 mg) in Freund's incomplete adjuvant into the base of the

tail. Rats were divided randomly into two groups and treated orally (gavage) with either HE3286 (25 mg/kg) dissolved in vehicle (HERF202) or with vehicle alone, in a final volume of 0.125 ml. Two experimental treatments were considered, one with a therapeutic regime that occurs early in the disease process, and the second with a late therapeutic regime. For the early regimen, the rats were treated on day 8 post-immunization and in the late regimen, the rats were treated on day 15 post-immunization. In both cases treatments was given twice daily for 15 consecutive days. On the first day of treatment the rats were randomly allocated to the different experimental groups. Post-randomization analysis showed no significant differences in the mean arthritic score or for paw edema between the different groups.

Clinical assessment of AIA. Rats were evaluated daily for arthritis using a macroscopic scoring system: 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. Arthritic index for each rat was calculated by adding the 4 scores of individual paws. Rats were weighed daily, beginning from the day of immunization.

Measurement of paw edema. Clinical disease severity was determined using plethysmography (model 7140; Ugo, Basel, Switzerland). The change in left hind paw volume was determined on a weekly basis.

Blood levels of IL-6 and TNF- α . Circulating levels of IL-6 and TNF- α were measured by specific solid phase ELISA kits in undiluted plasma samples from the different groups of rats on day 0 (prior to immunization), 15th (at the beginning of the treatment) and at the end of the study. Inter and intrassay of variations were below 10 and 5%, respectively. The assays were carried out according to the manufacturer's instructions (Bender MedSystems, Prodotti Gianni, Milan, Italy). The lower limit of sensitivity 31 pg/ml for IL-6 and 11.2 pg/ml for TNF- α . To allow evaluation of statistical analysis, those samples with levels below the limit of sensitivity were assigned a value corresponding to the lower limit of sensitivity.

Determination of myeloperoxidase activity. Neutrophil infiltration into the inflamed joints was indirectly quantified using an MPO assay in both groups of rats treated with HE3286 or its vehicle at the end of the treatment (day 30). Briefly, the left hind-paw tissue was removed and snap frozen in liquid nitrogen. Upon thawing, the tissue (0.1 g of tissue per 1.9 ml buffer) was homogenized in buffer (0.1 M NaCl, 0.2 M NaPO₄ and 0.015 M NaEDTA; pH 4.7), centrifuged at 3000 x g for 10 min and the pellet subjected to hypotonic lysis (1.5 ml of 0.2% NaCl solution followed 30 sec later by addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After further centrifugation, the pellet was resuspended in 0.05 M NaPO₄ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and rehomogenized. Aliquots (1 ml) of the suspension were transferred into 1.5-ml Eppendorf tubes followed by three freeze-thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 min at 3000 x g, the pellet was

resuspended to a volume of 1 ml and samples of hind paw were assayed. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM).

Collagen antibody induced arthritis (CAIA)

Animals. DBA/1Lac/J male mice (5 per group) purchased from Jackson Laboratories (Bar Harbor, Maine) were treated (gavage) daily with either 0.1 ml of vehicle (HERF202) or with HE3286 (1, 10 or 40 mg/kg), beginning on day 1. Animals were treated for 14 days.

Induction and evaluation of CAIA. To induce arthritis, mice were administered 1 mg of an anti- α II antibody cocktail (Chondrex, Redmond, WA) intravenously on day -2. On day 0, animals were treated intraperitoneally (IP) with LPS (12.5 μ g), as in a previous study (28). The arthritic severity of the mice was evaluated as in our AIA studies.

Human mixed lymphocyte response (MLR) studies. Human blood samples were obtained from 3 healthy, fasting male volunteers, ranging from 23-31 years of age, who gave informed consent. The subjects had not used immune modulating, anti-allergic drugs or antibiotics in the three months prior to the study. Samples were drawn between 9 and 10 a.m. to avoid fluctuations in the circulating levels of hormones or cytokines.

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque (density 1.077, Biochrom AG, Berlin, Germany) gradients and resuspended in culture medium (RPMI-1640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Invitrogen s.r.l., Milan, Italy). Autologous (responder) inactivated plasma was used as vehicle at 10% concentration.

