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Suppression of respiratory burst in human neutrophils by new synthetic pyrrolo-benzylisoquinolines

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Abstract

Reactive oxygen species produced by neutrophils contribute to the pathogenesis of inflammatory diseases. In this study, the inhibition of superoxide anion ($O_2^{\bullet-}$) generation in human neutrophils by new synthetic pyrrolo-benzylisoquinoline derivatives was determined. We found that KW-2, KW-5, and KW-7 (8,9-dimethoxyl-1-(*R*-phenyl)-5,6-dihydro-pyrrolo[2,1-*a*]isoquinoline-2,3-dione; where *R* is 3-chloro, 3-bromo, and 4-methoxy, respectively) were the most effective inhibitors of formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-induced $O_2^{\bullet-}$ release in human neutrophils. KW-2, KW-5, and KW-7 displayed no antioxidant or $O_2^{\bullet-}$-scavenging ability. The inhibition of $O_2^{\bullet-}$ generation was reversed by the protein kinase (PK)A inhibitor, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89), but not by the PKG inhibitor (8*R*,9*S*,11*S*)-(−)-2-methyl-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cyclocta(cde)trinen-1-one (KT5823), or the soluble guanylate cyclase (sGC) inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ). KW derivatives increased cellular cyclic AMP concentrations through the inhibition of phosphodiesterase (PDE) activity but not the elevation of adenylate cyclase (AC) activity. These results indicate that inhibition of FMLP-induced respiratory burst in human neutrophils by KW derivatives are cyclic AMP/PKA-dependent and are due to inhibition of PDE. The new chemical skeleton of PDE inhibitors may protect against the progression of inflammation.

Keywords: Adenylate cyclase; Cyclic AMP; Neutrophil; Phosphodiesterase; Protein kinase A; Pyrrolo-benzylisoquinoline

1. Introduction

Neutrophils play important roles in a host's defenses against invasion by microorganisms and are extensively involved in inflammatory processes. In response to diverse stimuli, activated neutrophils exhibit adhesion, chemotaxis, degranulation, and $O_2^{\bullet-}$ production [1,2]. Elevation of intracellular cyclic AMP levels is believed to suppress the activation of neutrophils. For example, agents that increase intracellular cyclic AMP have been widely recognized to diminish respiratory burst from stimulated neutrophils [3–6]. Increases in intracellular cyclic AMP concentrations by the inhibition of PDE have been shown to modulate the inflammatory response [7]. The clinical potential of cyclic AMP-elevating agents as inhibitors of neutrophil activities is supported by the suppression of endotoxin-induced acute lung injury in mice by the PDE

Abbreviations: AC, adenylate cyclase; CB, cytochalasin B; cyclic AMP, cyclic adenosine 3′,5′-monophosphate; cyclic GMP, cyclic guanosine 3′,5′-monophosphate; DMSO, dimethyl sulfoxide; FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine; H89, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; KT5823, (8*R*,9*S*,11*S*)-(−)-2-methyl-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cyclocta(cde)trinen-1-one; $O_2^{\bullet-}$, superoxide anion; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; Ro318220, 3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

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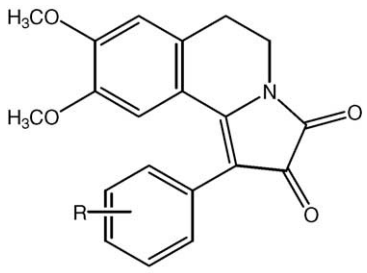
inhibitor, rolipram [8], and the anti-inflammatory activity of the new-generation PDE inhibitors, SB 207499 and AWD 12–281, in experimental asthma and chronic obstructive pulmonary disease [9,10].

