



The soluble guanylyl cyclase activator BAY 60-2770 inhibits murine allergic airways inflammation and human eosinophil chemotaxis



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ABSTRACT

Objectives: Activators of soluble guanylyl cyclase (sGC) act preferentially in conditions of enzyme oxidation or haem group removal. This study was designed to investigate the effects of the sGC activator BAY 60-2770 in murine airways inflammation and human eosinophil chemotaxis.

Methods: C57Bl/6 mice treated or not with BAY 60-2770 (1 mg/kg/day, 14 days) were intranasally challenged with ovalbumin (OVA). At 48 h, bronchoalveolar lavage fluid (BALF) was performed, and circulating blood, bone marrow and lungs were obtained. Human eosinophils purified from peripheral blood were used to evaluate the cell chemotaxis.

Results: OVA-challenge promoted marked increases in eosinophil number in BAL, lung tissue, circulating blood and bone marrow, all of which were significantly reduced by BAY 60-2770. The IL-4 and IL-5 levels in BALF were significantly reduced by BAY 60-2770. Increased protein expression of iNOS, along with decreases of expression of sGC ($\alpha 1$ and $\beta 1$ subunits) and cGMP levels were detected in lung tissue of OVA-challenged mice. BAY 60-2770 fully restored to baseline the iNOS and sGC subunit expressions, and cGMP levels. In human isolated eosinophils, BAY 60-2770 (1–5 μ M) had no effects on the cGMP levels and eotaxin-induced chemotaxis; however, prior incubation with ODQ (10 μ M) markedly elevated the BAY 60-2770-induced cyclic GMP production, further inhibiting the eosinophil chemotaxis.

Conclusions: BAY 60-2770 reduces airway eosinophilic inflammation and rescue the sGC levels. In human eosinophils under oxidized conditions, BAY 60-2770 elevates the cGMP levels causing cell chemotaxis inhibition. BAY 60-2770 may reveal a novel therapeutic target for asthma treatment.

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1. Introduction

Allergic asthma is a chronic inflammatory disease clinically characterized by bronchial hyperresponsiveness, airflow limitations, pulmonary inflammation and mucus secretion, which results in intermittent airway obstruction [1]. Airway inflammation in asthma is a multicellular process, and eosinophil recruitment to the inflamed lung is a hallmark of human and murine allergic asthma. The mechanism involved in eosinophil migration to the lung is dependent on the coordinated actions of different cytokines with a

prominent role for Th2-cytokines [2]. Moreover, interleukin-5 (IL-5) selectively mobilizes mature eosinophils from the bone marrow to the circulation, and in combination with the CC-chemokine eotaxin increases their migratory capacity and adhesiveness, promoting trafficking across the vasculature and recruitment into the lung [1].

Nitric oxide (NO) is a mediator involved in many diverse physiopathological signaling processes, and is produced by action of the classic family of NO synthases (NOS), namely the constitutive calcium-, calmodulin-dependent endothelial and neuronal NOS (eNOS and nNOS) and the inducible calcium-independent NOS (iNOS) [3]. The isotype iNOS is highly expressed in eosinophils and lung tissues of asthmatic patients and animals [4,5]. The airway eosinophilia during asthma exacerbation is correlated with high levels of iNOS-

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derived NO in exhaled air from asthmatic patients, contributing to loss of the lung capacity [5–8]. In addition, NO-reacting oxidants such as superoxide anion, hydrogen peroxide, and hydroxyl radicals have been implicated in allergic lung diseases [9,10]. Increased oxidative stress leads to inactivation of superoxide dismutase in asthmatic individuals, aggravating airway disease [11,12].

Soluble guanylyl cyclase (sGC) is a widely distributed signal-transduction enzyme that under stimulation by NO converts the nucleoside guanosine-5'-triphosphate (GTP) into cyclic GMP [13]. This enzyme is typically expressed as a heterodimer, consisting of two homologous subunits, namely α and β . The most studied and physiologically relevant isoform is $\alpha 1\beta 1$, which is also the most prevalent form in mouse lung tissue [14]. The β -subunit contains the prosthetic haem-moiety domain that is sensitive to NO in its Fe^{2+} reduced form, and its removal or oxidation abolishes NO-induced enzyme activation [15]. Although the detrimental effects of iNOS-derived NO in asthma are largely studied, little is known about the role of the downstream sGC-cGMP signaling in lung tissue and eosinophil activities in asthma [16]. Previous studies have shown that sGC expression and activity are reduced in lung tissues from non-allergic [17,18] and allergic mice [14]. In ovalbumin (OVA)-challenged guinea-pigs, inhibition of cGMP degradation significantly reduces eosinophil influx and NO levels [19]. In addition, in mouse mesenteric post-capillary venules [20] and human isolated eosinophils [21,22], NO-donor compounds inhibit cell rolling, adhesion and migration via cGMP dependent mechanisms, all suggesting that activation of sGC signaling plays an anti-inflammatory effect.

Advances in targeting sGC directly to enhance the production of cGMP independently of the endogenous ligand NO have been made through the use of NO-independent stimulators and activators of sGC [15]. The sGC stimulators like BAY 41-2272 activate the enzyme, acting synergistically with NO. Conversely, removal of the prosthetic haem moiety or its iron haem oxidation by ODQ largely diminishes the enzyme activation [23]. On the other hand, sGC activators like BAY 58-2667 and BAY 60-2770 preferentially activate sGC after enzyme oxidation or removal of the prosthetic haem group [24]. Consequently, the actions of sGC activators and resulting cGMP production in vascular and non-vascular smooth muscles are generally greater in experimental conditions of sGC oxidation or

Considering the potential anti-inflammatory role of sGC, we hypothesized in this study that the sGC activation could protect against the allergic eosinophilic inflammation, or at least partially inhibits eosinophil chemotaxis. Thus, the present study was aimed to investigate the effect of prolonged sGC activation by the sGC activator BAY 60-2770 in murine allergic eosinophilic inflammation and human isolated eosinophil chemotaxis.

2. Materials and methods

2.1. Animals

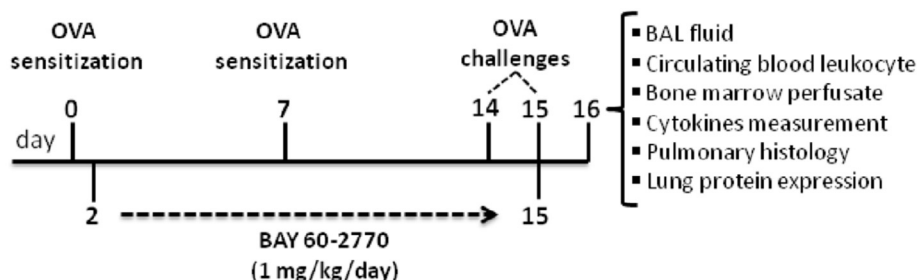
The experimental protocols were approved by the Ethical Principles in Animal Research adopted by The Brazilian College for Animal Experimentation (COBEA). Four-week-old male C57Bl/6 mice were provided by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science of State University of Campinas (UNICAMP). The animals were housed three per cage on a 12 h light-dark cycle in temperature-controlled rooms and received water and food *ad libitum* until used.

