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BAY 41-2272 inhibits human neutrophil functions

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ABSTRACT

BAY 41-2272 is a guanylyl cyclase (GC) stimulator derived from YC-1 (3-[(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole]). Previous studies by our group showed that BAY 41-2272 activates human monocytes via soluble guanylyl cyclase (sGC) and cGMP. In this study, we investigated the effect of BAY 41-2272 on human neutrophil function and found that 30 μM BAY 41-2272 inhibits neutrophil migration (1.82-fold lower than FMLP, $P < 0.05$ by one-way ANOVA followed by Tukey's test), oxidative burst (1.70-fold lower than PMA, $P < 0.05$ by one-way ANOVA followed by Tukey's test), and IL-8 cytokine production (1.80-fold lower than PMA, $P < 0.05$ by one-way ANOVA followed by Tukey's test). Our results suggest that these effects are independent of the sGC pathway but dependent instead on cGMP production, as the response induced by 30 μM BAY 41-2272 was 6.40-fold greater than that observed in our negative control ($P < 0.05$ by parametric *t*-test). 1H-[1, 2, 4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), which is an irreversible inhibitor of sGC, was unable to reverse the effects of BAY 41-2272 on human neutrophils, indicating that this drug acts independently of sGC. Our results confirm the immunomodulatory effect of BAY 41-2272 on human neutrophils.

1. Introduction

BAY 41-2272 is derived from 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1). It is a direct stimulator of soluble guanylyl cyclase (sGC) due to its capacity to bind an independent site of NO [1,2]. BAY 41-2272 activates the α2/β1 heterodimeric isoform of sGC. Amino acids 104 to 401 on the N-terminal region of the α2 subunit of sGC represent the binding site of this drug [3]. Nitric oxide (NO) is produced by the endothelium and performs an important protective function in blood vessels by preventing the adhesion of circulating cells, such as neutrophils and platelets [4]. These molecules negatively regulate the expression of different families of adhesion molecules and increase the rate of apoptosis in neutrophils [5–8].

BAY 41-2272 acts synergistically with NO, stabilizing the heme-nitrosyl complex [1,9,10]. Studies have demonstrated that BAY 41-2272 decreases neutrophil chemotaxis [11], inhibits the release of superoxide and the expression of NADPH oxidase components in cavernous tissue cells [12], and inhibits platelet aggregation. This final event and the hypotensive effect are regulated by the sGC pathway and occur independently of NO [13]. The mechanism by which NO and BAY 41-2272 exert their major anti-inflammatory effect is related to the activation and stimulation of sGC and therefore to the conversion of guanosine triphosphate (GTP) into the second messenger guanosine monophosphate (GMP) [14,15]. BAY 41-2272 increases the concentrations of both cGMP and cAMP in smooth muscle cells [16].

Our group demonstrated that in THP-1 cells and peripheral blood monocytes, BAY 41-2272 increases the release of superoxide anion (O_2^-), the expression of CYBB and NCF2 genes, the phagocytic microbicidal responses, the release of TNF-α and IL-12p70, and the intracellular levels of cGMP and cAMP [17,18].

Considering the potential role of sGC in the neutrophil response, our hypothesis is that BAY 41-2272 has immunomodulatory effects on neutrophils. In this study, we investigated the effect of BAY 41-2272 on human neutrophil functions such as the release of superoxide anion (O_2^-), cytokine production, cell migration, and cGMP production.

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2. Materials and methods

2.1. Isolation of neutrophils

Human neutrophils were obtained from healthy volunteers in accordance with the rules and regulations of the Ethics Committee of the University of São Paulo, which approved this protocol. All subjects provided written informed consent in accordance with the Declaration of Helsinki. Neutrophils were isolated from peripheral blood by sedimentation in dextran (Sigma-Aldrich, St. Louis, MO), followed by Ficoll-Hypaque (GE Healthcare., Chicago, USA) gradient centrifugation. The cells were washed three times with PBS and resuspended in RPMI 1640 medium (Thermo Fisher Scientific Inc., Massachusetts, USA). The purity of the cell suspension was routinely assessed by flow cytometry via analysis of the neutrophil-specific surface markers CD16 and CD66b; a yield above 95% was considered adequate for the subsequent experiments.

2.2. Cell viability

Cell viability was assessed using the LIVE/DEAD® Fixable Dead Cell Stain Protocol (Thermo Fisher Scientific Inc., Massachusetts, USA). After neutrophils (1×10^6 cells·mL^{-1}, 100 μL) were treated with BAY 41-2272 (3 and 30 μM), phorbol myristate acetate (PMA 50 nM) or dimethyl sulfoxide (DMSO, 0.1%; 0.2%; 0.3%; 0.4%; 0.5%; 2%; and 3% drug vehicle) for 60 min at 37 °C, the cells were analyzed using an Applied Biosystems Attune® NxT flow cytometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Live cells were determined using forward scatter (FSC) versus side scatter (SSC) parameters, and 50,000 events were collected from each sample. For data analysis, we used FlowJo software (Tree Star Inc.).

