Antiallergic and Antiasthmatic Effects of a Novel Enhydrazinone Ester (CEE-1): Inhibition of Activation of Both Mast Cells and Eosinophils


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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 antiallergic and antiasthmatic 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 and bone marrow–derived mouse mast cells. 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] versus 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 extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinases (MAPK). In vivo, topical application of 4.5–15 mg/kg of the compound significantly inhibited allergen-induced passive cutaneous anaphylaxis in mice. Similarly, in the mouse asthma model, the intranasal 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 abolishing 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 MAPK-activation pathways, and that these effects are translated into antiallergic and antiasthmatic effects in vivo. The compound, therefore, has 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 all mast cell and eosinophils. The compound, therefore, has potential application in the treatment of asthma and other allergic diseases.

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ABBREVIATIONS: 7-AAD, 7-amino-actinomycin D; AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; BMMC, bone marrow–derived mouse mast cell; BRG-14, ethyl 4-phenylhydrazinocyclohex-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; CI, confidence interval; DMSO, dimethyl sulfoxide; DNP, dinitrophenyl; EPO, eosinophil peroxidase; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FMLP, N-formyl-methionyl-leucyl-phenylalanine; LTC4, leukotriene C4; MAPK, mitogen-activated protein kinase; OPD, o-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis; RBL-2H3, rat basophilic leukemia cells-2H3.
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 and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases (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 promotes the activation of nuclear factor-κB or other transcription factors leading to the induction of gene transcription and cytokine generation (Adcock et al., 2008; Wu, 2011).

In view of the direct rules that mast cells and eosinophils play in the pathophysiology of allergic diseases, it is often believed that a good antiallergic 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, CEE-1 (ethyl 4-phenylhydrazinocyclohex-3-en-2-oxo-6-phenyl-1-oate), which possesses potent anti-inflammatory activities (Ezeamuzie and Edafiogho, 2012; Ezeamuzie et al., 2013). Although not much is known about the biologic activities of enhydrazinones, the enaminones, to which they are structurally related, have been shown to possess a number of pharmacologic activities, including anticonvulsant (Edafiogho et al., 2007, 2009), antitussive, and immunosuppressive activities (El-Hashim et al., 2010, 2011a).

Because allergic diseases are invariably inflammatory in nature, and because mast cells and eosinophils are proinflammatory 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

The synthesis of CEE-1 and its close analogs 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 (Edafiogho et al., 2009; Ezeamuzie and Edafiogho, 2012). Their chemical structures are shown in Fig. 1. Fresh solutions of the compounds were prepared each time, first dissolved in dimethyl sulfoxide (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% polyethylene glycol, and 19% phosphate-buffered saline (PBS)].

The following drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO): dexamethasone, HEPES buffer, p-nitrophenol-N-acyetyl-b-β-glucosaminide, human recombinant C5a, N-formyl-methionyl-leucyl-phenylalanine (FMLP), o-phenylenediamine (OPD), cytochalasin B, zileuton, ovalbumin, mouse monoclonal anti-dinitrophenyl (DNP) IgE (clone SPE-7), dinitrophenyl–bovine serum albumin conjugate, DMSO, fetal bovine serum (FBS), heparin, dinitro-fluorobenzene, acetone, demastig fumarate, methacholine, polyethylene glycol, t-glutamine, penicillin/streptomycin solution, RPMI 1640 medium, β-mercaptoethanol, bis-acrylamide N,N,N′′-methylene-bis-acryl-amide, SDS, histology stains, dithiothreitol, bromophenol blue, and Tris-base. Ketamine and xylazine were obtained from a local pharmacy.

Rat basophilic leukemia cells-2H3 (RBL-2H3) cell line was purchased from American Type Culture Collection (Manassas, VA); Flu-3 AM was obtained from Invitrogen (Carlsbad, CA). Pluronic acid (low UV) was obtained from Molecular Probes (Eugene, OR). Anti-Gel-S was obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany), and the digital micrometer was purchased from Silverline Tools Ltd (Sommerset, UK). Minitrans blot filter paper, immunoblot polyvinyladine fluoride membrane (0.2 μm) and protein assay reagents were all obtained from Bio-Rad (Hercules, CA). The leukotriene C4 (LTC4) enzyme-linked immunoassay kit was all purchased from Enzo Life Sciences Inc. (Farmingdale, NY). Dynabeads magnetic beads were obtained from Life Technologies (Grand Island, NY), and monoclonal anti-CD16 antibody (clone FeR gran 1) was obtained from CLB (Amsterdam, The Netherlands). Mouse monoclonal IgG antibodies to human ERK1/2, p38-MAPK, and β-actin, as well as the horseradish peroxidase–labeled goat anti-mouse IgG, were all obtained from Santa Cruz Biotechnology (Dallas, TX). Annexin V–fluorescein isothiocyanate (FITC)′7-aminoactinomycin D (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).

