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Synthesis of 3,3′-(1-piperidino)substituted methylene-bis-isoxazoles preventing stimulus-induced leukocytes activation

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Abstract

Some 3,3′-(1-piperidino)substituted methylene-bis-isoxazoles were prepared via Mannich base and tested to verify their antiinflammatory-related activity. Human neutrophils stimulated with either PMA and f-MLP were used as the cellular model. The efficiency of eight differently substituted compounds (**2**–**9**) was established on their capacity to reduce the O_2^- production by activated human neutrophils. The rising hydrophobicity in the side-chain of methylene-bis-isoxazoles leads to a distinction in the neutrophil response against the two stimuli, favoring the inhibition of the PMA elicited cell activation and leaving inaffected the f-MLP induced cell responses. Compounds **8** and **9** are particularly active and abolish almost completely the neutrophil activation in the presence of PMA stimulus.

Keywords: 3,3′-(1-Piperidino) substituted methylene-bis-isoxazoles; Mannich bases; Superoxide anion; Neutrophils; Ischemia; Reperfusion injuries

1. Introduction

A number of evidence are indicating that reperfusion of an ischemic tissue, such as heart, can cause further cellular damages [1,2]. This myocardial reperfusion injury [3,4] is mediated by the production of large quantity of reactive oxygen free radicals (ROS). This effect is supported by the reduction of infarct size and an increased functional recovery obtained in hearts treated with antioxidant enzymes. The presence of leukocytes in damaged tissues increases greatly the magnitude and the duration of ROS generation [5].

It has been suggested that leukocytes, namely neutrophils, mediate the reperfusion injury by chemotaxis and activation leading to the production of ROS and the release of proteolytic enzymes. Several studies are indicating that a decrease in the number of neutrophils can reflect a decrease in the infarct size [6,7].

It is well known that protein kinase C (PKC) isozymes are involved in many cell functions, through the activation of enzyme cascades leading to multiple cell responses [8]. Activation of these kinase forms take place following elevation of intracellular calcium, which promotes translocation and insertion of some PKC isozymes into the inner face of plasma membranes. Following this peculiar step, the enzyme acquires the active conformation by interacting with diacylglicerol and phosphatidylserine. The quaternary complex is required to reach the fully functional enzymes [9]. PKC activation occurs also in human neutrophils stimulated by natural or synthetic agents. Formylated peptides such as f-MetLeuPhe (f-MLP) are recognized by serpentine receptors which stimulation leads to the production of intracellular conditions causing the transient PKC translocation on the membrane and activation. The tumor promotor phorbol 12-myristate-13-acetate (PMA) activates directly PKC isozymes substituting the diacylglicerol moiety on the enzyme producing the permanent insertion of the kinase into the plasma membrane moiety. These differently activated PKC isoforms may be correlated to the neutrophil responses elicited by PMA or f-MLP. In fact, PMA is a potent stimulus of neutrophil oxidase activation and specific granule exocytosis, without any detectable

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cytoplasmatic Ca^{2+} flux. However, f-MLP mimics the effects of chemotaxins released by invading bacteria, through the binding to a G protein associated receptor. The two pathways, although promoting similar cell responses, utilize different mediators.

The presence of activated PKC in neutrophils is correlated to many cell defence responses, including promotion of ROS and secretion of granule content. However, neutrophil activation occurring at ischemic and inflammatory sites can favor a further progress of the cell damage, through the secretion of lytic enzymes and toxic ROS.

In previous works, naphthopyran-1-ones and benzopyran-4-ones bearing a dialkylamine in the position adjacent to the heteroatom were extensively tested as potential antiinflammatory agents because their ability to inhibit the PKC dependent signal transduction pathway [10]. In particular, these studies showed that the cited activity was prominent when the dialkylamino substituent was piperidine, diethylamine or bis(2-methoxyethyl)amine. In that context, we showed that some benzopyran-derived isoxazoles bearing a piperidino substituent in **3** were endowed with interesting antiinflammatory activity when tested against activated human neutrophils [11].