2.2. Ovalbumin (OVA) sensitization and challenge procedures

Six-week-old mice (25–30 g) were sensitized with two subcutaneous injections (0.4 ml) of 100 μg of OVA (grade V) mixed with 1.6 mg $\text{Al}(\text{OH})_3$ in 0.9% NaCl, at 0 and 7th day. Mice were intranasally challenged with 10 μg of OVA (in 50 μl saline) at 14th and 15th days, twice a day (6 h of intervals between challenges). The control group was intranasally instilled with saline (50 μl). Animals were sacrificed by a lethal dose of anesthesia (Isoflurane) followed by exsanguination and cervical dislocation, 48 h after the first OVA-challenge.

2.3. Oral long-term treatment with BAY 60-2770

BAY 60-2770 was given daily at a dose of 1 mg/kg (gavage) for 14 consecutive days [29], starting the first day after of OVA sensitization, as demonstrated on the schematic representation below. Animals receiving vehicle 150 μl of Transcutol/Cremophor/water (1/2/7 = v/v/v, microemulsion) served as controls.



haem group removal [25–30]. A recent study has dealt with the mechanisms underlying the bronchodilation in response to activation of the NO-sGC-cGMP pathway in allergic mice and human asthmatics [31]. Generally, authors found that inflamed asthmatic airway and associated excessive NO production damages the sGC in airway smooth muscle, leading to a diminished bronchodilation, which is restored by the sGC agonists BAY 41-2272 and BAY 60-2770 [31]. Nevertheless, to date the therapeutic potential of sGC activators have not been demonstrated in the allergic eosinophilic airway inflammation and human eosinophil chemotaxis.

2.4. Bronchoalveolar lavage (BAL) fluid

The trachea of animals was exposed and cannulated with a polyethylene tube connected to a syringe. The lungs were washed by flushing with phosphate-buffered saline (PBS) ($5 \times 300 \mu\text{l}$) through the tracheal cannula. The fluid recovered after each wash was combined and centrifuged (400 g for 10 min at 4°C), and BAL

fluid supernatant was stored at -80°C . The cell pellet was resuspended in 200 μl of PBS, and total (Neubauer) and differential (Diff-Quick stain) cell counts were done. A minimum of 100 cells were counted in triplicate and classified as eosinophils, neutrophils and mononuclear cells based on normal morphological criteria.

2.5. Histological analysis

Lungs were removed and post-fixed by immersion with buffered 10% formalin for 24 h and dehydrated in ethanol (70%), after which they were embedding in xylol and cut transversally into slices of approximately 3 mm. Only the middle third of the caudal portion of both lungs were included in paraffin. Histological sections (5 μm) were stained with haematoxylin-eosin, and evaluated for leukocyte infiltration to bronchiolar area. For each animal, the extent of the lung infiltrate was determined by establishing 5 compromised bronchiole, randomly selected at high-power fields (i.e. using a $4\times$ objective). The number of eosinophils in pulmonary parenchyma was quantified within 400 μm^2 peri-bronchiolar area. The quantification values represent the mean of 5 bronchioles chosen in a random manner, using a automated microscope (Leica DM5000 B, Wetzlar, Germany). The representative images were captured in $400\times$ magnification by Leica DFC 300FX CCD camera, and analyzed by coupled image software (Leica Q Win Plus v.3.2.0).

2.6. Circulating and bone marrow leukocytes

Blood samples were obtained from the abdominal vena cava. Bone marrow cells were collected by flushing the two femurs with PBS (2.5 ml per femur) over a Petri plate, by using a syringe attached to a 25×0.70 mm (or 22G1) needle. Cellular suspension obtained was transferred to 15-mL Falcon tube and homogenized. The total (Neubauer) and differential (Diff-Quick stain in cytospin slides) cell counts in circulating blood and bone marrow were done. A minimum of 300 cells were counted in each case. The leukocyte counts were differentiated as eosinophils, neutrophils, mononuclear cells and other cells, recognized by normal morphological criteria.

2.7. Western blotting in the mouse lung tissue

Lungs were removed and immediately homogenized in a SDS lysis buffer with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Inc., Westbury, NY) and centrifuged to remove insoluble material. Protein concentrations of the supernatants were determined by the Bradford assay, and equal amount of protein from each sample (50 μg) was treated with Laemmli buffer containing dithiothreitol (100 mM). Samples were heated in a boiling water bath for 10 min and resolved by SDS-PAGE. Electrotransfer of proteins to nitrocellulose membrane was performed for 60 min at 15 V (constant) in a semi-dry device (Bio-Rad, Hercules, CA, USA). Nonspecific protein binding to nitrocellulose was reduced by pre-incubating the membrane overnight at 4°C in blocking buffer (0.5% non-fat dried milk, 10 mM Tris, 100 mM NaCl, and 0.02% Tween 20). Detection was carried out using specific antibodies, HRP-conjugated secondary antibodies, luminal, *p*-Coumaric acid and H_2O_2 . One min after incubation, x-ray sensitive films were exposed to the membranes and marked by a chemiluminescent signal. Anti- α_1 and anti- β_1 sGC subunit antibodies were purchased from AbCam Technology (Cambridge, England, UK) and Novus Biologicals (Littleton, CO, USA), respectively. Anti-iNOS and anti-GAPDH antibodies were obtained from AbCam Technology and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Protein levels were normalized with GAPDH. Densitometry was performed using the Scion Image software (Scion Corporation, Frederick, MD, USA), and results represented as the percentage in relation to control protein expression.

2.8. Measurements of serum IgE and cytokines in BAL fluid

Serum mouse OVA-specific IgE was quantified by commercial ELISA kit (BioLegends Inc., San Diego, CA, USA), following the instructions of the manufacturers. Plates were read in a Hybrid Multi-Mode Microplate Reader Synergy™ H1 (BioTek Instruments, Winooski, VT, USA). Murine interleukin (IL)-4 and IL-5 were measured in BAL fluid using commercially available DuoSet ELISA kits (R&D, Minneapolis, USA).

2.9. Extraction and measurement of cGMP levels in the mouse lung tissue

Lungs from mouse instilled with saline and challenged with OVA, treated or not with BAY 60-2770 were removed and immediately frozen in liquid nitrogen and kept at -80°C until used. Frozen tissues were pulverized, homogenized in trichloroacetic acid (TCA; 5% wt/vol), and centrifuged for 10 min at 4°C at 1500 g and the supernatant was collected. TCA was extracted from the supernatant with three washes of water-saturated ether. Preparation of tracer, samples, standards, and incubation with antibody was performed as described in commercially available kits (Cayman Chemical Cyclic GMP EIA kit, Ann Arbor, MI, USA). The assays were performed in duplicates, and the protein concentration in supernatant was used to normalize data, which were expressed as pmol/mg protein.

