New Potential Targets to Modulate Neutrophil Function in Inflammation

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Abstract: The importance of neutrophils in human disease such as rheumatoid arthritis, asthma, adult respiratory distress syndrome, and COPD has prompted the search for drugs capable to slow down neutrophil-dependent inflammation, without interference with innate immune responses. In this review, we summarize new potential drugs targets against neutrophils mediated inflammatory responses.

Key Words: Neutrophil, drugs, innate immunity, inflammation.

INTRODUCTION

Neutrophils are originated from the myeloid line from the stem cells of the bone marrow [1] in response to specific growth factors. Neutrophils delivered for the circulation are mature forms of the initial progeny of myelocytic progenitors, the immature band cells. Unlike mature neutrophils, band cells possess few cytoplasmic granules and lack the segmented nucleus characteristic of the mature neutrophil, which is therefore also commonly referred to as the polymorphonuclear neutrophilic granulocyte. Several molecules needed for neutrophil responses are pre-packaged in the cells´ cytoplasmic granules or plasma membrane, and thereby being available for cellular functions quickly for response against microorganisms [2].

Neutrophil recruitment requires adhesion and transmigration through blood-vessel walls. Three steps have been described: rolling, activation and firm adhesion. Rolling, which is mediated by selectins; activation, which is mediated by chemokines; and adhesion, which is mediated by integrins. Progress has been made in defining additional steps: capture (or tethering), slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration [3].

Neutrophils can destroy pathogens, mediating engulfing and absorption of waste material, generation of ROS, antimicrobial peptides and proteolytic enzymes. These mechanisms not only assist in the killing and digestion of microorganisms but are potentially harmful to the host if released inappropriately. Several of these proteins are present in the granules Azurophilic (primary) and Specific (secondary), and are released in the presence of several proinflammatory stimuli, microorganism or as a strategy to destroy extracellular matrix (i.e. metalloproteinase) of the tissues to clean them from pathogens [4-6]. Although a vigorous response from neutrophils is necessary for host defense, overly aggressive or prolonged neutrophil responses can result in deleterious inflammatory conditions [7] and tissue destruction [8]. The pathogen killing is facilitated by oxygen-dependent and oxygen independent mechanism [9]. The former mechanism refers to the respiratory burst, an essential component of neutrophil biocidal function. The respiratory burst is defined as an increase in the oxidative metabolism of phagocytes following the uptake of particles, leading to the generation of reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). Recently has been proposed that NADPH oxidase pumps electrons into the phagocytic vacuole, thereby inducing a movement of compensating ions that activate bactericidal enzyme such as neutrophils elastase and cathepsin G [10].

Few currently available therapeutic agents, including corticosteroids, effectively down-regulate neutrophil proinflammatory activity. Insensitivity to corticosteroids may therefore be a feature of those disorders in which the neutrophil is the predominant inflammatory cell type. The relative insensitivity of neutrophils to corticosteroids is attributable to a combination of mechanisms. Neutrophils, which are now recognized to be an important source of newly synthesized cytokines [11, 12], particularly interleukin (IL)-8 and tumor necrosis factor (TNF)-α, contain comparatively high levels of the functionally inactive beta isoform of the glucocorticoid receptor (GR) [13, 14], the synthesis of which is further up-regulated on exposure of the cells to IL-8 [15], rendering them even less corticosteroid-sensitive. Moreover, neutrophils, unlike other types of immune and inflammatory cells, have been reported to be relatively insensitive to the apoptosis-inducing actions of corticosteroids [16, 17]. On the contrary, corticosteroids delay spontaneous apoptosis in neutrophils reducing Fas expression [18], caspase 9 activation and modulating Bcl-2 apoptosis regulatory proteins [19]. In this paper, we reviewed the potential new drug candidates in the control of neutrophil activity.

LTB4 ANTAGONISTS AND 5-LO INHIBITORS

Leukotriene B4 is a potent lipid mediator generated by 5'-lipoxygenase (5-LO) and exerts its effects *via* leukotriene

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B4 (BLT1) receptors in neutrophils. Potent 5-LO inhibitors have been difficult to develop; zileuton is a relatively weak 5-LO inhibitor that has a short duration of action. Its effect in asthma is greater than leukotriene receptor antagonists, particularly in severe asthma. In patients with COPD, five lipoxygenase activating protein (FLAP) inhibitor BAYx 1005, showed only a modest reduction in sputum LTB4 concentrations but no effect on neutrophil activation markers [20]. It has proven difficult to develop potent 5-LO inhibitors because many of these drugs are limited by their toxicity profiles. BLT1 antagonists inhibit the neutrophil chemotaxis in sputum samples from patients with COPD [21, 22], but clinical studies in patients with COPD have been disappointing.

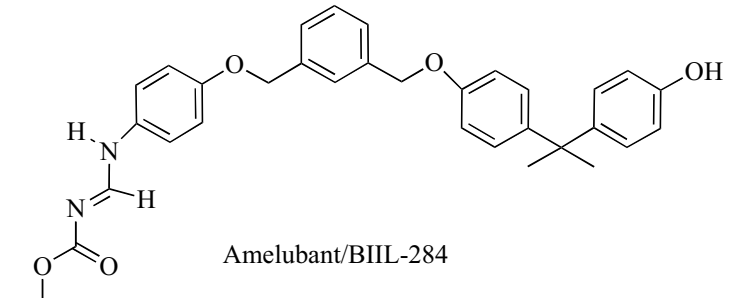
Amelubant (Fig. **1**) is a prodrug formulated for oral administration with negligible binding to the LTB4 receptor. *In vivo*, amelubant (BIIL-284) is converted by ubiquitous esterases to the active metabolites BIIL-260 the free guanidine and to BIIL-315, the henoxy α- D -glucuronidate conjugate (Fig. **1**). Both metabolites, BIIL-260 and BIIL-315, have high affinity for the LTB 4 receptor on isolated human neutrophil cell membranes (K_i =1.7 and 1.9 nM, respectively) and potently inhibit LTB 4 -induced chemotaxis of human neutrophils (IC_{50} =2.9 and 0.65 nM, respectively) [23]. BIIL-260 and BIIL-315 bind to LTB 4 receptors in a saturable, reversible and competitive manner. BIIL-260 has been reported as a dual BLT1 and BLT2 inhibitor. Several clinical development activities for amelubant for the potential treatment of inflammatory diseases, such as RA, COPD and cystic fibrosis have been reported [24].

LY-293111, a diaryl ether carboxylic acid derivative, is a reported LTB4 antagonist. The IC_{50} for inhibiting [^{3}H]LTB4 binding to human neutrophils was 17.6 ± 4.8 nM. LY293111 inhibited LTB4-induced human neutrophil aggregation (IC_{50} = 32 ± 5 nM), luminol-dependent chemiluminescence (IC_{50} = 20 ± 2 nM), chemotaxis (IC_{50} = 6.3 ± 1.7 nM), and superoxide production by adherent cells (IC_{50} = 0.5 nM) [25]. However, this compound is not selective, acting as a 5-LO inhibitor and PPARγ-agonist [24]. This compound has been approved for phase II in asthma, inflammatory bowel disease, rheumatoid arthritis (RA) and cancer, however, Eli Lilly reported that it had been discontinued for inflammatory indications for unknown reasons [24].

