

Biological evaluation of novel 6-Arylbenzimidazo [1,2-c]quinazoline derivatives as inhibitors of LPS-induced TNF-alpha secretion

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ABSTRACT

This study describes the effect of novel 6-Arylbenzimidazo[1,2-c]quinazoline derivatives as tumor necrosis factor alpha (TNF- α) production inhibitors. The newly synthesized compounds were tested for their *in vitro* ability to inhibit the lipopolysaccharide (LPS) induced TNF- α secretion in the human promyelocytic cell line HL-60. The compound 6-Phenyl-benzimidazo[1,2-c]quinazoline, coded as G1, resulted as the most potent inhibitor and with no significant cytotoxic activity. Thus, 6-Arylbenzimidazo[1,2-c]quinazoline derivatives may have a potential as anti-inflammatory agents.

Key words: anti-inflammatory agents; benzimidazoquinazoline derivatives; TNF- α inhibitors

INTRODUCTION

Originally described as a protein factor capable of killing tumor cells *in vitro*, tumor necrosis factor alpha (TNF- α) has been demonstrated to participate in several biological processes; however it is also a potent pro-inflammatory agent and has been implicated in a variety of acute and chronic inflammatory diseases (Beutler and Cerami, 1989). Consequently, the successful introduction of TNF- α neutralizing agents, Infliximab, Etanercept and Adalimumab for clinical applications, such as for the treatment of many inflammatory diseases including Crohn's disease, rheumatoid arthritis, ankylosing spondylitis, juvenile rheumatoid arthritis, psoriatic arthritis and psoriasis (Le and Abbenante, 2005), has ushered in a new era in the treatment of these chronic inflammatory conditions. The success of these

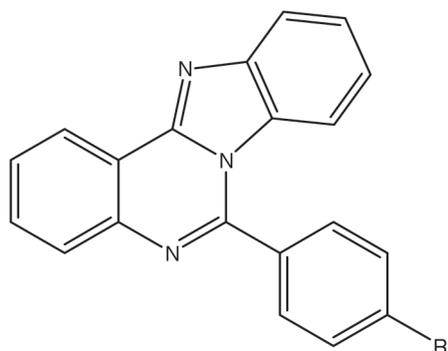
novel agents and their impressively demonstrated clinical benefits has stimulated the search for new anti-cytokine small molecules (Wagner and Laufer, 2006).

All of the anti-TNF- α drugs available to date are proteins, and therefore suffer to a varying degree from the general disadvantages associated with protein drugs, i.e., poor stability, poor cellular penetration, poor cellular activity, poor oral absorption and mandatory subcutaneous or intravenous administration, increasing the risk of allergic reactions, short half-life and rapid metabolism, and high costs of manufacturing. Therefore, orally active small molecules, targeting specific signaling and/or biosynthetic pathways of proinflammatory cytokines, would offer an attractive therapeutic alternative to anti-TNF- α biologicals in the treatment of inflammatory and autoimmune diseases.

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Previous reports described benzothiazolo- and benzimidazoquinazoline derivatives as cytotoxic compounds with potential antitumoral activity (Lunn et al., 1971; El-Sherbeny, 2000; Via et al., 2000), and particularly, there is one report that describes the antitumoral activity of benzimidazo[1,2-*c*]quinazolines (Brana et al., 1994).

In this paper we report the identification of 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives (Fig. 1), previously described by us (Pessoa-Mahana et al., 2004), as inhibitors of TNF- α production.



G1, R=H
G2, R=F
G3, R=OCH₃

Figure 1: Structure of the 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives

METHODS

Cell culture

The human promyelocytic leukemia cell line, HL-60, were cultured under standard conditions in DMEM, containing 10% fetal bovine serum (HyClone) and penicillin-streptomycin, at 37°C and 5% CO₂ atmosphere. Before each experiment, cells were starved over night in a serum-free medium.

Stimulation of HL-60

After the starvation period, the cells were stimulated simultaneously with lipopolysaccharide, LPS (Sigma) and phorbol 12-myristate 13-acetate, PMA

(Sigma), at a concentration of 10 μ g/mL and 1 μ M, respectively. Stimulation was for 6 hours both for TNF- α production and TNF- α mRNA expression. In experiments using inhibitors, each compound was added 30 minutes before stimulation. The compounds were dissolved in DMSO, and DMSO alone was used as a control. The n of subjects used for the experiments was 5.

