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Inhibitory effects on human eosinophil chemotaxis in vitro by BAY 41-2272, an activator of nitric oxide-independent site of soluble guanylate cyclase

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Abstract

This study was designed to investigate the effects of the 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272) on formyl-methionyl-leucyl-phenylalanine (fMLP; 10^{-7} M)-induced human eosinophil chemotaxis, cyclic guanosine-3′,5′-monophosphate (cGMP) and cyclic adenosine-3′,5′-monophosphate (cAMP) levels. Human eosinophils were pretreated or not with 3-isobutyl-l-methyl-xanthine (IBMX; 500 μM), and then exposed to BAY 41-2272 (0.1–10.0 μM) for either short (10 min) or prolonged (90 min) time periods. Exposition of eosinophils with BAY 41-2272 for either 10 min or 90 min markedly inhibited the eosinophil chemotaxis, independently of IBMX pretreatment. Inhibition of fMLP-induced eosinophil chemotaxis by BAY 41-2272 (in absence of prior treatment with IBMX) was about of the same irrespective if cells were exposed for 10 min or 90 min with this compound. In IBMX-pretreated eosinophils, the inhibition of fMLP-induced chemotaxis by BAY 41-2272 in the 10-min exposure protocols was even higher in comparison with the 90-min protocols. Incubation of IBMX-treated eosinophils for 90 min with BAY 41-2272 resulted in 2.0–2.5 times higher levels of cGMP and cAMP compared with the 10-min protocols. The BAY 41-2272-induced cGMP increases were abolished by pre-incubation of eosinophils with the soluble guanylate cyclase inhibitor 1*H*-[1,2,4]-oxidiazolo[4,3-*a*] quinoxalin-1-one (ODQ). No eosinophil toxicity was observed in any experimental condition, according to 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Our findings show that inhibitory effects of fMLP-induced human eosinophil chemotaxis by BAY 41-2272 at short-term or prolonged exposition time are accompanied by significant elevations of cGMP and cAMP, but we could not detect a clear correlation between chemotaxis inhibition and elevation of cyclic nucleotide levels.

Keywords: BAY 41-2272; cAMP levels; cGMP levels; Chemotaxis; Cytotoxicity; Eosinophil

1. Introduction

The soluble guanylate cyclase (sGC) acts as the principal intracellular receptor for nitric oxide (NO) and facilitates the formation of the second messenger cyclic guanosine-3′,5′-monophosphate (cGMP), which in turn governs many aspects of cellular function via interaction with specific kinases, ion channels and phosphodiesterases (PDE) [1,2]. This signal transduction pathway underlies the majority of physiological actions attributed to NO and is important in the regulation of the cardiovascular, gastrointestinal, respiratory, and nervous and immune systems [3,4]. Moreover, NO/cGMP is important in the regulation of leukocyte rolling, adhesion, and extravasation [5,6].

The compound 3-(5′-hydroxymethyl-2-furyl)-1-benzyl-indazole (YC-1) was described as a non-NO-based sGC activator able to relax vascular smooth muscle in vitro, to reduce systemic blood pressure in vivo and to inhibit platelet aggregation in vitro [7,8]. More recently, a more

Abbreviations: BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; cAMP, cyclic adenosine-3′,5′-monophosphate; cGMP, cyclic guanosine-3′,5′-monophosphate; fMLP, formyl-methionyl-leucyl-phenylalanine; IBMX, 3-isobutyl-l-methyl-xanthine; MEM, minimum essential medium Eagle; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; NO, nitric oxide; ODQ, 1*H*-[1,2,4]-oxidiazolo[4,3-*a*] quinoxalin-1-one; PDE, phosphodiesterase; sGC, soluble guanylate cyclase; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; YC-1, 3-(5′-hydroxymethyl-2-furyl)-1-benzyl-indazole