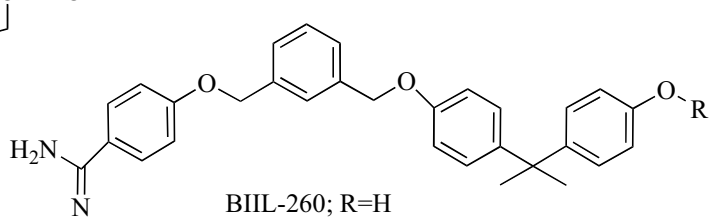
Pfizer reported two potent LTB4 antagonists, CP-105696 and CP-195543. CP-105696, a biphenylyl-substituted chroman carboxylic acid, was in development for rheumatoid arthritis and inflammatory bowel disease. The potency was significantly reduced when assays were performed in whole blood, demonstrating a high level of protein binding. This was the main reason of discontinuation of this molecule. A second generation was developed in order to reduce the protein binding, by replacement of the cyclopentyl group with an aromatic ring provided potent compounds. CP-195543, reduces binding to human neutrophils membrane with IC_{50} values of 6.8 nM. It also inhibited the LTB4 evoked human and mouse neutrophils chemotaxis at IC_{50} of 2.4 and 7.5 nM, respectively and blocked CD11b up-regulation on human neutrophils (pA_2 = 7.12) and murine neutrophils (pA_2 = 7.06) with a similar potency [26]. This compound possesses high affinity to BLT1 and BLT2 cloned human receptors [27], and has been reported to reduce the clinical symptoms and attendant weight loss in an IL-1-exacerbated murine model of collagen-induced arthritis [26]. In spite of these effects, a phase II clinical trial for the study in rheumatoid arthritis patients was discontinued, beacuse overall poor tolerability profile and high discontinuation rate when dual therapy with CP-195543 and Celecoxib was administered. Other LTB4 antagonists have been developed as LTB-019 (moxilubant maleate, CGS-25019C), and ONO-4057 however was discontinued due to lack of efficacy *in vivo* COPD or after Phase II trial of ulcerative colitis, respectively [24, 28].

CXCR2 ANTAGONISTS

CXCR1 and CXCR2 are G protein-coupled receptors for several chemoattractant cytokines (chemokines i.e. IL-8). Both CXCR1 and CXCR2 are expressed on human neutrophils and mediate both neutrophil chemotaxis and myeloperoxidase release [29-31]. CXCR2 expression has also been demonstrated on monocytes and alveolar macrophages [32].



Amelubant/BIIL-284



BIIL-260; R=H

BIIL-315; R=α-D-glucoronide

Fig. (1). LTB4 antagonists: BIIL-284, BIIL-260 and BIIL-315.

IL-8 activates neutrophils *via* a specific low-affinity G-protein coupled receptor (receptor for CXC chemokines [CXCR]) coupled to activation and degranulation and *via* a high-affinity receptor (CXCR2) shared with other members of the CXC family, which is important in chemotaxis [33]. GRO-a and ENA-78 may also be involved in neutrophilic inflammation and activate CXCR2. IL-8 levels are highly elevated in the sputum of patients with COPD and are correlated with disease severity [34, 35] and increase during exacerbations [36]. Blocking antibodies to IL-8 and related chemokines inhibit certain types of neutrophilic inflammation in experimental animals [37] and reduce the chemotactic response of neutrophils to sputum from patients with COPD [21, 38].

Small molecule inhibitors of CXCR2, such as SB225002 (Fig. **2**), have been developed and are entering clinical trials. SB 225002 (*N*-(2-hydroxy-4-nitrophenyl)-*N*'-(2-bromophenyl)urea) is the first reported potent and selective non-peptide inhibitor of a chemokine receptor. It is an antagonist of ^{125}I-IL-8 binding to CXCR2 with an IC_{50} = 22 nM. SB 225002 showed >150-fold selectivity for CXCR2 over CXCR1. *In vitro,* SB 225002 potently inhibited human and rabbit neutrophil chemotaxis induced by both IL-8 and GROα. *In vivo,* SB 225002 selectively blocked IL-8-induced neutrophil margination in rabbits [39]. Recently, Schering Plough introduced Sch527123 (2-hydroxy-*N*,*N*-dimethyl-3-{2-[[(*R*)-1-(5-methyl-furan-2-yl)-propyl]amino]-3,4-dioxo-cyclobut-1-enylamino}-benzamide) (Fig. **2**), that inhibited chemokine binding to (and activation of) both CXCR1 and CXCR2 receptors in an insurmountable manner and, as such, is categorized as an allosteric antagonist. Sch527123 inhibited neutrophil chemotaxis and myeloperoxidase release in response to CXCL1 and CXCL8 but had no effect on the response of these cells to C5a or formyl-methionyl-leucyl-phenylalanine. Sch527123 binding to CXCR1 and CXCR2 is saturable and reversible. Although Sch527123 bound to CXCR1 with good affinity (K_d = 3.9 ± 0.3 nM), the compound is CXCR2-selective (K_d = 0.049 ± 0.004 nM) [40]. This compound reduced the neutrophil recruitment, mucus production, and goblet cell hyperplasia in experimental animals models pulmonary inflammation [41]. Sch527123 is in Phase I clinical development for COPD treatment [42].

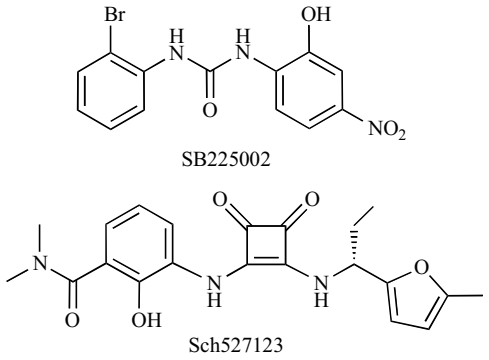


Fig. (2). Structural formula of CXCR antagonists: SB225002 and Sch527123.

E-SELECTIN INHIBITORS

Recruitment of neutrophils into the tissues is dependent on adhesion molecules expressed by these cells and on endothelial cells. Several adhesion molecules can now be inhibited pharmacologically. For example, E-selectin on endothelial cells interacts with sialyl-Lewis^x on neutrophils. A mimic of sialyl-Lewis^x, bimosiamose (TBC-1269), decreases neutrophils adhesion in P-selectin-coated plates [43]. Recently, it has been described as effective in a human allergen challenge model of asthma [44], however, there are concerns about this therapeutic approach for a chronic disease, because an impaired neutrophilic response may increase the susceptibility to infection.