2.10. Measurement of tail-cuff pressure in mice

The mouse was restrained and the tail was coupled to a pulse transducer connected to a oscilloscope (NIBP Controller, AD Instruments®, Australia), and systolic blood pressure was recorded using a data acquisition system (LabChart 7, Power Lab 4/35, AD Instruments) in a quiet room. Mice were trained for tail-cuff blood pressure procedures one week before starting the experiments.

2.11. Human eosinophil isolation and experimental protocols

Human blood eosinophil was isolated from healthy individuals free of medication after institution's Research Ethical Committee (CEP 44347) approval, and all individuals signed a free informed consent form. Whole blood was collected into 3.13% sodium citrate (w/v) anticoagulant, and diluted 1:1 with phosphate buffered saline (PBS) and 35 ml of diluted blood overlaid onto a 15 ml Percoll gradient (1.088 g/ml, pH 7.4, 340 mOsmol/Kg H_2O). Gradients were centrifuged at 1000 g for 23 min, 4°C (Thermo Fisher Scientific Centrifuge, Germany) and the red cell pellet collected. Granulocytes were separated by lysing the red cell pellet with lysing buffer (in mM: NH_4Cl 155, KHCO_3 10 and EDTA 0.1). Washed granulocytes were previously incubated (30 min) with anti-CD16 immunomagnetic microbeads (MACS; Miltenyi Biotec, Germany) before placing on a steel-matrix column in a magnetic field. The CD16-negative eosinophils (94–99% purity) were collected and then resuspended in Eagle's minimum essential medium (MEM; pH 7.2). Contaminating cells (1–6%) were mononuclear cells. After eosinophil purification, the in vitro assays of eosinophil chemotaxis, cellular viability (MTT reduction) and cGMP level measurement were carried out. These assays were performed in eosinophils

stimulated with eotaxin, in the absence and/or in the presence of BAY 60-2770 and/or the sGC inhibitor ODQ, as detailed below.

2.12. Human eosinophil chemotaxis assays

The chemotaxis assays were performed in 48-well microchemotaxis chamber (45). The eosinophil suspension was adjusted to 4×10^6 cells/ml in MEM and treated with BAY 60-2770 (1–10 μ M) for 30 min alone and/or after pre-incubation with ODQ (10 μ M) for 10 min. Thereafter, the bottom wells of the microchamber were filled with the chemoattractant agent eotaxin (300 ng/ml) in 27 μ l MEM, whereas the upper wells were filled with eosinophils (50 μ l) that had been treated or not with BAY 60-2770 and/or ODQ, and respective vehicles (DMSO 0.1%, only in the presence of BAY 60-2770, and DMSO 0.2% in presence of both BAY 60-2770 and ODQ). The bottom and upper cells were separated with a polycarbonate filter of 5 μ (25 $\times$ 80 mm; GE Water & Process Technologies, USA). The chamber was then incubated for 60 min at 37 °C with 5% CO₂ atmosphere. At the end of the incubation period, the filter was removed, washed, fixed and stained with Diff-Quik and mounted on a glass slide. Each one of the incubations was carried out in triplicate and migration was determined by counting eosinophils that had migrated completely through the filter in five random high-power fields (HPF; 1000 $\times$) per well.

2.13. Extraction and measurement of cGMP in human eosinophils

Human eosinophils were isolated and resuspended to a number of 1×10^7 cells/ml in PBS, and incubated with 3-isobutyl-1-methylxanthine (IBMX, 2 mM) for 30 min. Next, eosinophils were incubated (37 °C, 5% CO₂) with BAY 60-2770 (1–10 μ M) for 90 min in the absence and in the presence of ODQ (10 μ M, 10 min). The reaction was then interrupted by the addition of cold acidified absolute ethanol to a final concentration of 67% (v/v), and samples were vigorously agitated by hand for 30 s. Cell samples were then incubated on ice for 30 min before centrifuging at 4000 g for 30 min at 4 °C. The supernatants were collected and retained and the precipitates washed with 0.5 ml 67% (v/v) acidified ethanol before centrifuging again at 14,000 g for 5 min at room temperature. The supernatants from these washed samples were collected and combined with the first supernatants collected and dried at 55–60 °C under a stream of nitrogen in a water bath and stored at –20 °C until measurement of cGMP. Cyclic GMP in 1.5×10^6 cells/well were measured using Cayman kit (Cayman Chemical Company, Ann Harbor, MI, EUA), according to the instructions of manufacturer.

2.14. Cell viability in human eosinophils

Cell toxicity was evaluated by using the tetrazolium salt reduction test (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; MTT assay) by eosinophils after exposure to BAY 60-2770 at the conditions that were performed chemotaxis assays. Eosinophils were isolated and resuspended to a concentration of 4×10^2 cells/ml in MEM. Cells were exposed to BAY 60-2770 (1–10 μ M) for 90 min (30 min of pre-incubation with BAY 60-2770 plus 60 min of chemotaxis) at 37 °C and 5% CO₂. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (10 μ l/well; 5 mg/ml in PBS) was added in triplicates to 96-well plates. Cells were allowed to incubate for 3 h at 37 °C, 5% CO₂. After incubation, 100 μ l of 10% SDS acidified with 0.01 M HCl was added to each well. Cell samples were then incubated for 4 h (37 °C, 5% CO₂)

and absorbance measured at 540 nm in a microplate reader (Hybrid Multi-Mode Microplate Reader Synergy™ H1 BioTek Instruments, Winooski, VT, USA).

2.15. Drugs and materials

Ovalbumin (Grade V), 3-isobutyl-1-methylxanthine (IBMX), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), Percoll and dimethyl sulfoxide (DMSO) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). The chemokine rhCCL11/eotaxin was purchased from R&D System (Minneapolis, USA). BAY 60-2770 (acid 4-((4-carboxybutyl) [2-(5-fluoro-2-[[4-(trifluoromethyl) biphenyl-4-yl] methoxy] phenyl) ethyl] amino) methyl) benzoic) was provided by Pharma Research Center, Bayer AG (Wuppertal, Germany).

2.16. Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). The GraphPad Prism 5 Software was used for graphics confection and statistical analysis. Statistical comparisons were made using one-way analysis of variance (ANOVA), and the Tukey's method was chosen as a post-test. Unpaired Student's *t*-test was also used when appropriated. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. BAY 60-2770 reduces leukocyte migration to lung tissue and BAL fluid

Total and differential cell counts were evaluated in BAL fluid and lung tissue at 48 h of four groups of mice, namely: (i) vehicle-treated mice instilled with saline, (ii) vehicle-treated mice challenged with OVA, (iii) BAY 60-2770-treated mice instilled with saline and (iv) BAY 60-2770-treated mice challenged with OVA. Challenge with OVA in previously-sensitized mice promoted a significant increase (*p* < 0.001) in total leukocytes and eosinophils in BAL fluid compared with OVA-sensitized mice instilled with saline. Oral treatment with BAY 60-2770 markedly reduced (*p* < 0.001) cell infiltration in BAL fluid (*n* = 7–8; Fig. 1A and B). Histological examination of lungs from mice instilled with saline treated or not with BAY 60-2770 showed normal tissue with no inflammatory cells (Fig. 2A). However, challenge with OVA elevated significantly (*p* < 0.001) the number of eosinophils in lung parenchyma (Fig. 2B), which was significantly reduced (*p* < 0.001) by BAY 60-2770 treatment. Neutrophils and lymphocytes were not found in lung tissue in any group.