Following our interest in synthetic substances valuable in the antiinflammatory field, we now desire amplify our study synthesizing some symmetrical methylene derivatives of 3-(1-piperidino)isoxazoles useful to restrain the inflammatory response during ischemia/reperfusion injuries.

The synthetic pathway was already utilized in recent works to produce unsymmetrical methylene derivatives via Mannich bases [12–14]. Whereas in the cited papers some Mannich bases of coumarins, as an example, reacted in acetic anhydride with indoles forming unsymmetrical derivatives, for the present purpose, a Mannich base of a chromone was reacted with the same chromone to yield a symmetrical methylene dimer, which in turn was converted into an isoxazole dimer. Thus, the preparation of symmetrical derivatives is carried out as a particular case of a more general pathway yielding unsymmetrical methylene derivatives.

2. Chemistry

To obtain the title compounds, we followed the reaction pattern depicted in Fig. 1, starting from the 7-methoxy-2-(1-piperidinyl)chromone (**1**).

The chromone **1** was transformed in a Mannich base using 40% formaldehyde and piperazine. In turn, the Mannich base **2** and the chromone **1** were treated with acetic anhydride at 95 °C to yield the methylene-bis-derivative **3**. Then the compound **3** was converted into methylene-bis-isoxazole **4** by action of hydroxylamine hydrochloride in pyridine. Details of these three steps are given in Section 5.

The methylene-bis-isoxazole **4** was subjected to alkylation by action of dimethyl- and diethylsulfate yielding the alkyloxyderivatives **5** and **6**; then, the compound **4** was treated with acetic and propionic anhydride yielding the acyl derivatives **7** and **8**. Finally, by action of 2-(diethylamino)ethyl chloride hydrochloride in *N*,*N*-dimethylformamide (DMF), the compound **4** was transformed into the basic derivative **9** (Fig. 1).

All synthesized compounds (**2**–**9**) are white crystals and their structures are in agreement with elemental analyses and spectral data.

3. Experimental

Melting points (m.p.) were determined using a Electrothermal apparatus and are uncorrected. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer. The results of elemental analysis were within $\pm 0.3\%$ for C and ± 0.1 for H and N of the theoretical value. ^{1}H NMR spectra were performed on a Hitachi Perkin–Elmer R 600 (60 MHz) spectrometer using TMS as internal standard ($\delta = 0$). IR spectra were recorded on a Perkin–Elmer 398 spectrophotometer.

3.1. *N,N′([2-(1-piperidinyl)-7-methoxychromon-3-yl]methyl)piperazine* (**2**)

To 1.04 g (4.0 mmol) of chromone **1** dissolved in 15 ml of ethanol, 0.26 g (3.0 mmol) of piperazine, 1.5 ml of 40% formaldehyde and 0.06 ml of acetic acid were added. The resulting mixture was refluxed for 3 h. After cooling, the precipitate was filtered off and the solid was crystallized from acetone obtaining **2**; m.p.: 264–265 °C; 75.6% yield.

^{1}H NMR (DMSO-d_6): δ 1.54–2.00 (m, 12H, CH_2 β, γ-piperidine), 3.35–3.78 (m, 16H, CH_2 α-piperidine and piperazine), 3.95 (s, 6H, OCH_3), 4.30 (s, 4H, CH_2 bridge), 6.95–7.20 (m, 4H, H arom), 7.86–8.18 (d, 2H, H arom). IR (KBr) cm^{-1}: 1615, 1590, 1548. *Anal*. $C_{36}H_{44}N_4O_6$ (C, H, N).

3.2. *3,3′-Methylenbis[2-(1-piperidinyl)-7-methoxychromone]* (**3**)

To the solution of 1.04 g (4.0 mmol) of chromone **1** in 8 ml of acetic anhydride, 1.26 (2.0 mmol) of Mannich base **2** were added and the mixture was heated at 95 °C for 1.5 h. After cooling, the solution was poured onto crushed ice and the mixture was stirred for 1–2 h. The precipitate was filtered off and the solid was crystallized from ethyl acetate obtaining **3**; m.p.: 176–177 °C; 70.5% yield.