Benzylisoquinolines, a class of the most commonly isolated alkaloids in higher plants, have shown several biological activities such as muscle relaxation, analgesia, anticancer, cardiovascular activities, as well as anti-inflammation [11–16]. Among the benzylisoquinoline derivatives, pyrrolo-benzylisoquinolines were prepared as intermediates in the total synthesis of aporphines and protoberberines. The pyrrolo-benzylisoquinolines have been found to inhibit platelet aggregation [17]. However, the biological activities of these compounds in human neutrophils are unclear. Herein, we found that pyrrolo-benzylisoquinolines inhibited FMLP-induced $O_2^{\bullet-}$ production in human neutrophils. $O_2^{\bullet-}$ is considered to play an important role in injury to some organs in which inflammation occurs. Therefore, finding a modulator of $O_2^{\bullet-}$ generation to prevent human diseases would be beneficial. In the present study, cellular mechanisms of pyrrolo-benzylisoquinoline derivatives in the inhibition of respiratory burst in human neutrophils were investigated. The results indicated that the chemical skeleton of pyrrolo-benzylisoquinolines may protect against the progression of inflammation through the inhibition of PDE activities in human neutrophils.

2. Materials and methods

2.1. Materials

Pyrrolo-benzylisoquinolines were chemically synthesized as described previously (Fig. 1) [17] and were dissolved in DMSO to make stock solutions. Hanks' balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA). H89, KT5823 Ro318220 and rolipram were obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.4% and did not affect the parameters measured.



KW-1	R=4-Cl
KW-2	R=3-Cl
KW-3	R=2-Cl
KW-4	R=4-Br
KW-5	R=3-Br
KW-6	R=2-Br
KW-7	R=4-H_3CO
KW-8	R=3-H_3CO
KW-9	R=2-H_3CO

Fig. 1. Chemical structures of the KW derivatives.

2.2. Preparation of human neutrophils

Human neutrophils from venous blood of healthy, adult volunteers (18–32 years old) were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes [18]. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in HBSS buffer at pH 7.4, and kept at 4 °C before use.

2.3. Measurement of $O_2^{\bullet-}$ generation

The assay of the generation of $O_2^{\bullet-}$ was based on the SOD-inhibitable reduction of ferricytochrome *c* [19]. In brief, after supplementation with ferricytochrome *c* (0.5 mg/ml), neutrophils (10^6/ml) were equilibrated at 37 °C for 2 min and incubated with either control or KW derivatives for 5 min. Cells were activated by FMLP (100 nM) or PMA (10 nM) for 10 min. When FMLP was used as a stimulant, CB (1 μg/ml) was incubated for 3 min before activation by peptide. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\varepsilon = 21.1$/mM/10 mm).

2.4. $O_2^{\bullet-}$-scavenging activity

The $O_2^{\bullet-}$-scavenging ability of KW derivatives was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method [20]. After 0.1 mM xanthine was added to the assay buffer (50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase) for 15 min at 30 °C, the absorbance associated with $O_2^{\bullet-}$-induced WST-1 reduction was measured at 450 nm. The effects of KW derivatives on xanthine oxidase activity were also determined by measuring the absorbance of uric acid at 290 nm.

2.5. DPPH-scavenging activity

An ethanol solution of the stable nitrogen-centered free radical, DPPH (100 μM), was incubated with KW deriva-

tives or α-tocopherol for 16 min at 25 °C, and the absorbance was measured at 517 nM.

2.6. Cyclic AMP concentration assay

Cyclic AMP levels were assayed using enzyme immunoassay kits (Amersham Pharmacia Biotech). The reaction of neutrophils was terminated by adding 0.5% dodecytrimethylammonium bromide. Samples were then centrifuged at 3000 × *g* for 5 min at 4 °C. The supernatants were used as a source for the cyclic AMP samples. The assay was performed according to the manufacturer's instructions.

2.7. AC and PDE activity assay

Neutrophils (5×10^7 cells/ml) were sonicated in an ice-cold buffer, containing 25 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, and 10 μM pepstatin, and then cells were centrifuged at 100,000 × *g* for 40 min at 4 °C. The pellet and supernatant fractions were respectively used as sources for the AC and PDE enzymes. To assay AC activity, the reaction mixture contained 25 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM IBMX, 7.5 mM creatine phosphate, three units creatine phosphokinase, 0.5 mM dithiothreitol, 1 mM ATP, and the AC enzymes. The reaction was carried out for 20 min at 30 °C and was terminated by boiling for 3 min. Cyclic AMP contents were assayed using enzyme immunoassay kits.