3.2. Effect of BAY 60-2770 on blood and bone marrow eosinophilia

Eosinophil counts were performed in circulating blood and bone marrow at 48 h post-OVA challenge (or instillation with saline) in mice treated with BAY 60-2770 or its vehicle. A marked eosinophilia in blood (Fig. 3A) and bone marrow (Fig. 3B) was observed in OVA-challenged mice of vehicle group, which was nearly abolished by BAY 60-2770 treatment. The number of total leukocytes, neutrophils and mononuclear cells in blood and bone marrow did not significantly change between vehicle and BAY 60-2770 groups (Table 1).

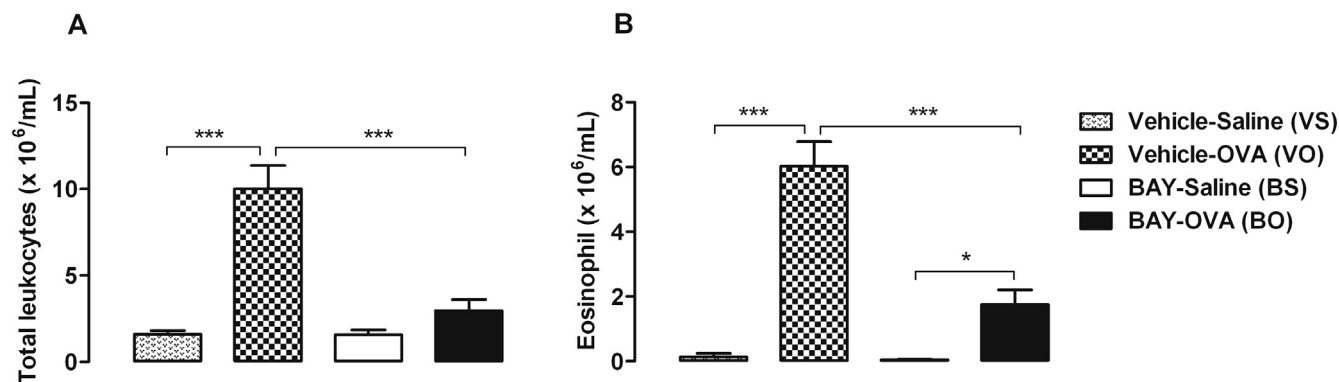


Fig. 1. Treatment with BAY 60-2770 significantly reduces the number of total leukocytes (A) and eosinophils (B) in bronchoalveolar lavage (BAL) fluid at 48 h after intranasal challenge with ovalbumin (OVA) or instillation with saline. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day in the drinking water for 14 days) or its correspondent vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v). Data are expressed as mean $\pm$ SEM for n = 7–8, **p* < 0.05, ****p* < 0.001.

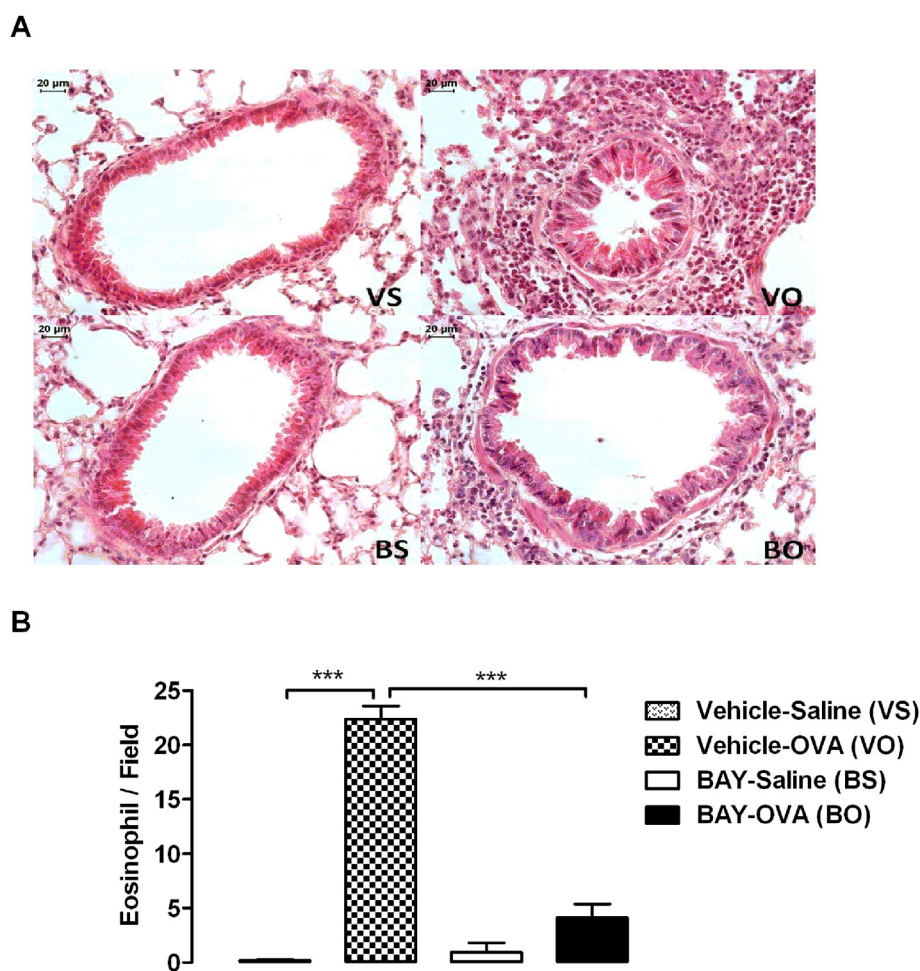


Fig. 2. Histological analysis of lung parenchyma from mice treated with BAY 60-2770. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day, 14 days) or its vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v). Histological analyses were performed at 48 h after intranasal challenge with ovalbumin (OVA) or instillation with saline. (A) Representative high-power fields of bronchiolar area, captured with optic microscope with magnification of 400 $\times$ (bar represents 20 μ m). (B) Frequency of eosinophils in peribronchiolar area (40 μ m²), expressed as number of eosinophil/field (mean of 5 randomized fields). Data represent mean $\pm$ SEM (n = 3). ****p* < 0.001.