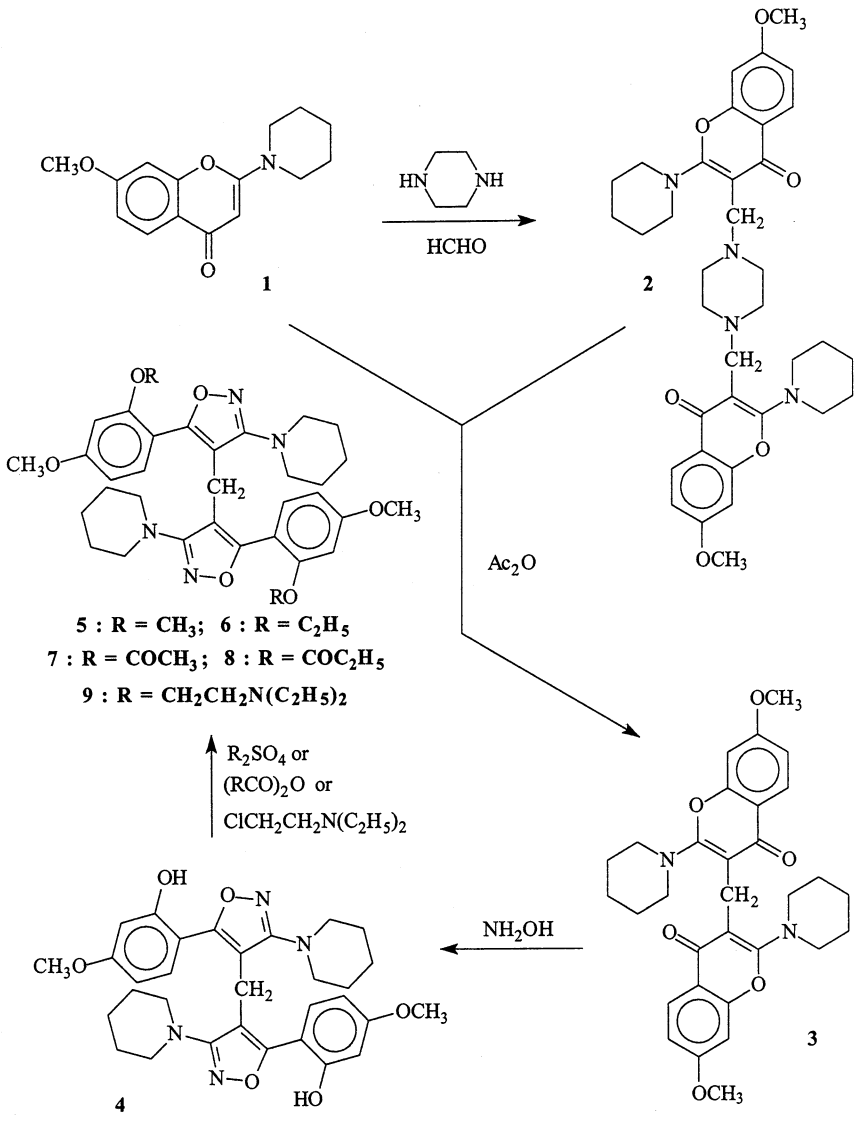

Fig. 1.

^{1}H NMR ($CDCl_3$): δ 1.20–1.65 (m, 12H, CH_2 β, γ-piperidine), 2.95–3.35 (m, 8H, CH_2 α-piperidine), 3.90 (s, 6H, OCH_3), 4.05 (s, 2H, CH_2 bridge), 6.70–7.05 (m, 4H, H arom), 8.10 (d, 2H, H arom). IR (KBr) cm^{-1}: 1605, 1547, 1499. *Anal*. $C_{31}H_{34}N_2O_6$ (C, H, N).