PDE activity was analyzed using a tritium scintillation proximity assay (SPA) system, and the assay was performed according to the manufacturer's instructions (Amersham Biosciences). Briefly, assays were performed at 30 °C for 15 min in the presence of 50 mM Tris–HCl (pH 7.5) containing 8.3 mM $MgCl_2$, 1.7 mM EGTA, and 0.3 mg/ml bovine serum albumin. Each assay was performed in a 100 μl reaction volume containing the above buffer, neutrophil supernatant fraction, and around 0.05 μCi [^{3}H]cyclic AMP or [^{3}H]cyclic GMP (for cyclic AMP PDE or cyclic GMP PDE activity, respectively). The reaction was terminated by the addition of 50 μl PDE SPA beads (1 mg) suspended in 18 mM zinc sulfate. Assays were performed in 96-well microtiter plates. The reaction mix was allowed to settle for 1 h before counting in a microtiter plate counter.

2.8. LDH release

Cytotoxicity was expressed as the percent LDH activity obtained in cell-free medium compared to the total LDH activity. The total LDH activity was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

2.9. Statistical analysis

Results are expressed as the mean ± S.E.M., and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant.

3. Results

3.1. Effects of KW derivatives on $O_2^{\bullet-}$ generation in human neutrophils

To investigate whether KW derivatives reduce respiratory burst in FMLP/CB-treated human neutrophils, the amount of $O_2^{\bullet-}$ generated was determined. As shown in Fig. 2A, KW derivatives inhibited $O_2^{\bullet-}$ release by human neutrophils in response to FMLP/CB. Among them, KW-2, KW-5, and KW-7 (8,9-dimethoxyl-1-(*R*-phenyl)-5,6-

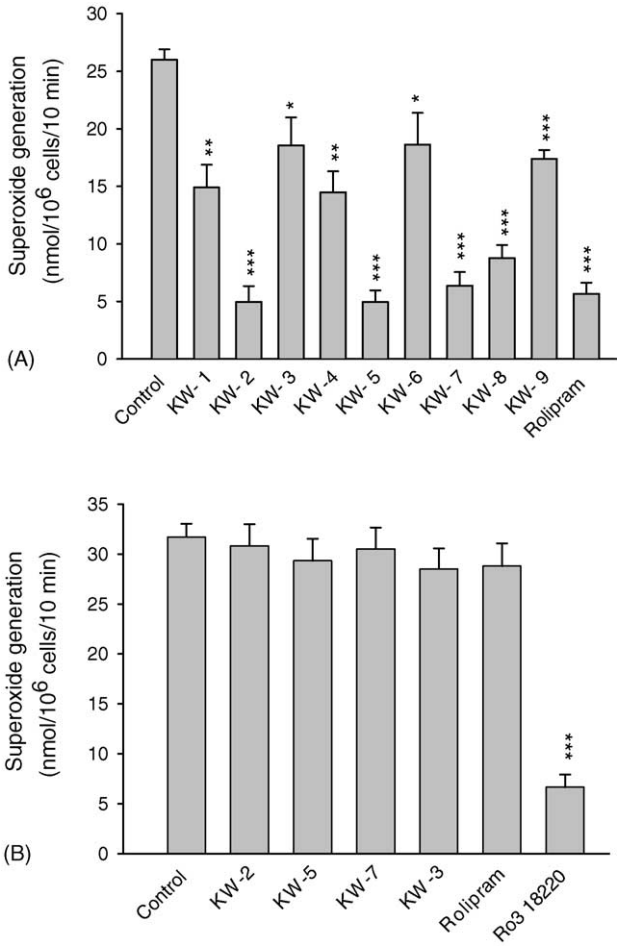


Fig. 2. Effects of the KW derivatives on $O_2^{\bullet-}$ generation from human neutrophils in response to FMLP/CB or PMA. $O_2^{\bullet-}$ generation was measured using SOD-inhibitable cytochrome *c* reduction as described in Section 2. Neutrophils were incubated with DMSO (control), KW derivatives (10 μM), rolipram (1 μM), or Ro318220 (0.3 μM) for 5 min and then activated by (A) FMLP/CB or (B) PMA. For all data, values are the mean ± S.E.M. ($n = 3$–4); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control.

