

**Anti-Allergic and Anti-Asthmatic Effects of a Novel Enhydrazinone Ester
(CEE-1): Inhibition of Activation of both Mast Cells and Eosinophils**

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Abbreviations

7-AAD, 7-amino-actinomycin D; BAL, bronchoalveolar lavage; BMMC, bone marrow-derived mouse mast cell; BRG-14, (ethyl 4-phenylethylaminocyclohex-3-en-2-oxo-6-phenyl-1-oate); C5a, complement fragment 5a; CB, cytochalasin B; CEE-1, ethyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate; CEE-2, methyl 4-

phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate; DMSO, dimethyl sulfoxide; DNFB, dinitro-florobenzene; DNP-BSA, dinitrophenyl-bovine serum albumin conjugate; ELISA, enzyme-linked immunosorbent assay; EPO, eosinophil peroxidase; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FMLP, N-formyl -methionyl-leucyl-phenylalanine; HRP, horseradish peroxidase; LTC₄, leukotriene C₄; MAPK, mitogen-activated protein kinase; OPD, O-phenylenediamine; PBS, phosphate-buffered saline;; PEG, polyethylene glycol; RBL-2H3, rat basophilic leukemia cells-2H3.

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ABSTRACT

Activation of mast cells and eosinophils is a fundamental process in the pathophysiology of allergic diseases. We have previously reported that the novel enhydrazinone ester CEE-1 (ethyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate) – possesses potent anti-inflammatory activity. We have now tested whether the compound also possesses anti-allergic and anti-asthmatic effects *in vitro* and *in vivo*. The compound significantly inhibited degranulation and leukotriene C₄ (LTC₄) release from activated human eosinophils, as well as IgE-dependent degranulation and LTC₄ release from passively-sensitized rat basophilic leukemia cells (RBL-2H3) and bone marrow-derived mouse mast cells (BMMC). In human eosinophils, the drug was more potent in inhibiting degranulation than LTC₄ release (IC₅₀ = 0.4 μM [confidence interval (CI) 0.1-0.9] vs. 3.8 μM (CI: 0.9-8.3), whereas, in mast cells the reverse was essentially the case. The drug did not affect stimulus-induced calcium transients in eosinophils, but significantly inhibited early phosphorylation of ERK1/2 and p38 MAP kinases. *In vivo*, topical application of 4.5-15 mg/kg of the compound significantly inhibited allergen-induced passive cutaneous anaphylaxis (PCA) in mice. Similarly, in the mouse asthma model, the intra-nasal administration of 6.5-12.5 mg/kg of the compound significantly inhibited bronchial inflammation and eosinophil accumulation in bronchial lavage fluid, as well as, abolished airway hyper-responsiveness to methacholine. These results show that CEE-1 inhibits the activation of both mast cells and eosinophils *in vitro*, probably by blocking MAP kinases activation pathways, and that these effects are translated into anti-allergic and anti-asthmatic effects *in vivo*. The compound, therefore, has a potential application in the treatment of asthma and other allergic diseases.

Introduction

The global prevalence of allergic diseases, including asthma, has been on the increase in recent times (Pearce et al., 2007). Mast cells and eosinophils are among the most important effector cells involved in the pathophysiology of allergic diseases (Stone et al., 2010; Holgate, 2012; Deckers et al., 2013). The primary trigger of allergic diseases is the interaction between an allergen and the allergen-specific IgE antibodies bound to the high affinity IgE receptors (FcεRI) on the surface of mast cells of sensitized individuals. This results in the degranulation of these cells and the consequent release of allergic and inflammatory mediators (Galli and Tsai, 2012; Stone et al., 2010). In response to chemotactic mediators generated during the primary allergic response, as well as from TH₂ cells and dendritic cells, eosinophils accumulate in large numbers in the affected tissues, where they are activated to release numerous mediators that contribute to bronchial inflammation, airway hyper-responsiveness (AHR) and airway remodeling, all of which are characteristic of the disease (Holgate, 2012; Fulkerson and Rothenberg, 2013). Although the role of eosinophils in clinical asthma has been questioned (Leckie et al., 2000), more recent studies, including one that employed eosinophil-deficient mice, as well as clinical studies with anti-eosinophil agents, strongly support an important role for eosinophils in asthma (Fulkerson and Rothenberg, 2013).

Mast cells and eosinophils are also known to co-exist in most tissues undergoing allergic inflammation (Minai-Fleminger and Levi-Schaffer, 2009). Recently, an interesting concept of physical interaction between both cells types, leading to mutual enhancement of their respective functional responses – the so called “Allergic Effector Unit (AEU)”, has been

developed (Elishmereni et al., 2011; Elishmereni et al., 2013). Such interplay may be critical in sustaining chronic allergic inflammation such as in asthma where it may be particularly relevant in the “eosinophilic” endotype of the disease.

To a large extent mast cells and eosinophils share similar signaling pathways leading to degranulation and other responses. For example, the earliest event in the stimulus-response coupling in the two cell types is the phosphorylation and activation of the Src tyrosine kinases Lyn and Syk (Adcock et al., 2008; MacGlashan, 2012). This is followed by the downstream activation of phospholipase C (PLC), and mitogen-activated protein kinases (MAPKs) kinases, such as ERK1/2 and p38 MAP kinases (Duan and Wong, 2006, Adcock et al., 2008; Shamri et al., 2013). Signaling through some of these kinases also promote the activation of NF- κ B or other transcription factors leading to the induction of gene transcription and cytokine generation (Wu, 2011; Adcock et al, 2008).

In view of the direct roles that mast cells and eosinophils play in the pathophysiology of allergic diseases, it is often believed that a good anti-allergic drug should be able to directly suppress the responses of both mast cells and eosinophils, especially their degranulation. Thus, drugs that are able to directly affect both cell types simultaneously would be expected to have potential advantages. This is particularly so given the idea that the recently described physical interplay between the two cell types may be the critical factor driving chronic allergic inflammation (Elishmereni et al., 2013).

Enhydrazinones are compounds that have in their structure a hydrazino (NH-NH) moiety that is joined to a keto group through a carbon-carbon double bond. Recently, we reported

the synthesis and characterization of a hydrazino benzyl enaminone derivative - ethyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate, code-named CEE-1, which possesses potent anti-inflammatory activities (Ezeamuzie and Edafiogho, 2012; Ezeamuzie et al., 2013). Although not much is known about the biological activities of enhydrazinones, the enaminones to which they are structurally related, have been shown to possess a number of pharmacological activities including anti-convulsant (Edafiogho et al., 2007; Edafiogho et al., 2009), antitussive and immunosuppressive activities (El-Hashim et al., 2010; 2011a).

Since allergic diseases are invariably inflammatory in nature, and since mast cells and eosinophils are pro-inflammatory cells, it was of interest to determine if these cells are also affected by CEE-1. Here we show that the drug is an effective inhibitor of the responses of both mast cell and eosinophil *in vitro*, and also effective *in vivo* in animal models of skin allergic disease and asthma.

Materials and Methods

Isolation and activation of human blood eosinophils

Human blood samples from healthy male adult donors (without allergic disease) were provided by the Kuwait Central Blood Bank, Jabriya, Kuwait, after obtaining informed written consent of donors. The Health Sciences Center Ethical Committee of Kuwait University approved the protocols for the study. Erythrocytes were sedimented using the methylcellulose method and the granulocytes separated by percoll gradient centrifugation. When eosinophils were to be cultured, diluted whole blood was centrifuged over Ficoll-Hypaque gradient and the resulting pellet was further subjected to hypotonic lysis of the erythrocytes to obtain the granulocyte fraction. Finally, eosinophils were purified from the granulocyte fractions to >98% purity by the immunomagnetic method (Hansel et al., 1991). For the induction of degranulation and release of LTC₄, the purified cells were re-suspended in the reaction buffer (same as described for mast cells) and aliquots containing 5×10^4 cells were added to each well of a 96-well microplate. The cells were then pre-incubated for 15 min with CEE-1 or the drug solvent (0.05% DMSO in reaction buffer) and subsequently stimulated with recombinant human C5a (30 nM) or PBS (for controls) in the presence of (5 µg/ml) cytochalasin B (CB) for 30 min at 37°C. All controls received vehicle (DMSO) in place of drug treatment. The amount of the granular EPO released into the supernatant (as index of degranulation) was determined by the o-phenylenediamine (OPD) method. Briefly, the OPD substrate solution containing 0.4 mg/ml OPD and 0.4 mg/ml urea hydrogen peroxide in PBS-citrate buffer (pH 4.5) was prepared and 100 µl of this was added to 50 µl of the samples in a microplate and incubated for 30 min at 37°C.