Quantification of TNF- α

TNF- α was determined in cell culture media after stimulation using a commercially available EIA kit for human TNF- α (Cayman Co.), according to the manufacturer's instructions.

RNA preparation and RT-PCR

After stimulation in the absence or presence of inhibitors (n=3), total RNA was isolated using TRIZOL reagent (Invitrogen). In RT reactions equal amounts of total RNA (500 ng) from HL-60 cells were incubated 3 min at 72°C, and were subsequently reverse transcribed into cDNA using random hexamer primers and MMLV Reverse Transcriptase (Invitrogen) for 1 h at 42°C. The specific mRNA expression was determined by PCR using specific primers for human TNF- α and normalized relative to GAPDH expression. TNF- α primers, sense: 5'-CTTCTCAAACACTCGAGTGAC AAGCC-3' and antisense: 5'-TGACGGTG TGGGTGAGGAGCACAT-3'; GAPDH primers, sense: 5'-CCATCACCATCTTCC AGGAG-3' and antisense: 5'-CCTGCTTC ACCACCTTCTTG-3'. Annealing conditions were 65°C for TNF- α (30 cycles, 231bp) and 60°C for GAPDH (20 cycles, 576bp). All reagents for PCR were from Invitrogen.

Cytotoxicity assay

Cytotoxicity of the new compounds was evaluated by measuring the activity of LDH released into the medium after 6 h exposure of the cells to the inhibitors (n=3). LDH activity was quantified using a commercially available kit (Roche). Additionally, the direct capacity of each compound to inhibit LDH was evaluated (n=3).

Statistics

Data are given as means \pm SEM, n indicates the number of independent experiments. Statistical analysis was performed using Student's *t*-test and a one way ANOVA, followed by a Newman-Keuls when corresponding. A value of $p < 0.05$ was considered statistically significant.

RESULTS

TNF- α secretion and mRNA expression in response to LPS, PMA and their combination

In order to establish an in vitro biological assay to determine the potential of new compounds to inhibit TNF- α production we used the human promyelocytic leukemia cell line, HL-60. HL-60 cells were first stimulated with LPS alone (10 μ g/mL) but no secretory answer was observed after 6 h (Fig. 2B). However, when PMA and LPS were added simultaneously to the HL-60 cells for 6 h, there was a high and significant response both in TNF- α secretion (Fig. 2B), as well as a significant increase in TNF- α mRNA expression (Fig. 2A). Therefore, this method, which describes the use of HL-60 cells, resulted in a fast way for in vitro evaluation of potential new compounds that inhibit TNF- α production.

Inhibition of PMA/LPS-mediated TNF- α production by 6-Arylbenzimidazo[1,2-c]quinazoline derivatives

In inhibition studies, HL-60 cells were stimulated simultaneously with PMA/LPS in the presence or absence of the new inhibitory benzimidazoquinazoline derivative at a concentration of 100 μ M. The new compound was added 30 min before stimulation and DMSO was used as a control. As shown in Figure 3, all three benzimidazoquinazoline derivatives described were able to inhibit the PMA/LPS-mediated TNF- α production by HL-60 cells. Compound G1 showed to be the most active, with a 93% inhibition followed by G3 with an 85% inhibition, in TNF- α stimulated secretion, while pentoxifylline, a drug with known TNF- α inhibitory properties and used as a control in this study, reached

only a 77% inhibition of PMA/LPS-mediated TNF- α production when used at 2 mM final concentration and no inhibition when used at 100 μ M, which is consistent with what is reported in the literature (Mander et al., 1997).

Effect of 6-Arylbenzimidazo[1,2-c]quinazoline derivatives on TNF- α mRNA expression

In order to get further insight into the inhibitory mechanism of the described benzimidazoquinazoline derivatives on TNF- α production, TNF- α mRNA expression was evaluated. The results showed that stimulation of HL-60 cells with PMA/LPS for 6 h in the presence of compound G1 showed a complete blockade of TNF- α mRNA expression (Fig. 4).

Cytotoxic effects of 6-Arylbenzimidazo[1,2-c]quinazoline derivatives and LDH inhibitory capacity

In order to evaluate potential cytotoxic activity of the newly described benzimidazoquinazoline derivatives, the LDH activity released into a conditioned medium after a 6 h exposure of the cells to the compounds was evaluated. Results showed no significant cytotoxicity as compared to DMSO alone (Fig. 5). Furthermore, when the ability of the benzimidazoquinazoline derivatives to inhibit LDH activity was evaluated, results showed no significant inhibition (Table I).