3.3. Effect of BAY 60-2770 on IL-4, IL-5 and IgE levels

The levels of IL-4 and IL-5 were measured in BAL fluid at 48 h after OVA challenge in vehicle and BAY 60-2770 groups. In vehicle group, high levels of IL-4 were detected after OVA-challenge, as

compared with its undetectable levels in mice instilled with saline (Fig. 4A). BAY 60-2770 treatment significantly reduced the IL-4 levels in OVA-challenged mice in comparison with vehicle group (*p* < 0.001). The levels of IL-5 did not significantly differ between saline-instilled and OVA-challenged groups; however, BAY 60-2770

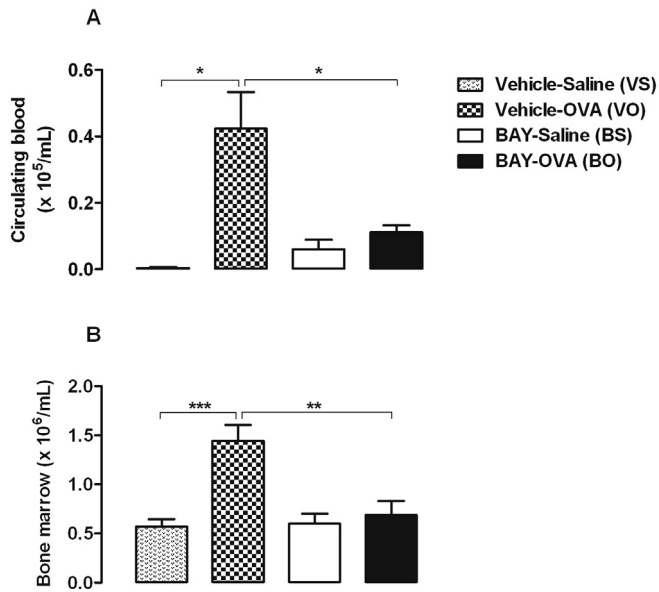


Fig. 3. Effects of treatment with BAY 60-2770 on the eosinophil number in circulating blood (A) and bone marrow (B) at 48 h after intranasal challenge with ovalbumin (OVA) or instillation with saline. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day in the drinking water for 14 days) or its correspondent vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v). Data are expressed as mean $\pm$ SEM (n = 8). * p < 0.05, ** p < 0.01, *** p < 0.001.

treatment significantly reduced (p < 0.05) the levels of this cytokine below baseline in OVA-challenged as compared with vehicle group (Fig. 4B).

Mice sensitized with OVA and treated with vehicle produced significant levels of serum OVA specific-IgE compared with naive non-sensitized mice (2227 ± 355 and 115 ± 70 pg/ml, respectively; p < 0.001). Treatment with BAY 60-2770 (1 mg/kg/day, 14 days) did not significantly alter the OVA specific-IgE levels (1826 ± 312 pg/ml) compared with its vehicle group.

3.4. Protein expressions of iNOS and α_1/β_1 subunits of sGC in the lung tissue, and cGMP levels

The expression of iNOS and of α_1 and β_1 subunits of sGC were determined in lung tissue at 48 h post-OVA challenge (or saline instillation) in mice treated or not with BAY 60-2770. In the vehicle group, OVA challenge elevated by 40% (p < 0.05) the expression of lung iNOS compared with mice instilled with saline, which was suppressed by treatment with BAY 60-2770 (Fig. 5A and B). BAY 60-2770 did not affect basal levels of iNOS in mice instilled with saline.

The levels of α_1 (Fig. 5A,C) and β_1 (Fig. 5A,D) subunits of sGC were respectively 75% and 38% lower in lung tissue of OVA-challenged mice compared with mice instilled with saline

(p < 0.05). The cGMP levels were also significantly reduced in lung tissue of OVA-challenged mice (Fig. 5E). Treatment with BAY 60-2770 fully restored to baseline the protein levels of α_1 and β_1 sGC subunits as well as the cGMP levels. In absence of OVA challenge (instillation with saline), the expressions of sGC subunits and cGMP levels did not significantly differ between groups.

3.5. BAY 60-2770 inhibits the eotaxin-induced human eosinophil migration in an ODQ-sensitive manner

Eosinophil suspensions were incubated (37 °C in 5% CO₂) for 30 min with BAY 60-2770 (1–10 μ M) or its vehicle (0.1% DMSO), after which cells were allowed to migrate in response to eotaxin (300 ng/mL) for another 60 min. A significant cell chemotaxis (p < 0.001) in response to eotaxin was observed, as compared with unstimulated eosinophils (random migration; MEM) (Fig. 6A). At concentrations up to 5 μ M, BAY 60-2770 had no significant effect on cell chemotaxis; however, at 10 μ M concentration, BAY 60-2770 produced a marked inhibition of eosinophil chemotaxis compared with vehicle groups (n = 3–5 individuals, p < 0.01; Fig. 6A). The random migration (MEM) was not affected by any experimental condition. In a separate set of experiments, eosinophil migration toward eotaxin was evaluated in cells pre-incubated for 10 min with the sGC oxidant ODQ (10 μ M) or its correspondent vehicle (0.1% DMSO), after which cells were treated to BAY 60-2770 (1–5 μ M) or further vehicle (0.1% DMSO). In the absence of ODQ, BAY 60-2770 had no effect on eosinophil chemotaxis. However, pre-incubation with ODQ led to a significant inhibition of eotaxin-induced chemotaxis by BAY 60-2770 at all concentrations (Fig. 6B).

3.6. Effect of BAY 60-2770 on cGMP levels in humans eosinophils

Isolated eosinophils were initially treated with IBMX to prevent cGMP degradation and to ensure detectable levels. Eosinophils were incubated with 10 μ M ODQ (or 0.1% DMSO) for 10 min followed by another 30-min incubation with 1–10 μ M of BAY 60-2770 (or 0.1% DMSO). Eosinophils were then stimulated with eotaxin (300 ng/ml, n = 3 to 4 individuals). The basal levels of cGMP did not differ in eosinophils stimulated or not with eotaxin, nor by the presence of DMSO (Fig. 7). Incubation of eosinophils with 10 μ M of BAY 60-2770 (but not with lower dose) significantly increased (p < 0.05) the intracellular levels of cGMP compared with vehicle (0.2% DMSO). When cells were previously incubated with ODQ, a greater increase in cGMP levels by BAY 60-2770 was observed in all concentrations assayed (Fig. 7).

3.7. BAY 60-2770 does not affect human eosinophil survival

The MTT assay showed that BAY 60-2770 (1–10 μ M) did not cause any toxic effect (not shown, n = 4 individuals). The vehicle used for BAY 60-2770 (DMSO 0.1%) also failed to affect cell viability.

Table 1

Effects of treatment with BAY 60-2770 on numbers of total leukocytes, neutrophils (NE) and mononuclear cells (MN) in circulating blood and bone marrow at 48 h after intranasal challenge with ovalbumin (OVA) or instillation with saline. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day in the drinking water for 14 days) or its corresponding vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v).