ANAPHYLATOXIN INHIBITORS

The complement fragments anaphylatoxins C3a and C5a are potent neutrophil chemoattractants. Both chronic obstructive pulmonary disease (COPD) and asthma are associated with an abnormal inflammatory response of the lungs. Previous studies suggest that the complement system may also participate in both disorders. In murine, blocking C5a receptors with a neutralizing antibody significantly reduced neutrophil inflammation in the lung after allergen exposure in sensitized animals, whereas blocking C3a with a specific antagonist, SB290157, (Fig. **3**) reduced airway hyperresponsiveness without affecting neutrophils influx [45]. A small peptide 3D53 AcPhe[Orn-Pro-DCha-Trp-Arg], showed an IC_{50} of 60 nM for the inhibition of C5a binding to whole PMNs and 30 nM for the inhibition of PMN degranulation [46]. This peptide has been the most intensively evaluated C5a antagonist with high affinity for dog, cat and rat PMN C5aR (IC_{50}=40 nM) but lower affinity for mouse PMN C5aR (IC_{50}>10 μM) [47]. In order to overcome the problems associated with peptides, some development of cheaper, orally more bioavailable and more target-selective non-peptidic antagonists compounds has taken place. Early non-peptidic ligands were of only low-moderate affinity antagonists for human C5aR, such as Merck's aminoquinolines [48] and Rhone-Poulenc's phenylguanidines such as RPR121154 (Fig. **3**) (IC_{50}=0.8 μM), which completely inhibited the respiratory burst response of human neutrophils to 100 nM C5a [49]. The basic nature of RPR121154 suggests that it may mimic a positively charged receptor-binding site in the core domain of C5a, although there is no evidence for this mechanism. Merck reported several other structural types of antagonists with submicromolar potencies [50], but they were not developed further due to partial agonist responses. The optimization of a series of substituted phenylguanidines led to Mitsubishi Pharma's to develop tetrahydronaphthalene-based compound W54011 (Fig. **3**), which is a competitive non-peptidic C5aR antagonist ([^{125}I]hC5a IC_{50}=2.2 nM) in human neutrophils, that inhibits intracellular Ca^{2+} mobilization, chemotaxis and production of reactive oxygen species with IC_{50}=3.1, 2.7 and 1.6 nM, respectively [51]. The combination of potency and oral availability appeared promising but its substantial hydrophobicity and problems with species specificity (active for human, cynomolgus monkey and gerbil but not mouse, rat, guinea pig, rabbit or dog neutrophils) complicated pre-clinical studies. NDT9520492 (Fig. **3**) is a member of a large series of compounds developed by Neurogen Corp with C5aR antagonist activity with a K_i of

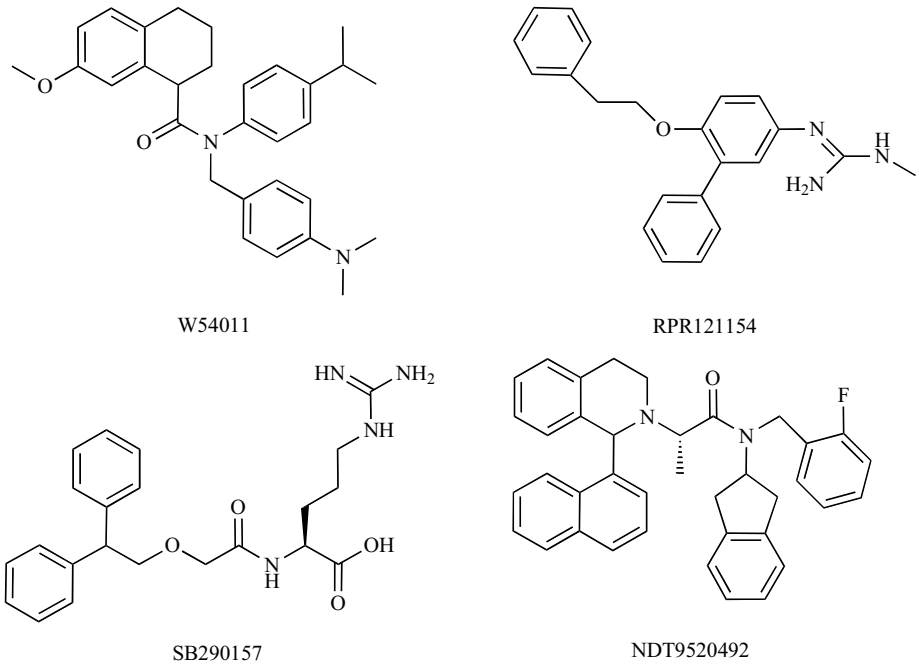


Fig. (3). Anafilotoxin antagonist: W54011, RPR121154, SB290157 and NDT9520492.

28.8 nM [52] in human and 108 nM in gerbil but not mouse C5aR.

LONG-ACTING β2-AGONISTS

In general, there are data reporting mast cell inhibitory effects of long acting β2-agonists (LABAs). β2-adrenergic receptors are present on neutrophils, and LABAs have been shown to affect neutrophil numbers, activity, and function [53]. Salmeterol is a long acting β2-receptor agonist, used in asthma and COPD because of its potent bronchodilatory properties in combination with inhaled corticosteroid therapy [54]. Clinical studies indicate that 50 μg of salmeterol *b.i.d* during 6-week [55] and/or 12-week period [56] may cause reduction in both the number and activation of neutrophils, concentrations of myeloperoxidase, soluble E-selectin and IL-8 in serum, each of which reflect neutrophil involvement, [55, 56]. Also, salmeterol may directly inhibit the stimulated respiratory burst of neutrophils [57, 58] independent of increase in cAMP levels [59] affecting the cell adhesion to human bronchial epithelial cells [60], accompanied by a reduced expression of CD11b on the plasmatic membrane [61]. A recent study showed that salmeterol with fluticasone does not influence the release of elastase, MMP-2 and MMP-9, but enhances the suppression of IL-8 and increases the translocation of glucocorticoid receptor in human neutrophils stimulated with cigarette smoke [62]. Finally, the response to salmeterol does not vary between ADRB2 genotypes after chronic dosing with an inhaled corticosteroid [63]. In rats, formoterol–another long acting β2-receptor agonist can reduce the amount of neutrophils that adhere to the vascular endothelium at sites of inflammation, and this effect was blocked by the β2-receptor antagonist ICI 181551 [64]. Formoterol appeared to reduce the inflammatory cell numbers in the bronchial submucosa and epithelium in mildly asthmatic patients [65] and significantly reduced sputum IL-8 concentrations and neutrophils number [66].

Recently, Novartis developed Indacaterol, a novel once-daily inhaled beta2-agonist, demonstrating sustained bronchodilator efficacy in patients with persistent asthma [67] and COPD [68].