Five hundred thousand responder PBMC (PBMCr) and 500,000 allogeneic irradiated (30 Gy) stimulator PBMC (PBMSc) were mixed (ratio 1:1 in 200 μ l medium) and cultured for 6 days in a flat bottom 96-well plate (Nunc, Roskilde, Denmark). Test compound was dissolved in ethanol and then diluted to test concentrations (0.3 or 0.03 μ M) with culture medium leading to a final solution containing 0.01% ethanol. Controls included vehicle, PBMCr and PBMScs cultured separately in the same vehicle at the same concentration. During the final 8 h, the PBMC were pulsed with 1 μ Ci/well [3 H]-thymidine (Amersham, Milan, Italy). Cells were harvested and incorporation of radioactivity into DNA measured with a beta cell counter.

Myocarditis induced by infection with Coxsackie B3 virus

Animals. Four- to five-week-old BALB/c male mice were purchased from Harlan Nossan (Correzzana, Italy). They were kept under standard laboratory conditions (non-specific pathogen free) with *ad libitum* food and water and were allowed to adapt one week to their environment before study commencement.

Treatment. Mice were randomized by weight into 3 groups of 10 and treated by oral gavage with 80 mg/kg HE3286 or vehicle HERF405, or IP with dexamethasone (Dex) at the

dose of 0.4 mg/kg for 14 consecutive days. After 14 days of treatment the mice were infected intraperitoneally with 10^5 plaque-forming units of purified CB3V. Animals were monitored twice daily, 7 days a week, for health status. Body weight was recorded daily. At day 28 post-infection, mice were sacrificed and the heart was weighed and submitted for histological analysis. The spleen was isolated and weighed.

Histology. At the end of the study, histopathological scores of hearts from groups of mice were evaluated as follows: 0 = no lesions; 1 = foci of mononuclear cell inflammation associated with myocardial cell reactive changes without myocardial cell necrosis; 2 = inflammatory foci clearly associated with myocardial cell reactive changes; 3 = inflammatory foci clearly associated with myocardial cell necrosis and dystrophic calcification; 4 = extensive inflammatory infiltration, necrosis and dystrophic calcification.

Statistical analysis

Rat model of AIA. All data are expressed as the mean \pm SD. The significance of differences in arthritic scores was evaluated by Student's t-test vs. vehicle-treated rats. $p < 0.05$ was considered significant.

DBA mouse model of CAIA. The various pair-wise comparisons between each active group and placebo (at each time point) were tested for significant differences via the exact Van Elteren's test wherein scored data were stratified based on the median baseline score. Data imputation is implemented via LOCF.

Human MLR. The mean cpm of quadruplicate wells was calculated. The hypothesis of non-inferiority was not analyzed formally due to the small sample size (three subjects). Instead, an exploratory three-step procedure was executed as follows: First, an exact 95% Hodges-Lehman confidence interval was constructed for the difference in population medians for each subject, regarding the replicates within that subject as independent. This step was then confirmed by means of the significance of non-inferiority of an active compound relative to a control via an exact one-sided Mann-Whitney test, per subject as above (30). Second, a decision was made as to whether each subject meets the non-inferiority criteria. Finally, the percentage of subjects adjudicated as cases of non-inferiority was calculated, with its attendant exact Blyth-Still-Casella confidence interval for the total fraction of subjects that, under similar experimental conditions, would show non-inferiority. This is a measure of the strength of the evidence at the population level inferred from the three cases (31).

Myocarditis. The various pairwise comparisons for body weight, spleen weight, and heart weight were tested for significant differences via the Wilcoxon-Mann-Whitney test. Histology scores were tested via the Freeman-Halton test.