After incubation, the reaction was stopped with 50 μ l of 4 M H₂SO₄ and the plate read at 490 nm. The released EPO was expressed as percentage of the total cell content (determined in cells lysed with 1% triton X-100).

For the release of LTC₄, eosinophils were treated as described above but were stimulated with 1 μ M N-formyl methionyl-leucyl-phenylalanine (FMLP). The supernatant containing released LTC₄ was collected by centrifugation and stored at -40°C pending assay.

Culture of Rat Basophilic Leukemia cells (RBL-2H3) and the generation of bone marrow-derived mouse mast cells (BMMC)

The RBL-2H3 cells - a well-characterized rat basophilic leukemia cell line having mucosal mast cell characteristics (Lin et al., 1991) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in our laboratory. They were cultured at a concentration of 1×10^5 /ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The culture was passaged at confluence and used between 4th and 10th passages.

Mouse bone marrow-derived mast cells (BMMC) were generated from 4-5 week old male Balb/c mice according to the method of Tertian et al., (1981). Essentially, bone marrow cells were harvested from the femoral bone marrow and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.0035% 2-mercaptoethanol, and 30 ng/ml mouse recombinant IL-3. One half of the culture medium was replaced every 3 days. BMMC was used after 4-8 weeks of culture, by

which time at least 97% of the cells had differentiated into mast cells as confirmed by the characteristic staining with toluidine blue stain.

IgE-dependent mast cell degranulation and LTC₄ release

RBL-2H3 cells or BMMC were re-suspended at a concentration of 1×10^6 cells/ml, seeded at 5×10^4 cells/well in a 96-well flat-bottom culture plate and passively sensitized overnight with 0.5 µg/ml anti-DNP monoclonal IgE antibody. The cells were then washed twice to remove unbound antibody and subsequently re-suspended in reaction buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.05% BSA and 20 mM HEPES, pH 7.4). They were then pre-incubated with CEE-1 (0.01 – 30 µM) or its solvent (0.05% DMSO) for 10-20 min before being stimulated with the specific antigen DNP-BSA (30 ng/ml) – a concentration that had been determined to give good, but sub-maximal response. After 30 min incubation, the amount of the granular enzyme β-hexoseaminidase released into the supernatant (index of degranulation) was determined colorimetrically using the p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. Briefly, 50 µl of sample was mixed with 50 µl of the substrate (5 mM in 0.2 M citrate buffer, pH 4.5), and incubated for 1 h at 37°C. The reaction was stopped with 100 µl of 0.05M carbonate buffer (pH =10) and the absorbance of the colored product was then read at 405 nm. Results were expressed as percentage of total cell content (determined in cells lysed with 1% triton X-100).

The same supernatants were stored at -40°C pending the determination of their LTC₄ content.

Assay of LTC₄

The level of LTC₄ released into the reaction supernatants in both mast cells and eosinophils was determined by ELISA method using commercially available kits obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA). The assay had a sensitivity of 26.6 pg/ml, and partially cross-reacting with other cysteinyl-leukotrienes, but not LTB₄ or other eicosanoids.

Determination of cell viability and apoptosis in human eosinophils

Cell viability was determined in freshly isolated eosinophils incubated with or without CEE-1 for 1 h (the total amount of time required for the completion of degranulation and acute mediator release experiments), by the trypan blue exclusion. For apoptosis, the annexin V binding/flow cytometric method was used. Freshly isolated cells were cultured in RPMI culture medium (supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) for 1 or 24 h without any eosinophil survival-enhancing agents, and then washed and incubated for a further 15 min with a mixture of FITC-labeled anti-annexin V antibody and the vital dye 7-amino-actinomycin D (7-AAD). Total apoptotic cells (annexin-positive/7-AAD-negative and annexin-positive/7-AAD-positive) were then determined by flow cytometry using Coulter-Beckman FC 500 flow cytometer (Beckman-Coulter, Brea, CA, U.S.A), and counting 10,000 events per sample.

Measurement of changes in cytosolic calcium levels in human eosinophils

Freshly isolated human eosinophils were re-suspended at a concentration of 2×10^6 /ml and incubated with the calcium-sensitive dye – Fluo-3 AM (2 µM) for 20 min at 37°C in the

presence of 0.03% pluronic acid. Cells were washed twice in phosphate-buffered saline, re-suspended in reaction buffer and kept on ice and in the dark. Aliquots of cell suspension (100 μ l) were then added to each well of a 96-well black microplate and incubated with 50 μ l of CEE-1 or vehicle for 15 min at 37°C. The plate was then transferred to the fluorimeter (Novostar^R, BMG Labtech, Ortenberg, Germany), where the fluorescence intensity (excitation 485 nm and emission 520 nm) was read every 5 s for 30 s before and 120 s after automatic injection of the stimuli (50 nM C5a). In order to normalize values against any changes in baseline, the response was expressed as a ratio of the peak fluorescence intensity at any time point to peak intensity at the beginning of measurement.

Western blotting

The Western blot analysis of human eosinophils was performed according to methods routinely employed in our laboratory (Ezeamuzie and Shihab, 2010). Essentially, 800 μ l of eosinophil suspension (2×10^6 /ml) was mixed with 100 μ l of CEE-1 in each well of a 6-well plate (or eppendorf tube) and incubated for 15 min before being stimulated with 100 μ l of the stimulus (30 nM C5a). At appropriate times after activation, the reaction was quickly stopped with excess ice-cold stopping solution (PBS containing 2 mM sodium orthovanadate) and quickly centrifuged. The pellet was lysed in 200 μ l of lysis buffer [2% SDS, 375 mM Tris/HCl (pH=6.8), 4% β -mercaptoethanol, 0.1% bromophenol blue, 25 mM dithiothritol and protease inhibitor mixture]. Protein content of samples was determined by the Bradford method. Protein equivalents of 7.5×10^5 cells were then boiled and electrophoresed per lane on 8% SDS-polyacrylamide gels. The separated proteins were subsequently transferred electrophoretically to nitrocellulose membranes, and the blots

probed with the appropriate primary antibodies (anti-phospho-p38 MAPK, anti-phospho-ERK1/2 and anti- β -actin), followed by the appropriate horseradish peroxidase (HRP)-linked secondary antibody. In all cases, blots were developed using chemiluminescent reagents and exposure times of 30 s - 30 min, depending on the intensity of the blot, and recorded on x-ray film. The density of each band was quantified using QUANTITY ONE software (Bio-Rad Laboratories, Hercules, CA, USA).

In vivo experiments

Male BALB/c mice (6-8 weeks old), obtained from Harlan Laboratories, Derby, UK, were used in these studies. All animals were maintained under temperature-controlled conditions with an artificial 12-h light/dark cycle and allowed standard chow and water *ad libitum*. All animal studies were carried out in compliance with the Regulations for the use of Laboratory Animals in the Health Sciences Centre, Kuwait University and complied with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The protocols were approved by the Animal Welfare Committee of the Health Sciences Center of Kuwait University.

Induction of mouse Passive Cutaneous Anaphylaxis (PCA)

Mouse ear PCA was performed according to the methods of Matsuda et al., (2004) but with a modification involving the measurement of ear thickness rather than dye exvasation as the index of cutaneous reaction. Six groups of mice, 7 animals per group, were used. Animals in the control group received intra-dermal injection of sterile PBS in both ears,

while those in all the other groups were injected in the right ear with 0.5 µg of anti-DNP IgE antibody in 50 µl of sterile PBS, and in the left ear with the same volume of sterile PBS. After 24 h, the baseline ear thickness of all mice was taken using a high precision digital micrometer with accuracy of 0.001mm [Silverline Tools Ltd, Somerset UK]. Immediately after, the right ears of the mice in the 4 drug treatment groups were pre-treated topically with 30 µl of one of the following: CEE-1 (4.5 mg/kg), CEE-1 (15 mg/kg), clemastine (15 mg/kg) or dexamethasone (1.5 mg/kg). The animals in the control and the drug-untreated (positive control) groups received the drug vehicle ((consisting of 5% DMSO, 76% polyethylene glycol (PEG) and 19% PBS)) in both ears. One hour after drug/vehicle pre-treatment, all mice were then treated topically with 30 µl of 0.2% dinitro-florobenzene (DNFB) in the right ear or its vehicle (acetone-olive oil, 4:1) in the left ear. Ear thickness was then measured again at various times (from 10 min to 8 days) after antigen application. The difference in thickness between the right and left ears of each animal was then determined as index of PCA reaction. After 24 h, drug treatment was continued, once daily, given after each measurement. Because of the time required for the measurements, especially at the early time points, 2 or 3 animals per group were run at the same time, and this was repeated 3 times.