dihydro-pyrrolo[2,1-*a*]isoquinoline-2,3-dione; where *R* is 3-chloro, 3-bromo, and 4-methoxy, respectively) were the most effective inhibitors. Rolipram (1 μM), a well-documented inhibitor of PDE4, inhibited FMLP/CB-induced $O_2^{\bullet-}$ release in human neutrophils (Fig. 2A). However, KW derivatives (10 μM) and rolipram (1 μM) failed to affect PMA-activated $O_2^{\bullet-}$ release. Ro318220 (0.3 μM), a well-documented inhibitor of PKC, was used as a positive control on PMA-caused $O_2^{\bullet-}$ generation (Fig. 2B). The IC_{50} values of KW-2, KW-5, and KW-7 were 5.76 ± 0.65, 5.08 ± 0.68, and 6.09 ± 0.78 μM, respectively (Fig. 3). These inhibitions were not due to cytotoxicity, since the cell viability was not changed by KW derivatives (up to 30 μM). Furthermore, the PKA inhibitor, H89 (3 μM) [21], reversed the inhibition of $O_2^{\bullet-}$ formation by KW-2, KW-5, and KW-7 (Fig. 3). In contrast, the PKG inhibitor, KT5823 (0.3 μM) [22], and the soluble guanylate cyclase (sGC) inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; 10 μM) [23], did not alter the inhibitory effects of KW derivatives (data not shown). These results suggest that PKA mediates the inhibition caused by the KW derivatives in human neutrophils.

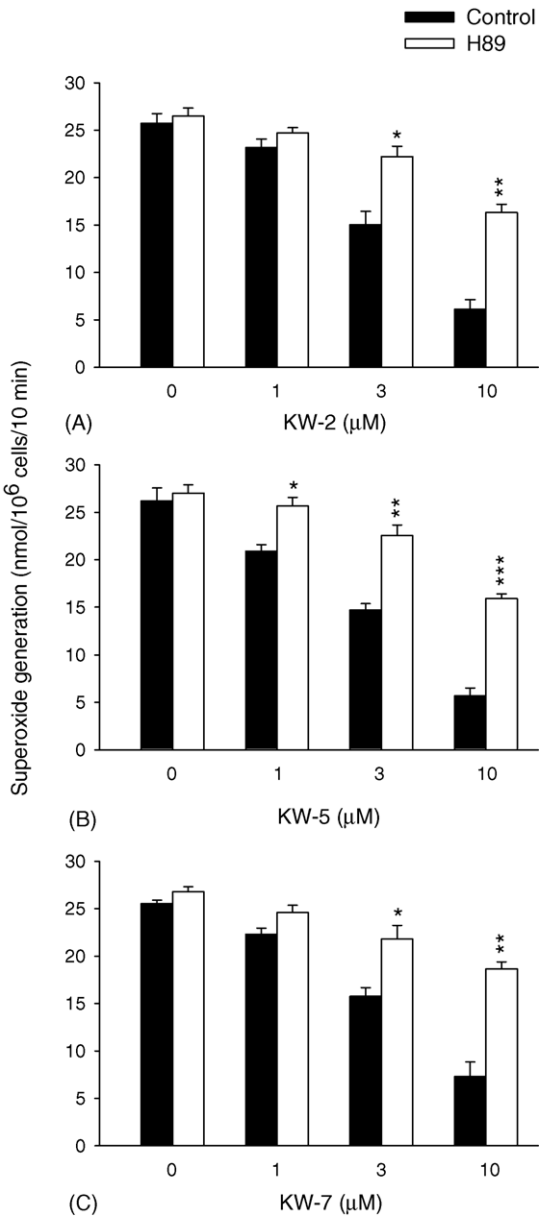


Fig. 3. Effects of the PKA inhibitor on KW-2-, KW-5-, and KW-7-caused inhibition of $O_2^{\bullet-}$ generation. H89 (3 μM) was preincubated for 5 min before the addition of (A) KW-2, (B) KW-5, or (C) KW-7 (1, 3, and 10 μM). For all data, values are the mean ± S.E.M. ($n = 4$); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the corresponding control.