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potent member of the non-NO-based sGC activator family has been reported, namely 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272) [9–13]. These studies suggested the existence of a new NO-independent regulatory site on sGC in the cysteine 238 and cysteine 243 regions of the α1-subunit of the enzyme that modulates the catalytic rate and the responsiveness towards the haem ligand [9,10]. BAY 41-2272 is a potent vasodilator in vitro of rabbit aortic rings [10,11,14], rabbit saphenous artery rings [11,12], human and rabbit corpus cavernosum [15–17], rabbit vaginal wall and clitoral smooth muscle [18]. Furthermore, this compound reduces the mean blood pressure in both normotensive and hypertensive rats, possesses antiplatelet activity in vitro and prolongs the tail-bleeding time in rats in vivo [10,13,14,19]. In a congestive heart failure model in dogs, BAY 41-2272 increases the cardiac output and preserves glomerular filtration rate without activating the renin–angiotensin–aldosterone system [20]. All of these effects is believed to be mediated by both NO-independent stimulation of the enzyme and sensitization of sGC towards endogenous NO. A recent study has demonstrated that the original compound YC-1 inhibits human neutrophil functions through a cyclic adenosine-3′,5′-monophosphate (cAMP)/protein kinase A-dependent pathway [21]. BAY 41-2272 represents therefore a useful pharmacological tool, to differentiate between cGMP-dependent and -independent actions of NO, and that may have significant therapeutic potential.

Eosinophils play an important role in host defense mechanisms in parasitic infestation and pathogenesis of allergic, immunological, and malignant disorders [22]. A functional NO–cGMP pathway in eosinophils that modulates the locomotion of this cell type has been demonstrated in isolated cells from rats and human [23,24]. Recently, the NO donors sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) have been shown to markedly inhibit the human eosinophil chemotaxis in vitro that is accompanied by intracellular elevation of cGMP levels [25], but the relationship between cGMP levels and inhibition of leukocyte locomotion is not fully understood [26–29]. Taking into consideration that BAY 41-2272 directly stimulates sGC and increases cGMP levels by NO-independent mechanisms, this study was carried out to investigate the effects of BAY 41-2272 in the human eosinophil locomotion in vitro and production of both cGMP and cAMP levels.

2. Materials and methods

2.1. Materials

VarioMACS system and microbeads were purchased from Miltenyi Biotec Inc. (Auburn, CA, USA). BAY 41-2272 was provided by Pharma Research Center, Bayer AG (Wuppertal, Germany). DMSO, fMLP, IBMX, MEM, MTT, ODQ, Percoll, and SNAP were purchased from Sigma (St. Louis, MO, USA). Polycarbonate filter (5 μm) was obtained from Nuclepore (Pleasanton, CA, USA). Diff-Quik was obtained from Baxter Healthcare Corp. (DE, USA). Kits for measurement of cGMP and cAMP were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Iloprost was kindly supplied by Schering (Germany).

2.2. Eosinophil isolation

Blood was collected from healthy volunteers (male and female volunteers, aged 18–50 years) who were not under medication. Informed consent and approval from the local ethical committee were obtained before the study.

Human eosinophils were isolated from peripheral blood using a method adapted from that of Hansel et al. [30]. Briefly, 120 mL blood collected in 3.13% (w/v) sodium citrate from a healthy subject was diluted 1:1 with PBS, and 35 mL diluted blood overlaid onto a 15 mL percoll gradient (1.130 ± 0.005 g/mL, pH 8.9 ± 0.3 at 20 °C, <20 mOsM/kg H_2O). Gradients were centrifuged at $1000 \times g$ for 20 min at 4 °C (Hermle model Z360k centrifuge, Germany) and the cell pellet was collected. Red cells contained in the granulocyte pellet were lysed with lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Washed granulocytes were incubated with anti-CD16 immunomagnetic microbeads before passing on a steel-matrix column in a magnetic field, and the CD16-negative eosinophils were collected. Eosinophils (92–99% purity) were then resuspended in minimum essential medium Eagle (MEM; pH 7.2). Contaminating cells were mononuclear cells.