Induction of mouse allergic lung inflammation and airway hyper-responsiveness

(AHR) – (mouse asthma model)

Immunization, challenge and drug treatment

Mice, as specified above, were immunized intraperitoneally (i.p.) with 10 µg ovalbumin mixed with 0.2 ml of aluminum hydroxide gel (Alu-Gel-S; SERVA Electrophoresis GmbH, Heidelberg, Germany), on day 0 and repeated on day 7. On day 14 after the start of immunization, the animals were challenged intra-nasally, once a day, over 4 consecutive days, with 50 µl of 0.06% ovalbumin solution in PBS. Control animals were similarly immunized with PBS and challenged intra-nasally with 50 µl PBS. All intra-nasal administrations were done following light anesthesia with halothane.

Five treatment groups (A-E, 11-24 animals per group) were established. Mice in groups A and B were pre-treated intra-nasally with 50 µl of the drug vehicle, 1 h before and 1 h after each intra-nasal challenge with PBS and ovalbumin, respectively. In the same manner, groups C and D, were pre-treated with the same volume of CEE-1 at 6.25 mg/kg and 12.5 mg/kg, respectively, and group E with dexamethasone (2.5 mg/kg), 1h before and 1h after each intra-nasal challenge with ovalbumin. The reason for choosing to administer CEE-1 twice per day, and close to the allergen challenge, was mainly to ensure sufficient local concentration of the drug during the induction stages of the response.

Different treatment groups were used out for the AHR and cytology/histology studies. Hence, 24 h after the last intra-nasal challenge, pulmonary function was measured in some animals while in others bronchoalveolar lavage (BAL), cytology and histology were performed after they were sacrificed with overdose of halothane.

Measurement of AHR

For the measurement of AHR, airflow was recorded in individual mice using a Finepoint Series RC site (Buxco Research Systems, Wilmington, NC, USA), according to the manufacturer's guidelines. In short, mice were anesthetized with an i.p. injection of ketamine/xylazine (1 mg/kg : 0.1 mg/kg) cocktail and tracheotomized with a steel 18-gauge cannula. Mice were subsequently mechanically ventilated at a rate of 150 breaths/min, and tidal volume of 0.15 ml, using a computerized small animal ventilator (Finepoint; Buxco Electronics, Wilmington, NC) as previously described [El-Hashim et al., 2011b]. After 5 min of stabilization followed by administration of PBS, airway resistance was measured by exposing mice to aerosolized methacholine (6.25–50.0 mg/ml) (5 μ l per delivery) delivered by nebulizer administration, and reported as total lung resistance (R_L) (cm H₂O/ml/s).

Bronchoalveolar lavage (BAL) fluid cell counts and lung histology

BAL fluid was collected by cannulating the trachea and washing the lungs with saline solution (4 $\times$ 0.3 ml each). BAL cells were counted using a particle size counter (Z1 series, Beckman Coulter, Florida, USA) and cytosmears were prepared for differential count. Cells were stained with Diff-Quik and a differential count of 200 cells was performed using standard morphological criteria. Results are expressed as total cell count/ml in BAL fluid. For lung histology, pieces of lung tissues were removed and fixed in 10% buffered formalin, embedded in paraffin wax and sectioned into 5 μ m thick slices. The sections were processed and stained with haematoxylin and eosin stain according to standard methods. Sections were examined under light microscope and the severity of pathological changes scored independently by two experienced histologists unfamiliar with the coding

of the slides. Score coding was as follows: (1 = normal, 2 = mild, 3 = moderate, 4 = severe and 5 =highly severe).

Materials

The synthesis of CEE-1 (ethyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate) and its close analogues, CEE-2 (methyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate) and BRG-14 (ethyl 4-phenylmethylaminocyclohex-3-en-2-oxo-6-phenyl-1-oate), were accomplished in-house according to methods described in detail elsewhere (Ezeamuzie and Edafiogho, 2012; Edafiogho et al., 2009). Their chemical structures are shown in figure 1. Fresh solutions of the compounds were prepared each time - first dissolved in DMSO and subsequently diluted down as appropriate. For *in vitro* experiments, the highest final solvent concentration in the wells did not exceed 0.05% - a concentration that had no significant effect on mast cells and eosinophils. For *in vivo* experiments, the compounds were made in drug vehicle (5% DMSO, 76% PEG and 19 % PBS).

The following drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.): dexamethasone, HEPES buffer, p-nitrophenyl-N-acetyl-B-D-glucosaminide, human recombinant C5a, FMLP, OPD, cytochalasin B, zileuton, ovalbumin, mouse monoclonal anti-DNP IgE (clone SPE-7), DNP-BSA, DMSO, FBS, heparin, DNFB, acetone, clemastine fumarate, methacholine, polyethylene glycol, L-glutamine, penicillin/streptomycin solution, RPMI 1640 medium, beta-mercaptoethanol, bis-acrylamide N, N'-methylene-bis-acryl-amide, sodium dodecyl sulfate (SDS), histology stains, DTT, bromophenol blue and tris-base. Ketamine and xylazine were obtained from a

local Pharmacy. RBL-2H3 cell line was purchased from American Tissue Collection Center (Manassas, VA, U.S.A), while Fluo-3AM was obtained from Invitrogen, Carlsbad, CA, USA. Pluronic acid (low UV) was obtained from Molecular Probes, Eugene, OR, USA. Alu-Gel-S was obtained from SERVA Electrophoresis GmbH, Heidelberg, Germany, while the digital micrometer was purchased from Silverline Tools Ltd, Somerset, UK. Minitrans blot filter paper, immuno-blot® PVDF membrane (0.2 µ) and protein assay reagents were all obtained from Bio-Rad Systems, (Herculus, CA, USA). The LTC₄ ELISA kits were all purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Dynabeads^R magnetic beads were obtained from Life Technologies (Carlsbad, CA, USA), while monoclonal anti-CD16 antibody (clone FcR gran 1) was obtained from CLB (Amsterdam, Netherlands). Mouse monoclonal IgG antibodies to human ERK1/2, p-38 MAPK and β-actin, as well as the HRP-labeled goat anti-mouse IgG, were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). Annexin V-FITC/7-AAD kit (containing mouse anti-human annexin V antibody and the vital dye 7-AAD), as well as the antibody isotype control (mouse IgG1-FITC) were obtained from Beckman-Coulter, Brea, CA, U.S.A. Finepoint Complete Lung Function Measurement System was obtained from Buxco Research Systems, Wilmington, NC, USA.

Data Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, U.S.A.). All results were expressed as the mean ± s.e.m. The IC₅₀ values were calculated from the concentration-response curves by non-linear regression analysis. For normally distributed data, values were evaluated by one-way ANOVA, followed by Bonferroni's

post-hoc test. For lung function measurements, absolute resistance (R_L) values were calculated and used as an index of the airway responsiveness to methacholine, while repeated measure analysis of variance, followed by a post hoc LSD test was used for statistical analysis. A Kruskal Wallis analysis of variance was used to compare differences between the different treatment groups, followed by multiple comparisons versus control group. Differences in means between groups were considered significant when $p \leq 0.05$. Where results were expressed in percentages, calculations were done on raw data before taking means.

Results

Effect of CEE-1 on the activation of human eosinophils

As shown in figure 2A, pre-treatment of human blood eosinophils with CEE-1 for 15 min before stimulation with C5a (in the presence of CB) resulted in a significant and concentration-dependent inhibition of the release of the granular EPO. The concentration causing 50% inhibition (IC_{50}) was 0.4 μ M (CI: 0.1-0.9), $n=8$, and at 10 μ M complete inhibition was achieved. CEE-2, a structurally very close analogue of CEE-1 (with a methyl group replacing the ethyl group of CEE-1), was also active (IC_{50} = 1.1 μ M (CI: 0.4-3.5), $n=8$, while another analogue BRG-14 [which lacks the hydrazino (-NH-NH-) moiety]] was essentially inactive. This confirms the specificity of the actions of CEE-1 and the importance of the hydrazino group for biological activity. In comparison, the steroid dexamethasone was without effect up to a concentration of 1 μ M.

When tested on the release of LTC_4 from eosinophils stimulated with FMLP + CB, a similar scenario was seen, with CEE-1 and CEE-2, but not BRG-14, being active (fig. 2B). However, the estimated IC_{50} value for CEE-1 was about one order of magnitude higher (3.8 μ M (CI: 0.9-8.3). Like zileuton - the prototype 5-lipoxygenase inhibitor, CEE-1 also abolished LTC_4 production, though at a higher concentration (30 μ M compared with 3 μ M for zileuton). Again dexamethasone, at concentrations of up to 1 μ M, had no significant effect on this response (fig. 2B). Since, from these results, CEE-1 appeared to be consistently more potent than its analogue CEE-2, the rest of the study was based on the former.