TABLE I

Inhibition of LDH activity by 6-Arylbenzimidazo[1,2-c]quinazoline derivatives^a.

Compound	% of maximal LDH activity ^b
DMSO	90,4 \pm 0,5
G1	88,04 \pm 4,3 ^c
G2	88,6,4 \pm 4,7 ^c
G3	70,9 \pm 11,4 ^c

^a Total LDH activity of a Triton X-100 cell lysate was evaluated in the presence or absence of 100 μ M of the each compound. DMSO alone was used as control.

^b Data are expressed as percentage of maximal LDH activity present in cell lysate.

^c Data are not statistically different versus DMSO.

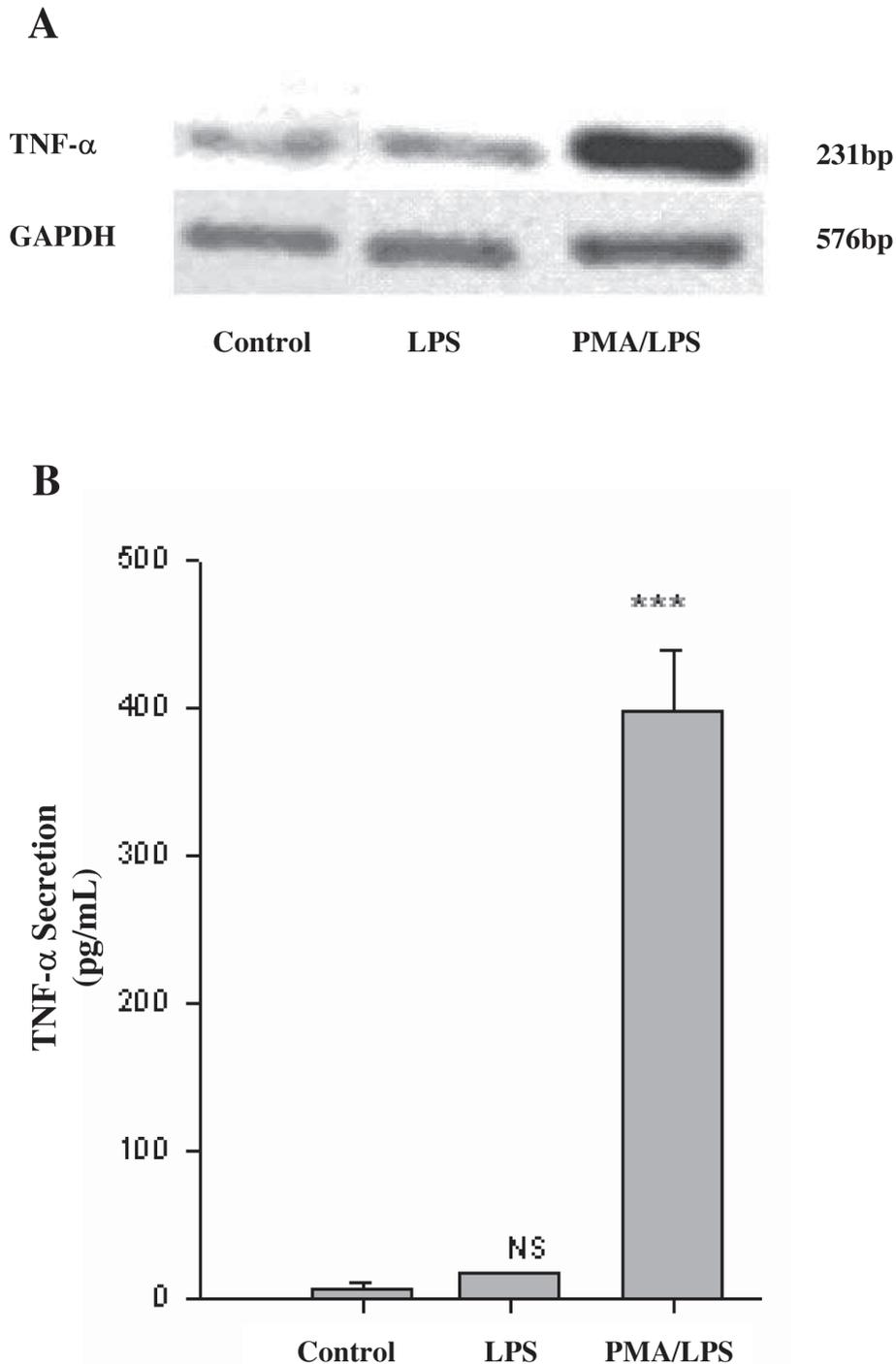


Figure 2: Effect of LPS, and the combination of PMA/LPS on TNF- α secretion and mRNA expression. HL-60 cells were stimulated either with LPS (10 μ g/mL) or PMA (1 μ M) + LPS (10 μ g/mL) for 6 h and (A) mRNA expression level was determined through RT-PCR using GAPDH as an internal control and (B) TNF- α was quantified in a conditioned medium by means of ELISA. Figure 2A shows a representative RT-PCR of three independent experiments for TNF- α mRNA expression in response to, LPS or PMA+LPS (PL). Figure 2B shows the TNF- α secretory response of HL-60 cells exposed to, LPS or PMA+LPS (PL). Results in B are presented as means \pm SEM of n= 5. ***p<0.001 vs control.

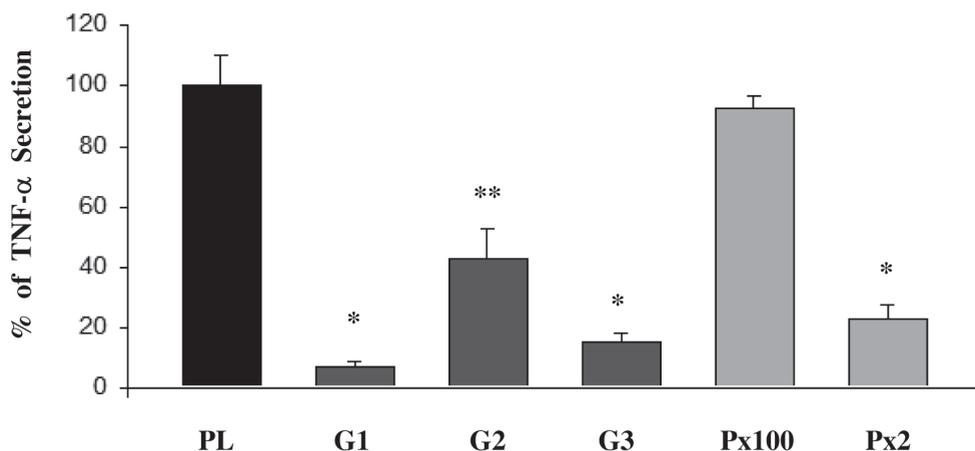


Figure 3: Inhibition of PMA/LPS-mediated TNF- α production by 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives. HL-60 cells were stimulated simultaneously for 6 h with PMA+LPS (PL) in the presence or absence of 100 μ M of the new 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives. Pentoxifylline (Px) was used at 100 μ M (Px100) and 2 mM (Px2) as a reference control. Each compound was added 30 min before stimulation and DMSO was used as a control. Results are presented as means \pm SEM of $n=4$. * $p<0.001$ vs PMA/LPS, ** $p<0.005$ vs PMA/LPS.

