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# Intracellular signaling modifications involved in the anti-inflammatory effect of 4-alkoxy-6,9-dichloro[1,2,4]triazolo[4,3-*a*]quinoxalines on macrophages

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ABSTRACT

Inflammation is part of a complex biological response directed by the immune system to fight pathogens and maintain homeostasis. Dysregulation of the inflammatory process leads to development of chronic inflammatory or autoimmune diseases. Several cell types, such as macrophages, and cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) are involved in the regulation of inflammation. The important role played by these cytokines as mediators of the inflammatory process and the side effects of current therapies have promoted the search of new therapeutic alternatives. Quinoxalines are important compounds allowing a wide range of chemical modifications in order to provide an extensive repertoire of biological activities. We have previously shown that a series of 4-alkoxy-6,9-dichloro[1,2,4]triazolo[4,3-*a*]quinoxalines exhibit potent anti-inflammatory activity, inhibiting the production of TNF-α and IL-6. Our aim here was to study the mechanism thereby this series of compounds act upon different intracellular signaling pathways to uncover their potential molecular targets. By using immunoblotting assays, we found that these compounds inhibit ERK 1/2 and JNK/c-Jun cascades, and reduce c-Fos expression, while activate the anti-inflammatory PI3K/Akt route. These results provide further information on their effect upon the intracellular signal transduction mechanisms leading to inhibition of TNF-α and IL-6 secretion. Our results may be of great interest for the pharmaceutical industry, and could be used as a starting point for the development of new and more potent anti-inflammatory drugs derived from the quinoxaline core.



## 1. Introduction

Inflammation is a complex biological response directed against noxious stimuli like pathogens, damaged cells, or irritant substances, in order to maintain homeostasis. While acute inflammation is a short duration response aiming to eliminate the stimulus, repair the tissues, and reestablish the functional status, chronic inflammation is a prolonged and dysregulated response involved in the physiopathology of multiple chronic diseases, such as psoriasis, rheumatoid arthritis, cirrhosis, type II diabetes, obesity and cancer (Feldmann et al., 2001; Tracey et al., 2008). Inflammation is elicited and regulated by several immune cells and soluble molecules including cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Feldmann et al., 2001). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin or ibuprofen, are frequently used regardless of their side effects, including gastrointestinal injury, ulcers, bleeding and nephrotoxicity (Fine, 2013). Thus, there is a constant demand to find new targets useful for the development of highly specific, potent and free from undesirable effects new anti-inflammatory drugs. Since pro-inflammatory cytokines such as IL-6 and TNF-α are relevant in the initiation and maintenance of the inflammatory process (Ruiz-Alcaraz et al., 2011), a good strategy would be to search for inhibitors of key intracellular signaling molecules that mediate the production of those cytokines (Guirado et al., 2012; Sundberg et al., 2014).

*Abbreviations:* ATF, activating transcription factor; CCM, complete culture medium; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NSAIDs, non-steroidal anti-inflammatory drugs; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAMP, pathogen-associated molecular patterns; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PRRs, PAMP recognition receptors; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.

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Immune cells express receptors able to recognize several pathogen-associated molecular patterns (PAMP), thus called PAMP recognition receptors (PRRs). Specific ligand recognition by PRRs triggers a series of intracellular signaling cascades that amplify the signal to induce the transcription of specific genes that lead to the synthesis of pro-inflammatory mediators, mostly cytokines (Newton and Dixit, 2012). In this study, we have focused in the analysis of crucial intracellular inflammatory routes, namely, PI3K-Akt, MAP kinases (MAPK), and transcription factors NFκB, c-Jun and c-Fos (Fig.1). PI3K (Phosphatidylinositol 3-Kinase) catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5 triphosphate (PIP3), which acts as a second messenger. Downstream PI3K, protein Kinase B (PKB) or Akt is activated in response to various stimuli, such as growth factors and insulin. The PI3K-Akt pathway regulates metabolism, stimulates cell proliferation, inhibits apoptosis (Cardone et al., 1998), and is involved in the synthesis of anti-inflammatory cytokines such as IL-10 (Antoniv and Ivashkiv, 2011). MAPK include a group of highly conserved kinases in eukaryotes activated by previous stimulation of tyrosine kinase receptors, G protein coupled receptors and integrins. Three main routes can be distinguished: ERK, JNK (c-Jun N-terminal Kinase) and p38 MAPK. The ERK (Extracellular signal-regulated kinase) pathway is activated by mitogens and growth factors. Upstream kinases phosphorylate and activate ERK. Phosphorylated ERK activates numerous substrates, such as Elk-1, c-Myc and c-Fos, which are essential for cell growth, differentiation, and inflammation. JNK and p38 MAPK pathways respond to environmental stimuli, including pro-inflammatory cytokines and stresses such as UV radiation, osmotic or heat shock. The main target of JNK is c-Jun (Angel and Karin, 1991), while one of the main substrates of the active p38 molecule is ATF (Activating Transcription Factor). Both of them are transcription factors that regulate important cellular functions, such as inflammation by inducing the synthesis of pro-inflammatory cytokines, as well as cell growth, differentiation, survival and apoptosis (Cook et al., 2007). Another transcription factor, c-Fos, binds to c-Jun forming the AP-1 heterodimer involved in the expression of several pro-inflammatory genes (Neimark et al., 2006). NFκB (Nuclear Factor kappa B) is a heterodimer, formed by p50 and p65 subunits, which remains inactive in unstimulated cells bound to its inhibitory molecule IκB. In activated cells, IκB is phosphorylated by IKK and addressed to ubiquitination and proteasome degradation. Free NFκB is then phosphorylated and translocates to the nucleus, where it regulates the transcription of genes involved in innate immunity, inflammation, survival, proliferation and cell differentiation. The main stimuli for this signaling route include LPS, viruses, pro-inflammatory cytokines and DNA damage.