Results

Effect of HE3286 in the rat model of AIA

Study I. Effect of early therapeutic treatment with HE3286 on

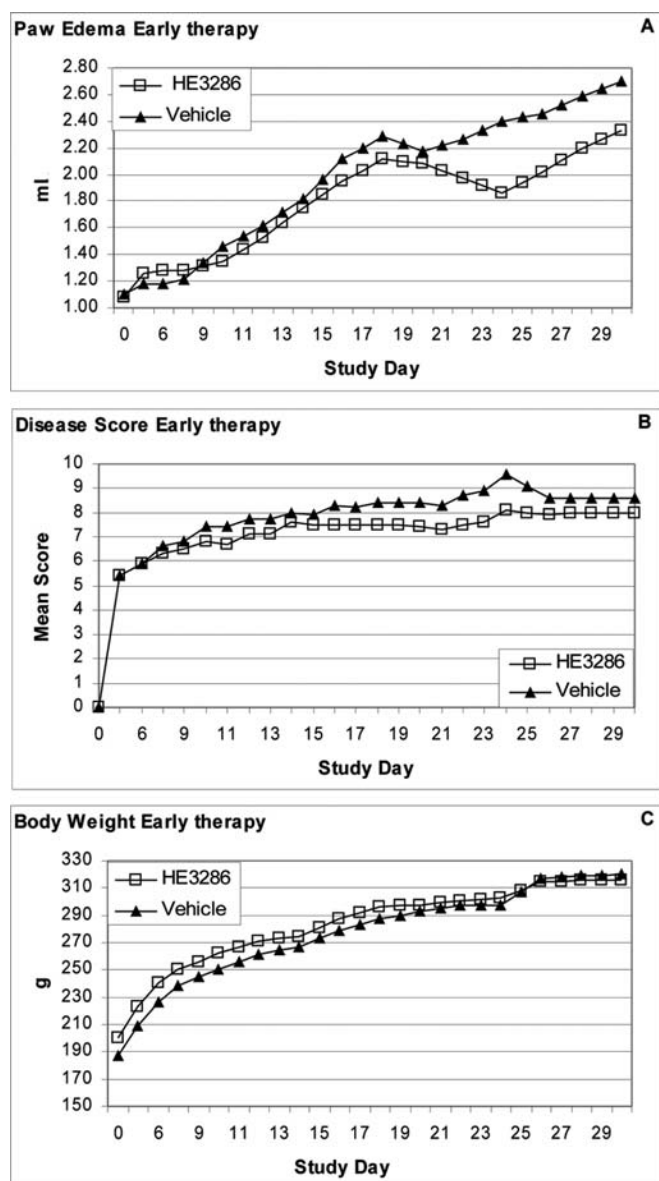


Figure 1. Effect of early therapeutic treatment with HE3286 in the rat model of AIA. Arthritis was induced in male Lewis rats (10 per group) by a single (s.c.) injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* H37Ra (0.3 mg) in Freund's incomplete adjuvant into the base of the tail. On day 8 (post-immunization to induce AIA), when the rats showed clinical signs of arthritis, twice-daily treatments (gavage) began with either HE3286 (25 mg/kg) or vehicle alone (125 μ l) and were continued for 15 consecutive days. Paw edema and disease score were assessed daily as described in Materials and methods. Data are expressed as median paw edema (A) and median disease score per animal (B) \pm standard deviation. (C) The body weight variation during the study period.

the development of AIA. The data in Fig. 1A and B demonstrated a time-dependent increase in both left hind paw volume (ml) and clinical arthritic score that were induced by adjuvant in vehicle-treated rats. The arthritis incidence in these animals was 100% (10/10). The rats treated with HE3286 exhibited significantly lower paw edema than vehicle-treated rats from day 22 to the end of the study on day 30 (Fig. 1A). HE3286 also significantly reduced the arthritic score of the treated rats as compared to vehicle from days 18-25 (Fig. 1B). The fluctuations of body weight seen in

