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Protective role of glycine and kolaviron on lipopolysaccharide-induced alterations of raw U937 cells and U937-derived macrophages

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Abstract

The effect of glycine and kolaviron on lipopolysaccharide-induced U937 cell damage and activation of U937-derived macrophages was studied. U937 cells were incubated with either glycine or kolaviron or both for 24 h before exposure to lipopolysaccharide. Cell viability and production of reactive oxygen species (ROS) were later assessed. In the other experiment, the U937 cells were transformed to the macrophage form using phorbol 12-myristate 13-acetate and incubated with or without glycine or kolaviron or both before exposure to lipopolysaccharide. Production of TNF-α, IL-1, IL-6 and NO were later assessed. The expression of the antioxidant enzymes- superoxide dismutase (SOD) and catalase (CAT) was also evaluated via reverse transcription polymerase chain reaction (RT PCR). It revealed that lipopolysaccharide caused significant cell death and production of reactive oxygen species that was reduced by glycine and kolaviron. Glycine and kolaviron also reduced lipopolysaccharide-mediated secretion of TNF-α, IL-1, IL-6 and NO in U937-derived macrophages. In some cases, pre-incubation of cells with both glycine and kolaviron was better than the individual responses. Glycine and kolaviron also reduced lipopolysaccharide-induced alterations in the expression of SOD and CAT (p<0.05). The study shows that both glycine and kolaviron (either separately or in combination) reduced lipopolysaccharide-mediated alterations in U937 cells and U937-derived macrophages.

Keywords: cell viability; glycine; kolaviron; lipopolysaccharide; macrophages

Introduction

Lipopolysaccharide (LPS) is an amphipathic molecule located at the outer membrane of Gram-negative bacteria. It is made up of a fatty acyl chain attached to a polypeptide up to 200 sugars, some of which bear phosphate groups (Okuda *et al.*, 2016). The LPS layer is part of the network that gives the Gram-negative bacteria a strong permeability barrier to enable them survive under harsh environmental conditions (Qiao *et al.*, 2014; Rios *et al.*, 2016).

The toxicity of Gram-negative bacteria is mediated largely due to the binding of LPS to specific targets of the innate immune system. This activates macrophages and neutrophils and, in the process, pro-inflammatory factors are elaborated (Huang and Kraus, 2016). This feature of the glycolipid has been used as a model for the study of infections and immune responses (Hwang *et al.*, 2011; Rhee, 2014).

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Glycine is a non-essential amino acid though it has been termed conditionally essential to enhance growth in humans and animals (Razak *et al*., 2017; Li and Wu, 2018). It has been suggested that the amount of glycine synthesized in the body may not be sufficient to meet metabolic needs. This simplest amino acid has been reported to play significant physiological roles such as anti-inflammatory, cytoprotection and metabolic regulation (Jain *et al*., 2012; Razak *et al*., 2017).

Garcinia kola Heckel (Clusiaceae) is an evergreen tree whose seeds are highly valuable and traditionally consumed as an antidote against some diseases (Iwu *et al*., 2009; Esiegwu *et al*., 2014). One of the important compounds isolated from *Garcinia kola* is kolaviron. Kolaviron is a biflavonoid complex widely reported to have various bioactivities in experimental models such as antimicrobial, antioxidant and also the modulation of some signaling pathways (Abarikwu, 2015; Adaramoye and Lawal, 2015; Akinmoladun *et al*., 2015; Oyagbemi *et al*., 2017; Apalowo *et al*., 2018).

This work is aimed at investigating the effect of glycine and kolaviron (separately and in combination) on LPS-induced toxicity on the proliferation of human monocyte cell line U937 and activation of U937-derived macrophages.

Materials and Methods

Materials

Glycine, fetal calf serum (heat inactivated), L-glutamine, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (from *E. coli* strain 055:B5), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl),-2,5-diphenyl-tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) and RPMI-1640 were purchased from Sigma-Aldrich (USA). The monocyte cell line U937 was obtained from the European Collection of Cell Cultures. All antibodies and biotinylated cytokines were products from Pharmingen (US). All other chemicals and reagents were of the highest purity and commercially available. Buffers and solutions were prepared using Milli-Q (18 $M\Omega cm^{-1}$) water and stored at room temperature unless stated otherwise.