2.3. Experimental designs for the chemotaxis assays

Eosinophils were initially incubated in absence or presence of the PDE inhibitor 3-isobutyl-l-methyl-xanthine (IBMX; 500 μM) for 30 min. Thereafter, in order to explore the effects of BAY 41-2272 on formyl-methionyl-leucyl-phenylalanine (fMLP)-induced eosinophil chemotaxis, two experimental protocols varying the exposure time of BAY 41-2272 with eosinophils were carried out. Basically, eosinophil suspensions were exposed to either 10 min or 90 min with BAY 41-2272 (0.1–10.0 μM) or the vehicle DMSO (0.007–0.7%) at 37 °C in 5% CO_2. For the 10-min exposure protocols, cells were previously maintained at 37 °C in 5% CO_2 for 20 min. BAY 41-2272 was then added to eosinophil suspension for 10 min, after which cells were washed and placed in the microchemotaxis chamber to carry out the chemotaxis assays for 60 min using fMLP (10^{-7} M) as a chemoattractant agent. For the 90-min exposure protocols, eosinophils were incubated for 30 min with BAY 41-2272, and maintained in

contact with drugs for a whole time period of 90 min, including the 60-min chemotaxis with fMLP (10^{-7} M).

2.4. Chemotaxis assay

Eosinophils were resuspended at a concentration of 5×10^6 cells/mL in MEM/ovalbumin, and migration assays were performed using a 48-well microchemotaxis chamber [31]. The bottom wells of the chamber were filled with the chemoattractant agent fMLP (10^{-7} M) in 28 μL MEM whereas the upper wells were filled with eosinophils (50 μL) that had been treated or not with BAY 41-2272. The bottom and upper cells were separated with a polycarbonate filter of 5 μm. The chamber was then incubated for 60 min at 37 °C with 5% CO_2 atmosphere. At the end of the incubation period, the filter was removed, washed, fixed in methanol for 2 min, 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×) per well.

2.5. Extraction and measurement of cGMP and cAMP from eosinophils

Eosinophils were isolated and resuspended to a concentration of 1×10^7 cells/mL in PBS. To achieve this number of eosinophils, a pool of three volunteers was used for each assay. Cells were incubated with IBMX (2 mM) for 30 min at room temperature before adding BAY 41-2272. In order to mimick the conditions employed in the chemotaxis assays (10- and 90-min protocols), eosinophils were incubated (37 °C, humidified atmosphere) with BAY 41-2272 (0.1–10.0 μM) for 10 min or 90 min, after which the reaction was 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 $4000 \times g$ 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 $14{,}000 \times g$ for 5 min at room temperature. The supernatants from these washed samples were collected and added to 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 or cAMP. In separate protocols, eosinophils were pre-incubated for 10 min with the sGC inhibitor 1*H*-[1,2,4]-oxidiazolo[4,3-*a*] quinoxalin-1-one (ODQ; 0.2 mM) before addition of BAY 41-2272. The NO donor SNAP (0.1 mM) and adenylate cyclase activator iloprost (3.0 μM) were used as positive control in the cGMP and cAMP assays, respectively. Cyclic GMP and cAMP in 1.5×10^6 cells/well were measured using Cayman kit, respectively [32,33]. The samples for measurement of cGMP (but not of cAMP) were previously acetylated, following the manufacturer recommendations.

2.6. MTT assay

Cell toxicity was estimated using the tetrazolium salt reduction test (MTT assay) by eosinophils after exposure to BAY 41-2272 [34,35]. Eosinophils were isolated and resuspended to a concentration of 2×10^6 cells/mL in MEM. Cells were exposed to BAY 41-2272 (0.1–10.0 μM) for either 10 min or 90 min at 37 °C in a humidified atmosphere. Eosinophils (100 μL/well), treated or not, and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; 10 μL/well; 5 mg/mL in PBS) were added in triplicate to a 96-well plate. Cells were allowed to incubate for 3 h at 37 °C, 5% CO_2. After incubation, 100 μL of 10% SDS in 0.01 M HCl were added to each well. Cell samples were then incubated for 18 h at 37 °C and 5% CO_2 and absorbance measured at 540 nm in a microplate reader (Multiscan MS, Labsystems, USA).