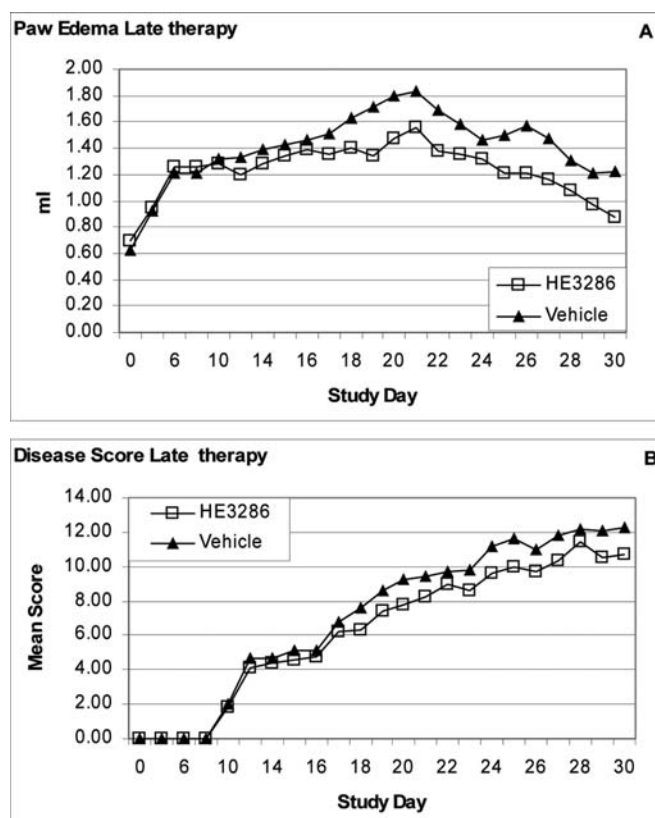


Figure 2. Effect of late therapeutic treatment with HE3286 in the rat model of AIA. Arthritis was induced in male Lewis rats (10 per group) by a single (s.c.) injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* H37Ra (0.3 mg) in Freund's incomplete adjuvant into the base of the tail. On day 15 (post-immunization to induce AIA), when the rats showed clinical signs of well-established arthritis, twice-daily treatments (gavage) began with either HE3286 (25 mg/kg) or vehicle alone (125 μ l) and were continued for 15 consecutive days. Paw edema and disease score were assessed daily as described in Materials and methods. Data are expressed as median paw edema (A) and median disease score per animal (B) \pm SD.

HE3286-treated control rats were comparable to those observed in the vehicle-treated controls (Fig. 1C).

Study II. Effect of late therapeutic treatment with HE3286 on the development of AIA. The efficacy of early therapeutic treatment on development of rat AIA prompted us to evaluate the efficacy of this compound when first applied under a fully therapeutic regime to rats with advanced clinical signs of arthritis. To this aim the rats were first treated with HE3286 from day 15 post-immunization. Also upon this late therapeutic treatment HE3286 exhibited powerful anti-arthritis effects and it was capable of favourably influencing the course of rat AIA. In fact, relative to vehicle-treated controls, the rats treated with HE3286 exhibited significantly lower paw edema from day 19 until the end of the study on day 30 (Fig. 2A) with exception of non-significant reductions vs. on days 23 and 24. HE3286 also significantly reduced the arthritic score of the treated rats as compared to vehicle from days 19-30 (Fig. 2B) with exception on day 22 where the reduction of the arthritic score was close to, but did not reach statistical significance. The fluctuations in body weight seen in HE3286-treated control rats were comparable to those observed in the vehicle-treated (data not shown).

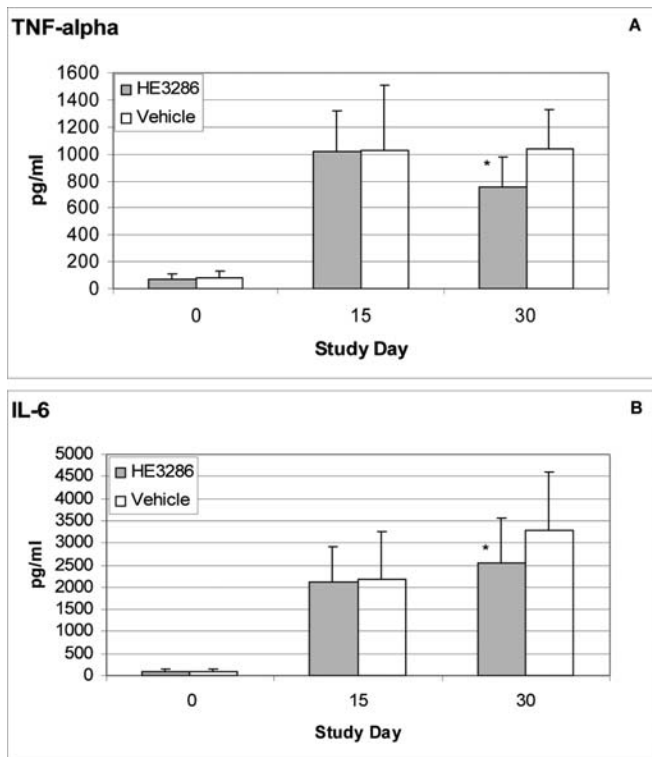


Figure 3. Effects of late therapeutic treatment with HE3286 on reduction of IL-6 and TNF- α blood levels in the AIA rat model. Cytokine blood levels in adjuvant-induced arthritis (AIA) on day 30 after adjuvant injection after 15 days of treatment with HE3286. (A) Tumor necrosis factor- α (TNF- α) levels in sera (n=10) and (B) IL-6 levels in sera (n=10). Values were measured by ELISA and are expressed as the mean \pm SD. *p<0.05.