Buxco FinePointe Complete Lung Function Measurement System was obtained from DSI (Wilmington, NC).

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), following informed written consent from the donors. The Health Sciences Center Ethical Committee of Kuwait University approved the protocols for the study. Erythrocytes were sedimented using the methylocellulose 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 resuspended in the reaction buffer (same as described for mast cells), and aliquots containing 5 × 10^5 cells were added to each well of a 96-well microplate. The cells were then preincubated for 15 minutes with CEE-1 or the drug solvent (0.05% DMSO in reaction buffer) and subsequently stimulated with recombinant human complement fragment 5a (C5a; 30 nM) or PBS, for controls, in the presence of 5 μg/ml cytochalasin B (CB) for 30 minutes at 37°C. All controls received vehicle (DMSO) in place of drug treatment. The amount of the granular eosinophil peroxidase (EPO) released into the supernatant (as index of degranulation) was determined by the OPRP method. Briefly, the OPRP 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 minutes at 37°C. After incubation, the reaction was stopped with 50 μl 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 μM FMLP. The supernatant containing released LTC4 was collected by centrifugation and stored at −40°C pending assay.

Culture of RBL-2H3 and the Generation of Bone Marrow-Derived Mouse Mast Cells

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 and maintained in our laboratory. They were cultured at a concentration of 1 × 10^7/ml in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The culture was passaged at confluence and used between 4th and 10th passages.

Mouse bone marrow–derived mast cells were generated from 4–5 week old 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% FBS, 100 IU/ml penicillin, and 100 μg/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 interleukin-3. One half of the culture medium was replaced every 3 days. Bone marrow–derived mast cells were 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 bone marrow–derived mast cell (BMMC) were resuspended 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 resuspended 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 preincubated with CEE-1 (0.01–50 μM) or its solvent (0.05% DMSO) for 10–20 minutes before being stimulated with the specific antigen dinitrophenyl-bovine serum albumin conjugate (30 ng/ml), a concentration that had been determined to give good, but submaximal response. After a 30-minute incubation, the amount of the granular enzyme β-hexosaminidase released into the supernatant (index of degranulation) was determined colorimetrically using 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 hour at 37°C. The reaction was stopped with 100 μl of 0.05 M 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 enzyme-linked immunosorbent assay method using commercially available kits obtained from Enzo Life Sciences Inc. The assay had a sensitivity of 26.6 pg/ml, and partially cross-reacts 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 hour (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 IU/ml penicillin, and 100 μg/ml streptomycin) for 1 or 24 hours without any eosinophil survival-enhancing agents, and then washed and incubated for a further 15 minutes with a mixture of FITC-labeled anti–annexin V antibody and the vital dye 7-AAD. Total apoptotic cells (annexin-positive/7-AAD-negative and annexin-positive/7-AAD-positive) were then determined by flow cytometry using Beckman Coulter FC 500 flow cytometer, and counting 10,000 events per sample.

Measurement of Changes in Cytosolic Calcium Levels in Human Eosinophils

Freshly isolated human eosinophils were resuspended at a concentration of 2 × 10^6/ml and incubated with the calcium-sensitive dye Fluo-3 AM (2 μM) for 20 minutes at 37°C in the presence of 0.03% pluronic acid. Cells were washed twice in PBS, resuspended in reaction buffer, and kept on ice and in the dark. Aliquots of cell suspension (100 μl) were then added to each well of a 96-well black microplate and incubated with 50 μl of CEE-1 or vehicle for 15 minutes at 37°C. The plate was then transferred to the fluorimeter (NOVOstar; BMG Labtech, Ortenberg, Germany), where the fluorescence intensity (excitation 485 nm and emission 520 nm) was read every 5 seconds for 30 seconds before and 120 seconds after automatic injection of the stimul (50 nM C5a). 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 μl of eosinophil suspension (2 × 10^7/ml) was mixed with 100 μl of CEE-1 in each well of a six-well plate (or Eppendorf tube) and incubated for 15 minutes before being stimulated with 100 μl 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 μl of lysis buffer (2% SDS, 375 mM Tris/HCl (pH 6.8), 4% β-mercaptoethanol, 0.1% bromophenol blue, 25 mM dithiothreitol, and protease inhibitor.
mixture). Protein content of samples was determined by the Bradford method. Protein equivalents of $7.5 \times 10^5$ 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–linked secondary antibody. In all cases, blots were developed using chemiluminescent reagents and exposure times of 30 seconds to 30 minutes, 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).