2.7. Statistical analysis

Data are expressed as the mean ± S.E.M. of at least three separate experiments carried out in triplicate. Data were analysed by analysis of variance (ANOVA) for multiple comparisons followed by Tukey's test, or unpaired Student's *t*-test when appropriate. A value of $P < 0.05$ was taken as significant.

3. Results

3.1. Effect of BAY 41-2272 on the fMLP-stimulated eosinophil chemotaxis

The eosinophil suspensions were exposed (37 °C in 5% CO_2) to either 10 min or 90 min with BAY 41-2272 (0.1–10.0 μM) and cells were allowed to migrate in response to the chemotactic agent fMLP (1×10^{-7} M). A significant cell chemotaxis ($P < 0.001$) in response to fMLP in both of these experimental conditions was observed, as compared with the random migration (MEM; Fig. 1A). However, the fMLP-induced response in the 90-min exposition protocols resulted in significantly higher cell chemotaxis compared with the 10-min exposition protocols ($P < 0.001$).

Exposition of eosinophils to BAY 41-2272 (0.1–10.0 μM; $N = 3$–5) for 10 min produced a significant inhibition of fMLP-induced chemotaxis (Fig. 1A). Similarly, exposition of eosinophils to BAY 41-2272 (0.1–10.0 μM; $N = 3$) for 90 min caused a concentration-dependent reduction of fMLP-induced chemotaxis ($P < 0.001$) in approximately the same extension as that seen in the short-exposure experiments (Fig. 1A).

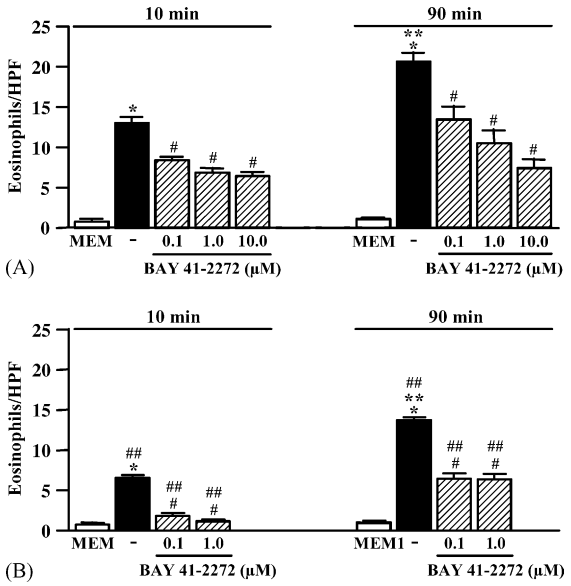

Fig. 1. Inhibitory effects of BAY 41-2272 on fMLP (1×10^{-7} M)-stimulated eosinophil chemotaxis. The human eosinophil suspension (5×10^6 cells/mL) was pre-incubated in absence (panel A) or presence of IBMX (500 μM; panel B), after which cells were exposed to BAY 41-2272 (0.1–10.0 μM) for either 10 min or 90 min. Control migration (fMLP) is represented by the solid column, whereas random chemotaxis (MEM, in absence of fMLP) is represented by the open column in both conditions. Each experiment was carried out in triplicate ($N = 3$–5 individuals). Eosinophil migration is expressed as the mean number of migrated cells per high power field (HPF). The results are shown as the mean ± S.E.M. $^{*}P < 0.001$ compared to respective MEM; $^{**}P < 0.001$ compared to fMLP alone at 10 min; $^{\#}P < 0.01$ compared to respective fMLP; and $^{\#\#}P < 0.05$ compared to respective groups in absence of IBMX (panel A).