Late therapeutic treatment with HE3286 reduced blood levels of IL-6 and TNF- α that were found to be elevated in vehicle-treated controls.

IL-6 and TNF- α were undetectable in most of these rats before immunization and increased thereafter with progression of the disease. A fluctuation similar to that seen in the vehicle-treated rats was also seen in the group of rats assigned to HE3286 treatment. However, when re-measured at the end of the study after 15 consecutive days of treatment with HE3286 the circulating levels of these cytokines were significantly lower in the treated rats as compared to those receiving the vehicle (Fig. 3A and B).

MPO activity was markedly increased, on day 30 in AIA-immunized rats treated with the vehicle. Late therapeutic treatment with HE3286 reduced the MPO activity in the hind-paw homogenate. The treatment with HE3286 for 15 consecutive days significantly reduced the MPO activity compared to vehicle-treated rats (Fig. 4).

Effect of HE3286 in the DBA mouse model of CAIA. In the CAIA model, daily treatment with HE3286 (40 mg/kg) produced benefit when compared to animals receiving either vehicle or 1 or 10 mg/kg HE3286 (Fig. 5). In this study, CAIA began to develop by day 3 (score <4) in both vehicle- and HE3286-treated. Disease peaked in all groups on days 7-8, where average scores were between 9 and 12 in both the

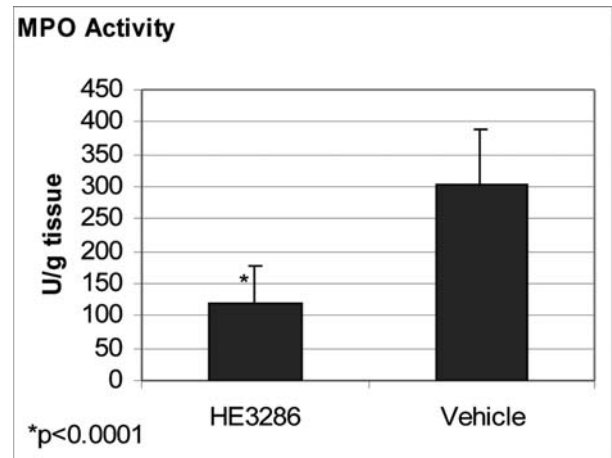


Figure 4. Effects of late therapeutic treatment with HE3286 on reduction of MPO activity in the AIA rat model. MPO activity in the left hind-paw tissue on day 30 after adjuvant injection after 15 days of treatment with HE3286. n=10 per group. Optical density values were measured using tetramethylbenzidine. Data are expressed as the mean \pm SD. *p<0.0001.

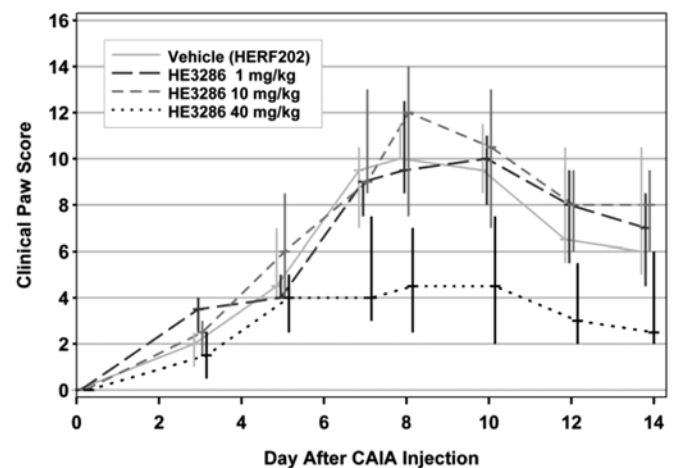


Figure 5. Effect of HE3286 in the DBA mouse model of CAIA. DBA male mice (5 per group) were administered 1 mg of an anti-bCII antibody cocktail intravenously on day -2. On day 0, animals were treated (IP) with LPS (12.5 μ g). Animals were treated daily with either 0.1 ml of vehicle or with HE3286 (1, 10 or 40 mg/kg) beginning on day 1. Data are expressed as the group's average clinical paw score \pm SD.

vehicle-treated and lower dose (1 and 10 mg/kg) HE3286-treated groups. In contrast, the high dose (40 mg/kg) became statistically lower on day 4 (p<0.031) and remained lower for the remainder of the study (day 14). Dose response trends were clearly detectable and statistically significant starting at day 4. This study therefore fully confirms a similar study with HE3286 in this model that has been recently reported (33).