Effect of CEE-1 on IgE-dependent activation of mast cells

To determine if the drug might also affect mast cell activation by allergen, the compound was tested on two mast cell types – the RBL-2H3 mast cell line and native bone marrow-derived mouse mast cells (BMMC). As shown in figure 3(A), CEE-1 significantly inhibited the release of the granular enzyme, β -hexoseaminidase, from RBL-2H3 cells in a concentration-dependent manner (IC_{50} = 5.1 μ M (CI: 1.6-9.7), n=6. As with degranulation, the drug also inhibited the release of LTC₄, but more potently (IC_{50} = 0.7 μ M (CI: 0.3-1.8), n=5, figure 3B. The maximal inhibition of LTC₄ release was comparable with that of zileuton, since at 30 μ M, the drug nearly abolished LTC₄ release, as did zileuton (10 μ M). A similar effect was seen with BMMC (fig. 3C&D). In general, the drug was more potent on human eosinophils than on rodent mast cells. A summary of the IC_{50} values for the inhibition of the various responses in the experimental models is shown in table 1.

Effect of CEE-1 on viability of human eosinophils

To verify that the inhibitory effect of CEE-1 was not due to toxicity to the cells, the effects of the compound on cell viability and apoptosis of human eosinophils were analyzed. As shown in figure 4, incubating the cells for 1 h (total time of contact of eosinophils with CEE-1 in all our *in vitro* experiments was 45 min), at the concentrations of 3 - 30 μ M did not significantly decrease the viability of eosinophils (fig. 4A). Essentially similar results were seen with apoptosis, except that in cells treated with the highest concentration of the compound (30 μ M) for 24 h, a small but statistically non-significant increase in apoptosis compared to vehicle-treated cells, was seen (fig 4B).

Effect of CEE-1 on calcium transients in human eosinophils

In order to determine the mechanism by which CEE-1 inhibited eosinophil activation, the effect of the drug on the early increase in intracellular Ca^{2+} following activation was studied. As shown in figure 5 A&B, pre-treatment of human eosinophils with CEE-1 (3 - 30 μM) for 15 min before stimulation with C5a (50 nM) did not significantly affect the C5a-induced intracellular Ca^{2+} transient. At the tested concentrations, the compound had no effect on un-stimulated cells. This suggests that the drug did not produce its inhibitory effect by blocking stimulus-induced elevation of intracellular Ca^{2+} concentrations.

Effect of CEE-1 on the phosphorylation of MAP kinases in human eosinophils

Phosphorylation of the MAP kinases ERK1/2 and p38 MAPK is among the most important early steps in the signaling pathways leading to activation of many cells including mast cells and eosinophils (Duan and Wong, 2006, Adcock et al., 2008; Shamri et al., 2013). We therefore studied the effect of CEE-1 on the phosphorylation of these enzymes during activation. As shown in figure 6A, C5a caused an extremely fast phosphorylation of both ERK1/2 and p38 MAPK in eosinophils. The effect on ERK1/2 was already pronounced by 6 s (0.1 min) after stimulation, peaked at 1 min and started to wane by 3 min. For p38 MAPK, phosphorylation was even faster as it peaked at 6 s (the earliest time that could be reliably measured in our protocol). Pre-treatment of the cells with CEE-1 for 15 min resulted in a statistically significant and concentration-dependent inhibition of phosphorylation of both ERK1/2 and p38 MAP kinases, with the highest concentration tested (30 μM) completely abolishing both responses (figs. 6 B&C). In this, and all other in

vitro experiments, the compound at the tested concentrations, had no effect on unstimulated cells. These results show clearly that inhibition of MAP kinases is the main mechanism by which CEE-1 inhibits the functions of human eosinophils.

Effect of CEE-1 on mouse ear Passive Cutaneous Anaphylaxis (PCA).

To test whether the *in vitro* effect of CEE-1 could be reproduced *in vivo*, the effect was studied on two animal disease models - mouse PCA and mouse allergic lung inflammation/AHR (asthma model). Preliminary experiments had revealed that systemically administered CEE-1 was largely ineffective due to rapid inactivation *in vivo*. For this reason, topical application was used for all further *in vivo* testing of the drug.

It has been established that when passively sensitized mouse ear is challenged with topical application of the antigen, the PCA reaction that develops is usually 2-phased: a rapid swelling of the ear that peaks in 30 min followed by a delayed (much greater) swelling of the same ear peaking at about the 3-6th day after challenge (Mukai et al., 2005). As shown in figure 7, topical application of CEE-1 to the ear 1 h before induction of PCA resulted in a dose-dependent inhibition of the early-phase ear swelling, with the higher dose (15 mg/kg) causing statistically-significant inhibition ($65.5 \pm 8.4\%$; $p < 0.01$, $n=7$), (fig. 7A). Similar application of clemastine (15 mg/kg) - a histamine H-1 receptor antagonist, but not dexamethasone (1.5 mg/kg), also produced significant inhibition of this component of the PCA reaction. In contrast, none of the drugs, including dexamethasone, significantly inhibited the late-phase PCA reaction when applied daily throughout the 6 days (fig 7B). Repeated application of 15 mg/kg CEE-1 to normal mouse ear produced no significant change compared to vehicle.

Effect of CEE-1 on allergic lung inflammation and AHR

As shown in figure 8A, ovalbumin (OVA)-sensitized mice that were treated intra-nasally with drug vehicle and subsequently challenged with ovalbumin (OVA/VEH group) had a significantly increased number of total inflammatory leukocytes in the BAL fluid 24 h after challenge compared with the control group (PBS/VEH group) that was treated with drug vehicle and challenged with PBS (49.7 ± 4.9 vs. 17.8 ± 1.5 ($\times 10^4$) cells/ml BAL fluid, respectively, $n=15$; $p<0.001$). Likewise, there were significant increases in the numbers of lymphocytes, neutrophils and eosinophils, but not monocytes. The corresponding increase in eosinophil count was most dramatic [23.2 ± 4.3 vs. 0.5 ± 0.2 ($\times 10^4$) eosinophils/ml BAL fluid, respectively; $p<0.001$, $n=12$). Intranasal instillation of CEE-1 (6.25 mg/kg and 12.5 mg/kg) to OVA-sensitized and challenged animals (OVA/CEE-1 groups) significantly inhibited the increase in eosinophils and neutrophils, but not lymphocytes. For example, with CEE-1 (12.5 mg/kg) treatment, eosinophil infiltration reduced from $23.2 \pm 4.3 \times 10^4$ cells/ml BAL fluid in OVA/VEH group to $6.9 \pm 2.3 \times 10^4$ cells/ml BAL fluid in OVA/CEE-1 (12.5 mg/kg) group, $p<0.001$, $n=15$). The effects of CEE-1 on eosinophil and neutrophils infiltrations were similar to those of the steroid, dexamethasone (2.5 mg/kg), which almost abolished both responses. However, unlike the latter, CEE-1 did not significantly affect the increase in lymphocytes numbers.

Similarly, 24 h after the last intra-nasal OVA challenge of sensitized mice, significant AHR, characterized as an increase in lung resistance (R_L) to methacholine was seen (fig. 8B). Specifically, the overall mean R_L was 6.58 ± 0.53 cm H_2O /ml/s compared to 3.48 ± 0.61 cm H_2O /ml/s for the PBS-challenged/vehicle-treated control group, $p<0.001$. The

groups treated with 6.25 mg/kg or 12.5 mg/kg doses of CEE-1 had significantly lower average R_L of 3.53 ± 0.62 cm $H_2O/ml/s$ and 3.81 ± 0.56 cm $H_2O/ml/s$, respectively, $p < 0.001$ for both, in comparison with the OVA-challenged/vehicle-treated group. The effects were comparable to that of dexamethasone-treated group, 3.1 ± 0.53 cm $H_2O/ml/s$. In effect, both doses of CEE-1 produced complete abolition of AHR in the same manner as dexamethasone (2.5 mg/kg). Treatment of normal mice with CEE-1 (12.5 mg/kg) had no significant effect on lung function values, when compared with untreated or vehicle treated groups.

The inhibitory effect of CEE-1 on bronchial inflammation was also seen in the histology of the lung tissues obtained from the different treatment groups 24 h after antigen (or PBS) challenge (fig. 9). In the OVA-sensitized/challenged, but vehicle-treated mice (OVA/VEH), there was severe inflammation characterized by massive infiltration of inflammatory cells in the lung tissues, especially around bronchioles and blood vessels, compared with the PBS/VEH-challenged group which lacked such inflammatory response (fig. 9; B vs. A). Intra-nasal instillation of CEE-1 (6.25 mg/kg and 12.5 mg/kg) resulted in significant and dose-dependent amelioration of inflammation, with the higher concentration being as effective as dexamethasone (2.5 mg/kg) in achieving almost complete attenuation of the inflammatory response (fig. 9; C-E and panel F).