In a separate set of experiments, eosinophils were pre-treated with the PDE inhibitor IBMX (500 μM), after which cells were exposed to BAY 41-2272 using the same experimental protocols above-mentioned. Fig. 1B shows that eosinophils treated with IBMX showed a significant inhibition of fMLP-induced chemotaxis in both 10-min and 90-min exposure protocols ($P < 0.001$), as compared with chemotaxis in absence of IBMX pretreatment (Fig. 1A). However, in these IBMX-treated eosinophils, the fMLP-induced response in the 90-min exposition protocols resulted in significantly higher cell chemotaxis compared with the 10-min exposition protocols ($P < 0.001$; Fig. 1B).

Exposition of IBMX-treated eosinophils to BAY 41-2272 (0.1 μM and 1.0 μM; $N = 3$) for either 10 min or 90 min produced a significant inhibition of fMLP-induced chemotaxis ($P < 0.001$; Fig. 1B). In terms of percentage of alterations, the inhibition of chemotaxis achieved with BAY 41-2272 in the 10-min exposure was higher (72% and 82% inhibition, for at 0.1 μM and 1.0 μM, respectively) than the same concentrations of BAY 41-2272 in the 90-min exposure protocols (52% and 53% inhibition, at 0.1 μM and 1.0 μM, respectively; $P < 0.05$).

In eosinophils treated with DMSO (vehicle used for BAY 41-2272 and IBMX), at concentrations up to 0.7%, no significant alterations in fMLP-induced responses were observed (not shown). Experimental protocols where BAY 41-2272 and IBMX (alone or combined) exceed 0.7% DMSO were not carried out to avoid interference of this vehicle in fMLP-induced chemotaxis.

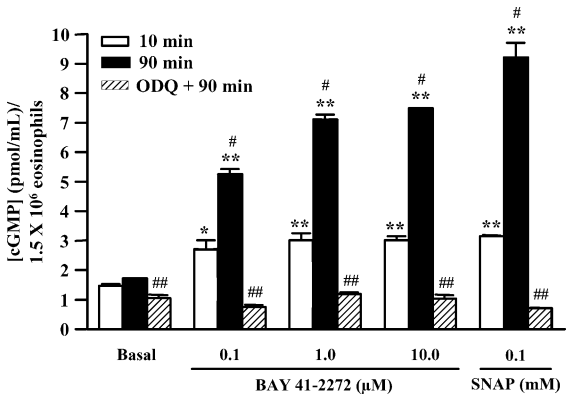

Fig. 2. Effect of BAY 41-2272 on intracellular levels of cGMP in human eosinophils. The human eosinophil suspension (1.5×10^6 cells/well) was incubated (37 °C) with BAY 41-2272 (0.1–10.0 μM) or SNAP (0.1 mM) for 10 min (open column) or 90 min (solid column). The effect of ODQ (0.2 mM) in the cGMP levels of eosinophils exposed to 90 min with BAY 41-2272 or SNAP is showed by the hatched columns. Each experiment was carried out in triplicate ($N = 3$ individuals). The levels of cGMP are expressed in pmol/mL in 1.5×10^6 eosinophils. The results are shown as the mean ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to respective basal; $^{\#}P < 0.001$ compared with respective 10-min protocols; and $^{\#\#}P < 0.001$ compared with respective 90-min protocols.