3.2. $O_2^{\bullet-}$- and free radical-scavenging activities of KW derivatives

To investigate the ability of KW derivatives to scavenge $O_2^{\bullet-}$ and free radicals, we examined the effects of KW-2, KW-5, and KW-7 in the cell-free xanthine/xanthine oxidase system and DPPH assay. The results showed that KW-2, KW-5, and KW-7 at concentrations of up to 30 μM failed to alter xanthine/xanthine oxidase-induced WST-1 reduction and uric acid production as well as the stability of DPPH radicals. SOD (100 U/ml; 100% inhibition) and α-tocopherol (1–50 μM; $IC_{0.2} = 13.55 \pm 0.30$ μM) were used as the positive control in the xanthine/xanthine oxidase system and DPPH assay, respectively (data not shown).

3.3. Effects of KW derivatives on cyclic AMP formation and AC activity

Cyclic AMP concentrations were measured to determine whether the inhibitory effects of KW-2, KW-5, and KW-7 are indeed mediated by cyclic AMP. KW-2, KW-5, and KW-7 (3, 10, and 30 μM) as well as IBMX (300 μM)

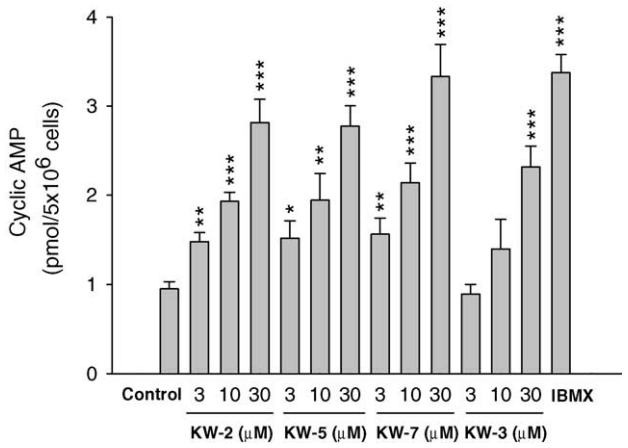


Fig. 4. Effects of KW derivatives and IBMX on cyclic AMP levels. Human neutrophils were incubated with KW-2, KW-3, KW-5, KW-7 (3, 10, and 30 μM), or IBMX (300 μM) for 5 min before stimulation with FMLP/CB for another 5 min. The reaction was stopped by adding 0.5% dodecytrimethylammonium bromide, and cyclic AMP was assayed using enzyme immunoassay kits. For all data, values are the mean ± S.E.M. ($n = 3$–6); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control.

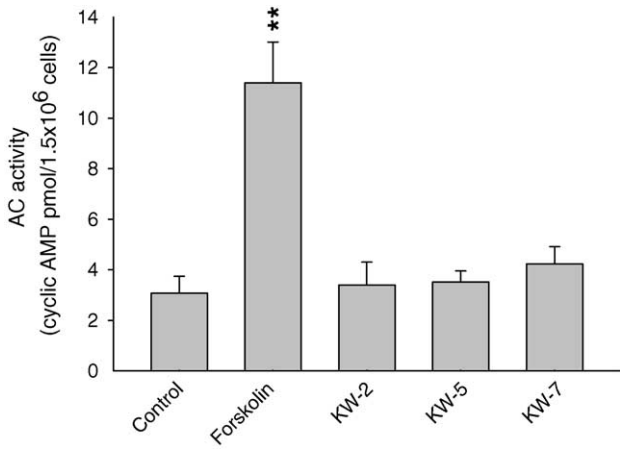


Fig. 5. Effects of KW-2, KW-5, KW-7, and forskolin on AC activities. Neutrophil membrane fractions were incubated with KW-2, KW-5, KW-7, or forskolin (30 μM) at 30 °C for 20 min in the presence of 1 mM ATP. Cyclic AMP was assayed using enzyme immunoassay kits. For all data, values are the mean ± S.E.M. ($n = 3$–4); $^{**}p < 0.01$ compared with the control.