Quinoxaline derivatives constitute an interesting class of heterocyclic compounds with two aromatic rings, benzene and pyrazine (Pereira et al., 2015). Although these compounds are rare in nature some of them have been described (Sato et al., 2013). Nevertheless, since their chemical synthesis is relatively simple, a variety of derivatives have been generated, displaying a wide range of activities. Besides being used in industry, for instance as sensors for the detection of $Cu^{2+}$ (Kumar et al., 2013), multiple biological properties have been reported (Tristán-Manzano et al., 2015). Regarding the anti-inflammatory effects, some of the identified molecular targets include cyclooxygenase (COX) (Singh et al., 2004) and lipoxygenase (LOX) enzymes (Burguete et al., 2011), which are involved in prostaglandins and leukotrienes synthesis. We have previously synthesized and analyzed a new series of 4-alkoxy-6,9-dichloro [1,2,4] triazolo [4,3-a] quinoxalines that exhibit a potent inhibitory effect on the production of pro-inflammatory cytokines TNF-α and IL-6 in an *in vitro* model of macrophage-like cells, with very low levels of cytotoxicity (Guirado et al., 2012).

Given the high therapeutic potential of these quinoxaline derivatives, we aim now to reveal their anti-inflammatory mechanism of action and molecular targets by analyzing their effect upon signal transduction pathways involved in the regulation of the inflammatory activity in macrophages. Concretely, we have analyzed the pattern of activation of key signaling molecules of the MAPK, NFκB and PI3K/Akt pathways, as well as the expression of the transcription factor c-Fos.

## 2. Materials and Methods

### 2.1. Quinoxaline Derivatives

Quinoxaline compounds were synthesized by a novel high throughput method (Guirado et al., 2012), obtaining the series 8, in which from a core quinoxaline structure (Fig. 2A) new molecules were obtained by the incorporation of different radicals (R) as indicated in Fig. 2B. The series contains eight compounds, named from compound 8a to compound

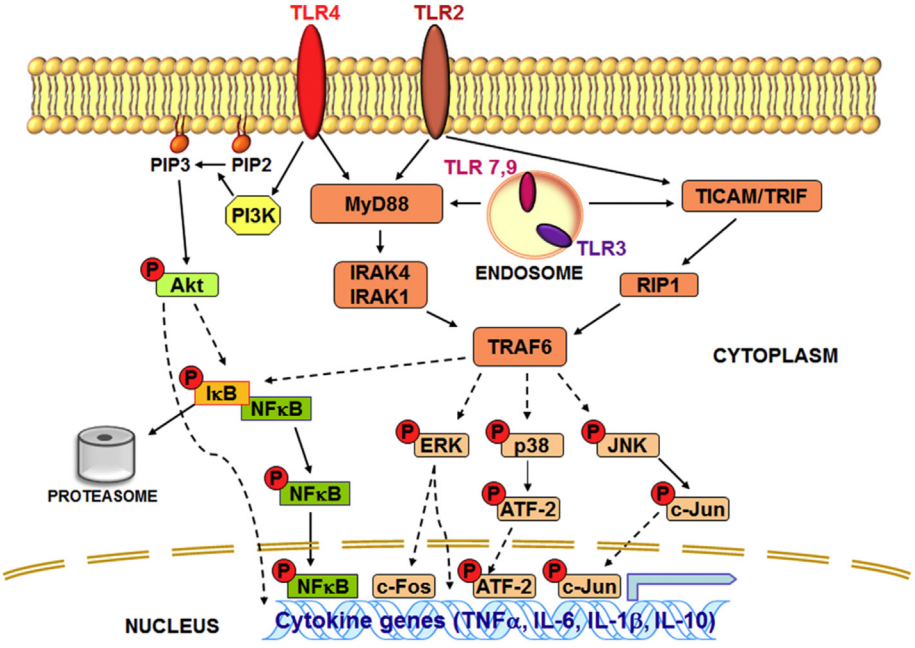
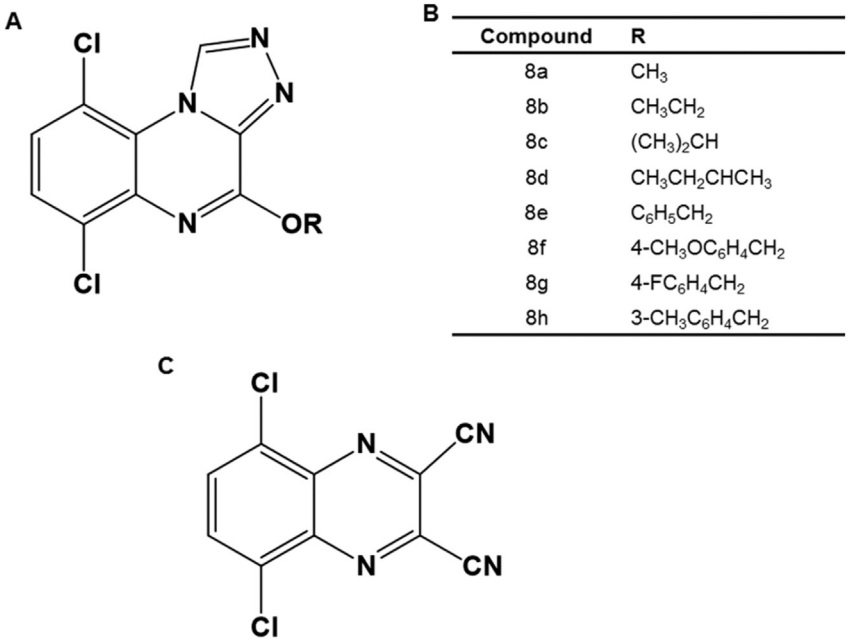