DISCUSSION

To investigate the inhibitory properties on TNF- α production of newly described benzimidazoquinazoline derivatives we used HL-60 cells instead of standard peripheral blood mononuclear cells (PBMC) because HL-60 cells undergo differentiation towards a macrophage like phenotype upon PMA stimulation (Schultz et al., 1997) and because a main feature of macrophages is their elevated capacity to produce TNF- α in response to LPS.

We report here that HL-60 cells did not secrete TNF- α in response to PMA or LPS alone, but their TNF- α secretion capacity was extremely increased when PMA and LPS were used simultaneously to stimulate them for only 6 h. Although not shown, we could also observe that a 30 min pulse of PMA was enough to commit HL-60 cells to differentiate towards a macrophage phenotype, and thereby responding to LPS stimulation with increased TNF- α production.

Benzimidazoquinazoline derivatives were first described for their antitumoral activities (Lunn et al., 1971; El-Sherbeny, 2000; Via et al., 2000) including

benzimidazo[1,2-*c*]quinazolines (Brana et al., 1994). Here we report a novel anti-cytokine activity for 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives (Pessoa-Mahana et al., 2004), which have the ability to inhibit TNF- α production in response to LPS. Additionally, the new 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives showed no potential cytotoxic activity as determined by their poor LDH releasing capacity into a conditioned medium.