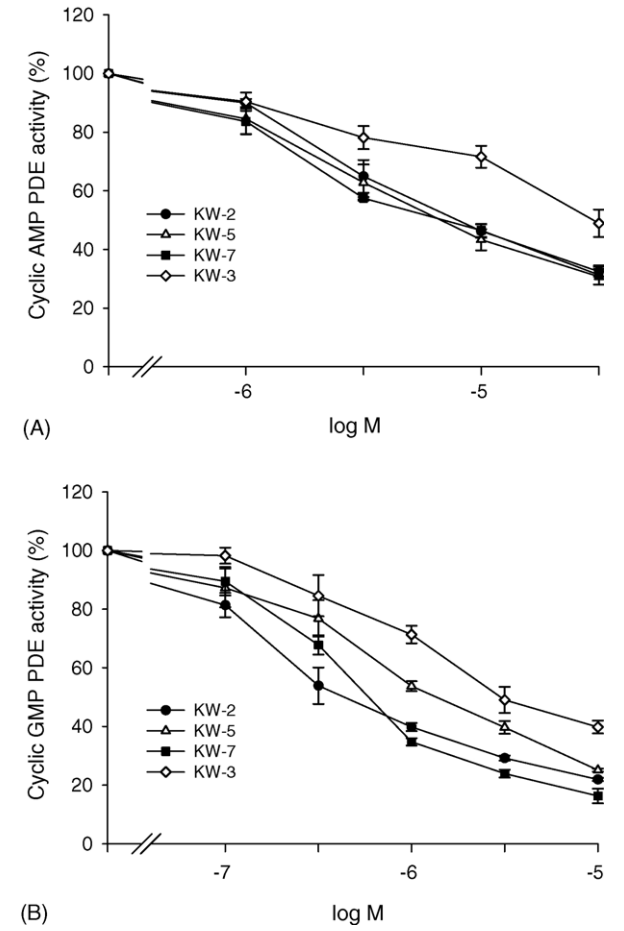


Fig. 6. Concentration-dependent effects of the KW derivatives on the inhibition of cyclic AMP and cyclic GMP PDE activities. Human neutrophil homogenates were incubated with KW-2, KW-5, KW-3, or KW-7 and then [^{3}H] cyclic AMP or [^{3}H] cyclic GMP was added to the reaction mixture at 30 °C for 15 min. PDE activity was measured as described in Section 2. Values are the mean ± S.E.M. ($n = 3$).

notably increased cyclic AMP levels in FMLP/CB-stimulated human neutrophils (Fig. 4). On the other hand, KW-3 caused minor effect on the elevation of cyclic AMP accumulation except at concentrations up to 30 μM. Cellular cyclic AMP levels are modulated either by synthesis, via AC, or by degradation, via PDEs. Our data showed that forskolin (30 μM), but not KW derivatives (30 μM), increased the activity of AC (Fig. 5).

3.4. Effects of KW derivatives on PDE activity

Human neutrophils contain two major cytosolic isozymes of PDE, subtypes PDE4 (cyclic AMP-specific) and PDE5 (cyclic GMP-specific) [3]. As shown in Fig. 6, KW-2, KW-3, KW-5, and KW-7 inhibited cyclic AMP-specific PDE and cyclic GMP-specific PDE activities with IC_{50} values of 11.61 ± 2.23 and 0.75 ± 0.18 μM, 29.48 ± 4.28 and 5.56 ± 1.08 μM, 9.02 ± 1.80 and 1.92 ± 0.19 μM, and 9.96 ± 2.01 and 0.82 ± 0.08 μM, respectively. The KW-3 had a less potency than KW-2, KW-5, or KW-7. This result was consistent with the observation on cyclic AMP and $O_2^{\bullet-}$ production. IBMX also inhibited both PDE activities with IC_{50} values of 17.38 ± 1.08 and 9.76 ± 1.49 μM, respectively.

4. Discussion

In the present study, we investigated the effects of KW derivatives, newly synthetic pyrrolo-benzylisoquinoline compounds on respiratory burst in human neutrophils, which play an important role in the pathogenesis of sepsis, myocardial ischemia-reperfusion injury, atherosclerosis, and other inflammatory processes [2]. Of the nine compounds, KW-2, KW-5, and KW-7 were the most powerful inhibitory agents. Investigation of the signal transduction pathways indicated that the inhibitory effects of KW derivatives were closely associated with elevation of cyclic AMP levels through inhibition of cyclic AMP PDE activities.