PDE4 INHIBITORS

Phosphodiesterase 4 (PDE4) is the predominant PDE expressed in neutrophils [69, 70]. The selective inhibition of PDE would be effective in the treatment of inflammatory diseases [71]. PDE4 inhibitors suppress multiple neutrophil responses through the modulation of cAMP and Ca^{2+} levels [69, 70, 72], including the production of IL-8, LB4, superoxide anions, degranulation, chemotaxis, adhesion [71, 73] and CD11b expression [70]. PDE4 inhibitors also antagonize proapoptotic signals in neutrophils [70, 74] and this effect was enhanced by salbutamol [75]. The two main orally active PDE4 inhibitors are cilomilast and roflumilast (Fig. **4**). The potency of cilomilast is similar to rolipram, with a PDE4 IC_{50} of 95 nM and TNF-α production IC_{50} of 110 nM. This compound has also been shown to inhibit degranulation of neutrophils [76]. An *in vitro* study showed that PDE4 inhibitors significantly suppressed the myeloperoxidase, elastase and MMP-9 release from neutrophils in presence and absence of TNF-α, and this effect was not shared by PDE3 inhibitors or theophylline [77]. COPD patients treated with cilomilast showed a significant reduction in sub-epithelial neutrophils number [78]. Roflumilast followed cilomilast into the clinic and it is in development for asthma as well as COPD. Roflumilast is more selective and potent with a superior therapeutic ratio. It is approximately 100-fold more potent than cilomilast at the enzymatic level [79], with a PDE4 IC_{50} of 800 pM [71]. In the sputum of COPD patients, roflumilast significantly reduced the absolute number of neutrophils, soluble interleukin-8 and neutrophil elastase [80]. The dose of PDE4 inhibitors is limited by side effects, particularly nausea, diarrhea and abdominal pain [69, 76].

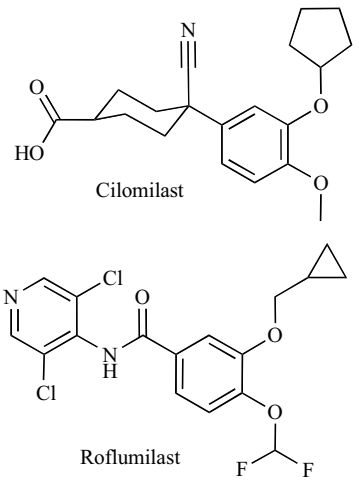


Fig. (4). Compound PDE4 inhibitors: Cilomilast and Roflumilast.

NF-κB INHIBITORS

NF-κB is a transcription factor that controls gene expression during inflammation, immunity, cell proliferation, stress response, and apoptosis [81-83]. NF-κB activation increases expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1, while NF-κB inhibition reduces leukocyte adhesion and transmigration [84].

Its participation has been well demonstrated and its activation has been shown to have a role in rheumatoid arthritis [85-87] and in human inflammatory airway disease [88]. In these patients an increased number of neutrophils in arthritic joints and bronchial tissue have also been reported.

The basic molecular biology of the NF-κB activation pathway is well described. NF-κB is sequestered within the cytosol by the inhibitory protein IκB (inhibitor of NF-κB) that masks the nuclear localization signal present within the NF-κB protein sequence. Treatment of cells with proinflammatory cytokines or with bacterial products leads to the activation of a specific IκB-kinase (IKK) complex that phosphorylates IκB and thereby tags it for ubiquitination and degradation by the proteasome. The degradation of IκB thus allows NF-κB to translocate into the nucleus where it can act as a transcription factor [89, 90]. Approaches to modify this axis have involved inhibition of various components of the classical activation pathway, including ubiquitination and proteosomal degradation of IκB. Several drugs used to treat human inflammatory disease have been described to inhibit NF-κB activation [91]. Nonsteroidal anti-inflammatory drugs decrease IKKß kinase activity or inhibit the binding of NF-κB to DNA [92], salycilates block the ATP binding site of IKKß [93], glucocorticoids inhibit the ability to transactivate the expression of proinflammatory genes [94] and antioxidants reduce reactive oxygen species production, which can activate NF-κB [81]. Compounds that inhibit proteasome decrease proteasome-dependent degradation of IκBα, and antisense oligodeoxynucleotides targeting the NF-κB p50 and p65 subunit inhibit expression of NF-κB proteins, have also been reported to inhibit NF-κB [81]. Natural compounds such as Andrographolide (Fig. **5**), a labdane diterpenic from *Andrographis paniculata*, at 50 and 100 μM interferes with binding of NF-κB to DNA in HL-60-derived neutrophilic cells stimulated with PAF or fMLP [95] and endothelial cells [96] reducing COX-2 expression. One of the main goals to control NF-κB activity has been to develop small molecular inhibitors of IKK2, because it is considered to be the functionally important kinase and it also plays a pivotal role in transmitting various different stimuli into an NF-κB response. Most of the inhibitors identified can inhibit both IKK1 and IKK2, often inhibiting IKK2 in the nano-molar range and IKK1 in the micro-molar range. These inhibitors are considered to be selective for IKK2 at therapeutic doses. In contrast, no specific inhibitors of IKK1 have been described. Several IKK2 inhibitors have been identified, such as ATP-competitive inhibitors SPC-839 and SC-514, non-competitive inhibitor BMS-345541, TPCA-1 and ML120B (Fig. **5**) [97-100]. One concern about long-term inhibition of NF-κB is that effective inhibitors may result in immune suppression and impair host defenses, because mice that lack NF-κB genes succumb to septicemia. Moreover, knockout mice experiments in animals lacking IKK2 die early during embryogenesis with liver degeneration [101]. It may therefore be necessary to deliver IKK-2 inhibitors *via* the inhaled route [69].

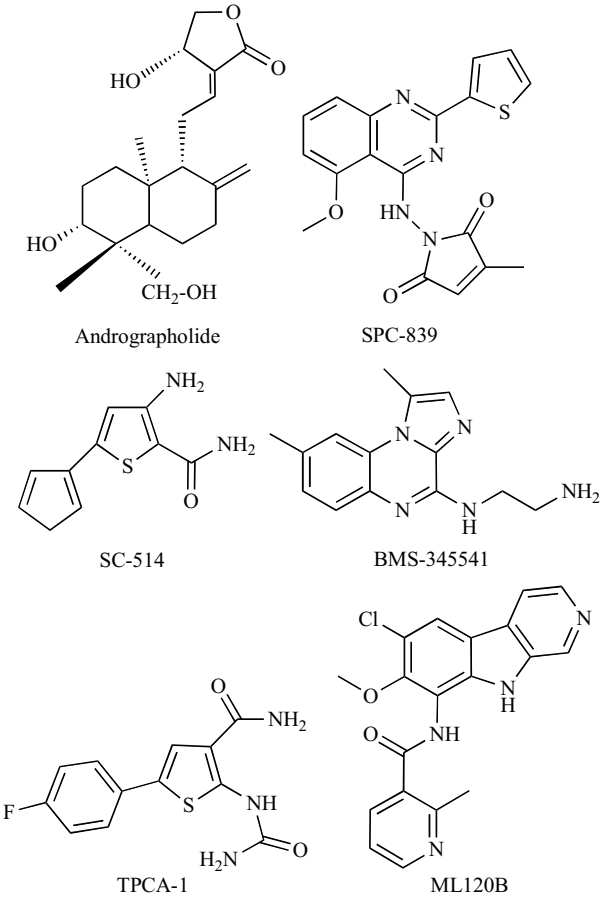


Fig. (5). NFκB pathway inhibitors: andrographolide (DNA binding inhibitor), IKK2 ATP-competitive inhibitors SPC-839 and SC-514, IKK2 non-competitive inhibitor BMS-345541, TPCA-1 and ML120B.