3.2. Effect of BAY 41-2272 on intracellular levels of cGMP in eosinophils

Isolated eosinophils were initially treated with IBMX to get cGMP levels detectable (see Section 2.5), and then were exposed to BAY 41-2272 (0.1–10.0 μM; $N = 3$) for either 10 min or 90 min. Fig. 2 shows that BAY 41-2272 markedly increased the cGMP levels compared with vehicle-treated cells (Fig. 2). However, the 90-min protocol resulted in significantly larger cGMP levels compared with the 10-min protocols (94%, 136%, and 148% higher for 0.1 μM, 1.0 μM, and 10.0 μM, respectively). The vehicle DMSO (0.7%) alone had no significant effect upon intracellular level of cGMP in both of the protocols used compared with MEM (not shown). The NO donor SNAP (0.1 mM), used as a positive control, was able to markedly increase the cGMP levels in both of the protocols used, but the amounts achieved with 90-min exposition was 192% larger ($P < 0.001$) than the 10-min protocols (Fig. 2). The increases in cGMP levels observed after treatment of eosinophils with BAY 41-2272 or SNAP (90-min protocols) were prevented by pre-incubating (10 min) cells with the sGC inhibitor ODQ (0.2 mM; Fig. 2).

3.3. Effect of BAY 41-2272 on intracellular levels of cAMP in eosinophils

Isolated eosinophils were initially treated with IBMX, and then were exposed to BAY 41-2272 (0.1–10.0 μM;

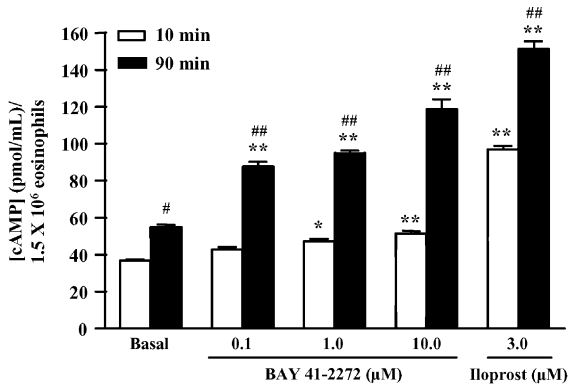

Fig. 3. Effect of BAY 41-2272 on intracellular levels of cAMP in eosinophils. The human eosinophil suspension (1.5×10^6 cells/well) was incubated (37 °C) for 10 min (open column) or 90 min (solid column) with BAY 41-2272 in concentrations of 0.1–10.0 μM or with iloprost (3.0 μM). Each experiment was carried out in triplicate ($N = 3$). The levels of cAMP are expressed in pmol/mL in 1.5×10^6 eosinophils. The results are shown as the mean ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to respective basal; and $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ compared with respective 10-min protocols.

$N = 3$) for either 10 min or 90 min. Fig. 3 shows that exposition of eosinophils with BAY 41-2272 (0.1–10.0 μM; $N = 3$) for either 10 min or 90 min significantly increased the cAMP levels compared with vehicle-treated cells (Fig. 3). However, the latter protocol resulted in significantly larger cAMP levels compared with 10-min protocols (105%, 100%, and 131% higher for 0.1 μM, 1.0 μM, and 10.0 μM, respectively). The basal production of cAMP in the 90-min exposition protocols was significantly higher ($P < 0.01$) compared with the 10-min protocols (Fig. 3). The adenylate cyclase activator iloprost (3.0 μM), used as a positive control, was able to markedly increase the cAMP levels in both of the protocols used, but the amounts achieved with 90-min exposition was 56% larger ($P < 0.001$) than the 10-min protocols (Fig. 3).

3.4. Effect of BAY 41-2272 on the eosinophil toxicity

The MTT reduction assay showed that neither the short (10 min) nor the prolonged (90 min) exposure-time of BAY 41-2272 (0.1–10.0 μM; $N = 3$) to human eosinophils caused any toxic effect (Table 1). The vehicle used for BAY 41-2272 (0.7% DMSO) also failed to affect cell viability.