In the model described here, pentoxifylline reached only a 77% inhibition of PMA/LPS-mediated TNF- α production when used at 2 mM final concentration, as compared with >90% inhibition of the 6-Phenyl-benzimidazo[1,2-*c*]quinazoline derivative (G1) tested at 100 μ M. Pentoxifylline is a well-known vasoactive drug with proven clinical efficacy in various circulatory disorders, and is also a commonly used drug during sepsis (Staubach et al., 1998) and to improve the outcome from sepsis in premature infants (Kirsch and Giroir, 2000). Therefore, the 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives described here might be potential candidates for anti-

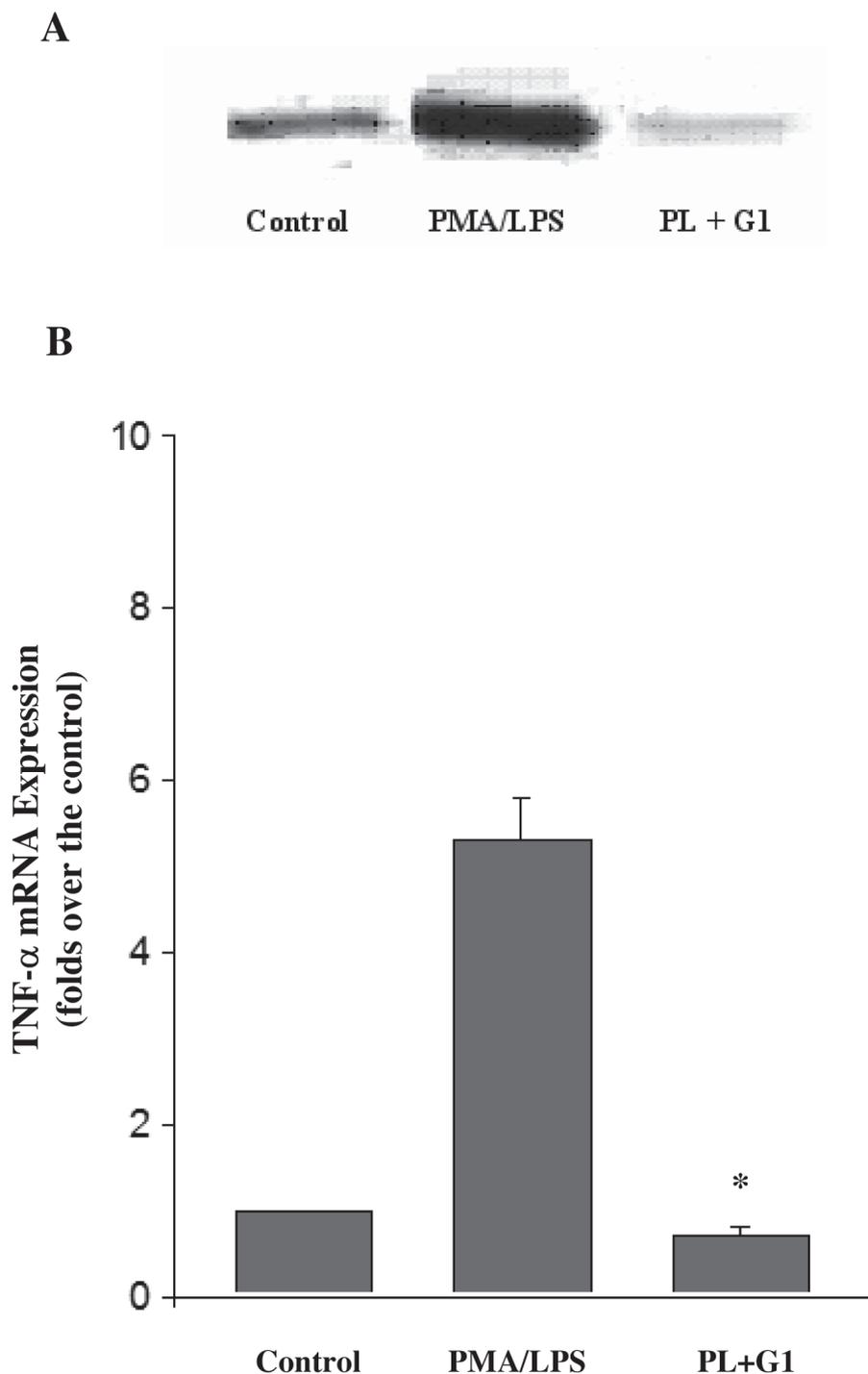


Figure 4: Effect of 6-Phenyl-benzimidazo[1,2-*c*]quinazoline on TNF- α mRNA expression. HL-60 cells were stimulated simultaneously for 6 h with PMA/LPS (PL) in the presence or absence of 100 μ M of 6-Phenyl-benzimidazo[1,2-*c*]quinazoline (G1). The compound G1 was added 30 min before stimulation and DMSO was used as a control. Figure 4A shows a representative RT-PCR of three independent experiments. Figure 4B shows the TNF- α mRNA expression, expressed as folds over the control. Results are presented as means \pm SEM of n= 3. *p<0.001 vs PMA/LPS. C, control; PL, PMA/LPS.