HDAC2 ACTIVATORS

Transcription factors such as NF-κB or AP1 regulate the expression of multiple inflammatory genes and play a pivotal role in chronic inflammatory diseases, such as asthma and COPD [102, 103]. Glucocorticoids activate glucocorticoid receptors (GR), which act as transcription factors and inhibit transcription induced by NF-κB and AP-1. Activation of genes involves hyperacetylation of core histones to open up the chromatin structure to initiate transcription. GR recruit histone deacetylase-2 (HDAC2) to the activated inflammatory gene to switch off transcription [104, 105]. Ito and coworkers demonstrated that recruitment of HDAC2 by activated GR to the hyperacetylated coactivators reverses the upregulation of genes induced by NF-κB [106, 107]. Corticosteroid resistance is one of the typical features of COPD. An alternative therapeutic strategy is therefore to reverse the molecular mechanism of this resistance, which seems to be due to a defect in the nuclear HDAC2 [108]. This can be achieved *in vitro* with theophylline, which is an HDAC activator, or by inhibiting oxidative or nitrative stress [105]. Therapeutic concentrations of theophylline (10^{-6} – 10^{-5} M) significantly reduced the number of neutrophils and enhanced the suppression of IL8 by dexamethasone in patients with COPD. The effect was blocked by the HDAC inhibitor trichostatin A [109]. In a clinical study, patients with COPD were treated with theophylline, at plasma concentrations of 9-11 mg/L for 4 weeks. Induced sputum inflammatory cells, neutrophils, IL-8, myeloperoxidase, and lactoferrin were all significantly reduced by about 22% by theophylline. Moreover, neutrophils from a healthy donor also showed reduced chemotaxis (~30%) [110, 111]. The mechanism whereby theophylline activates HDAC is currently being explored, but it is known that it is not mediated *via* inhibition of phosphodiesterases [69, 111].

P38 MAP KINASE INHIBITORS

p38 MAPK is a family of proteins with four distinct isoforms identified in mammalian cells, of which only p38α and p38δ were detected in neutrophils [112]. p38α MAPK activation induced by LPS regulates at least three distinctly different functions in neutrophils: adhesion, activation of NF-κB, and the synthesis of TNF-α [112] .

The inhibition of p38 MAPK may be an attractive target to limit inflammatory responses because this pathway has an important role in neutrophil polarization and motility. Inflammatory stimulus initiates a stop signal through a p38 MAPK pathway, which may promote the retention of neutrophils in inflammatory sites. In fact, inhibition of p38 MAPK by the pyridinyl imidazoles SB203580 blocked LPS-induced adhesion, NF-κB activation, and synthesis of TNF-α [112]. SB202190, another p38 MAPK inhibitor, reduced neutrophil spreading and enhanced neutrophil polarization and neutrophil migration rates induced by TNF-α and IL-8 [113, 114]. SB239063, a potent and selective inhibitor of p38 MAPK, reduces proinflammmatory cytokine production that leads to diminished neutrophil trafficking and activation in the lung [115].

Activation of the p38 MAPK pathway appears to be involved in the pathogenesis of COPD. Recently, an increase in the active form of p38 MAPK in the lungs of COPD patients compared with controls has been described [116], suggesting the potential role of p38 MAPK inhibitors as a therapy for COPD. In this sense, two clinical trials have been completed to evaluate efficacy and safety of the p38 MAPK inhibitor SB-681323 (NCT00144859, from US National Institutes of Health), however at present, the results have not been revealed. SD-282, a selective inhibitor of p38α MAPK, markedly reduced inflammatory responses in a model of tobacco smoke-induced pulmonary inflammation in A/J mice. SD-282 inhibited tobacco smoke-induced increases in macrophages, neutrophils, cyclooxygenase-2 and interleukin-6 levels, and phospho-p38 expression in the lungs [117].

Several p38 MAPK inhibitors have progressed to testing in clinical trials in rheumatoid arthritis, Crohn's disease, pain, acute coronary syndrome and psoriasis, however, some of these candidates has failed, for safety, but several have reported clinical data [118]. VX-745 and VX-702, selective p38 MAPK inhibitors being developed by Vertex and Kissei have been evaluated. VX-745 was discontinued because side-effects were reported such as rash, infection, and gastrointestinal intolerance [118]. VX-702 was evaluated in a Phase 2 clinical study of approximately 130 patients with moderate to severe RA in combination with methotrexate for 3 months [119], however, the results have not been published yet by the company.

PI3Kγ INHIBITORS

There are multiple isoforms of PI3K, divided into four classes (Ia, Ib, II and III) [120]. Of these four classes, only class Ia and Ib have been implicated in chemotaxis. In particular, one member of the class Ia group (PI3Kδ) and the sole class Ib member (PI3Kγ) have been identified as playing central roles in neutrophil chemotaxis. Although PI3K has been established to be a central pathway in the chemotaxis of some cell types, however, the role in chemotaxis of neutrophils has been controversial. Some authors have proposed that PI3K activity is a total or partial requirement for migration towards formyl-met-leu-phe peptide (fMLP), a commonly used peptide to study chemotaxis. However, it has also been demonstrated that chemotaxis towards fMLP is independent of PI3K. Recently, it has been described that PI3K can enhance early responses to the bacterial chemoattractant fMLP, and it is not required for migration towards this chemoattractant [121]. More clearly, it has been extensively established that PI3K regulates the activation of NADPH oxidase in neutrophils [122-124].

The biochemical and physiologic role of PI3Kγ has been assessed using PI3K$\gamma^{-/-}$ mice. In neutrophils from these mice, IL-8 is unable to stimulate accumulation of PtdIns(3,4,5)P3, which correlates with a marked reduction of neutrophil recruitment in response to IL-8. Chemoattractant-stimulated PI3K$\gamma^{-/-}$ neutrophils displayed impaired respiratory burst and motility. The genetic knock-out of PI3Kγ has resulted in varying and incomplete inhibition of chemokine-stimulated cell migration, suggesting that alternative mechanisms can still sustain varying amounts of cell migration depending on the chemokine investigated [125].

Recent studies demonstrate the role of PI3Kγ in autoimmune disorder, in which neutrophil infiltration is required for tissue injury. The blockade of PI3Kγ has potential therapeu-

tic value in the treatment of chronic inflammatory conditions where neutrophil infiltration is observed, such as inflammatory arthritis [126].

PI3K inhibitors reduce chemoattractant-induced motility of four kinds of leukocyte, including neutrophils, macrophages, T cells and natural killer cells [127]. Classical inhibitors of PI3K are Wortmannin and LY294002. Wortmannin is a well studied isoform non-selective PI3K inhibitor; in neutrophil it abolishes several responses such as the formyl peptide-induced stimulation of respiratory-burst, cytoskeletal rearrangements, MAPK activation induced by PAF, and intracellular alkalinization [95, 128-130]. LY294002 (2-(4-morpholinyl)-8-phenylchromone) completely abolishes PtdIns 3-kinase activity in fMet-Leu-Phe-stimulated human neutrophils [131]. *In vivo* studies using a murine model of peritoneal chemotaxis demonstrated that LY294002 reduces neutrophil chemotaxis during early steps of inflammation [132].