**Fig. 1.** Scheme of intracellular signaling pathways that control the expression of cytokines in macrophages. The binding of TLR receptors to their correspondent ligands triggers a series of signaling cascades that will induce the synthesis of several pro-inflammatory (such as TNF-α, IL-6 and IL-1β) and anti-inflammatory (IL-10) cytokines.




| Compound | R |
|---|---|
| 8a | $CH_3$ |
| 8b | $CH_3CH_2$ |
| 8c | $(CH_3)_2CH$ |
| 8d | $CH_3CH_2CHCH_3$ |
| 8e | $C_6H_5CH_2$ |
| 8f | $4\text{-}CH_3OC_6H_4CH_2$ |
| 8g | $4\text{-}FC_6H_4CH_2$ |
| 8h | $3\text{-}CH_3C_6H_4CH_2$ |

**Fig. 2.** Chemical structure of quinoxaline compounds. (**A**) Basic Structure of 4-alkoxy-6,9-dichloro [1,2,4] triazolo [4,3-a] quinoxaline. Derivative Series 8 varies in radical R. (**B**) List of radicals of the correspondent quinoxaline derivatives included in the series. (**C**) Structure of the synthesis intermediate 5,8-dichloro-2,3-diacyanoquinoxaline (Compound 3).

8h. Compound 3: 5,8-dichloro-2,3-diacyanoquinoxaline (Fig. 2C), is an intermediate of the synthesis process that was also tested in the biological *in vitro* assays.

### 2.2. In Vitro Assays

As an *in vitro* model, we used the myeloid cell line HL-60 (ATCC® CCL-240™) from a human's acute promyelocytic leukemia. These cells were first cultured in suspension in complete culture medium (CCM) formed by RPMI 1640 culture medium (Biowest, Nuaillé, France) containing 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin/streptomycin (GIBCO, Invitrogen, Paisley, UK) into an incubator at 37 °C and 5% $CO_2$. Once cells were growing at exponential rate they were differentiated into macrophage-like cells by culturing them in 6-well plates at $2 \cdot 10^6$ cells/well in the presence of the previously optimized dose of 16.2 nM (10 ng/ml) phorbol myristate acetate (PMA) during 24 h. Next, for resting purposes, cells were cultured for 24 h with CCM in the absence of PMA. Quinoxaline derivatives were diluted in dimethylsulfoxide (DMSO; MERK, Whitehouse Station, NJ, USA) and used in the cell assays at the optimal dose of 10 μM, which was previously established by dose-response assays. After differentiation and resting periods, cells were first pretreated with the optimal dose of the different compounds for 30 min prior being stimulated with 0.1 μg/ml LPS (*Escherichia coli* 0111.B4; Sigma Chemical Co. Saint Luis, MO, USA) for 24 h. All tests were carried out after cell passage 4 and before passage 20. Finally, cell lysates from three different experiments were obtained for immunoblotting assays.

### 2.3. Immunoblotting

Protein extracts from cells were obtained and treated as described elsewhere (Ruiz-Alcaraz et al., 2011). The activation levels of the potential target molecules were analyzed by denaturing electrophoresis (SDS-PAGE) and Western blotting, using primary antibodies against specific epitopes of phosphorylated proteins ERK 1/2 (Thr202/Tyr204), ATF-2 (Thr71), c-Jun (Ser63), and Akt (Ser473), and against non-phosphorylated epitopes of proteins IκBα, c-Fos (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and β-Actin (Sigma-Aldrich Co, Saint Luis, MO, USA), followed by addition of the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) enzyme. The activity of membrane-bound HRP was detected by using an enhanced chemiluminescent detection method (Enhanced ChemiLuminescence system; ©Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein bands were quantified by digital densitometry using ImageJ software and expressed relative to β-Actin.