2.3. Chemotaxis assay

Neutrophil chemotaxis in response to chemoattractant factors was assessed according to Paulo-Neto et al. [11]. After 1 h of incubation with BAY 41-2272 (0.3, 3 or 30 μM) or SNAP (10 μM) and with or without ODQ, neutrophils (5×10^5 cells) were transferred to a 5-μm pore plate (Corning, USA) containing the chemoattractant stimulus N-For-Met-Leu-Phe (FMLP) and incubated for another 60 min in a 37 °C incubator with CO_2. Cells were quantified using Atune NxT flow cytometry to visualize the total amount of cells that migrated through the transwell.

2.4. Immunophenotyping

Neutrophils (1×10^6 cells·mL^{-1}, 100 μL) were treated with BAY 41-2272 (3 and 30 μM), 8-Br-cGMP (100 μM), 8Br-cAMP (100 μM) or SNAP (10 μM), followed by 50 nM PMA, and incubated with PBS containing 10% human AB serum and 2% FBS for 10 min to prevent nonspecific binding. Then, the cell suspensions (100 μL, 0.5–1 $\times 10^6$ cells) were transferred to 5-mL round-bottomed polystyrene tubes (cytometry tubes). In each tube, the antibodies were pooled (to prevent the fluorescence values from interfering in the analysis) and added to the samples, which were then incubated for 30 min at 4 °C in the dark. The cells were washed twice with PBS containing 2% FBS (centrifugation at 900 $\times g$ for 5 min), collected in tubes, fixed in PBS containing paraformaldehyde (1%) and analyzed using a FACSCanto® II flow cytometer (Becton Dickinson). The live cell population was determined using FSC versus SSC parameters, and 50,000 events were collected from each sample. For the data analysis, we used FlowJo software (Tree Star Inc.). Based on this analysis, we determined the percentages of the cell population expressing the markers and the density of each marker per cell (suggested by the median fluorescence intensity).

2.5. Superoxide anion production by chemiluminescence

The production of superoxide anion by neutrophils was measured as previously described by Hatanaka et al. [19]. After 1 h of incubation with BAY 41-2272 (0.3, 3 or 30 μM), 8-Br-cGMP (100 μM) lucigenin suspension (1 mM) or PMA (50 nM) was added. The chemiluminescence was monitored for 1 h using a microplate luminometer (EG & G Berthold LB96V, Bad Wildbad, Germany). The results are expressed in relative light units (RLUs), and the maximum RLU levels were recorded to calculate the average maximum superoxide production during the observation period.

2.6. Reactive oxygen species production by DHR

The cytometric dihydrorhodamine (DHR) assay was performed as previously described [47]. The samples were treated with BAY 41-2272 (0.3, 3 and 30 μM), 8-Br-cGMP (100 μM), 8Br-cAMP (100 μM) or SNAP (10 μM), then assessed with an Applied Biosystems Attune® NxT flow cytometer (Thermo Fisher Scientific Inc., Massachusetts, USA) and analyzed by determining the FSC versus SSC parameters after 50,000 events were collected. For the data analysis, we used FlowJo software (Tree Star Inc.).

2.7. Cytokine assay

The neutrophils (1×10^6 cells·mL^{-1}) from human blood were treated for 1 h with BAY 41-2272 (3 and 30 μM), 8-Br-cGMP (100 μM) or SNAP (10 μM), followed by stimulation with PMA (50 nM) for an additional 4 h and culturing in 96-well plates. The supernatant was collected, and an ELISA for detecting IL-8 (BD Biosciences) was performed as previously described by Pitzurra et al. [20].

2.8. cGMP production

Neutrophils were treated with 0.3 μM, 3 μM, and 30 μM BAY 41-2272 or 10 μM SNAP for 10 min. After that, we added two conditions, ODQ or phosphodiesterase type 5 inhibitor (iPDE5). After stimulation, the cells were centrifuged, and the supernatant was discarded. The concentration of cGMP was measured using a Cyclic GMP EIA Kit (Cayman Chemical, No. 581021, Ann Arbor, Michigan) according to the manufacturer's instructions. The concentration of the samples was estimated via comparison with a standard curve derived from a serial dilution of 30 to 0.23 pmol/mL. The absorbance results were analyzed by linear regression, and the cGMP concentrations in the samples were given as pmol/mL.