Discussion

In the present study, we have shown that the novel enhydrazinone ester, CEE-1, possesses a unique ability to directly inhibit the activation of both mast cells and eosinophils *in vitro*, and to produce anti-allergic and anti-asthmatic effects *in vivo*. In the *in vitro* studies, the drug inhibited both the degranulation and LTC₄ release in activated human eosinophils, as well as in two different mast cells types, following IgE-dependent activation. In human eosinophils, the drug was about one order of magnitude more potent in inhibiting degranulation than in inhibiting the release of LTC₄, whereas, in rodent mast cells the reverse was the case. In contrast to the above effect of CEE-1 on human eosinophils, dexamethasone – a clinically used anti-allergic steroid, had no significant effect on both degranulation and LTC₄ release, even at a high concentration of 1 μ M. This confirms our previous observation that glucocorticoids have no direct short-term inhibitory effect on human eosinophils (Ezeamuzie and Al-Hage, 1998), although they inhibit these cells *in vivo* or during prolonged culture *in vitro* (Strehl et al., 2011). On LTC₄ release from both eosinophils and mast cells, CEE-1 had comparable efficacy (maximal inhibition) to zileuton – the prototypical 5-lipoxygenase inhibitor and clinically used anti-asthma drug, though it was slightly less potent. Like zileuton, the compound also inhibited LTC₄ generation by both IgE-dependent and independent stimuli. Thus, the compound appears to have a unique spectrum of biological activities (inhibition of both degranulation and LTC₄ release in both mast cells and eosinophils), which are relevant to the treatment of allergic diseases, including asthma.

The use of structural analogues further confirms the importance of the hydrazino moiety in the biological activity of CEE-1, in that the close analogue, CEE-2, which retains this moiety, had comparable activity, whereas another close analogue, BRG-14, which lacks this moiety had no activity. These structure-activity relationships, which were seen both in the degranulation and LTC₄ release experiments, confirm the specificity of action of the drug on the test systems and support our earlier observations (Ezeamuzie et al., 2013).

We further investigated the mechanism by which CEE-1 inhibited eosinophil responses by focusing on two early signaling events – stimulus-induced Ca²⁺ transients and phosphorylation of MAP kinases (ERK1/2 and p38-MAPK). It has been established that both of these events are critical steps in the signaling pathways of activation for both mast cells and eosinophils (MacGlashan, 2012; Shamri et al., 2013), Adcock et al., 2008). While CEE-1 failed to block C5a-induced increase in intracellular Ca²⁺ concentration, it significantly attenuated the phosphorylation of both ERK1/2 and p38 MAPK, thus suggesting that the drug may produce its effect by blocking the phosphorylation of these critical MAPKs. There is ample evidence that both ERK1/2 and p38 MAPK can phosphorylate phospholipase A₂ (PLA₂), leading to the generation of arachidonic acid necessary for leukotrienes synthesis (Zhu et al., 2001; Cho et al., 2004). They are also required for the activation of 5-LO - the enzyme responsible for the conversion of arachidonic acid to the leukotrienes (Zhu et al., 2001). Our results are in agreement with those of other workers who have shown the importance of MAPKs in eosinophil functions in vitro (Duan and Wong 2006; Langlois et al., 2009; Shamri et al., 2013), as well as the effectiveness of

MAPK inhibitors in *in vivo* models of allergic lung inflammation and clinical asthma (Liu et al., 2008; Adcock et al., 2008; Chung, 2011).

Cellular toxicity as a contributory mechanism to the action of CEE-1 can be ruled out since incubation of the cells with CEE-1 for 1 h (representing 15 min pre-incubation and 30-45 min release times) had no significant effect on the viability of the eosinophils nor in the percentage of apoptotic cells (even when culture was extended to 24 h) as determined by annexin V-binding/flow cytometric analysis. Moreover, when eosinophils were pre-treated with CEE-1 (10-30 μ M) - a concentration that abolished degranulation, they still responded normally to C5a with completely undiminished Ca^{2+} transient. This confirms that the drug treatment did not affect the functional integrity of the cells.

The activation of mast cells and eosinophils, and the consequent release of their allergic and inflammatory mediators, are the major etiological factors involved in most allergic diseases. In the context of the newly developed concept of physical interaction between both cells types, leading to mutual enhancement of their respective functional responses (Elishmereni et al., 2011; Elishmereni et al., 2013), the ability of CEE-1 to directly block the activation of both cell types would appear to confer it with potentially superior clinical utility.

To test for the above potential, we conducted studies on two *in vivo* disease models – the mouse ear PCA and the mouse allergic lung inflammation/AHR. In both studies the drug was administered topically because preliminary *in vivo* tests had shown that the drug was poorly effective following systemic administration, which may have been due to rapid inactivation (data not shown). The observed ability of topically applied CEE-1 (15 mg/kg)

to significantly inhibit the 30 min mouse ear PCA reaction is consistent with its *in vitro* action as an inhibitor of IgE-dependent mast cell activation. This is supported by the fact that clemastine - a histamine H-1 receptor antagonist (which presumably blocked the access of mast cell-derived histamine to its receptors), but not dexamethasone, also produced significant inhibition of the PCA reaction. This suggests that topical CEE-1 possesses potentially useful anti-allergic properties, presumably arising from its ability to inhibit mast cell activation *in vivo*. However, repeated application of CEE-1 or the other drugs failed to block the late PCA that peaked at the 6th day after induction. Previous studies have shown that the late PCA - a rather poorly understood phenomenon, appears to be totally different in characteristics from the early phase, and are thought to be independent of mast cells (Mukai et al., 2005; Obata et al., 2007).

In the mouse asthma model, intra-nasally administered CEE-1 showed a powerful efficacy in the suppression of antigen-induced cellular infiltration, especially of eosinophils, in BAL fluid and lung tissues. There was also a dramatic abolition of the associated AHR to methacholine. At a dose of 12.5 mg/kg, CEE-1 was as effective as dexamethasone (2.5 mg/kg), thus, supporting the view that eosinophil accumulation in the lung plays important roles in the development of AHR in the asthmatic lung (Fulkerson and Rothenberg, 2013; Trivedi and Llyod, 2007). Limitations to this aspect of the study include the lack of direct evidence of the effect of CEE-1 on degranulation of mast cells and eosinophils in the asthma model, since both cell types do contribute to the allergic lung inflammation. Our attempt at measuring BAL fluid EPO was frustrated by the rather low levels of its release following antigen challenge in mice. Denzler and co-workers (2001) have previously encountered and reported this problem. Another limitation was that the two mast cell types

used in our *in vitro* experiments, being of mucosal phenotype, may not accurately represent the connective tissue mast cell types predominantly involved in the *in vivo* responses.

Given the important roles that mast cells and eosinophils are believed to play in the pathophysiology of allergic diseases, it is not surprising that a drug that is capable of inhibiting the activation of both cell types is effective in animal models of allergic diseases as has been demonstrated in the PCA and asthma models. We have previously shown that CEE-1 has significant anti-inflammatory effect, based on the analysis of its effect on the release of inflammatory cytokines by activated monocytes/macrophages (Ezeamuzie et al., 2013). Thus, like the steroids, CEE-1 appears to combine general anti-inflammatory effect with anti-allergic effects. On the other hand, the drug has the distinct disadvantage of loss of activity when administered systemically. Although the mechanism of loss of activity has not been specifically investigated, it is likely to be due to spontaneous or enzymatic hydrolysis of the ester or the hydrazino bonds within the molecule. However, the fact that it is effective topically may be an advantage when treating allergic diseases of the skin, eye or lung, because systemic adverse effects are likely to be mitigated. In fact, the development of rapidly metabolized drugs, or drugs developed to be pharmacokinetically restricted to the point of application (the so called ‘antedrugs’), are current strategies to reduce systemic adverse effects of new drugs (Kurimoto et al., 2010; Biffen et al., 2012).

To the best of our knowledge, this is the first report describing a potentially useful anti-allergic and anti-asthmatic effect of any enhydrazinone ester, although we have recently shown that CEE-1 has significant anti-inflammatory activity *in vitro* (Ezeamuzie et al., 2013).