### 2.4. Cytotoxicity Test

Compounds-derived citotoxicity was evaluated by MTT cell viability assays (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, to purple formazan by cellular mitochondrial enzymes) (Guirado et al., 2012). Briefly, MTT was added to the cells at a final concentration of 0.3 mg/ml and incubated at 37 °C within 5% $CO_2$ for 1 h, then a solubilization solution (an acidified isopropanol solution containing 0.1% NP-40 detergent and clorhidric acid 0.04 M) was added to dissolve the insoluble purple formazan product, retained in the cells, into a colored solution. The absorbance in each well was measured at 550 nm by a spectrophotometer. The percentage of compound cytotoxicity was obtained as compared to control conditions (100% viability, 0% cytotoxicity).

### 2.5. Statistical Analysis

Results are reported as histograms representing mean ± standard error of the mean (SEM). Statistical differences were determined by using Mann-Whitney *U* test and T-Student test. Statistical significance was considered for $p$ values lower than 0.05. Calculations were performed using the SPSS 21.0 software (Chicago, IL, USA).

## 3. Results

### 3.1. Quinoxalines Effect on the ERK 1/2 Signaling Route

Treatment with LPS activated ERK 1/2 by inducing a 50% increase in its phosphorylation level. Combined treatments with most of the compounds studied plus LPS produced a marked decrease in the phosphorylation profile of ERK 1/2 compared with the LPS-stimulated positive control, with the only exception of compound 8h. The highest inhibitory

effect on ERK 1/2 phosphorylation was obtained with compounds 3, 8a and 8e, which significantly reduced the level of ERK 1/2 phosphorylation below 50% compared to the baseline of activation observed in control untreated cells (Fig. 3A, Table 1).

### 3.2. Quinoxalines Effect on the JNK Signaling Cascade

As shown in Fig. 3B, treatment with LPS increased slightly, but significantly ($p = 0.001$), the phosphorylation of transcription factor c-Jun (1.12-fold), which is the main substrate of activated JNK. A variable pattern of c-Jun phosphorylation was observed depending on the quinoxaline compound assayed concomitantly with LPS, highlighting the inhibitory effect of compounds 3, 8c, 8e, 8f, 8g and 8h, which reduce in each case the LPS-induced phosphorylation effect on c-Jun below the control baseline level, while compounds 8a, 8b and 8d did not lower the activation effect induced by LPS (Table 1).

### 3.3. Quinoxalines Effect on the p38 MAPK Signaling Pathway

Regarding the effect upon the phosphorylation pattern of the transcription factor ATF-2, substrate of p38 MAPK, when cells were treated with the quinoxaline derived compounds in the presence of LPS, we found that the activation of ATF-2 was generally higher than the baseline level, or similar to that induced by LPS alone. Surprisingly, we observed a significant positive effect of compound 8d, which strongly induced phosphorylation of ATF-2 about 10-fold above baseline. The only exception in the series was observed with the compound 8a, which showed a non-significant trend to reduce ATF phosphorylation down to the baseline level (Fig. 3C, Table 1).

### 3.4. Effect of Quinoxalines on the PI3K/Akt Signaling Route

The study of the effect of quinoxaline derivatives on the PI3K/Akt route showed that stimulation with LPS not only did not produce any increase over the basal level of phosphorylation of Akt, but also slightly reduced it. However, treatment of cells with derivatives of quinoxalines plus LPS induced in all cases the activation of this route, with increments ranging between 2 and 9-fold in Akt phosphorylation compared to that registered at baseline. Specially remarkable is the rise up to 9-fold in Akt phosphorylation induced by compound 8c, and the 6-fold activation produced by compounds 8b and 8f, followed by compounds 8a, 8d, 8e and 8h, which reached over 4-fold of Akt phosphorylation (Fig. 4, Table 1).

### 3.5. Quinoxalines Effect on the p65 NFκB Signaling Route

Related to this signaling pathway, our results showed that neither LPS alone nor any of the quinoxaline combined with LPS treatments were able to modify the relative expression levels of IκBα, which is the natural inhibitor of p65 NFκB, thus displaying protein expression level similar to baseline in all cases (Fig. 5A, Table 1).

### 3.6. Quinoxalines Effect on the Expression of c-Fos Transcription Factor

Analyses of the transcription factor c-Fos showed that LPS presents a trend to slightly induce its protein expression up to 1.25-fold compared to control. Treatments with LPS plus quinoxaline derivatives revealed that most of the assayed compounds decreased the expression of c-Fos below basal levels (Fig. 5B). Thus, c-Fos protein levels were significantly reduced in the presence of compound 8d (65% reduction) and to a lesser extent, by compounds 8f (40%), 8g (37%), 8e (30%) and 8a (25%). Exception to this lowering effect was observed with compounds 8b and 8c that did not vary the expression of c-Fos with respect to those obtained with LPS alone, as well as with the intermediate 3, which induced the expression of c-Fos by 1.9-fold (Table 1).

### 3.7. Compounds Toxicity

Cytotoxicity of the quinoxalines derivatives tested by MTT assays is shown in Table 1. None of the tested compounds showed a significantly higher cytotoxicity compared to control conditions.