Groups	Circulating leukocytes ($\times 10^5$ /mL)			Bone marrow leukocytes ($\times 10^6$ /mL)		
	Total	NE	MN	Total	NE	Others
Vehicle-saline	13.8 $\pm$ 1.2	2.04 $\pm$ 0.3	11.0 $\pm$ 0.9	11.6 $\pm$ 0.6	4.4 $\pm$ 0.4	6.7 $\pm$ 0.4
Vehicle-OVA	13.0 $\pm$ 1.9	2.50 $\pm$ 0.5	9.8 $\pm$ 1.6	11.0 $\pm$ 0.6	4.5 $\pm$ 0.4	5.5 $\pm$ 0.3
BAY-saline	12.0 $\pm$ 0.8	2.30 $\pm$ 0.3	9.4 $\pm$ 0.9	10.8 $\pm$ 0.6	4.5 $\pm$ 0.2	5.7 $\pm$ 0.4
BAY-OVA	12.1 $\pm$ 0.6	2.40 $\pm$ 0.4	9.2 $\pm$ 0.5	9.7 $\pm$ 1.6	4.0 $\pm$ 0.4	5.1 $\pm$ 0.7

Data are shown as mean $\pm$ SEM for n = 7–8.

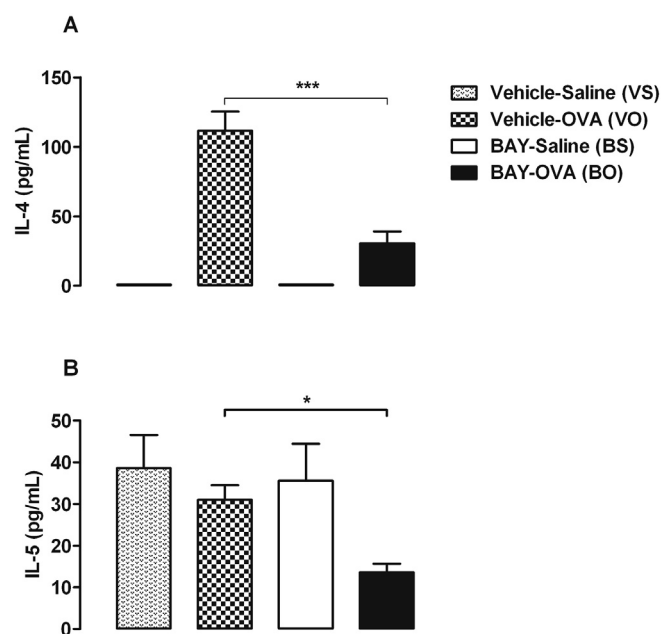


Fig. 4. Effects of BAY 60-2770 on the levels of IL-4 (A) and IL-5 (B) in bronchoalveolar lavage (BAL) fluid from mice challenged with ovalbumin (OVA) or instilled with saline. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day in the drinking water for 14 days) or its correspondent vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v). Data are shown as mean $\pm$ SEM ($n = 6$). * $p < 0.05$, *** $p < 0.001$.

3.8. Tail-cuff pressure

The tail-cuff pressure was evaluated at 16 h before the first OVA-challenge. Treatment with BAY 60-2770 did not significantly affect tail-cuff pressure compared with OVA-challenged mice receiving vehicle (83.1 ± 2.2 and 82.1 ± 3.7 mmHg, respectively; $n = 11$ – 12).

4. Discussion

This study shows that treatment with the NO- and heme-independent sGC activator BAY 60-2770 significantly reduces OVA-induced murine eosinophil trafficking to the lung tissue and that this is accompanied by decreased levels of the IL-4 and IL-5 in BAL fluid. BAY 60-2770 also normalizes the increased iNOS expression, as well as the reduced α_1 and β_1 sGC subunit protein expressions and cGMP levels in lung tissue of OVA-challenged mice. Moreover, BAY 60-2770 inhibited eotaxin-induced human eosinophil migration in a cGMP-dependent manner, an effect potentiated by the sGC oxidant ODQ.

A fundamental feature of asthma associated with allergic sensitization is the ability of the airways to recognize allergens and to generate the so-called Th2 cytokines, a process related to IgE production, eosinopoiesis, and bronchial eosinophilic inflammation [32]. In our study, the leukocyte profile of BAL fluid and lung tissue from OVA-challenged mice showed a marked presence of eosinophils, which was greatly reduced by 14-day therapy with BAY 60-2770, suggesting that sGC activation results in a deficient eosinophil transmigration from vascular bed through endothelium to bronchial mucosa. IL-4 is classically known to mediate the isotype B cell production of IgE and eosinophil recruitment, and is produced by activated CD4⁺ T cells, mast cells and eosinophils, leading to differentiation and expansion of Th2 cells [32]. We found that the serum levels of OVA-specific IgE in previously sensitized mice remained significantly elevated in BAY 60-2770-treated animals, which is suggestive that the inhibitory effects of this sGC activator

on the allergic eosinophilic inflammation do not rely on alterations or suppression of circulating Th2 lymphocytes. On the other hand, the increased levels of IL-4 levels in BAL fluid of OVA-challenged mice were markedly attenuated by BAY 60-2770 treatment. BAL fluid comprises the pulmonary microenvironment surrounded by recruited and resident cells; therefore, resident mature mast cells and migrated cells like eosinophils and basophils might effectively have contributed to IL-4 release upon OVA-challenge. Previous studies showed that IL-4 and IL-5 induce differentiation of Th2 cytokine-producing eosinophils, where IL-5 (also reduced in BAY 60-2770-treated mice) can drive bone marrow progenitor cells into IL-4-producing eosinophils [33,34]. Furthermore, IL-5 acts synergistically with eotaxin, promoting selective recruitment of eosinophils to allergen-challenged lungs [35]. IL-5 is considered to be specific to eosinophilic lineage and for priming eosinophils, inducing their growth, differentiation, activation and survival [36]. Under allergen challenge, the bronchial mucosa communicates with bone marrow leading to an increased pool of mature eosinophils that became available to be mobilized and recruited [1,36]. In accordance, a marked bone marrow and blood eosinophilia were observed in OVA-challenged mice, both of which were normalized by BAY 60-2770. Although OVA-challenge itself did not increase the levels of IL-5 in BAL fluid, BAY 60-2770 treatment significantly reduced its levels when compared with vehicle group. Thus, the inflammatory communications between bronchial mucosa, eosinophils and bone marrow may become disconnected via IL-4 down-regulation and deficient IL-5 signaling.