Effect of HE3286 in human MLR. Minimal proliferative responses (>2000 CPM) were observed at the end of the culture period in individual cultures of control responder and stimulator PBMCs (obtained from each of three donors R1, R2 and R3). In contrast, a marked proliferative response was observed when PBMCr and PBMCs were cultured together with or without vehicle (Fig. 6). Overall, there was no

Table I. Effects of HE3286 on body weight variations, spleen and heart weights and heart histological score in male BALB/C mice infected with Cocksackie B3 virus.

	Variation body weight (%)			Spleen weight (mg)	Heart weight (mg)	Histo score
	Day 0 Start of treatment	Day 14 End of treatment before infection	Day 42 sacrifice	Day 42	Day 42	Day 42
HE3286	0	-1.0±8.3	27.1±8.9	99.6±20.9	139.4±12.6	0.0±0.0
Dex	0	-3.6±3.4	24.3±5.5	96.8±9.8	143.7±13.4	0.1±0.3
Vehicle	0	13.1±4.3	28.7±7.6	98.6±10.4	152.9±18.1	0.6±0.8
SHAM	0	18.2±12.1	37.7±10.5	82.1±3.8	147.1±9.9	0.0±0.0

BALB/c male mice (n=10 per group) were treated orally (gavage) with HE3286 (80 mg/kg) or with vehicle alone for 14 consecutive days; Dex was administered IP under the same experimental conditions. Body weight was measured daily and, after 14 days of treatment, the mice were infected intraperitoneally with 10^5 plaque-forming units of purified virus. Animals were monitored twice daily, 7 days a week for health status. At day 28 post-infection, the mice were sacrificed, the spleen and heart were weighed and the heart histologically analyzed.

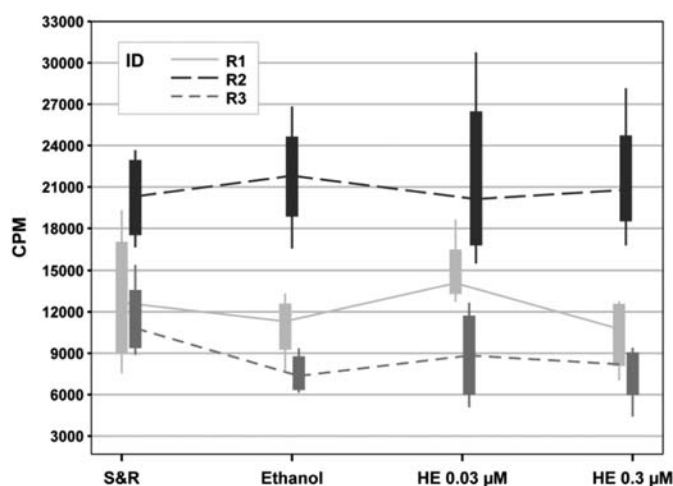


Figure 6. Effect of HE3286 in human MLR. Five hundred thousand responders PBMC (PBMCr) and 500,000 allogeneic irradiated (30 Gy) stimulator PBMC (PBMCs) from three different volunteers (R1, R2 and R3) were mixed (at a ratio of 1:1 in 200 μ l medium) and cultured for 6 days in a flat bottom, 96-well plates (± 0.3 μ M or 0.03 μ M HE3286). HE3286 was dissolved in ethanol and then diluted to desired concentration with culture medium leading to a final solution containing 0.01% ethanol. Controls included 0.01% ethanol alone, and PBMCr and PBMCs cultured separately. During the last 8 h of the culture period the PBMCs were pulsed with 1 μ Ci/well [3 H] thymidine. Cells were harvested and radioactivity incorporation measured with a beta cell counter. Data are expressed as CPM. CPMs, counts per minute; ID, subject identification code; S&R, stimulator and responder jointly; HE, HE3286. The bottom and top edges of the box are located at the sample 25th and 75th percentiles. The vertical lines, or whiskers, are drawn from the box to the most extreme point within 1.5 interquartile ranges. The line connecting the treatments passes through the respective median CPM for each treatment.

profound suppressive influence of any HE3286 concentration tested.