## 4. Discussion

In recent years it has been shown that chronic inflammatory processes are closely involved in many diseases, not only in those of a clear inflammatory nature such as rheumatoid arthritis, psoriasis or cirrhosis, but also in others such as diabetes or cancer (Feldmann et al., 2001; Tracey et al., 2008). Since IL-6 and TNF-α are among the most crucial pro-inflammatory cytokines related to these pathologies, they have become relevant targets for the development of new therapies. Based on that, a major effort is being focused in the development of new synthetic drugs capable of inhibiting those cytokines without producing severe adverse side effects (Fine, 2013).

Our group has developed and described a new series of quinoxaline derivatives with a potent inhibitory activity on the secretion of IL-6 and TNF-α in a human macrophage-like cell model *in vitro*, with very low

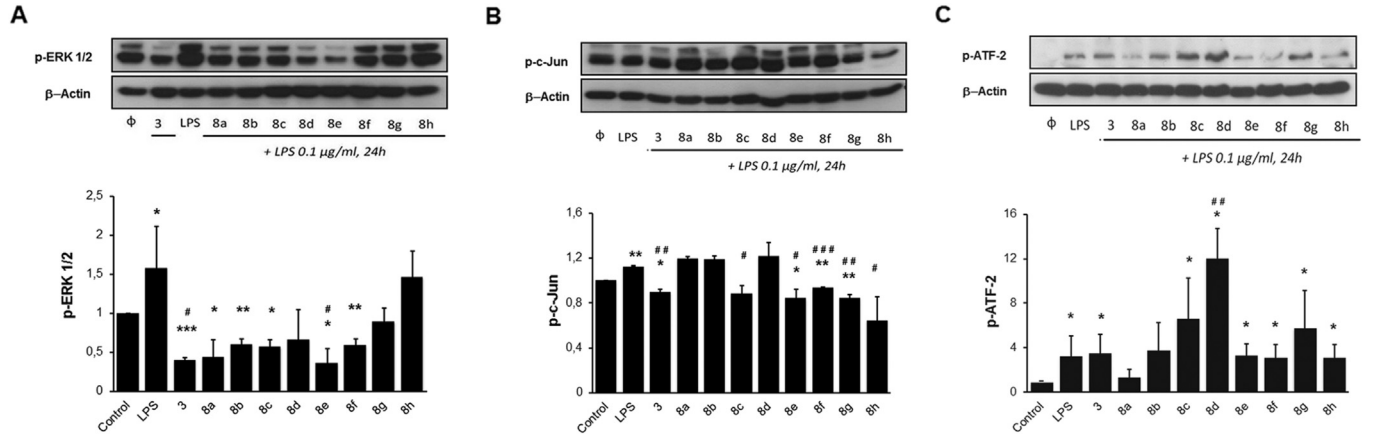



**Fig. 3.** Activation patterns of MAP kinases pathways. HL-60 macrophage-like differentiated cells were either treated with vehicle (DMSO) as control (ϕ), LPS alone (0.1 μg/ml), or pre-incubated (30 min) with different quinoxaline derivatives (10 μM) prior LPS treatment, and incubated at 37 °C with 5% $CO_2$ for 24 h. Cells extracts were obtained for immunoblotting assays to test the phosphorylation state of MAP kinase ERK 1/2 (**A**), transcription factor c-Jun (**B**), and p38 MAPK (**C**). Phosphorylation levels were quantified using β-Actin protein expression as loading control and data referred to control conditions (normalized as 1). Representative Western blots are shown. Histograms represent mean ± SEM from three different experiments. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$ and $^{***}$ $p < 0.001$, treatments compared with control. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$, quinoxalines treatments plus LPS compared to LPS alone.