2.9. Chemical reagents

BAY 41-2272, dimethyl sulfoxide (DMSO, vehicle), phorbol 12-myristate 13-acetate (PMA, a PKC (protein kinase C) activator), N-formyl-methionine-leucine-phenylalanine (FMLP, a chemoattractant stimulus), 8-Br-cGMP (a cGMP agonist), 8-Br-cAMP (a cAMP agonist), and 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, an irreversible sGC inhibitor) were obtained from Sigma (St. Louis, MO, USA). S-nitroso-*N*-acetyl penicillamine (SNAP, NO donor) was purchased from Cayman Chemical Company (Michigan, USA). The concentrations of FMLP, PMA, 8-Br-cAMP, 8-Br-cGMP, ODQ, and SNAP were based on Hwang et al. [21] and Paula-Neto et al. [11].

2.10. Statistical analysis

The results of the experiments were expressed as the mean standard deviation. The statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test [22]. GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) was used to perform the statistical analysis, and the results were considered statistically significant if

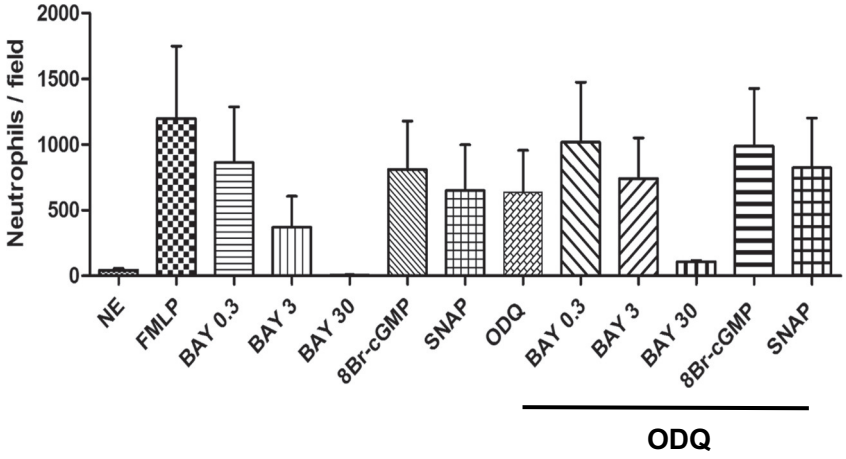

Fig. 1. Treatment with BAY 41-2272 inhibits the chemotaxis of neutrophils. Peripheral blood neutrophils were pre-incubated with 10 μM ODQ for 10 min and then treated with BAY 41-2272 (0.3 μM, 3 μM and 30 μM), 8-Br-cGMP (100 μM), SNAP (10 μM) and FMLP (100 nM) for 2 h. After treatment, we counted the cell migration by flow cytometry. NE: nonstimulated. *P < 0.05 compared to FMLP treatment, $n = 3$. *P < 0.05 compared to NE treatment, ANOVA one-way test followed by Tukey's test. BAY 41-2272 (30 μM) inhibited 100 nM FMLP-induced chemotaxis (one-way ANOVA, n = 6, P < 0.05). This inhibition was also observed with 10 μM SNAP (NO donor) alone or in combination with 100 nM FMLP. Treatment with ODQ was also unable to reverse this effect.