The increased oxidative stress in the lung has been shown to play an important role in the mouse allergic airway disease [37]. Evidence shows that sGC downregulation in inflamed lungs may be secondary to high formation of reactive-oxygen species (ROS) [38]. Therefore, excess NO production, as a consequence of iNOS upregulation within the oxidative pulmonary environment is associated with the high local levels of ROS that could shift the redox state of sGC from a reduced (Fe^{2+})-NO-sensitive to an oxidized (Fe^{3+})-NO-insensitive state. The initial interaction of NO with sGC causes enzyme desensitization by haem-assisted S-nitrosation, also suggesting that high NO production itself may downregulate the sGC-cGMP signaling pathway [39]. In the present study, the protein expressions of α_1 and β_1 subunits of sGC along with the cGMP levels in the lung tissue were significantly reduced after OVA challenge. A previous study in BALB/c mice also reported a diminished expression of sGC subunits after OVA challenge [14]. In addition, similar reductions of sGC subunit expressions and/or activity were reported in non-allergic pulmonary inflammatory conditions, such as lipopolysaccharide-induced lung injury and chronic obstructive pulmonary disease (COPD) and newborn lung injury models similar reductions of sGC subunit expressions were reported [17,18,40]. We found that BAY 60-2770 treatment fully restored both the protein expressions of α_1 and β_1 subunits of sGC and cGMP levels in OVA-challenged mice to the levels of saline group, possibly by preventing sGC degradation. BAY 60-2770 has been shown to displace the haem, acting as a haem mimetic via carboxylate-mediated interactions with the conserved YxSxR motif, and via hydrophobic interactions in sGC [41]. Therefore, the protection given by BAY 60-2770 on sGC degradation could be of potential interest in a microenvironment with ongoing oxidative and nitrosative stress, such as that observed in allergic asthma. Recently, the mouse airway hyperresponsiveness (AHR) toward the bronchoconstrictor methacholine was markedly reduced by BAY 60-2770 given intratracheally 30 min before OVA or house dust mite extract (HDME) challenges, which was accompanied by an increased catalytic (cGMP production) response toward this sGC activator [31].

The sGC expression in human eosinophils has never been

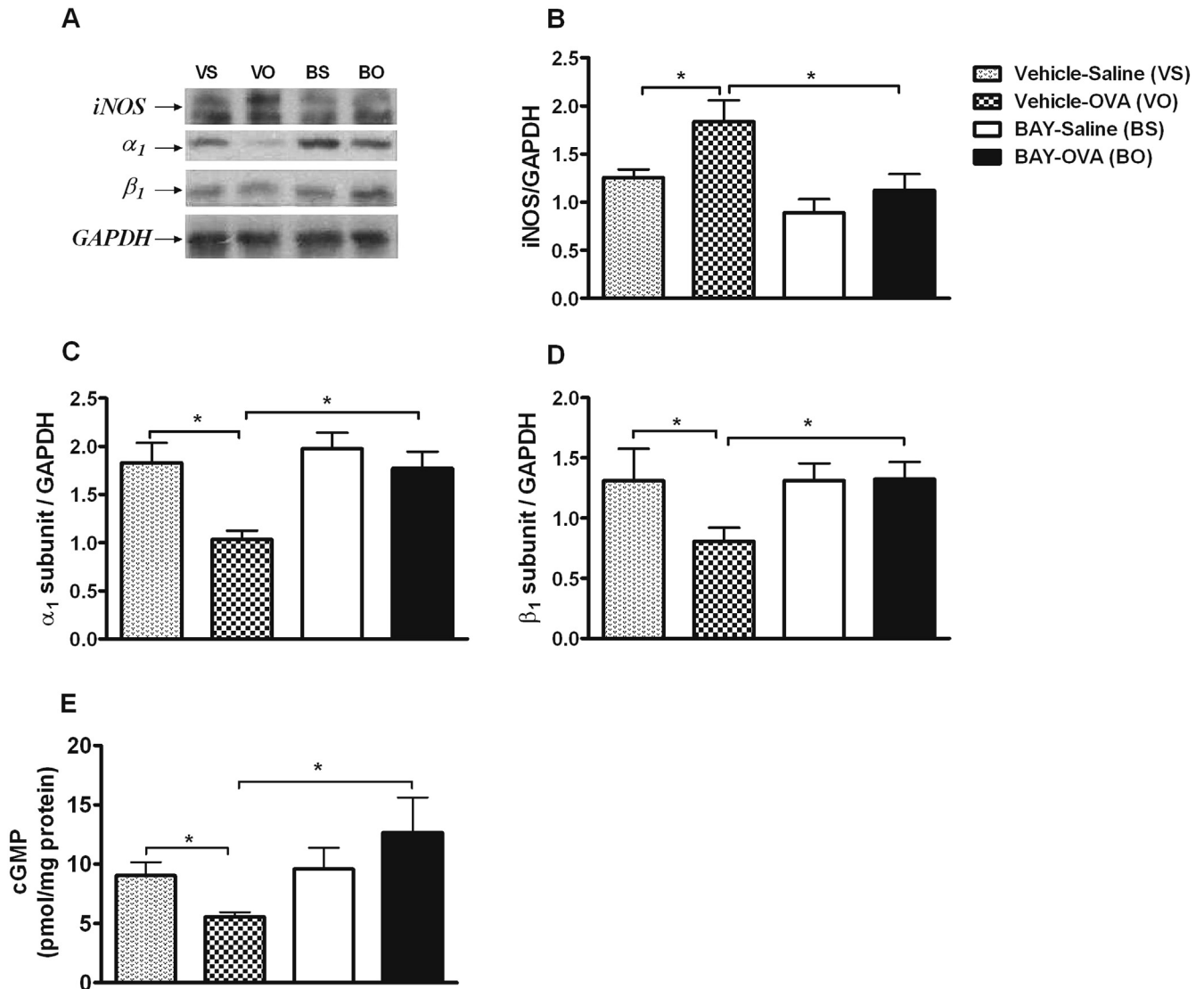


Fig. 5. Effects of BAY 60-2770 in the protein expressions of inducible nitric oxide synthase (iNOS; A,B) and of α_1 and β_1 subunits of soluble guanylyl cyclase (sGC; A,C,D), and cGMP levels (E) in lung tissue from mice challenged with ovalbumin (OVA) or instilled with saline. Mice were treated with BAY 60-2770 (BAY; 1 mg/kg/day in the drinking water for 14 days) or its correspondent vehicle (150 μ l of Transcutol/Cremophor/water; 1/2/7 v/v/v). The protein expression of iNOS (A, B), and α_1 (A, C) and β_1 (A, D) subunits of sGC were quantified in lung homogenates by Western blotting ($n = 6-9$). Protein levels expression were quantified by densitometric analysis and normalized for constitutive GAPDH. The cGMP levels were determined by EIA kits ($n = 3-4$), and expressed as pmol mg protein⁻¹. Data are shown as mean $\pm$ SEM * $p < 0.05$.

demonstrated, and that the function of this enzyme remains uncertain. The NO-cGMP pathway is functionally present in human eosinophils, where intracellular elevations of cGMP levels act to inhibit human activated eosinophil chemotaxis and adhesion [21,22]. Interestingly, human primed eosinophils show elevated ROS production [42,43]. BAY 60-2770 significantly inhibited the eotaxin-induced human eosinophil migration concomitantly with elevation of intracellular levels of cGMP. Furthermore, prior incubation of eosinophils with ODQ markedly enhanced BAY 60-2770-induced cGMP production. Accordingly, a greater inhibition of eotaxin-induced eosinophil migration compared with BAY 60-2770 alone was seen with the lower concentrations of BAY 60-2770 (1, 3 and 5 μ M) in the presence of ODQ.