**Table 1**
Effect of quinoxaline derivatives on signaling pathways, and correspondent cytotoxicity levels.

| Treatment | Level of phosphorylation | | | | Level of expression | | Cytotoxicity (%) |
|---|---|---|---|---|---|---|---|
| | ERK 1/2 | c-Jun | ATF-2 | Akt | IκBα | c-Fos | |
| LPS | 1.58 ± 0.53$^{*}$ | 1.12 ± 0.01$^{**}$ | 3.36 ± 1.74$^{*}$ | 0.86 ± 0.09$^{*}$ | 1.02 ± 0.08 | 1.25 ± 0.19 | 0.0 |
| +3 | 0.41 ± 0.02$^{***,\#}$ | 0.90 ± 0.03$^{*,\#\#}$ | 3.57 ± 1.66$^{*}$ | 2.42 ± 0.49$^{**,\#\#}$ | 0.95 ± 0.05 | 1.86 ± 0.52$^{*}$ | 13.1 ± 15.5 |
| +8a | 0.45 ± 0.22$^{*}$ | 1.19 ± 0.02 | 1.42 ± 0.62 | 4.01 ± 1.03$^{**,\#\#}$ | 1.04 ± 0.03 | 0.75 ± 0.12$^{*,\#}$ | 0.0 |
| +8b | 0.61 ± 0.06$^{**}$ | 1.19 ± 0.03 | 3.86 ± 2.42 | 6.24 ± 1.78$^{**,\#\#}$ | 1.01 ± 0.02 | 1.20 ± 0.02$^{***}$ | 6.1 ± 5.2 |
| +8c | 0.58 ± 0.08$^{*}$ | 0.88 ± 0.07$^{\#}$ | 6.68 ± 3.56$^{*}$ | 8.88 ± 2.68$^{**,\#\#}$ | 1.11 ± 0.02 | 1.02 ± 0.21 | 8.2 ± 6.5 |
| +8d | 0.67 ± 0.38 | 1.21 ± 0.13 | 12.04 ± 2.68$^{*,\#\#}$ | 4.34 ± 1.20$^{**,\#\#}$ | 1.09 ± 0.11 | 0.35 ± 0.07$^{***,\#\#}$ | 0.0 |
| +8e | 0.37 ± 0.18$^{*,\#}$ | 0.84 ± 0.08$^{*,\#}$ | 3.40 ± 0.93$^{*}$ | 4.40 ± 1.18$^{**,\#\#}$ | 1.02 ± 0.02 | 0.70 ± 0.11$^{*,\#}$ | 0.0 |
| +8f | 0.60 ± 0.07$^{**}$ | 0.94 ± 0.01$^{**,\#\#\#}$ | 3.19 ± 1.13$^{*}$ | 5.94 ± 1.67$^{**,\#\#}$ | 0.99 ± 0.10 | 0.60 ± 0.20$^{*,\#}$ | 0.0 |
| +8g | 0.90 ± 0.16 | 0.84 ± 0.03$^{**,\#}$ | 5.80 ± 3.37$^{*}$ | 2.02 ± 0.39$^{**,\#\#}$ | 0.99 ± 0.07 | 0.63 ± 0.11$^{**,\#}$ | 0.0 |
| +8h | 1.47 ± 0.33 | 0.65 ± 0.21$^{\#}$ | 3.17 ± 1.14$^{*}$ | 4.51 ± 1.28$^{**,\#\#}$ | 1.06 ± 0.11 | 0.88 ± 0.29 | 0.0 |

Data represents relative levels of phosphorylation, or protein expression, as Mean ± SEM compared to control conditions (Normalized to 1). Cytotoxicity levels are presented as Mean percentage ± SEM compared to control conditions (0%).
$^{*}$ $p < 0.05$.
$^{**}$ $p < 0.01$.
$^{***}$ $p < 0.001$, treatments compared with control.
$^{\#}$ $p < 0.05$.
$^{\#\#}$ $p < 0.01$.
$^{\#\#\#}$ $p < 0.001$, quinoxalines treatments plus LPS compared to LPS alone.

cytotoxic activity (Guirado et al., 2012). Also, we have recently reviewed the described targets of compounds with quinoxaline structure and their therapeutic potential (Tristán-Manzano et al., 2015). Herein, we have studied the effect of these compounds on the activation pattern of crucial intracellular signaling routes involved in the expression of the inflammatory function of human macrophages, MAPK (ERK 1/2, JNK and p38 MAPK), PI3K/Akt, p65 NFκB, as well as the expression of the transcription factor c-Fos, in an attempt to identify potential molecular targets that would allow us to develop new compounds with greater inhibitory potential and higher specificity.

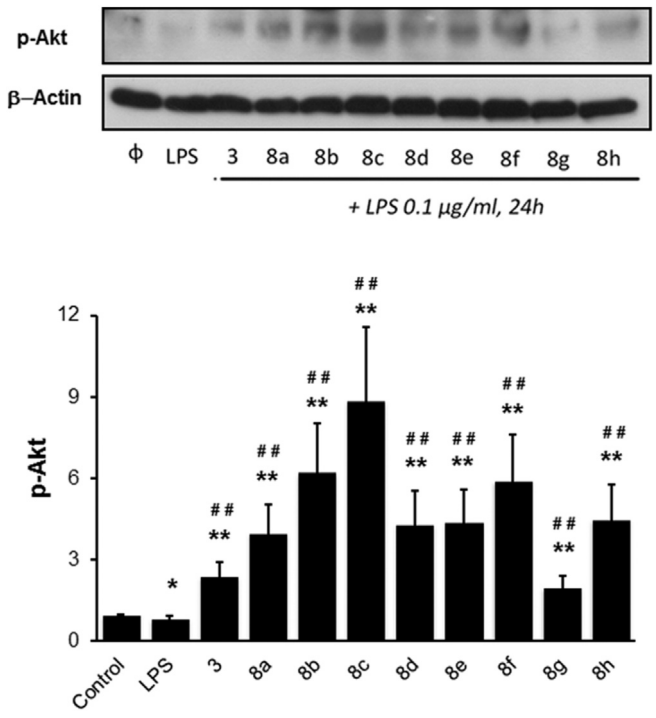



**Fig. 4.** Activation pattern of Akt. HL-60 macrophage-like differentiated cells were either treated with vehicle (DMSO) as control (ϕ), LPS alone (0.1 μg/ml), or pre-incubated (30 min) with different quinoxaline derivatives (10 μM) prior LPS treatment, and incubated at 37 °C with 5% $CO_2$ for 24 h. Cells extracts were obtained for immunoblotting assays to test the phosphorylation state of Akt. Phosphorylation levels were quantified using β-Actin protein expression as loading control and data referred to control conditions (normalized as 1). Representative Western blots are shown. Histograms represent mean ± SEM from three different experiments. $^{*}$ $p < 0.05$ and $^{**}$ $p < 0.01$, treatments compared with control. $^{\#\#}$ $p < 0.01$, quinoxalines treatments plus LPS compared to LPS alone.