*3.3. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2-hydroxy-4-methoxyphenyl)isoxazole] (**4**)*

To a solution of 0.53 g (1.0 mmol) of **3** in 18 ml of ethanol, 0.5 g of hydroxylamine hydrochloride and 0.7 ml of pyridine were added. The mixture was refluxed for 24 h. The final solution was evaporated under reduced pressure to leave a pale yellow solid. The solid was dissolved in a small amount of 2 N NaOH, filtering off any impurity of the unreacted starting product. Then the alkaline solution was acidified with 6 M HCl obtaining a white precipitate which was filtered off and washed with water. The crude product was crystallized from ethanol obtaining **4**; m.p.: 251–252 °C; 72.5% yield.

^{1}H NMR ($CDCl_3$): δ 1.00–1.70 (m, 12H, CH_2 β, γ-piperidine), 2.73–3.20 (m, 8H, CH_2 α-piperidine), 3.57 (s, 2H, CH_2 bridge), 3.81 (s, 6H, OCH_3), 6.36–6.72 (m, 4H, H arom), 7.30 (d, 2H, H arom), 9.03 (s, 2H, OH). IR (KBr) cm^{-1}: 3110, 1620, 1510. *Anal*. $C_{31}H_{36}N_4O_6$ (C, H, N).

*3.4. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2, 4-dimethoxyphenyl)isoxazole] (**5**)*

To 0.28 g (0.5 mmol) of methylene-bis isoxazole **4** dissolved in 3 ml of NaOH 1 N, 0.20 g of dimethylsulfate were added and the mixture were heated for 2 h at 70 °C. After cooling, the solution was poured onto crushed ice and the mixture was stirred for 1–2 h and extracted with $CHCl_3$. The organic phase was evapo-

rated under reduced pressure leaving a solid. The solid was crystallized from ethyl acetate yielding **5**, m.p.: 190–191 °C; 74.4% yield.

^{1}H NMR ($CDCl_3$): δ 1.28–1.75 (m, 12H, CH_2 β, γ-piperidine), 2.78-3.16 (m, 8H, CH_2 α-piperidine), 3.44 (s, 2H, CH_2 bridge), 3.66 (s, 6H, OCH_3-orto), 3.86 (s, 6H, OCH_3-*para*) 6.32-6.75 (m, 4H, H arom), 7.35 (d, 2H, H arom). IR (KBr) cm^{-1}: 1635, 1600, 1580. *Anal*. $C_{33}H_{40}N_4O_6$ (C, H, N).

*3.5. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2-ethoxy-4-methoxyphenyl)isoxazole] (**6**)*

To 0.28 g (0.5 mmol) of methylene-bis isoxazole **4** dissolved in 3 ml of NaOH 1 N, 0.25 g of diethylsulfate were added and the mixture were heated for 3 h at 90 °C. With the same procedure as above the derivative **6** was obtained; m.p.: 192–193 °C; 85.3% yield.

^{1}H NMR ($CDCl_3$): δ 1.05–1.34 (t, 6H, CH_2CH_3), 1.37–1.72 (m, 12H, CH_2 β, γ-piperidine), 2.82–3.20 (m, 8H, CH_2 α-piperidine), 3.38–3.65 (q, 4H, CH_2CH_3), 3.76 (s, 2H, CH_2 bridge), 3.86 (s, 6H, OCH_3), 6.48–6.72 (m, 4H, H arom), 7.38 (d, 2H, H arom). IR (KBr) cm^{-1}: 1638, 1605, 1577. *Anal*. $C_{35}H_{44}N_4O_6$ (C, H, N).

*3.6. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2-acetoxy-4-methoxyphenyl)isoxazole] (**7**)*

A 0.28 g quantity (0.5 mmol) of methylene-bis isoxazole **4** was dissolved in 5 ml of acetic anhydride and heated for 1.5 h at 95 °C. At the end, after cooling, the solution was poured onto crushed ice and the mixture was stirred for 1–2 h obtaining a solid. The solid was filtered off and crystallized from ethyl acetate yielding **7**; m.p.: 237–238 °C; 71.1% yield.