A new chemical series of small-molecule inhibitors of PI3Kγ has been described; AS-604850 and AS-605240 are isoform-selective inhibitors of PI3Kγ, in contrast to the well-known class I PI3K inhibitors wortmannin and LY294002, and are ATP-competitive inhibitors. Oral treatment with a PI3Kγ inhibitor suppresses the progression of joint inflammation and damage in two distinct mouse models of rheumatoid arthritis [133]. AS041164, (5-benzo[1,3]dioxol-5-ylmethylene-thiazolidine-2,4-dione), a selective PI3Kγ inhibitor, showed three times more potent ability than LY294002 in reducing neutrophil recruitment *in vivo* [132].

Only a few clinical trials with Akt inhibitors have been reported up to now, while no clinical trials using PI3K inhibitors have been published. The PI3K/Akt pathway is a prototypic survival pathway that is constitutively activated in many types of cancer, therefore PI3K/Akt pathway inhibitors have been used as single agents and in combination with other therapies [134]. However, the activity of single Akt inhibitors, in solid tumors has been disappointing, and gastrointestinal and constitutional toxicities were problematic, particularly in a trial in advanced pancreatic cancer [134, 135].

ANTIOXIDANTS

Overproduction of free radicals, specifically ROS and release of proteolytic enzymes are common features of response produced by neutrophils in the lungs of COPD patients. Pharmacological interventions to reduce the inflammatory processes can be obtained using molecules known to affect these responses, such as N-acetylcysteine (NAC). NAC inhibits the release of elastase, IL-8 and respiratory burst in phorbol myristate acetate (PMA) and fMLP-induced neutrophil [136], transepithelial migration in response to LTB4 [137], and TNF-α and macrophage inflammatory protein-2 production induced by paraquat, an agent that specifically increases intracellular superoxide [138]. The compounds 4-(2-aminoethyl)benzenesulphonyl fluoride and 4-hydroxy-3-methoxyaceto-phenone, two NADPH oxidase inhibitors, cancelled the anti-IgE-induced COX-2 protein up-regulation in neutrophils [139]. Several flavonols (fisetin, morin and quercetin) also reduce oxidative burst in human polymorphonuclear neutrophils stimulated by fMLP [140]. The NF-κB pathway, reported to be activated by ROS, represents an attractive therapeutic target for antioxidants as a strategy to control neutrophilic inflammation and lung injury. Treatment *in vivo* with NAC reduces NF-κB activation and chemoattractant mRNA expression in lung tissue, and neutrophilic alveolitis [136, 141]. The treatment of neutrophils with NAC or α-tocopherol inhibits the production of proinflammatory cytokines (TNF-α, macrophage inflammatory protein-2, and IL-1β), as well as the degradation of IκBα and increased nuclear accumulation of NF-κB induced by paraquat [138], activation of the kinases IKKα, IKKβ, Akt and p38 and ERK1/2 MAPK in response to Toll-like receptor 4 (TLR4) [142]. Resveratrol, a polyphenol present in red wines and vegetables, reduces generation of superoxide anion, hypochlorous acid and nitric oxide production, and chemotaxis in neutrophils [143, 144].

METALLOPROTEINASE INHIBITORS

Acute respiratory distress syndrome (ARDS), which is the most severe form of acute lung injury (ALI), involves the disruption of the alveolar-capillary barrier, infiltration of inflammatory cells, and production of inflammatory mediators. Matrix metalloproteinases (MMPs), have been reported to increase during the course of ARDS. In ALI, and mainly in ARDS, increased levels of MMP-2 and MMP-9 in the bronchoalveolar lavage or fluid have been suggested to play a role in basement membrane disruption [145]. A histological hallmark of ARDS is the accumulation of neutrophils in the microvasculature of the lung, considered to be central in the pathogenesis of ALI. It has been reported that MMP-9 is a major factor involved in neutrophil migration across basement membranes [146] and that MMP-9 inhibition by MMP-I reduces neutrophil transmigration [147]. Fast degranulation of considerable amounts of intracellularly stored gelatinase B from neutrophils is induced by various types of chemotactic factors. Increased expression of gelatinase B by inflammatory cells e.g. neutrophils and macrophages is correlated with a variety of processes that cause lung damage and is important in cytokine and protease modulation and therefore represents a potential target in COPD and rheumatoid arthritis treatment [148]. Several non peptidic drugs (Fig. **6**), such as BMS-275291 (Bristol-Myers Squibb) and Col-3 (CMT-3) (CollaGenex Pharmaceutical) are effective against MMP-2 and MMP-9; however nonspecific MMP inhibitors, (CGS 27023A, Novartis Pharmaceuticals) have also been discovered. Despite massive research and development efforts, only one MMP inhibitor (Periostat) has been approved by the FDA for the treatment of periodontal disease (Fig. **6**). Possible reasons for the low success rate of MMP inhibitors in the clinic include unwanted side effects caused by their lack of selectivity, poor oral bioavailability and decreased potency *in vivo* [149]. Col-3 (CMT-3) 6-deoxy-6-demethyl-4-dedimethylamino-tetracycline is the most potent gelatinase inhibitor obtained from chemically modified tetracycline [150]. Col-3 inhibits MMP-9 and protects against the development of ventilator-induced lung injury in rats by down-regulation of neutrophil-mediated inflammation [151].

STORE OPERATED CALCIUM ENTRY INHIBITORS

Store-operated calcium (Ca^{2+}) entry" (SOCE) or "capacitative Ca^{2+} entry" is a critical mechanism involved in the

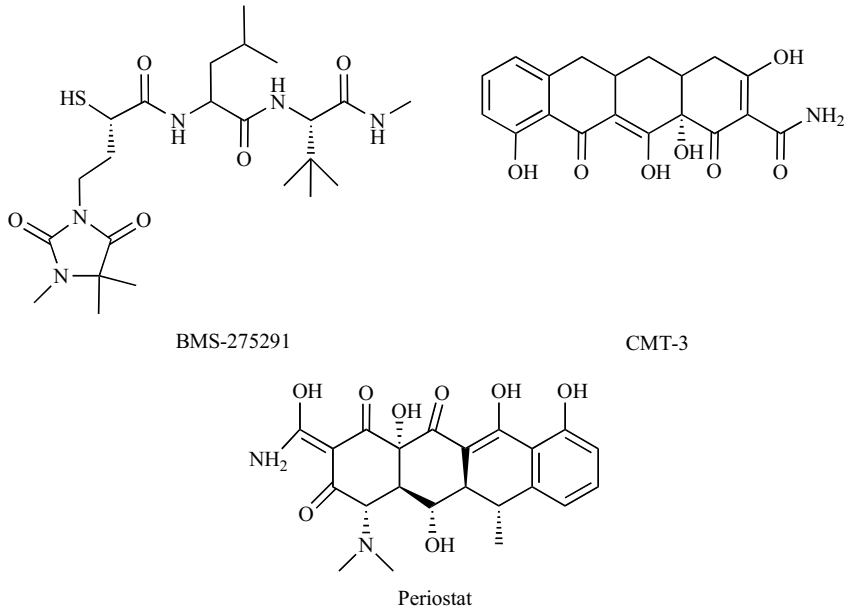


Fig. (6). MMP9 inhibitors BMS-275291, CMT-3 and Periostat.

regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration in nonexcitable cells [152]. The fall in Ca^{2+} concentration within the lumen of the Ca^{2+}-storing organelles (most commonly, components of the endoplasmic reticulum) activates plasma membrane Ca^{2+} channels. The retrograde process by which plasma membrane Ca^{2+} channels are signaled by the endoplasmic reticulum has been called SOCE [153]. The roles of the SOCE phenomenon in polymorphonuclear neutrophil (PMN) functions as well as other nonexcitable cells have remained controversial, as the potential mechanisms have been almost exclusively studied in heterologous expression systems rather than in native cells.