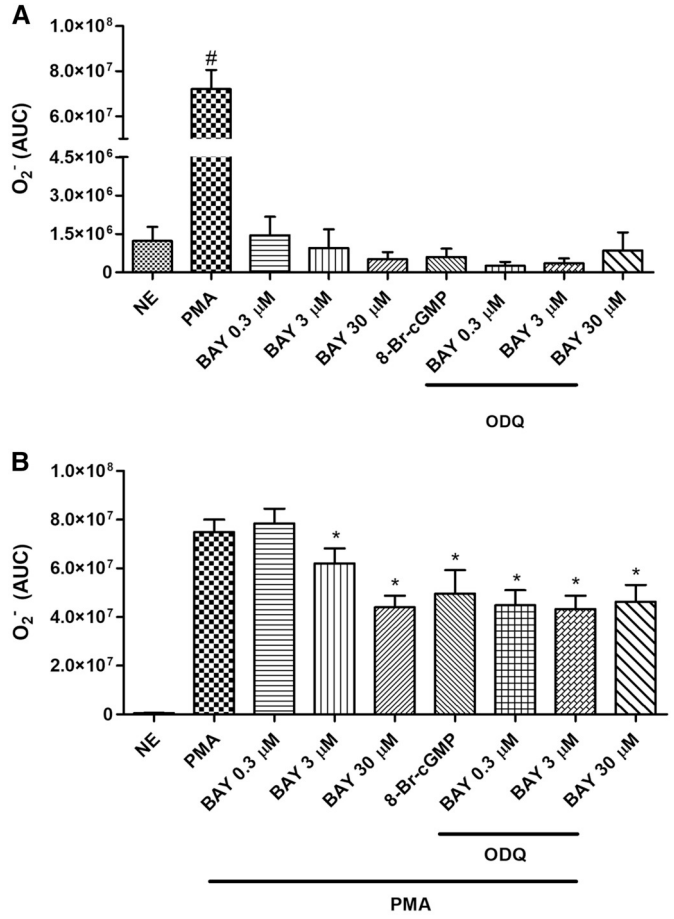

Fig. 2. Superoxide anion production by neutrophils after treatment with BAY 41-2272. Peripheral blood neutrophils were preincubated with ODQ for 10 min and treated with BAY 41-2272 (0.3 μM, 3 μM and 30 μM) for 1 h (2A), followed by treatment with 50 nM PMA (2B). After treatment, the cells were incubated with lucigenin (1 mM) for an hour to evaluate superoxide anion (O_2^-) production by chemiluminescence. The results are expressed as the area under the curve (AUC) ($n = 9$ healthy individuals in duplicate). $^{\#}p < 0.05$ compared to NE (nonstimulated) and *p < 0.05 compared to PMA treatment, ANOVA one-way test followed by Tukey's test. ODQ: inhibitor of soluble guanylate cyclase. 8-Br-cGMP: cGMP analog. BAY 41-2272, as a direct form of activation, did not induce superoxide anion (O_2^-) production (panel A). However, compared to the cells in the PMA-stimulated control group, following pretreatment with BAY 41-2272 (3 μM and 30 μM doses) and activation with 50 nM PMA (PKC activator), the cells exhibited a significant decrease in superoxide anion (O_2^-) production (panel B).

$P < 0.05$.

3. Results

3.1. Treatment with BAY 41-2272 inhibits chemotaxis in neutrophils

Neutrophil migration is an essential effector mechanism. BAY 41-2272 (30 μM) inhibited 100 nM FMLP-induced chemotaxis (one-way ANOVA, $n = 6$, P < 0.05; Fig. 1). This inhibition was also observed with 10 μM SNAP (NO donor) alone or in combination with 100 nM FMLP. Treatment with ODQ was also unable to reverse this frame.

We also checked the cell viability after treatment with BAY 41-2272 at 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM and 30 μM for 1 h. BAY 41-2272 did not alter the percentage of live cells (one-way ANOVA, $n = 6$, $P < 0.05$, Supplementary Fig. 1). Moreover, treatment with DMSO, which was the vehicle used for BAY 41-2272, did not alter the cell viability (Supplementary Fig. 2). The phenotypic characterization also showed that BAY 41-2272 did not alter the expression of the neutrophil-specific surface molecules CD16 and CD66b, suggesting the maintenance of the phenotypic profile of neutrophils (Supplementary Fig. 3). Thus, BAY 41-2272 did not affect cell viability or alter the phenotypic profile of neutrophils. We also checked the effect of SNAP on neutrophils and the sensitivity to ODQ ($n = 3$ individuals in duplicate, $p < 0.05$ compared to treatment with PMA, ANOVA one-way test followed by Tukey's test). ODQ increased the production of superoxide anion when added along with SNAP compared to the results of SNAP treatment alone.

3.2. Pretreatment with BAY 41-2272 inhibits the oxidative burst in human neutrophils

Reactive oxygen species (ROS) are known to play an important role in mediating microbial death. BAY 41-2272, as a direct form of activation, did not induce oxidative burst (Fig. 3A) or superoxide anion (O_2^-) production (Fig. 2A). However, compared to the cells in the PMA-stimulated control group, following pretreatment with BAY 41-2272 (3 μM and 30 μM doses) and activation with 50 nM PMA (PKC activator), the cells exhibited a significant decrease in superoxide anion (O_2^-) production (Fig. 2B; one-way ANOVA, $n = 8$, $P < 0.05$) and oxidative burst activity (Fig. 3B; one-way ANOVA, n = 8, P < 0.05). Treatment with ODQ did not change this inhibitory effect. DMSO did not interfere with the production of superoxide anion (Supplementary Fig. 4). SNAP also inhibits superoxide anion, but ODQ did change this effect (Supplementary Fig. 5).