^{1}H NMR ($CDCl_3$): δ 1.49–1.90 (m, 12H, CH_2 β, γ-piperidine), 2.37 (s, 6H, $COCH_3$), 3.12–3.50 (m, 8H, CH_2 α-piperidine), 3.85 (s, 8H, CH_2 bridge, OCH_3), 6.56–7.10 (m, 4H, H arom), 7.80 (d, 2H, H arom). IR (KBr) cm^{-1}: 1766, 1618, 1572. *Anal*. $C_{35}H_{40}N_4O_8$ (C, H, N).

*3.7. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2-propyloxy-4-methoxyphenyl)isoxazole] (**8**)*

A 0.28 g quantity (0.5 mmol) of methylene-bis-isoxazole **4** was dissolved in 5 ml of propionic anhydride. With the same procedure as above the derivative **8** was obtained: m.p.: 232–233 °C; 71.5% yield.

^{1}H NMR ($CDCl_3$): δ 1.04 (t, 6H, CH_2CH_3), 1.42–1.73 (m, 12H, CH_2 β, γ-piperidine), 2.25 (q, 4H, $COCH_2$), 2.95–3.25 (m, 8H, CH_2 α-piperidine), 3.84 (s, 8H, CH_2 bridge, OCH_3), 6.67–6.98 (m, 4H, H arom), 7.69 (d, 2H, H arom). IR (KBr) cm^{-1}: 1768, 1620, 1515. *Anal*. $C_{37}H_{44}N_4O_8$ (C, H, N).

*3.8. 4,4′-Methylenbis[3-(1-piperidinyl)-5-(2-[2-N,N-diethylaminoethoxy]-4-methoxy-phenyl)isoxazole] (**9**)*

To 1.90 g (3.4 mmol) of methylene-bis isoxazole **4** dissolved in 19 ml of DMF, 1.75 g (10.2 mmol) of 2-(diethylamino)ethyl chloride hydrochloride and 1.87 g of anhydrous potassium carbonate were added and the mixture was heated at 120 °C for 7 h. After cooling, 30 ml of water were added obtaining a precipitate that was crystallized from diethyl ether/light petroleum ether. The derivative **9** was obtained; m.p.: 82–83 °C: 21.7% yield.

^{1}H NMR ($CDCl_3$): δ 0.93 (t, 12H, NCH_2CH_3), 1.31–1.70 (m, 12H, CH_2 β, γ-piperidine), 2.30–2.72 (m, 12H, NCH_2), 2.80–3.15 (m, 8H, CH_2 α-piperidine), 3.30–3.96 (m, 12H, CH_2 bridge, OCH_2, OCH_3), 6.34–6.75 (m, 4H, H arom), 7.19 (d, 2H, H arom). IR (KBr) cm^{-1}: 1638, 1605, 1515. *Anal*. $C_{43}H_{62}N_6O_6$ (C, H, N).

4. Biology

4.1. Isolation of neutrophils

Freshly collected heparinized human blood (100 ml) from healthy donors was treated with 1.6% dextran (final concentration) and left at 25–28 °C to sedimentate for 1 h. The supernatants (40 ml) were collected and layered onto 10 ml of 6% Ficoll 400 solution containing 0.17% (v/v) Urovison and centrifuged at $800 \times g$ for 20 min. The pellets containing mostly neutrophils and contaminating red cells were resuspended in 10 ml of hypotonic 0.2% NaCl. After 30 s, 10 ml of hypertonic 1.6% NaCl were added to normalize the osmotic pressure. This treatment lyses all contaminating red cells. The white cells were recovered and washed three times with 0.01 M sodium phosphate (pH 7.4), 5 mM KCl, 0.12 M NaCl, 24 mM $NaHCO_3$, 5 mM glucose. Prior to use, the cells were maintained in an ice bath in the same medium at a concentration of $15–20 \times 10^6$ cells per ml. The cell population obtained consisted of more than 96% neutrophils, as evaluated by microscopic examination. The few remaining cells were eosinophils and monocytes. Cell viability, evaluated by Trypan Blue dye exclusion, resulted to be more than 97%.