Data obtained from the analysis of MAPK activation profiles showed that most of the assayed compounds inhibit the ERK 1/2 pathway, reducing the phosphorylation of this intermediate molecule to levels below its baseline state in the absence of pro-inflammatory stimulus. Related to the analysis of the JNK pathway, we found that several compounds are able to reduce the phosphorylation of the transcription factor c-Jun, substrate of JNK. While in the case of p38 MAPK the phosphorylation levels of its substrate ATF-2 are not reduced, but remain unchanged or even increased in one case (Table 1). In this regard, our previous data obtained in a cellular model of human chronic inflammatory pathology, such as peritoneal macrophages obtained from ascites of patients with cirrhosis revealed that the level of activation of ERK 1/2 directly correlated with the production of IL-6. Furthermore, we showed that the ERK 1/2 and JNK/c-Jun signaling routes are the most important for the induction of IL-6 and TNF-α secretion in response to LPS (Ruiz-Alcaraz et al., 2016, 2011; Tapia-Abellán et al., 2013). Consistent with those, our present results suggest that the inhibitory effect of our quinoxaline derivatives on ERK 1/2 and JNK/c-Jun, would be one of the main causes of the corresponding inhibitory effect on the production of IL-6 and TNF-α.

Related to the effect of quinoxalines on p38 MAPK, the pattern of activation of this pathway does not change significantly with respect to that induced by LPS, showing a tendency to be activated in some cases, as it significantly happened with the compound 8d, but also to be inhibited to baseline levels only in the case of compound 8a (Table 1). This suggests that the inhibitory effect of quinoxalines on IL-6 and TNF-α secretion would not be significantly related to the pattern of activation of p38 MAPK. Related to this, we have previously found that the p38 MAPK pathway is weakly activated in macrophages from cirrhotic ascites compared with macrophages derived from blood monocytes of healthy donors, and that its regulation in the presence of stimuli such as LPS, did not correlate with the production of IL-6 or TNF-α, pointing to a minor role in the regulation of these cytokines in macrophages present in an chronic inflammatory scenario (Ruiz-Alcaraz et al., 2016, 2011; Tapia-Abellán et al., 2013). Furthermore, analysis of the effect of quinoxaline compounds on the p65 NFκB pathway, by studying the expression of its inhibitor IκBα, did not reveal significant changes. This fact indicates that this pathway is not target of the assayed compounds, and therefore it is not responsible for their inhibitory effect on IL-6 and TNF-α. Note that we have previously described, as with p38 MAPK, that NFκB