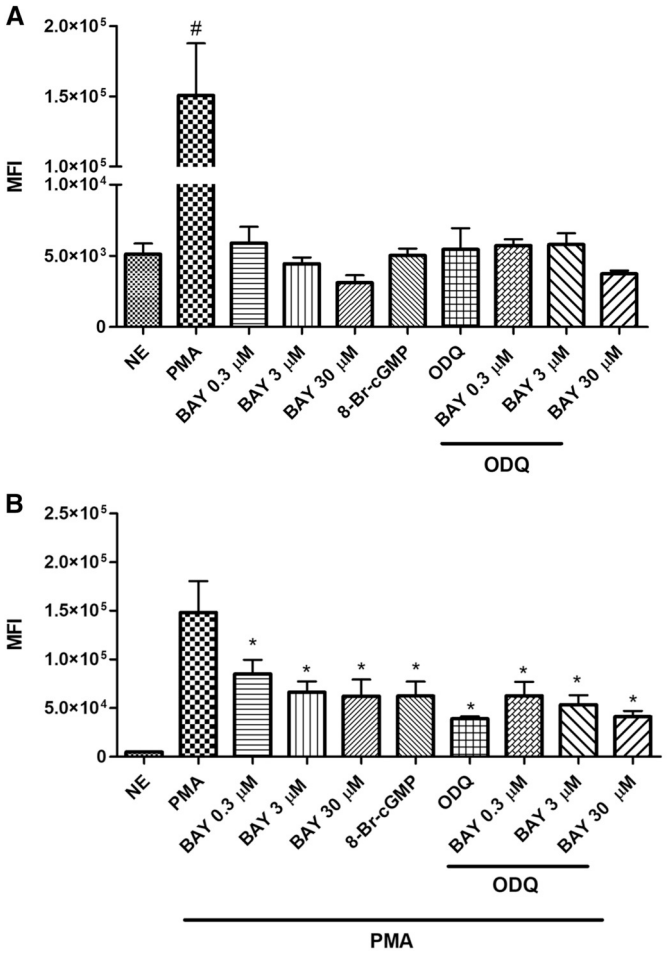

Fig. 3. Oxidative burst evaluation of neutrophils after treatment with BAY 41-2272. Peripheral blood neutrophils were preincubated with 10 μM ODQ for 10 min and then treated with BAY 41-2272 (0.3 μM, 3 μM and 30 μM) for 1 h (3A), followed by activation with 50 nM PMA (3B). After stimulation, the cells were incubated for 10 min with DHR (10 μg/mL) to evaluate hydrogen peroxide (H_2O_2) production by flow cytometry. The results are expressed as the mean fluorescence intensity (MFI) ($n = 8$ individuals, in duplicate). $^{\#}p < 0.05$ compared to NE (nonstimulated) and $^{*}p < 0.05$ compared to PMA treatment, ANOVA one-way test followed by Tukey's test. ODQ: inhibitor of soluble guanylate cyclase. 8-Br-cGMP: cGMP analog. BAY 41-2272, as a direct form of activation, did not induce oxidative burst (panel A). However, compared to the cells in the PMA-stimulated control group, following pretreatment with BAY 41-2272 (3 μM and 30 μM doses) and activation with 50 nM PMA (PKC activator), the cells exhibited a significant decrease in the oxidative burst activity (panel B).

3.3. Pretreatment with BAY 41-2272 inhibits the production of IL-8 by human neutrophils

The production of cytokines is another important function performed by neutrophils, and IL-8 is the main cytokine produced by these cells. We verified the production of this chemokine and observed a decrease in IL-8 release in neutrophils pretreated with 30 μM BAY 41-2272 followed by PMA activation (in the absence and presence of ODQ) compared to that in the cells stimulated with PMA alone (one-way ANOVA, $n = 12$, $P < 0.05$, Fig. 4B). Similar to the results observed in the superoxide anion and oxidative burst assays, ODQ (10 μM) did not reverse the inhibitory effect of BAY 41-2272 on cytokine production (Fig. 4B). In addition, direct treatment with BAY 41-2272 did not alter IL-8 production (Fig. 4A). IL-8 release was also assessed in the presence of the NO donor drug SNAP. Similar to BAY 41-2272, SNAP inhibited the production of this cytokine, and compared to neutrophil treatment with PMA alone, concomitant treatment with SNAP and BAY 41-2272 resulted in a significant decrease in IL-8 production (one-way ANOVA, $n = 6$, $P < 0.05$, Fig. 4C).

3.4. BAY 41-2272 induces cGMP production

Cyclic GMP (cGMP) is the product of sGC activation; thus, we evaluated the cGMP levels after 10 min of stimulation with BAY 41-2272 (one-way ANOVA, $n = 6$, $P < 0.05$, Fig. 5). Compared to the unstimulated control, 30 μM BAY 41-2272 and 10 μM SNAP induced the production of cGMP. We also verified whether additional treatment