4.2. Activation of neutrophils and assay of superoxide anion (O_2^-)

Cells (10^6) were diluted in 1 ml of 10 mM HEPES, pH 7.4, containing 0.15 M NaCl, 5.0 mM glucose, 1.0 mM Ca^{2+} and 0.625 mg ml^{-1} of cytochrome *C* (Fe^{3+}) and incubated at 37 °C for 2 min. PMA (100 ng) or f-MLP, (0.1 μM final concentration) were then added. Superoxide production was evaluated by measuring the superoxide dismutase-inhibitable reduction of ferricyto-

chrome *C* followed continuously at 550 nm in an automatic recording spectrophotometer [15,16]. The rates of O_2^- production (100% values) were 6.2 and 3.7 nmol per 10^6 cells min^{-1} for cells activated with PMA or f-MLP, respectively.

4.3. Samples

The compounds were dissolved in DMSO at the appropriate concentration. When tested on neutrophils, 1 μl of standard solution was added to 1 ml of cell suspension. As the control, 1 μl of DMSO was added to the blank sample. Each compound was tested at concentrations of 2, 20, 200 μM. Only the data obtained with 20 μM compound were utilized, being more indicative of their efficiency in inhibiting neutrophil function. To establish if these compounds can hydrophobically interact with the lipid stimulus PMA, cells were incubated with 100 ng PMA and 20 μM compound and immediately centrifuged at $2000 \times g$ for 1 min. Supernatans were removed and replaced with fresh medium containing cytochrome *C*. The amount of O_2^- produced in these conditions was not affected by the presence of the synthesized compounds in the incubated mixture, indicating that no subtraction of stimulus occurred during neutrophil activation. Each determination was done in triplicate and the results were the arithmetical mean of the three values.

5. Results

In recent works we pointed out our attention at the mechanism of the reaction between Mannich bases and acidic hydrogen-containing compounds (in particular chromones, coumarins and indoles) to obtain unsymmetrical methylene derivatives [12–14]. Formerly, we had shown that the formation of symmetrical derivatives may also occur, although with poor yield, treating a proper Mannich base with acetic anhydride without the presence of a acidic hydrogen-containing compound [17].

Now, our results suggest that the formation of symmetrical methylene-bis-derivatives, following the route proposed in this paper, must be considered a special case of the more general pattern leading to the production of unsymmetrical methylene derivatives. In fact, our synthetic pathway relies on these considerations:

- As the type of amine is not significant, due to the removal of the amine in the next step, the piperazine was preferred. Indeed such amine yields good amount of base **2**, which is manageable more easily than the corresponding morpholinomethyl or piperidinomethyl derivatives. In fact, compound **2**, having a high molecular weight, precipitates better than morpholinomethyl or piperidinomethyl derivatives from the reaction mixture or from the crystallization solvent (data not shown).
- Following the reaction mechanism proposed for the unsymmetrical methylene derivatives [12], the yield of the methylene-bis-derivative **3** was increased adding the chromone **1** to the Mannich base **2** in molar ratio 2:1. In fact, one molecule of Mannich base **2** produces two carbocations, which in turn react with two molecules of chromone **1**.
- The nucleophilic attack of hydroxylamine at the position 2 of the bis-chromone **3** produces the opening of the chromone ring and the loss of a molecule of water, leading to bis-isoxazole **4** [18].

To evaluate the antiinflammatory activity of compounds bearing a double piperidine substitution, the synthesized molecules were tested for their ability to affect the O_2^- production by human neutrophils stimulated with either PMA and f-MLP. The results are reported in Table 1.