Effect of HE3286 in myocarditis induced by CB3V. During the 14-day treatment period, Dex- and HE3286-treated mice showed relatively no weight gain while sham-treated mice and vehicle-treated mice averaged 13 and 18% gain in body

weight, respectively (Table I). This difference may have been due to a genuine effect of HE3286. If so, this effect of HE3286 appears to be strain-specific, since it was not observed in other strains [e.g., DBA mice, SJL mice or NOD mice (unpublished data)]. However, beginning two days following CB3V infection through the end of the study, the increased change in body weight from day 14 significantly ($p < 0.05$) favoured both the Dex- and HE3286-treated mice when compared to the vehicle-treated mice.

As expected, the infection with CVB3 induced a significant ($p < 0.05$) increase in spleen weight in vehicle-treated mice as compared to the healthy (sham) mice. However, two weeks of daily pre-treatments with either HE3286 or Dex prior to viral challenge did not appear to exert any profound effects on the subsequent course of the infection as no significant differences were observed in spleen or heart weights compared to the mice pre-treated with the vehicle alone. However, there was a trend ($p = 0.093$) towards reduced heart weights (suggestive of reduced inflammation) in the HE3286-treated group compared to the vehicle-treated group (139.4 mg \pm 12.6 vs. 152.9 mg \pm 18.1). This difference could not be attributed to any significant difference in body weights between these groups at the end of the study.

The histological examination of the heart showed only low numbers of cellular infiltration foci and some calcified areas in the group of mice that had been pre-treated with vehicle (Table I). However, histology did show a trend ($p = 0.087$) toward lower scores in hearts of animals that had been pre-treated with HE3286 compared to those treated with vehicle alone, also suggesting a slight benefit from pre-treatment.

Discussion

The data presented herein show for the first time that HE3286, a synthetic derivative of AET, provided clinical benefit in rodent models of AIA with a simultaneous reduction of MPO activity and diminished blood levels of IL-6 and TNF- α . We also confirm the finding of a previous study (28) on the

efficacy of this compound to ameliorate the course of CAIA. In addition, we show herein that HE3286 was not immune suppressive in MLR studies and that extended pre-treatments were safe and may even have provided benefit in the murine model of myocarditis induced by CB3V infection. The present studies confirm the anti-inflammatory activity reported for HE3286 in our previous publications (23,24,26).

Our observation that HE3286 provides benefit in the rat model of AIA, even when treatment is delayed until disease is well established, is in agreement with our previous studies in the type II CIA DBA mouse model of arthritis (28). This observation has strong significance for the potential translation of these findings to the clinical setting as it has been shown that a compound's therapeutic efficacy, rather than prophylactic efficacy, in AIA and CIA models is more predictive of clinical efficacy in human RA than data from either model alone (29).

In contrast to CIA, no single antigen has been identified that is deemed responsible for the arthritogenic response in the AIA model (32). Rather, the induction of adjuvant arthritis has been attributed to a mycobacterial cell wall component, muramyl-dipeptide, which is immunostimulatory but does not evoke a specific immune response (33). In the rat model of AIA, the chief pathological features of adjuvant arthritis include edema, mononuclear and polymorphonuclear cell joint infiltration, pannus formation, periostitis and erosion of cartilage and bone. Hence the reduced MPO activity seen in HE3286-treated rats as compared to vehicle-treated controls is probably secondary to reduced neutrophil influx into the inflamed paws. This is consistent with the clinical observation of both a reduced arthritic score as well as paw edema observed upon treatment with HE3286.