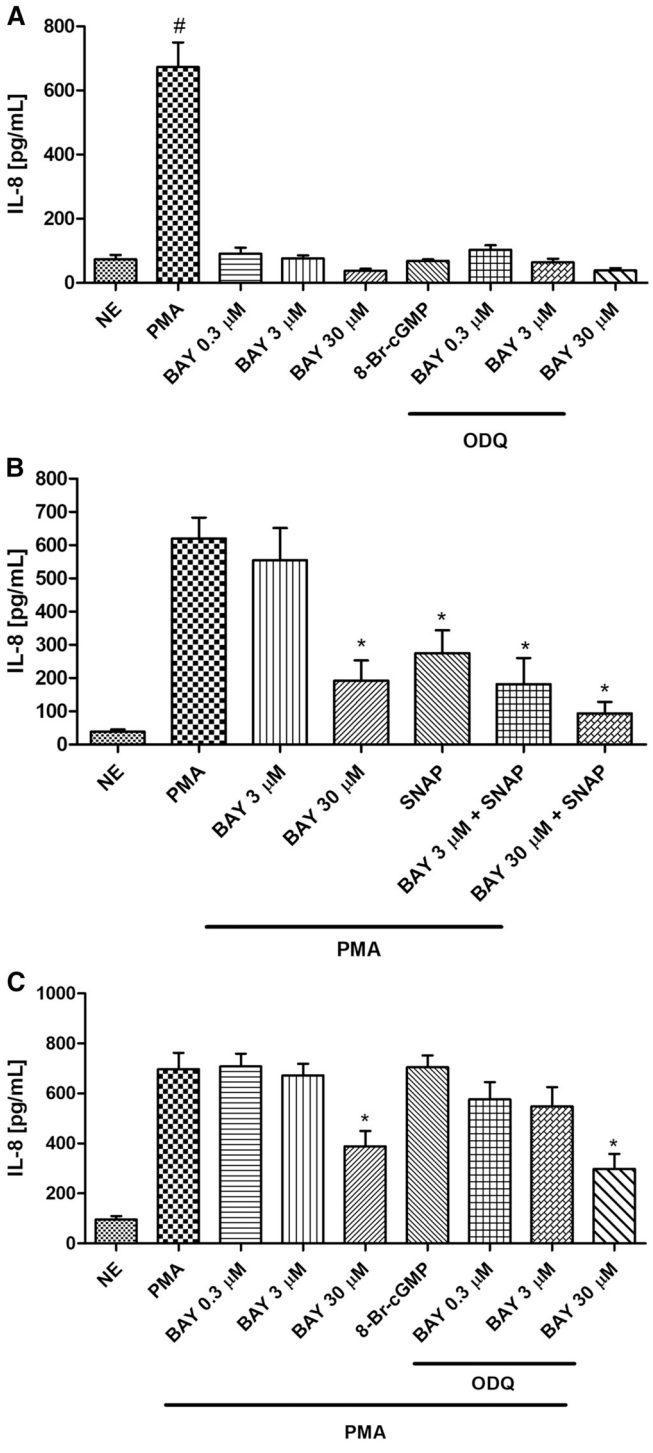

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Fig. 4. Production of IL-8 by neutrophils after treatment with BAY 41-2272 and SNAP. Peripheral blood neutrophils were preincubated with 10 μM ODQ for 10 min and treated with BAY 41-2272 (0.3 μM, 3 μM and 30 μM) for 4 h (4A) or for 1 h followed by activation with 50 nM PMA for 4 h (4B) or treated with SNAP (10 μM) and BAY 41-2272 (0.3 μM, 3 μM and 30 μM) for 1 h followed by stimulation with 50 nM PMA for 4 h (4C). After treatment, the culture supernatant was collected to measure IL-8 levels by ELISA ($n = 11$ subjects (4A and 4B); $n = 6$ subjects (4C); all in duplicate). $^{\#}p < 0.05$ compared to NE (non-stimulated) and $^{*}p < 0.05$ compared to PMA treatment, ANOVA one-way test followed by Tukey's test. ODQ: inhibitor of soluble guanylate cyclase. 8-Br-cGMP: cGMP analog. SNAP: NO donor. We verified the production of IL-8 and observed a decrease in the release of this cytokine in neutrophils pretreated with 30 μM BAY 41-2272 followed by PMA activation (in the absence and presence of ODQ) compared to that in the cells stimulated with PMA alone (one-way ANOVA, $n = 12$, $P < 0.05$, panel B). Similar to the results observed in the superoxide anion and oxidative burst assays, ODQ (10 μM) did not reverse the inhibitory effect of BAY 41-2272 on cytokine production (panel B). In addition, direct treatment with BAY 41-2272 did not alter IL-8 production (panel A).

with ODQ and iPDE5 affected cGMP production, and they did not reverse the inducer effect of BAY 41-2.

4. Discussion

Pharmacological agents have been synthesized to interact with sGC independently of nitric oxide to generate cGMP, which is an important second messenger involved in cellular processes such as host defense, cell growth, proliferation, homeostasis, vascular growth and neuronal transmission [23,24]. Based on their mechanisms of action, these pharmacological agents can be classified as stimulators, such as BAY 41-2272, or guanylate cyclase activators, such as BAY 60-2770 [25,26]. Our group demonstrated that in THP-1 cells and peripheral blood monocytes, BAY 41-2272 and its downstream signaling increase the release of superoxide anion (O_2^-), the expression of *CYBB* and *NCF2* genes, the phagocytic and microbicidal activities, the release of TNF-α and IL-12p70, and the intracellular levels of cGMP and cAMP [17,18].