Despite the above, it has been proposed that SOCE controls a wide variety of cellular functions such as cell proliferation, cell death, and enzymatic activity [153], and in PMN, it controls respiratory burst, degranulation, and motility [154-157]. The transient receptor potential (TRP) and related proteins are the candidate channels for SOCE phenomenon. Recent work has implicated calcium channel proteins of the TRP superfamily in the mediation of SOCE in human neutrophils [158-162]. However, recently two new proteins STIM1 (the ER Ca^{2+} sensor) and Orai1 (the Ca^{2+} channel), have been proposed as the missing links in SOCE [163, 164].

SOCE putative inhibitors such as 2-APB, BTP2, SKF96365, capsaicin and flufenamic acid have been proposed (Fig. **7**). 2-APB reduces the influx of calcium induced by PAF [165] or fMLP [166] in human neutrophils, however, these authors proposed that the effect of 2-APB is by inhibiting $InsP_3$ receptors from the endoplasmic reticulum. Nevertheless, several reports indicate that the principal antagonistic effect of 2-APB is on Ca^{2+} entry rather than Ca^{2+} release, and for this reason, this compound is also used as SOCE inhibitors in nonexcitable cells [167]. We recently demonstrated that 2-APB selectively reduced SOCE in bovine neutrophils activated by PAF and thapsigargin, reducing the PI3K and ERK1/2 pathways activation [168]. The inhibition of SOCE also reduced the intracellular alkalinization induced by PAF or propionate in bovine neutrophils [168, 169].

Capsaicin is a well known SOCE inhibitor in neutrophils-like HL-60 (human promyelocytic leukemia) cells; moreover, this compound can interfere with the superoxide production induced by PAF [170], a SOCE dependant response [156].

Flufenamic acid also inhibits SOCE phenomenon and has been described earlier in human neutrophils treated with fMLP, A23187 and scarcely with thapsigargin [155, 171]. Recently, BTP-2 has been proposed as a SOCE inhibitor in human neutrophils at doses of 10 μM, however, in bovine neutrophils, BTP2 did not reduce the area under curve or maximum $[Ca^{2+}]_i$ peak induced by PAF. On the contrary, it produced an increase of $[Ca^{2+}]_i$ peak in the absence of external calcium thus, suggesting an effect on intracellular calcium release [168], limiting its potential use. Another SOCE putative inhibitor described in neutrophils is SKF96365 [172, 173]. This compound reduced $[Ca^{2+}]_i$ peak at doses of 25 μM in the presence or absence of external calcium, but did not inhibit the area under curve induced by PAF. Recently, SKF96365 has been considered as a non selective SOCE inhibitor, because it also produces inhibition of voltage operated calcium entry, receptor operated calcium entry and $[Ca^{2+}]_i$ release [173], and shows contradictory effect such as calcium influx or intracellular calcium increase [174].

Although 1,4-dyhidropyridines (DHPs) have been proposed as inhibitors of SOCE in HL-60 cells, the most potent DHPs were those containing a 4-phenyl group with an electron-withdrawing substituent, such as *m*- or *p*-nitro or *m*-trifluoromethyl (IC_{50} values: 2-6 μM). The most suitable compounds for the development of selective compounds

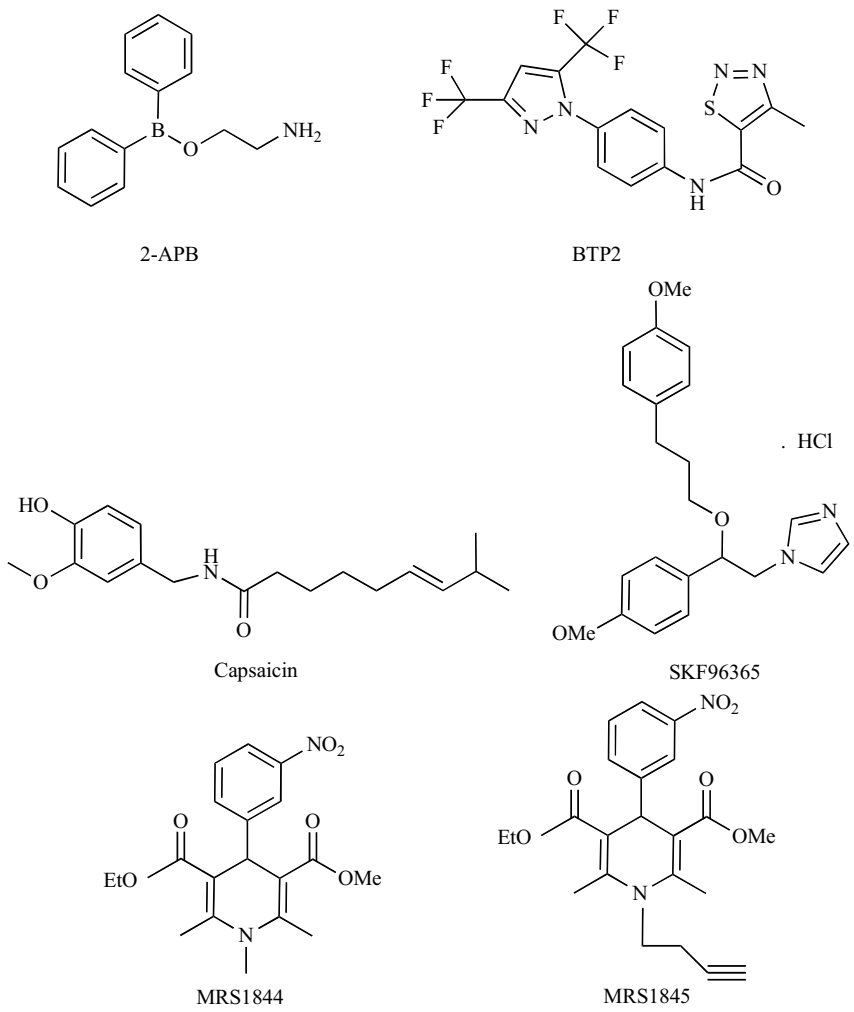


Fig. (7). SOCE putative inhibitors: 2-APB, BTP2, capsaicin, SKF96365, MRS1844 and MRS1845.

were N-Methylnitrendipine (MRS1844) and N.propargylnifrendipine (MRS1845) [175] (Fig. **7**).