During phagocytosis of microorganisms, neutrophils increase their oxygen consumption through the activity of an NADPH-oxidase that generates $O_2^{\bullet-}$. This phenomenon is the so-called respiratory burst [24]. The $O_2^{\bullet-}$ is converted to various species of oxygen radicals that are strongly antimicrobial but which also directly or indirectly cause damage by destroying surrounding tissue. The formation of $O_2^{\bullet-}$ in neutrophils can be inhibited by modulating the cellular signaling pathways, but also by directly scavenging $O_2^{\bullet-}$. KW-2, KW-5, and KW-7 at concentrations of up to 30 μM neither scavenged $O_2^{\bullet-}$ not acted as antioxidants in cell-free systems, indicating that the cellular signaling pathways in human neutrophils are mediated by the action of KW derivatives. Increases in cellular cyclic AMP concentration are associated with

suppression of the functions of inflammatory cells. For example, agents that stimulate intracellular cyclic AMP levels have been demonstrated to inhibit several neutrophil functions including respiratory burst [3–6]. Our results are in line with those previous findings, showing that PDE4 inhibitor, rolipram, can diminish FMLP/CB-induced $O_2^{\bullet-}$ release in human neutrophils. The PKA inhibitor, H89, significantly restored KW-2-, KW-5-, and KW-7-caused inhibition. KW-2, KW-5, and KW-7 concentration-dependently elevated cyclic AMP concentrations in human neutrophils. Accordingly, these results indicate that inhibition of respiratory burst in human neutrophils by KW derivatives occurs through a cyclic AMP/PKA-dependent pathway. Cellular cyclic nucleotides are modulated either by synthesis, via AC, or by degradation, via PDEs, a group of enzymes that catalyze the hydrolysis of 3′,5′-cyclic nucleotides to inactive 5′-nucleotide metabolites. Our data showed that KW derivatives did not increase the AC functions, but attenuated the PDE activities. These results suggest that the cyclic AMP-elevating effects of KW derivatives result from inhibition of PDE activities but not from stimulation of cyclase functions.

KW derivatives and rolipram inhibited $O_2^{\bullet-}$ release by human neutrophils in response to FMLP/CB, but not to PMA. Cyclic AMP inhibition of FMLP-induced but not PMA-induced $O_2^{\bullet-}$ generation by neutrophils has been previously reported and is confirmed here [25]. Human neutrophils contain two major cytosolic isozymes of PDE, subtypes PDE4 (cyclic AMP-specific) and PDE5 (cyclic GMP-specific) [3]. KW-2, KW-5, and KW-7 preferentially inhibited cyclic GMP-specific PDE over cyclic AMP-specific PDE by about 15.48-, 4.70-, and 12.15-fold, respectively. Although the cyclic GMP-specific PDE activity was reduced, neither the sGC inhibitor, ODQ, nor the PKG inhibitor, KT 5723, was able to reverse the inhibitory effects of the KW derivatives. These data rule out a role for cyclic GMP in the KW derivative-caused inhibition. These findings are in agreement with our recent study demonstrating that cyclic GMP-elevating agents increase sGC activity and cyclic GMP formation but fail to alter the FMLP-induced $O_2^{\bullet-}$ release in human neutrophils [26]. Previous paper demonstrated that pyrrolo-benzylisoquinolines inhibit platelet aggregation [17], which could be contributed to the inhibition of cyclic GMP-specific PDE (PDE5) in platelet by these compounds. Interestingly, pentoxifylline, a nonselective PDE inhibitor, has been used to treat septic patients, as it improves cardiopulmonary function and reduces the mortality rate [27,28]. Additionally, Rocco et al. (2003) showed that LASSBio596, a PDE4 and PDE5 inhibitor, displays an important anti-inflammatory function in a mouse model of *Escherichia coli* lipopolysaccharide-induced acute lung injury [29]. Therefore, experimental studies and limited clinical experience suggest that the use of agents that simultaneously inhibit PDE 4 and 5 may be useful adjunct therapy for lung injury. Clearly, it will now be of interest to further elucidate the beneficial effects of KW derivatives in in vivo lung inflammation.

In summary, the newly synthetic pyrrolo-benzylisoquinoline derivatives inhibit FMLP-induced respiratory burst in human neutrophils. These effects are attributed to the elevation of cellular cyclic AMP through the inhibition of PDE. Our data suggest that KW derivatives are new types of PDE inhibitors and may have anti-inflammatory potential.

Acknowledgments

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