In the mouse model, BAY 41-2272 significantly inhibits the

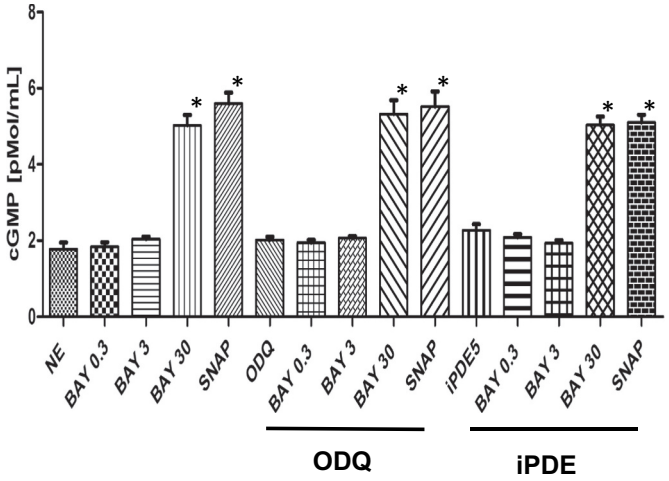

Fig. 5. Production of cGMP by neutrophils. Peripheral blood neutrophils were preincubated with 10 μM ODQ or phosphodiesterase type 5 inhibitor (iPDE5) at the same concentration for 10 min and then treated with SNAP (10 μM) or BAY 41-2272 (0.3 μM, 3 μM and 30 μM) for 10 min. After treatment, cells were lysed, and the supernatant was collected to measure cGMP levels by ELISAs ($n = 3$ subjects, in duplicate). $^{*}p < 0.05$ compared to NE, one-way ANOVA followed by Tukey's test. SNAP: NO donor. Compared to the unstimulated control, 30 μM BAY 41-2272 and 10 μM SNAP induced the production of cGMP. We also verified whether treatment with ODQ and iPDE5 affected cGMP production: they did not reverse the inducer effect of BAY 41-2272.

polarization and adhesion of neutrophils to endothelial cells by decreasing the expression of P-selectin; this inhibition can be reversed by the sGC inhibitor ODQ, indicating that this effect is dependent on sGC activation and subsequent cGMP synthesis [27]. Dal Secco et al. [27] demonstrated that NO inhibits the migration of neutrophils by negatively modulating ICAM-1 (CD54) in the endothelium, and treatment with ODQ, which blocks sGC, is able to reverse this negative modulation of ICAM-1. Paula-Neto et al. [11] observed a decrease in neutrophil chemotaxis in vitro after treatment with BAY 41-2272 followed by stimulation with IL-8 or FMLP; decreased chemotaxis was also observed after lipopolysaccharide (LPS) treatment. The authors also verified that the inhibition of sGC by ODQ in a mouse sepsis model restores the migration of neutrophils without altering the function of neutrophil killing, contributing to the increased survival of mice treated with sGC inhibitors. This outcome suggests that the inhibitory effect on neutrophil migration depends on the sGC-cGMP-PKG signaling axis; thus, we investigated whether the effects of BAY 41-2272 treatment on human neutrophils occur through the sGC-cGMP pathway. Our analysis revealed that the in vitro chemotaxis of neutrophils after treatment with BAY 41-2272 and chemotactic stimulation by FMLP was significantly inhibited compared to that in untreated cells and FMLP-stimulated cells, corroborating the data described in the literature and supporting our hypothesis that in vitro, neutrophil chemotaxis is reduced due to the inhibition of the chemotactic response (in this work, FMLP) in neutrophils. The addition of ODQ was not able to reverse the inhibition of migration caused by BAY 41-2272. As already mentioned and demonstrated in our results, the effect of BAY 41-2272 is dependent on the cGMP pathway; however, another factor may be involved in this pathway, since it was not inhibited in our experiments on chemotaxis and IL-8 dosage.

Klink M. et al. [28] demonstrated the direct influence of NO on inflammatory cells, especially neutrophils, because this gas prevents the activation, adhesion and expression of the CD11b/CD18 (Mac-1) molecule in neutrophils stimulated by PMA or FMLP. Analysis of the influence of NO on neutrophils showed that the inhibition of the oxidative burst of neutrophils after treatment with NO donors was independent of cGMP because even after the neutrophils were treated with sGC inhibitors, the significant reduction in ROS production was maintained. By analyzing effector functions, Klink et al. showed that the effects of NO adhesion on neutrophils are not mediated by cGMP formation. Studies have also shown that NO donors inhibit PMN effector functions, such as chemotaxis, degranulation, adhesion and ROS production [29–31]. In addition to the role of ROS production as a primordial response in the elimination of pathogens, ROS are involved in the release of other mediators during the inflammatory process. Several tissues constitutively express NADPH oxidases (NOXs), and ROS produced by this family of enzymes can regulate the expression of adhesion molecules in the endothelium and inflammatory cells, affecting cellular recruitment to the inflammatory site [32–34]. In addition, the production of ROS plays an important role in inflammatory processes because it is an important mechanism during the death process in pathogens, and in the long term, excessive ROS can result in endothelial damage, increased vascular permeability, and cell death [35,36]. The pretreatment of neutrophils with YC-1, the precursor of BAY 41-2272, inhibits the production of superoxide anion (O_2^-) after treatment with PMA or FMLP, and pretreatment with ODQ followed by YC-1 and PMA cannot reverse the inhibitory effect caused by YC-1 [21].