BRADYKININ B_1 RECEPTOR ANTAGONISTS

Kinins are an important group of short-lived peptide hormones that act close to their site of formation in a paracrine manner. The nonapeptide bradykinin has been recognized as an inflammatory mediator, since it can reproduce the four classic signs of inflammation. It produces a local endothelium-dependent vasodilatation and widening of intercellular junctions between endothelial cells forming postcapillary venules, an effect that results in edema formation. Additionally, bradykinin has been considered the most potent pain-producing substance when applied to a blister base or when injected intradermally [176]. Kinins bind to two types of G protein-coupled receptors known as B_1 (B_1R) and B_2 receptors. Their stimulation triggers a number of common intracellular routes that may include phospholipase C, inositol 3-phosphate, Ca^{2+} mobilization and prostaglandins and nitric oxide formation [177].

The B_1R is over-expressed during inflammation or by cytokines such as IL-1β and TNF-α. Bradykinin (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) is the preferred ligand for B_2R whereas the B_1R has major affinity for Des-Arg^9 metabolites of the parent bradykinin molecule, being Lys-DesArg^9-bradykinin its natural ligand. Experiments in the mouse indicated that a B_1R antagonist [178] attenuates neutrophil accumulation, in response to IL-1β application. Furthermore, B_1R knockout mice have a decreased number of cells migrating into the inflamed tissue [179] and *in vitro* stimulation with 10^{-10} M of a B_1R agonist specifically induces neutrophil chemotaxis [180].

Since the description of the bradykinin structure in the 60s' many analogs has been synthesized. Some of them potent and/or resistant antagonists such as Lys-DesArg^9[Leu^8]-bradykinin, B-9858 (Lys-Lys[Hyp^3, Igl^5, D-Igl^7, Oic^8]Des Arg^9-bradykinin), and B-9958 (Lys-Lys[Hyp^3, CpG^5, D-Tic^7, CpG^8]DesArg^9-bradykinin) have been developed [181-183]. In addition, orally presented non-peptide B_1R antagonists have been constructed. Two examples are the antagonists called compound 12 and SSR240612 [184]. Construction of a B_1R knockout mouse has provided key evidence to implicate the B_1R as a key player of inflammation [185] that may be involved in the complex network controlling diseases, such as asthma, arthritis, sepsis and pain. Experimental studies have shown that B_1R antagonists or B_1R gene deletion

can reduce inflammatory and hemodynamic events following exposure to LPS or prevent endotoxic shock, respectively [186-188]. The B_1R antagonists can also reduce arthritis induced by peptidoglycan-polysaccharide injection [189] and lung inflammation caused by ovalbumin, immune complexes or carrageenan and can block nasal hyperresponsiveness to leukotriene D4 [190-193]. Despite these and many other experimental studies, human trials using B_1R antagonists have not yet been accomplished and it is not possible to envisage the efficacy of B_1R antagonists, including those orally active in human pathological processes as well as the side effects that its long-term use may produce.

CONCLUSIONS AND PERSPECTIVES

Current research on the mechanisms of neutrophilic inflammation is opening an exciting era of new experimental therapeutics. Characterization of innate immune response during inflammation in COPD and rheumatoid arthritis is contributing to our understanding about the role of neutrophils in the onset of tissue injury produced in these diseases. The effects of various different compounds (Table **1**), each with different chemical structures, on neutrophils functions indicate potentially fruitful therapeutic approaches to reverse or to stop the tissue damage, and give rise to the assumption

Table 1. Summary of Potential New Drug Candidates in the Control of Neutrophil Activity

Compound	Target	Status	Reference
BAYx1005	5-LO inhibitor	Clinical development (COPD)	[20-22]
Amelubant	LTB 4 receptor antagonist	Preclinical and clinical developments (RA, COPD and cystic fibrosis)	[23, 24]
LY293111	LTB4 antagonist	Preclinical and clinical developments (asthma, inflammatory bowel disease, RA and cancer)	[24, 25]
CP-195543	LTB4 antagonist	Preclinical and clinical developments (RA)	[26, 27]
SB225002	CXCR2 inhibitor	Preclinical development	[39]
Sch527123	CXCR2 antagonist	Preclinical and clinical developments (COPD)	[40-42]
3D53 AcPhe	C5aR antagonist	Preclinical development	[46, 47]
NDT9520492	C5aR antagonist	Preclinical development	[52]
Salmeterol	LABA agonist	Preclinical and clinical developments (asthma)	[54-63]
Formoterol	LABA agonist	Preclinical and clinical developments (asthma)	[64-66]
Indacaterol	LABA agonist	Clinical developments (asthma, COPD)	[67, 68]
Cilomilast, roflumilast	PDE4 inhibitors	Clinical developments (COPD)	[76- 80
Andrographolide	NF-κB DNA binding inhibitor	Preclinical development	[95, 96]
SPC-839, SC-514, BMS-345541, TPCA-1 , ML120B	IKK2 inhibitor	Preclinical development	[97-100]
Theophylline	HDAC activator	Clinical development (COPD)	[109-111]
SB203580, SB202190, SB239063, SD-282	p38 MAPK inhibitor	Preclinical development	[112-115, 117]
SB-681323	p38 MAPK inhibitor	Clinical development (COPD)	NCT00144859, NIH
VX-745 and VX-702	p38 MAPK inhibitor	Clinical development (rheumatoid arthritis)	[118, 119]
Wortmannin, LY294002, AS-604850, AS-605240 AS041164	PI3K inhibitor	Preclinical development	[95, 128-133]
BMS-275291, Col-3	MMPs inhibitor	Preclinical development	[150, 151]
Periostat	MMPs inhibitor	Clinical development (periodontal disease)	[149]
2-APB, SKF96365, capsaicin, flufenamic acid	SOCE inhibitor	Preclinical development	[165-174]
R 892, B-9958, SSR240612	B1R antagonist	Preclinical development	[181-184]

that more safety and new classes of targets may soon emerge.

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ABBREVIATIONS

5-LO	=	5'-lipoxygenase
ALI	=	Acute lung injury
ARDS	=	Acute respiratory distress syndrome
BLT	=	Leukotriene B4 receptor
COPD	=	Chronic obstructive pulmonary disease
COX-2	=	Ciclooxygenase-2
fMLP	=	formyl-met-leu-phe peptide
GR	=	Glucocorticoid receptor
HDAC2	=	Histone deacetylase-2
IκB	=	Inhibitor of NF-κB
IKK	=	IκB-kinase
IL	=	Interleukin
LTB4	=	Leukotriene B4
MMPs	=	Matrix metalloproteinases
NAC	=	N-acetylcysteine
PAF	=	Platelet activating factor
RA	=	Rheumatoid arthritis
ROS	=	Radical oxygen species
SOCE	=	Store-operated calcium entry
TNF	=	Tumor necrosis factor

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