From these data it is possible to draw the following conclusions:

- Compounds retaining the chromone moiety (**2** and **3**) are enoughly active in inhibiting the neutrophil activation and their action is similar to that already found for analogous substances [11].
- The methylene-bis-isoxazole **4** having free hydroxy groups is poorly active.
- The methylene-bis-isoxazoles **5**–**8** with the hydroxy groups bearing short alkyl and acyl substituents are endowed with an interesting behavior. These compounds differently affect the cell response elicited by the two stimuli. Thus, the substituted bis-isoxazoles almost completely are devoid of activity against the f-MLP stimulus whereas they are highly efficient against PMA stimulation, particularly if the acyl moiety is present at the position 2′. Maximal efficiency, in this group of compounds, is observed

Table 1
Effects of compounds **2**–**9** on O_2^- production by human neutrophils stimulated with f-MLP and PMA

Comp.	PMA% inhibition	f-MLP% inhibition
2	58	71
3	76	62
4	8	36
5	40	4
6	34	2
7	62	5
8	94	3
9	100	0

Neutrophil stimulation was carried out as described in Section 4. The concentration of each compound was 20 μM.

with derivative **8** substituted in 2′ by the propionyl group.
- The methylene-bis-isoxazoles **9** with the hydroxy groups bearing a 2-(diethylamino)ethyl group shows an 100% efficiency against PMA stimulated neutrophils whereas is deprived of activity against f-MLP stimulated neutrophils.

6. Discussion

It has been established that polymorphonuclear (PMN) leukocytes play a primary role in the setting of myocardial injury due to ischemia/reperfusion, contributing to microvascular plugging, cardiac contractile dysfunction, and enhanced cardiomyocyte necrosis [19–21]. On reperfusion, PMN leukocytes accumulate in the coronary microvasculature and infiltrate into cardiac tissue [20,22]. These transmigrated PMN leukocytes can provoke tissue injury by the release of cytotoxic substances, including ROS, chemotactic factors and proteolytic enzymes [20,23]. These activated cells can contributed also to endothelial dysfunction [24]. Therefore, it is likely that compounds inhibiting in one site PMN leukocytes adherence to the vascular endothelium [25], and on the other site production of ROS and secretion of toxic agents by activated leukocytes, can exert tissue-protective effects against the PMN leukocyte induced-injury.

In our experimental design, we have considered the possibility to counteract neutrophil activation with specific bis-isoxazoles derivatives as a tool to protect tissues from further cellular damages. Neutrophil activation was induced with two different stimuli (PMA and f-MLP) in order to better classify the action of the synthesized compounds.

The chain extension in the bis-isoxazoles **5**–**9** is clearly addressed in the distinction of the response against the two stimuli. In fact, whereas the tested bis-isoxazoles seem do not affect the neutrophil activation induced by f-MLP, the cell response elicited by PMA is highly affected. Such biological activity increases from compound **5** to **9**, compounds **8** and **9** not only are those showing the highest efficiency, but they are also specific in inhibiting PMA stimulated neutrophils. This selectivity could be due to the fact that PMA and f-MLP utilize different intracellular signals in promoting the neutrophil responses. The differences in intracellular Ca^{2+} mobilzation and in translocation of PKC isozymes to the plasma membranes induced by PMA or f-MLP can further provide an explanation for the observed effects [26–28].

These results are indicating that compounds **8** and **9** are selectively addressed against specific signal transduction pathways. The possibility that a PKC isozyme can be the target of these agents is also supported by the fact that these kinases undergo translocation to the plasma membranes, the site at which the tested hydrophobic compounds are accumulated.

The specificity shown by these compounds could be attributed to the dimerization of 3-(1-piperidinyl)isoxazoles found previously active on neutrophils stimulated with both PMA and f-MLP. The highest degree of molecular complexity and hydrophobicity of methylene-bis-isoxazoles **8** and **9**, as compared with previously tested isoxazoles [11], can explain the obtained results.

Chemokines potentially released from damaged cells or tissues, such as IL-8, can stimulate neutrophils promoting the secretion of toxic agents, including ROS, amplifying the injury [29]. This hypothesis is supported by the fact that treatment with antioxidant enzymes increases the functional recovery and decreases the heart infarct size. Furthermore, high number of leukocytes are present in tissue damaged by ischemia and reperfusion.

The present results could be interesting in designing new agents endowed with specific activity against a particular step in the complex mechanism leading to neutrophil activation in damaged tissues.

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