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-hour 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 US 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

Mouse ear passive cutaneous anaphylaxis (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 exavasation as the index of cutaneous reaction. Six groups of mice, seven animals per group, were used. Animals in the control group received intradermal injection of sterile PBS in both ears, whereas 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 hours, the baseline ear thickness of all mice was taken using a high precision digital micrometer with accuracy of 0.001 mm (Silverline Tools Ltd, Somerset, UK). Immediately after, the right ears of the mice in the 4 drug treatment groups were pretreated topically with 50 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, and 19% PBS) in both ears. One hour after drug/vehicle pretreatment, all mice were then treated topically with 30 μl of 0.2% diflunisal in olive oil 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 minutes to 8 days) after antigen application. The difference in thickness between the right and left ears of each animal was then determined as the index of PCA reaction. After 24 hours, drug treatment was continued, once daily, given after each measurement. Because of the time required for the measurements, especially at the early time points, two or three animals per group were run at the same time, and this was repeated three times.

Induction of Mouse Allergic Lung Inflammation and Airway Hyper-Responsiveness (Mouse Asthma Model)

Immunization, Challenge, and Drug Treatment. Mice, as specified above, were immunized intraperitoneally with 10 μg ovalbumin mixed with 0.2 ml of aluminum hydroxide gel (Alu-Gel-S; SERVA Electrophoresis GmbH) on day 0 and repeated on day 7. On day 14 after the start of immunization, the animals were challenged intranasally, once a day, over 4 consecutive days, with 50 μl of 0.06% ovalbumin solution in PBS. Control animals were likewise immunized with PBS and challenged intranasally with 50 μl PBS. All intranasal 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 pretreated intranasally with 50 μl of the drug vehicle, 1 hour before and 1 hour after each intranasal challenge with PBS and ovalbumin, respectively. In the same manner, groups C and D were pretreated with the same volume of CEE-1 at 6.25 and 12.5 mg/kg, respectively, and group E with dexamethasone (2.5 mg/kg), 1 hour before and 1 hour after each intranasal 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 for the AHR and cytology/histology studies. Hence, 24 hours after the last intranasal challenge, pulmonary function was measured in some animals, whereas 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 Buxco FinePointe series RC site (DSI, Wilmington, NC), according to the manufacturer’s guidelines. In short, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (1:0.1 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 (FinePointe site), as previously described (El-Hashem et al., 2011b). After 5 minutes of stabilization followed by administration of PBS, airway resistance was measured by exposing mice to aerosolized methacholine (6.25–50.0 mg/ml, 5 μl per delivery) delivered by nebulizer administration, and reported as total lung resistance ($R_L$) (centimeters H2O per milliliter per second).

BAL Fluid Cell Counts and Lung Histology. BAL fluid was collected by cannulating the trachea and washing the lungs with saline solution (4 × 0.3 ml each). BAL cells were counted using a particle-size counter (Z1 Single Threshold; Beckman Coulter) and cytosemears were prepared for differential count. Cells were stained with Diff-Quik and a differential count of 200 cells was performed using standard morphologic criteria. Results are expressed as total cell count per milliliter 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-μm-thick slices. The sections were processed and stained with H&E stain according to standard methods. Sections were examined under light microscope and the severity of pathologic 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).