In conclusion, we have shown that the novel enhydrazinone ester CEE-1 potently inhibits the activation of both mast cells and eosinophils in vitro, perhaps by preventing the phosphorylation of ERK1/2 and p-38 MAP kinases, which are early events in the activation of both cell types. Using two animal disease models, these effects were shown to translate into in vivo anti-allergic and anti-asthma effects. These results show that CEE-1 has the potential to be developed into a useful drug for the prophylactic and acute treatment of allergic diseases.

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Authorship Contributions:

Participated in research design: Ezeamuzie, Edafiogho and El-Hashim.

Conducted experiments: Ezeamuzie, Edafiogho, El-Hashim and Renno.

Contributed new reagents or analytical tools: Ezeamuzie and Edafiogho.

Performed data analysis: Ezeamuzie, El-Hashim and Renno.

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Foot Notes

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Legends to figures

Figure 1. Chemical structure of CEE-1 and two of its analogues.

Chemical structure of the enhydrazinone ester, CEE-1, and two of its close analogues, CEE-2 (ortho-methyl ester substituted CEE-1) and BRG-14 (phenyl-methylamino analogue).

Figure 2. Effect of CEE-1 on human eosinophil activation.

Concentration-dependent effect of CEE-1, its analogues - CEE-2 and BRG-14, as well as the steroid dexamethasone, on the activation of purified human eosinophils *in vitro*. A: Effect on degranulation (release of granular EPO) following stimulation with C5a (30 nM) in the presence of 5 µg/ml cytochalasin B (CB). B: Effect on the release of LTC₄ following activation with FMLP (1 µM) in the presence of CB (5 µg/ml). In both cases, cells were pre-incubated with the drug(s) for 15 min before stimulation. Values are mean ± SEM, n=8 for EPO and n=6 for LTC₄. The mean EPO release in un-stimulated/vehicle-treated and stimulated/vehicle-treated cells were 4.6 ± 0.6% and 23.2 ± 4.8% of total cell content, respectively. **p<0.01; ***p<0.001.

Figure 3. Effect of CEE-1 on mast cell activation.

Concentration-dependent effect of CEE-1 on IgE-dependent degranulation and the release of LTC₄ from RBL-2H3 mast cells (A & B, respectively), as well as from bone marrow-

derived mouse mast cells (BMMC) (C & D, respectively). Cells were passively sensitized with 0.5 µg/ml mouse monoclonal anti-DNP IgE overnight, washed and pre-incubated with CEE-1, its vehicle or zileuton for 15 min before stimulation for a further 30 min with the specific antigen DNP-BSA (30 ng/ml). Values are mean ± SEM, n=5-6. *p<0.05; **p<0.01; p<0.001.

Figure 4. Effect of CEE-1 on cell viability and apoptosis.

The effect of CEE-1 on the viability (A) and apoptosis (B) of purified human blood eosinophils. Cells were incubated with the drug for 1 h, washed and then tested for viability by the trypan blue exclusion method. For apoptosis, cells were cultured for 1 h (washed and incubated for an additional 3 h) or they were cultured with drug for 24 h before apoptosis was determined by the annexin V binding/7-AAD uptake analysis using the flow cytometry method. Values are means ± SEM, n=4.

Figure 5. Lack of effect of CEE-1 on calcium transients.

Effect of CEE-1 on C5a-induced increase in intracellular Ca^{2+} in purified eosinophils. Cells pre-loaded with fluo-3 dye were washed and then incubated with the drug for 15 min before stimulation with C5a (50 nM) or vehicle (DMSO) by direct automatic injection. Fluorescence intensity was monitored 30 s before and 120 s after stimulus injection. Response is expressed as the ratio $\text{FU}_{(t)}/\text{FU}_{(0)}$, where $\text{FU}_{(t)}$ = fluorescence intensity at any time point after the start of measurement and $\text{FU}_{(0)}$ = fluorescence intensity at the start of the measurement. A: Time-course; B: Quantitation by area under the curve (AUC) of the

effect of CEE-1 on calcium transients, $n=4$. *** $p<0.001$, with respect to unstimulated/vehicle control.

Figure 6. Effect of CEE-1 on phosphorylation of MAP kinases

Western blot analysis of the effect of CEE-1 on the phosphorylation of ERK1/2 and p-38 MAK kinases in purified human eosinophils stimulated with C5a. A: time-course for the phosphorylation of the MAPKs. B: effect of 15 min pre-treatment with CEE-1 on phosphorylation determined 30 s after stimulation with C5a. C: Drug effect quantitation. ### $p<0.001$, compared with control (veh); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with C5a-stimulated and vehicle-treated.

Figure 7. Effect of CEE-1 on PCA reaction *in vivo*

Effect of topically applied CEE-1 on mouse ear passive cutaneous anaphylaxis (PCA) reaction measured at the peak of the early-phase reaction at 30 min (A), and at the peak of the delayed, and much more intensive reaction measured on day 6 after induction (B). The right ears of mice that were sensitized the previous day with anti-DNP IgE, were pre-treated topically with CEE-1, its vehicle (Veh) or other test drugs – clemastine (CLEM) and dexamethasone DEXA), for 1 h before PCA reaction was induced with topical application of 0.2% dinitro-florobenzene (DNFB). Ear thickness was then measured serially over 8 days. Results are presented as the change in thickness between right and left ear (which were pre-treated with the drug vehicle and PBS only). For the delayed response, drug or vehicle treatment was repeated every day for 8 days. Data presented are for the 30

min and 6th day measurements, and represent mean $\pm$ SEM, n=7 per group. ##p<0.01, ###p<0.001, compared with the control (PBS/Veh/Veh) group, and *p<0.05, **p<0.01, compared with PCA-induced but vehicle-treated (IgE/Veh/DNP) group.

Figure 8. Effect of CEE-1 on allergic lung inflammation and AHR.

The effect of intra-nasally-administered CEE-1 on the cellular contents of bronchoalveolar lavage (BAL) fluid and on airway hyper-responsiveness (AHR) in OVA-sensitized and intra-nasally-challenged mice. A: Number of each cellular component of BAL from mice pre-treated with vehicle and challenged with PBS (PBS/VEH), pre-treated with vehicle and challenged with OVA (OVA/VEH), pre-treated with CEE-1 (6.25 mg/kg) or (12.5 mg/kg) and challenged with OVA [OVA/CEE-1 (0.25%) or (OVA/CEE-1 (12.5 mg/kg))] and pre-treated with 2.5 mg/kg dexamethasone and challenged with OVA [(OVA/DEXA (2.5 mg/kg)]. Animals were treated with drug or vehicle 1 h before and 1 h after each intra-nasal challenge and were lavaged 24 h after the last challenge. B: Effect of CEE-1 on AHR to aerosolized methacholine in some of the mice from the above treatment groups. Lung function measurements were done 24 h after the last challenge. For cell count, ##p<0.01, ###p<0.001, compared with control (PBS-challenged and vehicle pre-treated mice) and *p<0.05, **p<0.01, ***p<0.001, compared with OVA-challenged and vehicle-treated mice. For lung function, ***p<0.001, compared with either PBS-challenged and vehicle-treated, or OVA-challenged and vehicle-treated mice. Values are mean $\pm$ SEM, n=12-20 (for cell counts) and n=9-12 (for lung function).

Figure 9. Effect of CEE-1 on allergic lung inflammation-histology.

Representative histology of hematoxylin and eosin-stained sections of lung tissues from the various treatment groups, showing the effect of intra-nasal administration of CEE-1, its vehicle or dexamethasone. Samples were obtained 24 h after the last intra-nasal challenge in mice challenged with PBS following pre-treatment with vehicle (A), pre-treated with vehicle and challenged with OVA (B), pre-treated with CEE-1 (6.25 mg/kg) and challenged with OVA (C), pre-treated with CEE-1 (12.5 mg/kg) and challenged with OVA (D), and pre-treated with dexamethasone (2.5 mg/kg) and challenged with OVA (E). Scale bar, 500 μ m. Panel F: Quantitation of the severity of inflammation. ### $p < 0.001$, compared with group A and * $p < 0.05$; $p < 0.01$; $p < 0.001$, compared with group B.

TABLE 1: Summary of IC_{50} values for the inhibition of the responses of various cell types.

Human eosinophil EPO and LTC₄ releases were induced by C5a (30 nM) and FMLP (1 μ M), respectively, both in the presence of cytochalasin B (5 μ g/ml). For BMMC and RBL-2H3 mast cells, activation was with antigen after passive sensitization of the cells with IgE.