Rat AIA exhibits immunopathogenic and pharmacological similarities with human RA including the increased blood levels of IL-6 and TNF- α during the course of the disease (34,35) and the favourable response to immunosuppressive and anti-inflammatory drugs used in RA such as cyclosporin A (36), leflunomide, methotrexate and indomethacin (37,38). As in human RA (39), cyclooxygenase (COX) II appears to be involved in the pathogenesis of rat AIA since occurrence of arthritis is associated with local production of PGE₂ and up-regulated expression of COX-2, IL-6 and TNF- α mRNAs in the affected paws (38). Selective inhibition of COX-2 reverses clinical occurrence of AIA and expression of COX-2 and IL-6. This resembles our present observation of concomitant inhibition of clinical signs of AIA and reduced blood levels of IL-6 (and TNF- α) in the rats treated with HE3286. These findings are also consistent with our studies in murine type II CIA where HE3286-mediated clinical benefit was associated with decreased production of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-17 (28). The above findings may indicate that the anti-arthritogenic capacities of HE3286 may be at least partly mediated by its capacity to down-regulate the synthesis of proinflammatory cytokines involved in arthritogenic pathways such as IL-6 and TNF- α .

In agreement with our earlier studies (28), we report herein the significant benefit of HE3286 treatment in the CAIA model, where the typical disease course involves a short period of acute disease followed by gradual resolution in joint inflammation (40). The CAIA model permits an analysis of the contribution of non-T and B cell compartments to the

pathogenesis of experimental RA. The observation that HE3286 provides benefit in these 3 models of arthritis such as CIA, AIA, and CAIA that recognize differences in pathogenetic pathways probably relates to a multifaceted mechanism of action and suggests that there is good potential for success in the treatment of human RA.

Our previous studies with AET showed benefit in rodent models of ulcerative colitis and multiple sclerosis (23,24). Thus, a broad based anti-inflammatory activity is perhaps central to the activities of HE3286 in auto-immune disease. However, the exact mechanism by which HE3286 mediates these effects remains elusive. Potential mechanisms of action for 7-hydroxy steroids have been grouped into four broad categories, including gating (ligand inactivation), modulation of ion channels, interaction with atypical receptors, and modulation of steroidogenic enzymes (41). Non-genomic cell surface receptors are also a possible target, as may be the case with DHEA (42,43) and estradiol (44). Potential targets within each of these categories are currently under consideration. In terms of potential cellular targets, our previous studies (28) associated the activity of HE3286 with increased regulatory T cells, thought to play pivotal roles in the maintenance of immune homeostasis and in the prevention of autoimmune disease (45). We also found reductions in key pro-inflammatory cytokines including IL-17 and IL-6. The role of these cytokines in the biology of regulatory T cells (46,47), the transition from acute to chronic inflammation (48), and in IMID (45) is well established.

HE3286 was not found to be immune suppressive *in vivo* or *in vitro* in the present studies. Also while no immune suppressive effect of Dex could be demonstrated at the end of our CVB3 study, HE3286 was not found to be immune suppressive in any of the classic models of immune suppression, such as mitogen-induced lymphocyte proliferation, delayed type hypersensitivity responses or the popliteal lymph node assay, as reported previously (28). HE3286 does not appear to interact (either via binding or transactivation) with any of the known nuclear hormone receptors, including the glucocorticoid or sex steroid receptors (Riveros-Flores *et al*, unpublished data).

Whatever the exact mechanism of action, HE3286 has demonstrated attractive pharmaceutical properties including surprisingly good oral bio-availability (25%) in rodents, primates and humans and resistance to metabolism (HollisEden Pharmaceuticals, unpublished data). Recent observations in ongoing phase I/II trials suggest an attractive half-life in humans (8 h) compared to rodents, and demonstrate that putative efficacious blood levels are attainable with doses in the same range as commonly prescribed glucocorticoids. The anti-inflammatory activity of HE3286 resembles glucocorticoids, but has a distinctly different safety profile consistent with use in disease settings of chronic inflammation, where administration over extended periods of time is desirable. In this regard, HE3286 appears to be bone-sparing in both *in vitro* and *in vivo* models (Malik *et al*, unpublished data). Taken together with the anti-inflammatory activity of HE3286 observed in several animal models, the data presented herein strengthen the application of this compound to RA patients. In addition, the apparent lack of immunosuppressive effects of HE3286 may represent an important advantage for the

prolonged use of this compound in other both systemic and organ-specific autoimmune diseases including systemic lupus erythematosus, multiple sclerosis, Guillain Barré syndrome, inflammatory bowel disease and type 1 diabetes. HE3286 is currently in phase I/II clinical trials in RA and ulcerative colitis, and may represent a novel, first in class anti-inflammatory agent, with similar applications as the corticosteroids and cyclooxygenase inhibitors, but with an improved safety profile.

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