Data Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). All results were expressed as the mean ± S.E.M. The IC50 values were calculated from the concentration-response curves by nonlinear regression analysis. For normally distributed data, values were evaluated by one-way analysis of variance, 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, whereas repeated measure analysis of variance, followed by a post-hoc least significant difference 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 $P < 0.05$. 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 Fig. 2A, pretreatment of human blood eosinophils with CEE-1 for 15 minutes 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 IC$_{50}$ was 0.4 μM (confidence interval [CI]: 0.1–0.9), n = 8, and at 10 μM complete inhibition was achieved. CEE-2, structurally a very close analog of CEE-1 (with a methyl group replacing the ethyl group of CEE-1), was also active (IC$_{50}$ = 1.1 μM; CI: 0.4–3.5, n = 8), whereas another analog, BRG-14 [which lacks the hydrizino (NH-NH) moiety], was essentially inactive. This confirms the specificity of the actions of CEE-1 and the importance of the hydrizino group for biologic activity. In comparison, the steroid dexamethasone was without effect up to a concentration of 1 μM.

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 μM; 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 μM compared with 3 μM for zileuton). Again dexamethasone, at concentrations of up to 1 μM, had no significant effect on this response (Fig. 2B). Because, from these results, CEE-1 appeared to be consistently more potent than its analog CEE-2, the rest of the study was conducted with 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 BMMC. As shown in Fig. 3A, 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 μM; CI: 1.6–9.7; n = 6]. As with degranulation, the drug also inhibited the release of LTC$_4$, but more potently [IC$_{50}$ = 0.7 μM; CI: 0.3–1.8; n = 5; Fig. 3B]. The maximal inhibition of LTC$_4$ release was comparable with that of zileuton, since at 30 μM, the drug nearly abolished LTC$_4$ release, as did zileuton (10 μM). A similar effect was seen with BMMC (Fig. 3, C and 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 attributable to toxicity to the cells, the effects of the compound on cell viability and apoptosis of human eosinophils were analyzed. As shown in Fig. 4, incubating the cells for 1 hour (total time of contact of eosinophils with CEE-1 in all our in vitro experiments was 45 minutes) at the concentrations of 3–30 μM did not significantly decrease the viability of eosinophils (Fig. 4A). Essentially similar results were seen with apoptosis, with the exception that in cells treated with the highest concentration of the compound (30 μM) for 24 hours, a small but statistically insignificant increase in apoptosis compared with vehicle-treated cells was seen (Fig. 4B).

**Effect of CEE-1 on Calcium Transients in Human Eosinophils.** 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 Fig. 5, A and B, pretreatment of human eosinophils with CEE-1 (3–30 μM) for 15 minutes 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 unstimulated 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 Fig. 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 seconds (0.1 minutes)
after stimulation, peaked at 1 minute, and started to wane by 3 minutes. For p38-MAPK, phosphorylation was even faster, as it peaked at 6 seconds (the earliest time that could be reliably measured in our protocol). Pretreatment of the cells with CEE-1 for 15 minutes resulted in a statistically significant and concentration-dependent inhibition of phosphorylation of both ERK1/2 and p38-MAPK, with the highest concentration tested (30 μM) completely abolishing both responses (Fig. 6, B and 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 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 because of 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 two-phased: a rapid swelling of the ear that peaks in 30 minutes followed by a delayed (much greater) swelling of the same ear peaking at about the third to sixth day after challenge (Mukai et al., 2005). As shown in Fig. 7, topical application of CEE-1 to the ear 1 hour 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 ± 8.4%; P < 0.01, n = 7) (Fig. 7A). Similar application of clemastine (15 mg/kg), a histamine H-1 receptor

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<th>IC50 Values, Geom. Mean (95% CI)</th>
<th>CEE-1</th>
<th>Dexamethasone</th>
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<td>Human eosinophils</td>
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<td>EPO release (n = 8)</td>
<td>0.4 (0.1–0.9)</td>
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<td>LTC4 release (n = 6)</td>
<td>3.8 (0.9–8.3)</td>
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<td>RBL-2H3 mast cells</td>
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<td>β-Hexoseaminidase release (n = 6)</td>
<td>5.1 (1.6–9.7)</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>LTC4 release (n = 5)</td>
<td>0.7 (0.3–1.8)</td>
<td>&gt;1.0</td>
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<td>BMMC</td>
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<tr>
<td>β-Hexoseaminidase release (n = 3)</td>
<td>6.5 (2.1–11.8)</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>LTC4 release (n = 3)</td>
<td>3.3 (0.7–7.5)</td>
<td>&gt;1.0</td>
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Geom, geometric.
agonist, 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 with vehicle.