Furthermore, the literature reports that the treatment of cells from cavernous tissue with BAY 41-2272 inhibits the formation of superoxide anion (O_2^-) and NADPH oxidase expression by downregulating p22phox and gp91phox [12]. Our data show that compared to the PMA stimulus alone, pretreatment with BAY 41-2272 followed by stimulation with PMA significantly inhibited superoxide anion (O_2^-) production and oxidative burst in neutrophils, confirming the anti-inflammatory profile of BAY 41-2272. Interestingly, in our work, pretreatment with ODQ was unable to reverse the inhibitory effect of

BAY 41-2272; thus, we speculate that a possible pathway independent of sGC and dependent on cGMP exists because 8-Br-cGMP also inhibited the functions of PMN in our work. ROS are also able to indirectly increase the expression of cytokines and chemokines produced by neutrophils [37,38]. ODQ was not able to inhibit cGMP production in cells pretreated with ODQ + SNAP. This confirms our hypothesis that the cGMP path participates independently of GC, although the canonization of activation of BAY 41-2272 is GC-GMPc-PKG. This modulation of adhesion molecules and cytokine production occurs because ROS, mainly oxygen peroxide (H_2O_2), can act as second messengers in the transduction of the inflammatory signal and stimulate the activity of MAP kinases, leading to the activation of several transcription factors with proinflammatory functions [34,39]. In addition, one of the main functions of the cells in the immune system is the production of cytokines, and the main chemokine produced by neutrophils is IL-8. Thus, in analyzing cytokine production, we found that BAY 41-2272 maintains its inhibitory profile because IL-8 production is decreased following the treatment of neutrophils with BAY 41-2272 and is stimulated following treatment with PMA. Other cytokines, such as IFN-ɣ, TNF-α, and IL-6, were measured but not detected (data not shown).

To elucidate the possible mechanisms by which BAY 41-2272 exerts its effects, we investigated the production of cGMP. One anti-inflammatory mechanism of NO is the synthesis of cGMP in muscles, endothelial cells and platelets [14,15]. In some pathological processes, a reduction in NO bioavailability may occur, compromising NO-cGMP-PKG pathway signaling; therefore, several molecules, such as BAY 41-2272, currently used as NO-independent stimulators and agonists [40]. sGC pathway activation leads to cGMP production, which is an important mediator regulating various cellular functions. Once produced, cGMP can be rapidly hydrolyzed by PDEs and exerts its function through protein kinases, mainly protein kinase G (PKG)-I and PKG-II, which share several targets with protein kinase A (PKA) [41]. BAY 41-2272 increases cAMP and cGMP levels and the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a cytoskeletal protein that exhibits several effects, such as motility, migration and cell adhesion, in mouse muscle cells [42,43]. BAY 41-2272 can inhibit Ca^{2+} influx, Na +/K+ ATPase enzyme activity and, at high concentrations, phosphodiesterase 5 activity [44–46]. In smooth muscle, BAY 41-2272 can inhibit the growth of muscle cells and increase the concentrations of both cGMP and cAMP [16]. We analyzed the production of cGMP by neutrophils and observed a significant increase in cGMP production following treatment with 30 μM BAY 41-2272 and 10 μM SNAP (a NO donor). The cGMP agonist 8-Br-cGMP had the same effect as BAY 41-2272, i.e., inhibition of the effector functions of neutrophils, suggesting that the effect of BAY 41-2272 on neutrophils occurs through the cGMP pathway and is independent of sGC. The production of maintained the increase in cGMP production previously observed to result from the use of BAY 30 and SNAP, and there was no decrease in cGMP production when ODQ was applied, showing that it did not reverse the effect, neither in BAY 30 nor SNAP, practically confirming our hypothesis that neutrophil functions are inhibited by via cGMP but independent of sGC. The use of iPDE5 prevents the degradation of cGMP; if cGMP were degraded by PDE5, the cGMP values when using phosphodiesterase type 5 inhibitor would be higher, especially in BAY 3, which showed basal production, and would increase further in BAY 30. In this way, the use of iPDE5 confirms that the cGMP is not being degraded since the cGMP production by BAY 30 and the SNAP were kept the same as when we did not use iPDE5.

We conclude that BAY 41-2272 inhibits the effector functions of human neutrophils, such as cell chemotaxis, cytokine production, and oxidative burst and that BAY 41-2272 could act as an immunomodulatory agent in neutrophils.

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Declaration of Competing Interest

None.

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