The oral dose of 1 mg/kg/day of BAY 60-2770 for 14 days was used based on our previous experience [29]. In female spontaneous hypertensive rats (SHR), the dose of 1 mg/kg of BAY 60-2770 resulted in a 10% reduction of arterial pressure, which was restored after 9 h thereafter [44]. In our study, prolonged BAY 60-2770

treatment did not affect arterial blood pressure in awake conditions, but further studies would be required to evaluate the regional blood flow in the lungs in control and treated mice. In isolated human eosinophils, it is noteworthy that cGMP elevations and inhibition of eotaxin-induced eosinophil chemotaxis were achieved at micromolar ranges of BAY 60-2770 (even when eosinophils were pre-incubated with ODQ), which are unlikely to be achieved at a steady-state plasma concentration. Therefore, strategies of delivering BAY 60-2770 by inhalation into the airways to obtain effective concentrations locally may be of interest.

5. Conclusions

This study shows that two-week therapy with the sGC activator BAY 60-2770 promotes an anti-inflammatory effect on OVA-challenged mice through inhibition of eosinophil recruitment to airways, accompanied by reduced levels of IL-4 and IL-5 in BAL fluid and restoration of pulmonary levels of iNOS and sGC. The

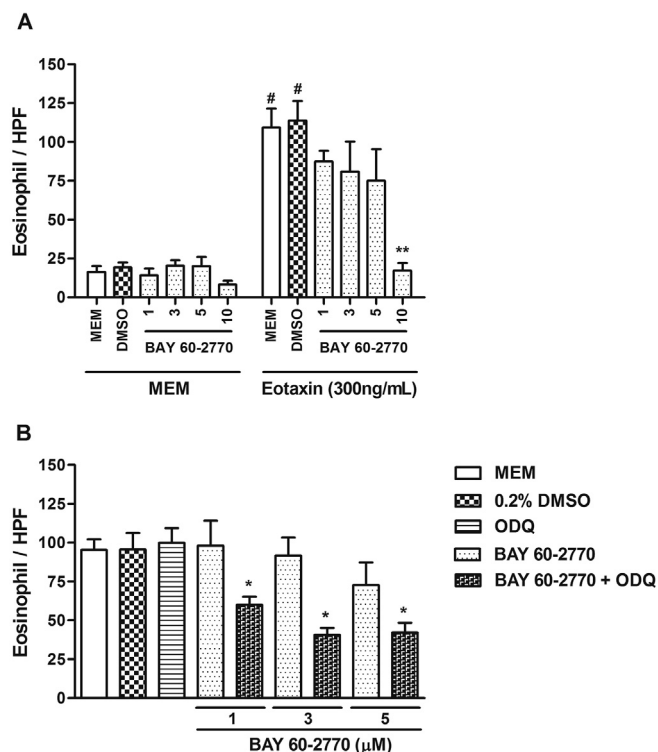


Fig. 6. Effects of BAY 60-2770 on eotaxin-induced human eosinophil chemotaxis. (A) Eosinophil suspension (4×10^6 cells/ml) was exposed for 30 min with BAY 60-2770 (1, 3, 5 and 10 μ M) and/or vehicle (0.1% DMSO), after which cells were left to migrate toward eotaxin (300 ng/ml, 1 h) or left unstimulated (MEM; minimum essential media). (B) Eosinophils (4×10^6 /ml) were pre-incubated with ODQ (10 μ M; 10 min) and exposed to BAY 60-2770 (1, 3 and 5 μ M; 30 min), after which cells were allowed to migrate toward eotaxin (300 ng/ml, 1 h). The eosinophil chemotaxis is expressed as the mean number of migrated cells per high power field (HPF). Data are shown as the mean $\pm$ SEM ($n = 3-5$ individuals, each assay done in triplicate). Panel A: [#] $p < 0.001$ compared with MEM in non-stimulated eosinophils; ^{**} $p < 0.01$ compared with respective vehicle (0.1% DMSO). Panel B: ^{*} $p < 0.05$ compared with BAY 60-2770 in absence of ODQ.

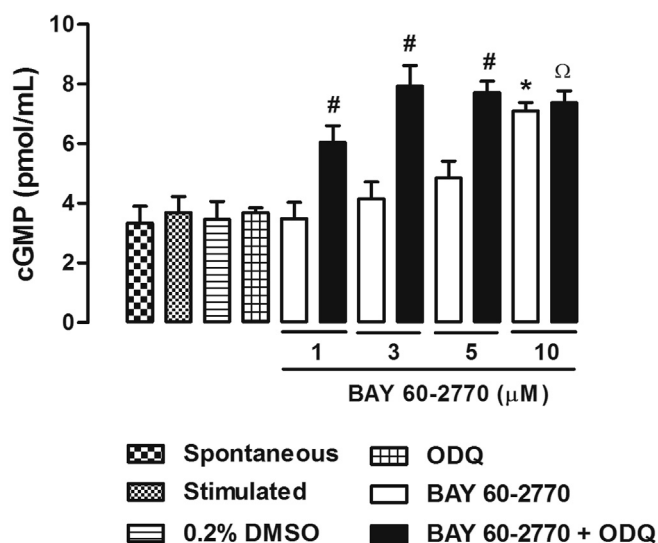


Fig. 7. Effects of BAY 60-2770 on cyclic GMP levels in human eosinophils treated with BAY 60-2770 in absence and presence of ODQ. Eosinophils (1×10^6 cells/ml) were treated with ODQ (10 μ M, 10 min), and then treated with BAY 60-2770 (1, 3, 5 and 10 μ M). Thereafter, eosinophils suspensions were stimulated with eotaxin (300 ng/ml, 1 h) or left unstimulated (minimum essential media; MEM). Data are shown as the mean $\pm$ SEM ($n = 3-4$ individuals, each assay done in triplicate). ^{*} $p < 0.05$ compared with vehicle (0.2% DMSO). [#] $p < 0.05$ compared with respective BAY 60-2770 concentrations in the absence of ODQ. ^Ω $p < 0.05$ compared with ODQ alone.

chemotaxis of human isolated eosinophils towards eotaxin was also inhibited by BAY 60-2770 in a cGMP dependent manner, an effect potentiated by the previous sGC oxidation with ODQ. Together, our findings show an anti-inflammatory effect of BAY 60-2770 and reveal the sGC as a potential and novel therapeutic target in allergic asthma.

Conflict of interests

We all authors have declared no conflict of interests.

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