Table 1
Effect of BAY 41-2272 upon the eosinophils by MTT assay

Treatment	MTT reduction (% control)	
	10 min	90 min
0.1 μM BAY 41-2272	98.27 ± 1.73	101.34 ± 4.70
1.0 μM BAY 41-2272	98.84 ± 1.16	95.30 ± 1.34
10.0 μM BAY 41-2272	105.20 ± 1.16	104.03 ± 0.67
0.7% DMSO	104.05 ± 3.47	106.04 ± 2.01

The human eosinophil suspension (2×10^6 cells/mL) was incubated (37 °C) for 10 min or 90 min with the BAY 41-2272 in concentrations of 0.1–10.0 μM. Each experiment was carried out in triplicate ($N = 3$). The result is expressed in MTT reduction (% control). The results are shown as the mean ± S.E.M.

4. Discussion

We demonstrated in this study that BAY 41-2272 exhibits a marked inhibitory and non-cytotoxic effect on fMLP-induced human eosinophil chemotaxis that is accompanied by significant elevations in both cGMP and cAMP levels. The role of the NO–cGMP pathway in modulating cell locomotion in vivo and in vitro has been shown to be very complex, and opposite effects have been described depending on the experimental models and animal species employed. In vitro studies have shown that treatment of human monocytes [26], polymorphonuclear cells [27,29] or eosinophils [25] with pure NO or NO-donor compounds such as GEA 3162, GEA 5024, SNP, SIN-1, and SNAP inhibits the fMLP-induced cell chemotaxis and markedly increase the cellular cGMP production, but is still unclear whether both of these phenomena (cGMP production and cell chemotaxis inhibition) are closely linked to each other. In human eosinophils, inhibition of fMLP-induced chemotaxis by SNP, SIN-1, and SNAP seems not to correlate with the elevated levels of this nucleotide since a similar pattern of cell inhibition is observed in conditions of low and high intracellular levels of cGMP [25].

We have now used BAY 41-2272 to further explore the relationship between cellular cGMP production and cell chemotaxis inhibition. In contrast to the classical NO donors, BAY 41-2272 is reported to generate significant amounts of cGMP by stimulating the sGC via NO-independent mechanisms [9–13]. Besides, a recent study has reported that physiological effects of BAY 41-2272 in human platelets are due to synergism of sensitization of NO-sensitive sGC and inhibition of PDE-5 [36].

In our study, the eosinophils (pretreated or not with IBMX) were exposed to BAY 41-2272 for short (10 min) or prolonged (90 min) time-periods, after which nucleotide production and functional assays (cell chemotaxis) were evaluated. Both of these protocols resulted in significant elevations of cGMP above basal levels and inhibition of cell chemotaxis. However, the prolonged incubation resulted in much higher increase in cGMP levels, but no additional inhibition in cell chemotaxis could be observed. If one assumes that inhibition of eosinophil locomotion by BAY 41-2272 is due to elevation of cellular cGMP production, then no direct correlation exists between cGMP production and chemotaxis inhibition. This seems to weaken previous suggestions that human neutrophil chemotaxis undergo a biphasic regulation by cGMP levels where high concentrations of NO donors inhibit, whereas lower NO concentrations increase the cell chemotaxis [28,29].

Eosinophils exposed for 90 min in MEM exhibited an amplified chemotactic response to fMLP compared with cells exposed for only 10 min, as observed in both untreated and IBMX-treated cells. It is suggested that

the long-term exposure protocols promote a self-activation of eosinophils causing the release of endogenous factors [e.g. platelet-activating factor (PAF) and interleukin 5 (IL-5)] that prime these cells to further addition of chemoattractants such as fMLP [25]. Accordingly, a previous study showed that brief (5–15 min) or prolonged exposition (1–2 h) of human neutrophils with the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) increases the fMLP-induced chemotactic responses due to an enhancement in the receptor (and/or affinity) population for this chemoattractant [37]. However, our findings that BAY 41-2272 inhibited the eosinophil chemotaxis independently of the exposition time (10-min and 90-min exposition) indicate that its inhibitory action is independent of the signaling mechanisms triggered by fMLP in both of these conditions.