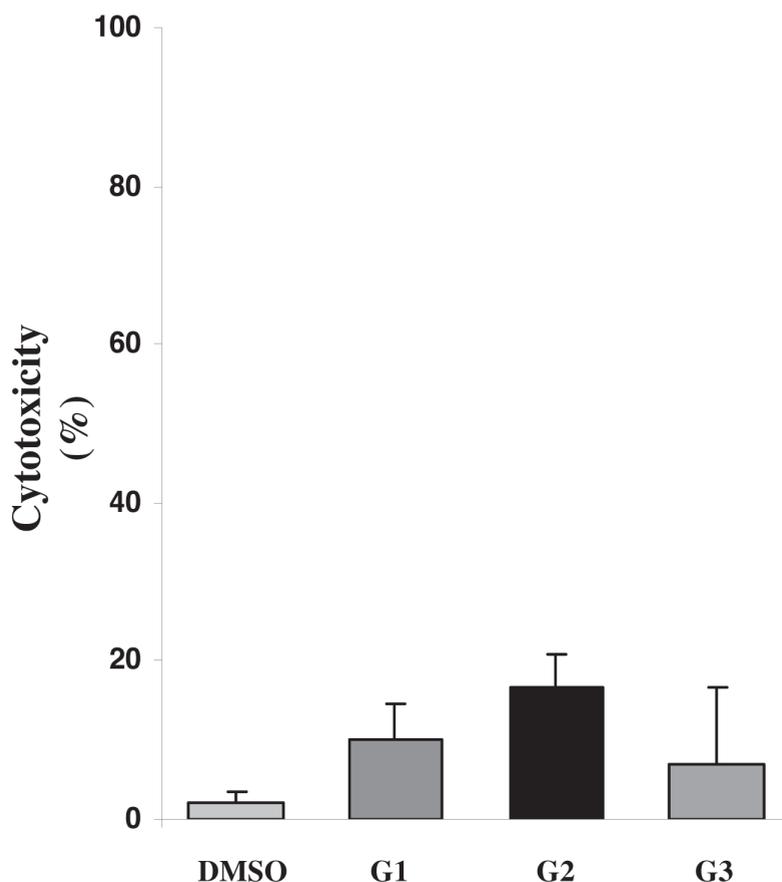


Figure 5: Cytotoxic effects of 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives. HL-60 cells were incubated for 6 h in the presence or absence of 100 μ M of the new inhibitory 6-Arylbenzimidazo[1,2-*c*]quinazoline derivatives and the LDH activity released into a conditioned medium was evaluated. Cytotoxicity is expressed as the % of LDH activity compared to the maximal released activity. Results are presented as means \pm SEM of $n=3$. No statistically differences versus control (DMSO) were observed.

inflammatory drugs as an alternative to protein biologicals. Nevertheless, further confirmation of their anti-inflammatory properties in an animal model of sepsis is needed. Thus, the next step of the present work will explore the *in vivo* effect of the 6-Phenyl-benzimidazo[1,2-*c*]quinazoline derivative in an animal model of sepsis.

Finally, the interesting anti-cytokine properties of all derivatives tested strongly indicate that this heterocyclic system represents an active framework for this activity. Although conscious that only three compounds are described, the substituents used allowed us to consider that the dropping in activity, observed when R=H is

replaced with F, suggest that the *para* position in the 6-phenyl substituent is a key position for the interaction between this framework and the corresponding target. Considering the similar molecular ratio between H and F, it seems clear that the electron-withdrawing effect of the fluorine atom reduces bioactivity. This effect can also explain the attenuation in the activity observed when R is a methoxyl group. Nevertheless, further studies are needed in order to clarify the effect of electron-donor substituents at the same position.

In summary, we have shown a rapid assay for evaluating new anti-cytokine inhibitors and identified novel 6-Arylbenzimidazo[1,2-

c]quinazoline derivatives as TNF- α production inhibitors with less cytotoxic activity, thereby providing potential new alternatives to protein biologicals to fight inflammatory diseases.

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