IC ₅₀ Values [Geom. mean (95% CI), μ M]		
	CEE-1	Dexamethasone
Human Eosinophils		
EPO release (n=8)	0.4 (0.1 - 0.9)	> 1.0
LTC ₄ release (n=6)	3.8 (0.9 - 8.3)	> 1.0
RBL-2H3 mast cells		
β -hexoseaminidase release (n=6)	5.1 (1.6 – 9.7)	> 1.0
LTC ₄ release (n=5)	0.7 (0.3 - 1.8)	>1.0
BMMC		
β -hexoseaminidase release (n=3)	6.5 (2.1 – 11.8)	> 1.0
LTC ₄ release (n=3)	3.3 (0.7 - 7.5)	>1.0

Fig. 1

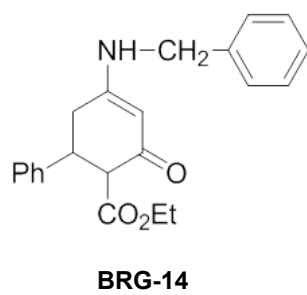
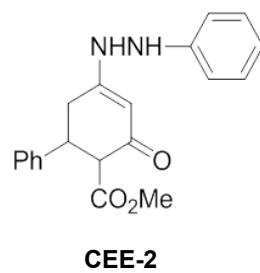
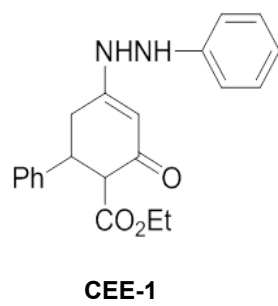


Fig. 2

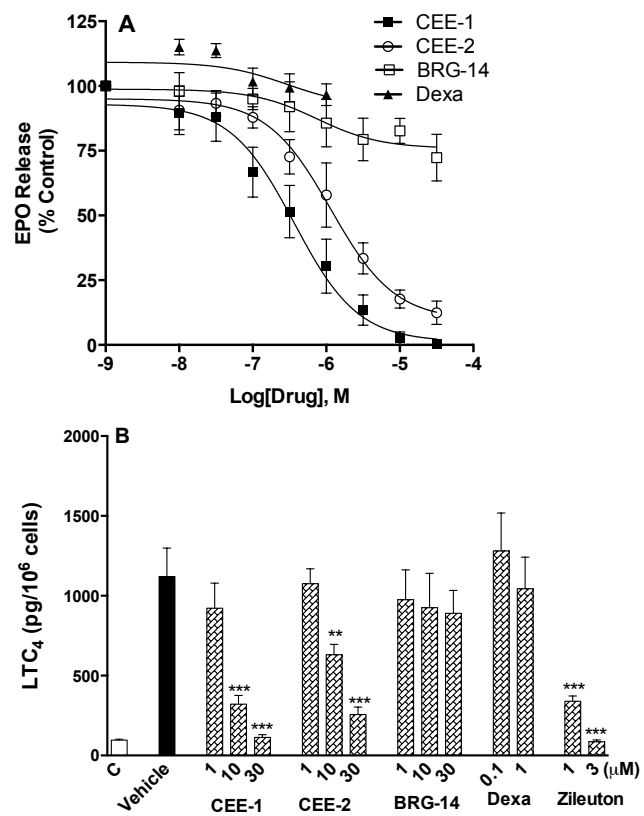


Fig. 3

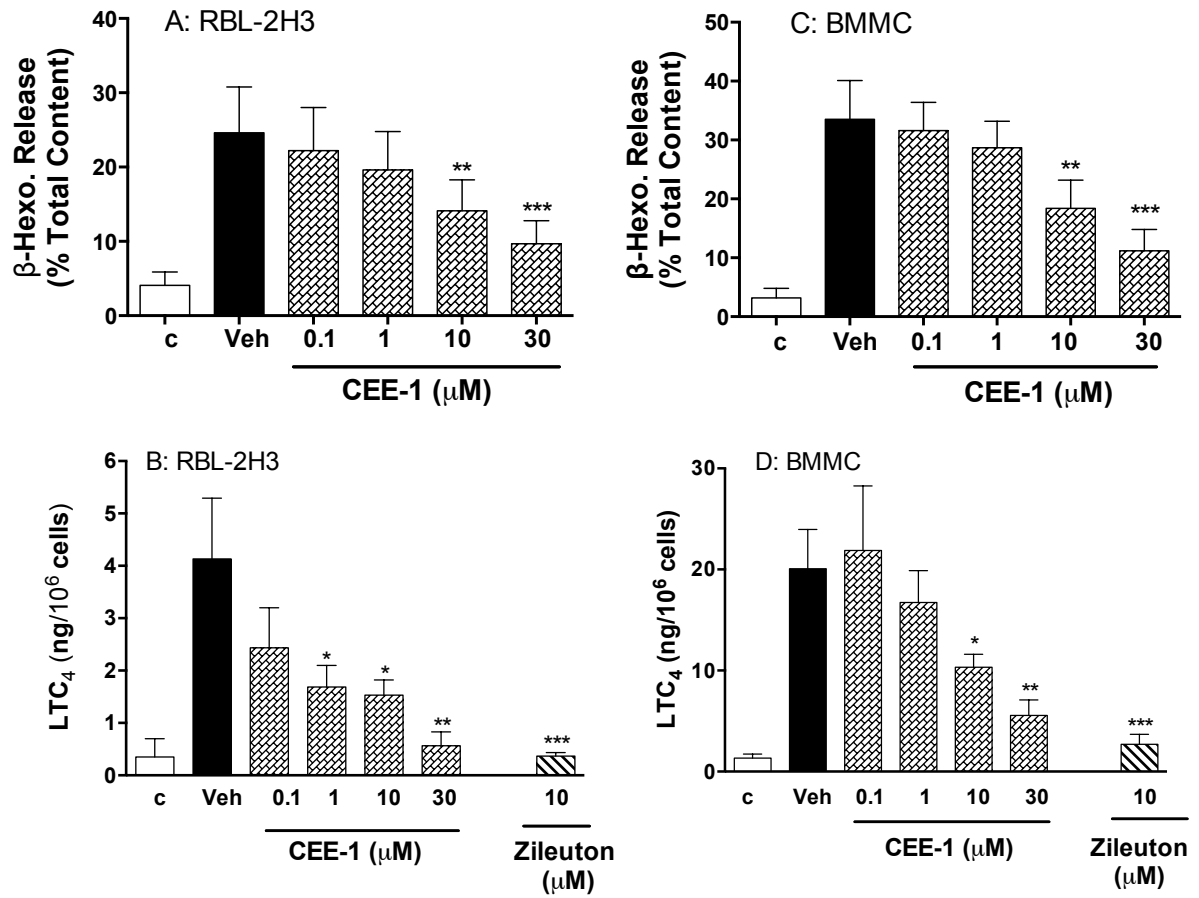


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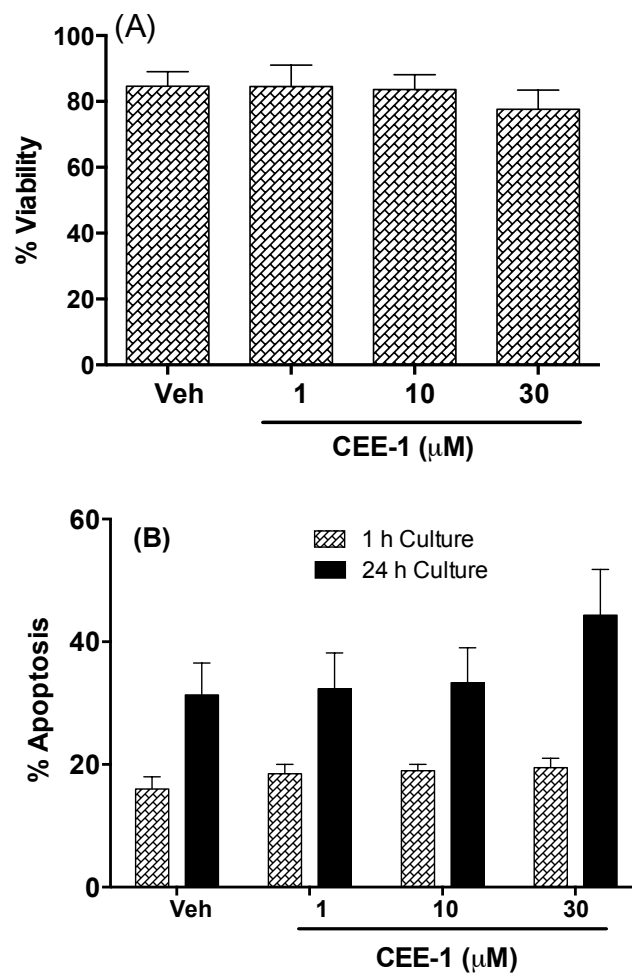