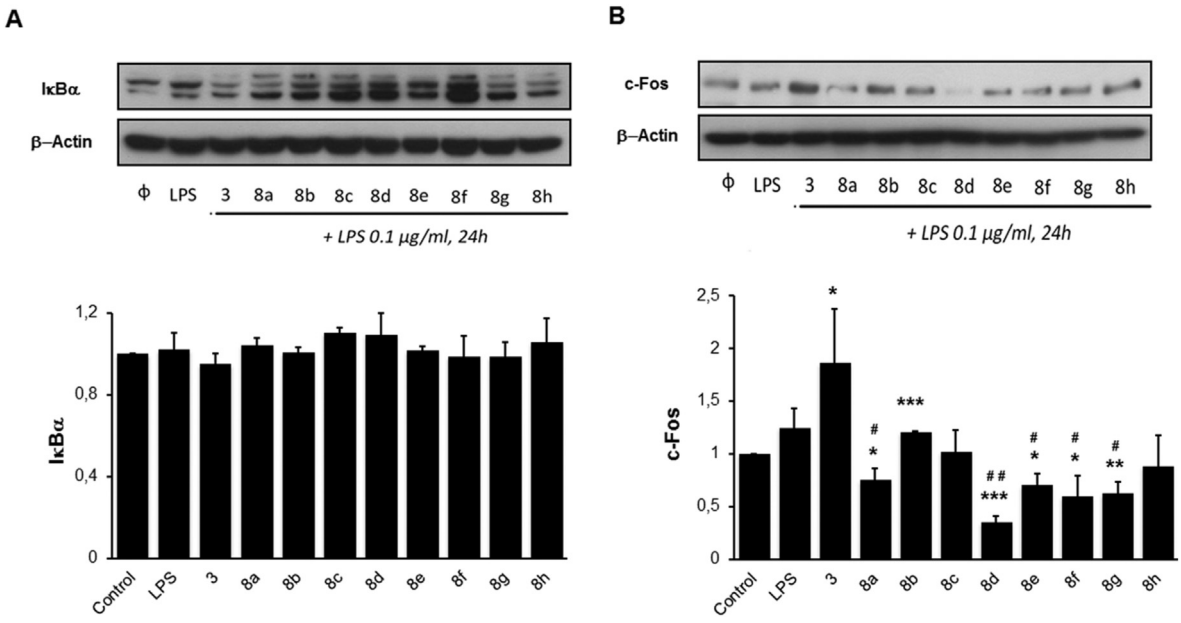



**Fig. 5.** Protein expression pattern of IκBα and c-Fos. HL-60 macrophage-like differentiated cells were either treated with vehicle (DMSO) as control (ϕ), LPS alone (0.1 μg/ml), or pre-incubated (30 min) with different quinoxaline derivatives (10 μM) prior LPS treatment, and incubated at 37 °C with 5% $CO_2$ for 24 h. Cells extracts were obtained for immunoblotting assays to test the protein expression of the NFκB cell signaling modulator IκBα (**A**), and transcription factor c-Fos (**B**). Phosphorylation levels were quantified using β-Actin protein expression as loading control and data referred to control conditions (normalized as 1). Representative Western blots are shown. Histograms represent mean ± SEM from three different experiments. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$ and $^{***}$ $p < 0.001$, treatments compared with control. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$, quinoxalines treatments plus LPS compared to LPS alone.

has also very low levels of activation in ascites macrophages, which suggests that is not significantly involved in the regulation of IL-6 and TNF-α production in this cell type (Ruiz-Alcaraz et al., 2011).

When we analyzed the expression levels of transcription factor c-Fos, which is part of the transcription complex AP-1 when it is bound to c-Jun, we observed that most of the assayed compounds were able to reduce the expression of this protein (Table 1). This indicates that c-Fos is indeed a molecular target of quinoxalines, and that the reduction of its expression may have an important role, together with the inhibition of ERK 1/2 and c-Jun, in the inhibitory effect of these compounds on IL-6 and TNF-α production.

Also, we analyzed the Akt phosphorylation profile, which is a key molecule of the signaling PI3K/Akt pathway directly involved in the up-regulation of the anti-inflammatory cytokine IL-10 (Antoniv and Ivashkiv, 2011) and the down-regulation of pro-inflammatory IL-1β (Tapia-Abellán et al., 2013), playing therefore an important anti-inflammatory role. Our data showed that all derivatives of quinoxalines effectively induced Akt phosphorylation, and so the activation of this anti-inflammatory route, which could be playing an important role in the final effect of the assayed compounds (Table 1).

Finally, it is important to mention that, in this study, none of the tested quinoxaline derivatives showed significant levels of cytotoxicity (Table 1).

Our results partially agree with those from Park et al. (Park et al., 2011), who showed how a different series of quinoxaline derivatives (4-chlorotetrazolo[1,5-*a*]quinoxaline) present an anti-inflammatory function by reducing both TNF-α and IL-4 production and mast cell degranulation *in vitro* and in animal models. This anti-inflammatory effect upon mast cells was mediated by a dose-dependent inhibition of major MAP kinases (ERK1/2, p38 MAPK, and JNK) and PI3K/Akt pathway, through the inhibition of the upstream kinase Syk (Park et al., 2011). In this macrophage model with our particular quinoxaline series, we also observed an inhibition of ERK and JNK/c-Jun, although we did not find significant modifications in the p38 activation pattern, and, on the contrary, an enhancement of the PI3K/Akt pathway was detected. Other authors have also shown how quinoxaline derivatives can inhibit other upstream molecules that will in turn inhibit MAP kinases and PI3K/Akt pathways. This is the case of pyrrolo[1,2-*a*]quinoxaline derivatives, which act as non-cytotoxic inhibitors of the human protein kinase CK2 (Guillon et al., 2013). While others have described how series of these compounds can directly interact and inhibit JNK, opening the door to a hit-to lead optimization of their activity and selectivity (Li et al., 2013).

## 5. Conclusions

We have successfully identified a number of intracellular signaling targets of a new series of 4-alkoxy-6,9-dichloro[1,2,4]triazolo[4,3-*a*]quinoxaline derivatives. Our data point out that the main targets involved in its inhibitory effect on the secretion of IL-6 and TNF-α, would include the inhibition of ERK 1/2 and JNK/c-Jun cascades and the reduction of the protein expression of transcription factor c-Fos. Furthermore, the p38 MAPK pathway would play a secondary role on the effect of these compounds, while the NFκB route is not target of these compounds. Finally, another target identified in this study is the PI3K/Akt route, which is actually induced by these compounds and may fulfill an important role as anti-inflammatory effector.

The observed anti-inflammatory potential of quinoxalines is therefore the result of a synergy effect, which comprehends different signaling alterations (activation or inhibition) of the studied intracellular pathways that will, in turn, down-regulate the expression of pro-inflammatory cytokines IL-6 and TNF-α. The identification of the modifications on these target routes can be useful for the rational design of new compounds derived from quinoxaline core, or other synthetic compounds, with the intention of increasing their anti-inflammatory potential.

## Conflict of Interest Disclosure

The authors declare no conflict of interest.

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