Effect of CEE-1 on Allergic Lung Inflammation and AHR. As shown in Fig. 8A, ovalbumin (OVA)-sensitized mice that were treated intranasally 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 hours after challenge compared with the control group (PBS/VEH group), which was treated with drug vehicle and challenged with PBS [49.7 ± 4.9 versus 17.8 ± 1.5 (×10⁴) 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 versus 0.5 ± 0.2 (×10⁴) eosinophils/ml BAL fluid, respectively; n = 12; P < 0.001]. 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 ± 4.3 × 10⁴ cells/ml BAL fluid in OVA/VEH group to 6.9 ± 2.3 × 10⁴ cells/ml BAL fluid in OVA/CEE-1 12.5-mg/kg group (n = 15; P < 0.001). 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 lymphocyte numbers.

Similarly, 24 hours after the last intranasal OVA challenge of sensitized mice, significant AHR, characterized as an increase in lung resistance (Rₐ) to methacholine, was seen (Fig. 8B). Specifically, the overall mean Rₐ was 6.58 ± 0.53 cm H₂O/ml per second compared with 3.48 ± 0.61 cm H₂O/ml per second 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 Rₐ of 3.53 ± 0.62 cm H₂O/ml per second and 3.81 ± 0.56 cm H₂O/ml per second, 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 H₂O/ml per second. 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 hours 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 versus A). Intranasal 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–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 antiallergic and antiasthmatic 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 antiallergic steroid, had no significant effect on both degranulation and LTC4 release, even at a high concentration of 1 μM. 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 LTD₄ release from both eosinophils and mast cells, CEE-1 had efficacy (maximal inhibition) comparable to zileuton, the prototypical 5-lipoxygenase inhibitor and clinically used antiasthma drug, though it was slightly less potent. Like zileuton, the compound also inhibited LTD₄ generation by both IgE-dependent and independent stimuli. Thus, the compound appears to have a unique spectrum of biologic activities (inhibition of both degranulation and LTD₄ release in both mast cells and eosinophils), which are relevant to the treatment of allergic diseases, including asthma.

The use of structural analogs further confirms the importance of the hydrazino moiety in the biologic activity of CEE-1, in that the close analog, CEE-2, which retains this moiety, had comparable activity, whereas another close analog, BRG-14, which lacks this moiety, had no activity. These structure-activity relationships, which were seen both in the degranulation and LTD₄ 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 (Adcock et al., 2008; MacGlashan, 2012; Shamri et al., 2013). Although 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 MAP kinases. There is ample evidence that both ERK1/2 and p38-MAPK can phosphorylate phospholipase A₂, leading to the generation of arachidonic acid necessary for leukotriene synthesis (Zhu et al., 2001). 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 others who have shown the importance of MAP kinases in eosinophil functions in vitro (Duan and Wong 2006; Langlois et al., 2009; Shamri et al., 2013), as well as the effectiveness of MAP kinase inhibitors in vivo models of allergic lung inflammation and clinical asthma (Adcock et al., 2008; Liu 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 hour (representing 15-minute preincubation and 30- to 45-minute 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 hours) as determined by annexin V–binding/flow cytometric analysis. Moreover, when eosinophils were pretreated with CEE-1 (10–30 μM), a concentration that abolished degranulation, they still responded normally to C5a with completely undiminished Ca²⁺ 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, 2013), the ability of CEE-1
To directly block the activation of both cell types would appear to confer 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 owing to rapid inactivation (data not shown). The observed ability of topically applied CEE-1 (15 mg/kg) to significantly inhibit the 30-minute 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 demastin, 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 antiallergic 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 sixth 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, intranasally 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 an important role in the development of AHR in the asthmatic lung (Trivedi and Lloyd, 2007; Fulkerson and Rothenberg, 2013). 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 coworkers (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, on the basis of 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 antiallergic 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 probably attributable 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 would probably 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 antiallergic and antiasthmatic 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 p38-MAPK, 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 antiallergic and antiasthma 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, El-Hashim
Conducted experiments: Ezeamuzie, Edafiogho, El-Hashim, Renno
Contributed new reagents or analytic tools: Ezeamuzie, Edafiogho
Performed data analysis: Ezeamuzie, El-Hashim, Renno
Wrote or contributed to the writing of the manuscript: Ezeamuzie, Edafiogho, El-Hashim

References


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