Fig. 5

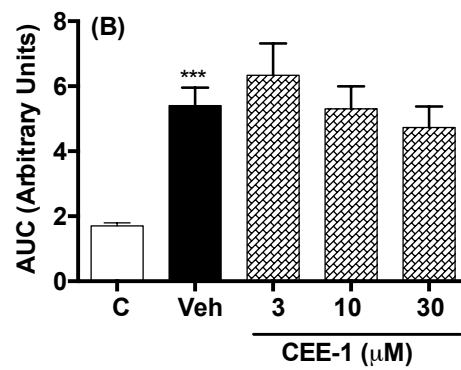
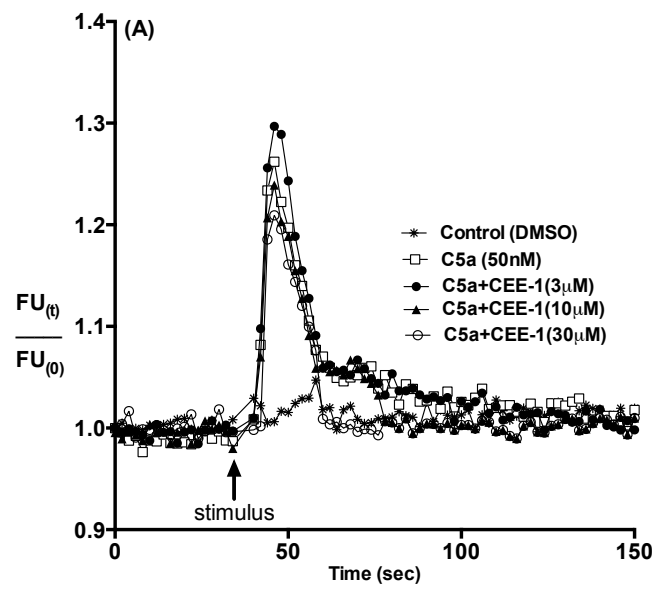


Fig. 6

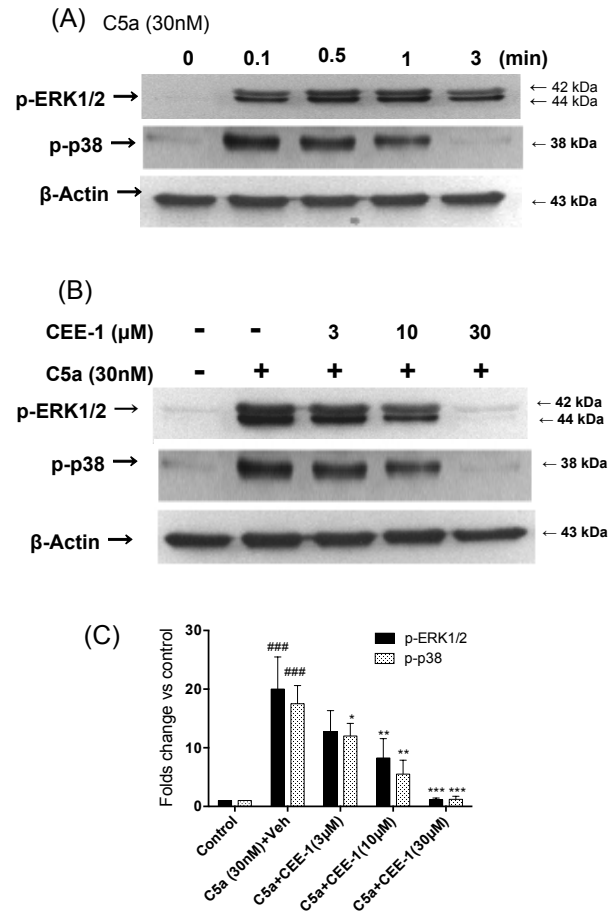


Fig. 7

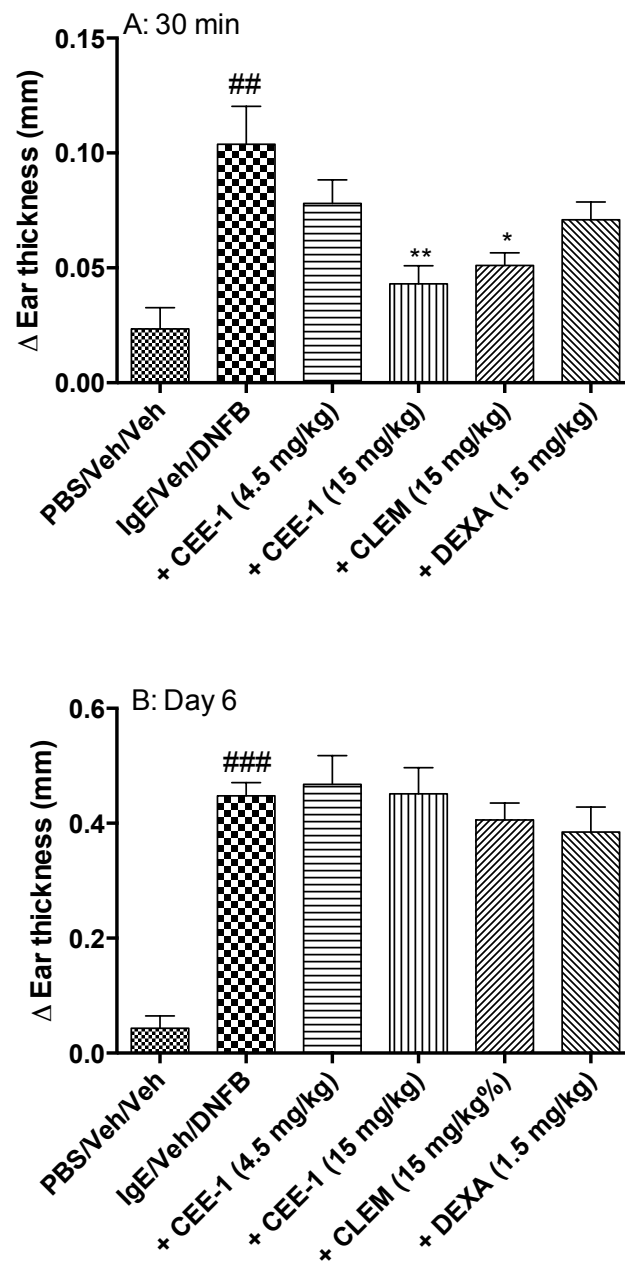


Fig. 8

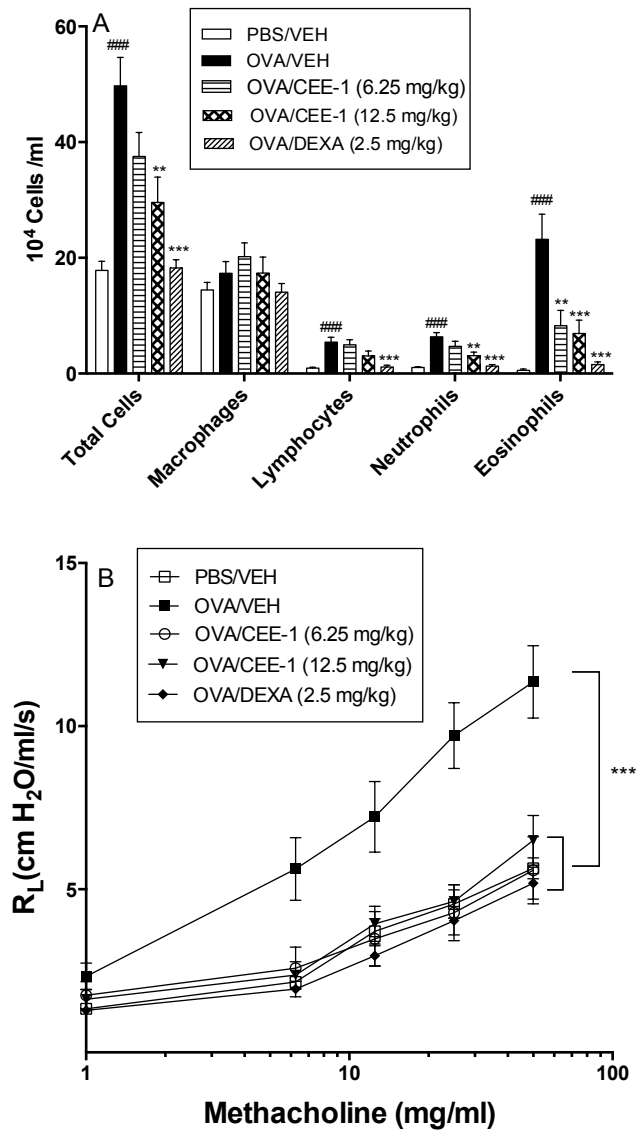


Fig. 9