It is well established that NO binds to the haem site of sGC, activating the enzyme and catalyzing the conversion of GTP to cGMP [38]. The compound ODQ inhibits NO-stimulated sGC activity [39] and has been extensively used to study the function of the NO–cGMP transduction pathway. The inhibitory effect of ODQ on NO-stimulated sGC is due to changes in the oxidation state of the haem moiety, without adversely affecting the catalytic activity of the enzyme [40]. The compound ODQ fails to reduce the BAY 41-2272-induced sGC stimulation in purified enzyme [10], and only partly reduces the corpus cavernosum relaxations induced by BAY 41-2272 [15]. However, in our study, the increases in eosinophil cGMP levels induced by BAY 41-2272 were completely prevented by pre-incubation with ODQ. Based on the observations that ODQ and YC-1 (parent compound of BAY 41-2272) binds to NO-bound sGC leading to identical perturbations in the secondary structure of the sGC [41], one may suggest that in eosinophils, besides oxidizing the haem moiety of sGC, ODQ has an allosteric mechanism by interfering with the binding of BAY 41-2272.

Elevation of intracellular cAMP levels can be achieved through the activation of adenylate cyclase either directly or through appropriately coupled membrane receptors, as well as by preventing the hydrolysis of cAMP by the cyclic nucleotide phosphodiesterases [42]. Prostaglandin I_2 or prostaglandin E_2 increases cAMP levels and suppress the chemotaxis of guinea pig [43] and human eosinophils [44]. Furthermore, PDE IV inhibitors such as rolipram and Ro 20-1724 have been shown to reduce in vitro stimulated eosinophil-induced chemotaxis [45] and in vivo airways eosinophil infiltration by antigen challenge, PAF or IL-5 exposure [46,47]. Our present findings that eosinophils treated with the non-selective PDE inhibitor IBMX undergo a significant reduction of fMLP-induced chemotaxis in both of the protocols reinforce these studies. Furthermore, a recent study showed that YC-1 inhibits human neutrophil functions (generation of superoxide anion, release of β-glucuronidase and membrane-associated p47phox) and accelerates resequestration of cytosolic calcium in fMLP-activated human cells through activation of the cAMP/protein kinase A pathway independently of cGMP [21]. In our study, BAY 41-2272 significantly increased the intracellular levels of cAMP in eosinophils in both of the exposure conditions used (10 min and 90 min). It is, therefore, plausible that cAMP rather than cGMP plays a major role in modulating the inhibitory effects of BAY 41-2272 on the fMLP-induced eosinophil chemotaxis. This suggestion is weaken, however, by our findings that inhibition of cell chemotaxis achieved with BAY 41-2272 is roughly the same in conditions of low (10-min assays) or high levels of cAMP (90-min assays). Actually, in eosinophils pretreated with IBMX, the inhibition of chemotaxis achieved with BAY 41-2272 at 0.1 μM and 1.0 μM was higher in the 10-min exposure (72–82% inhibition) in comparison with the same concentrations of BAY 41-2272 in the 90-min exposure protocols (52–53% inhibition). Nevertheless, we cannot neglect the possibility that small concentrations of cAMP and cGMP achieved with the short exposition to BAY 41-2272 were already sufficient to produce maximal inhibition of the fMLP-induced eosinophil chemotaxis. If so, small amounts of cAMP and cGMP generated by BAY 41-2272 could be acting synergistically in the eosinophils leading to maximal inhibition of cell migration. Synergism between cAMP and cGMP leading to maximal cell inhibition has been clearly observed in rabbit platelets in vitro [48].

In conclusion, BAY 41-2272 inhibits the fMLP-induced human eosinophil chemotaxis and that is accompanied by significant elevations in both cGMP and cAMP levels. However, inhibition of eosinophil chemotaxis was obtained in conditions of low or high nucleotide levels, showing a non-correlation between chemotaxis inhibition and nucleotide production.

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