Cell culture

Monocyte U937 cells were grown in RPMI-1640 supplemented with fetal calf serum (heat inactivated), penicillin (100 U/L), streptomycin (100 mg/ml) and L-glutamine (2 mM). Cells were kept in an incubator at a temperature of 37 °C gassing up to 5% CO_2. For differentiation induction (i.e. transformation to the macrophage form), the cultured cells were seeded at a density of 5 x 10^4 cells/ml and supplemented with phorbol 12-myristate 13-acetate (PMA) as described (Okoko and Oruambo, 2009).

Extraction of kolaviron

Fresh *Garcinia kola* seeds (4.5 kg) were sun-dried, seed coats were removed and later pulverized using a warring blender. Kolaviron was isolated from the resulting powder according to Iwu (1985). Briefly, powdered seeds were extracted with light petroleum ether (bp 40-60 °C) in a Soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The ethylacetate fraction was concentrated to give a golden yellow solid known as kolaviron. Percent recovery was 5.6%.

Cytotoxicity and ROS production

Cell cultures (seeded at 5 x 10^4 cells/ml) were supplemented with either glycine (300 μM in RPMI-1640) or kolaviron (25 μg/ml in RPMI-1640) or both for 24 h before exposure to 1 μg/ml lipopolysaccharide (LPS). For control experiments, cultures were not supplemented with glycine (GLY) or kolaviron (KVR) but

with an equivalent amount of RPMI-1640. One hour later, cell death was determined via MTT assay according to the method of Zhou *et al.* (2006). Briefly, 10 µl of MTT solution was added to each culture to a final concentration of 0.5 mg/ml and incubated for 1 h at 37 °C. The MTT was removed and each culture supplemented with DMSO (Dimethyl sulfoxide). Absorbance was measured at 570 nm using a microplate reader. Values are expressed as percentage of readings of cells not treated with LPS. The production of ROS was assessed based on the oxidation of 2′,7′-dichlorodihydrofluorescein by intracellular peroxides as reported (Koga and Meydani, 2001) with a slight modification. Following the incubation with GLY and KVR for 24 h, media was aspirated and replaced with 50 µM DCHF-DA (in RPMI-1640) and incubated for 30 min at 37 °C. Cells were later washed with 0.02 M phosphate buffered saline (pH 7.4) and incubated with LPS (1 µg/ml) for 1 h. Fluorescence of cells was measured at excitation and emission wavelength at 485 nm and 530 nm respectively. Final value was expressed as % production of ROS. Production of ROS in cells incubated with only LPS was arbitrarily assigned 100%.

Cytokine and NO production

After 24 h of PMA-induced differentiation, media was aspirated and replaced with GLY or KVR or both for 24 h before exposure to LPS (1 µg/ml). The supernatants of each cell culture were analyzed for the production of TNF-α, IL-1 and IL-6 via cytokine capture ELISA as described (Okoko and Oruuambo, 2009) while nitric oxide production was determined according to Hwang *et al.* (2002) as modified by Hsieh *et al.* (2007).

Quantitative RT-PCR

Following PMA-induced induction and subsequent treatment of cells with GLY, KVR and LPS (as described previously), total RNA was purified from cell pellets using TRIzol reagent (Invitrogen) and quantified by NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). Preparation of cDNA was done using a Revert Aid cDNA synthesis kit according to the manufacturer's protocol. For RT-PCR, 1 µg of the resulting cDNA was used to amplify regions specific to superoxide dismutase (SOD) and catalase (CAT) in an ABI Prism 7500 system (Applied Biosciences) with primer pairs listed in table 1. The thermal cycler was set at an initial denaturation at 95 °C for 5 min and 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min (35 cycles) and a final extension step at 72 °C for 10 min. Real-Time PCR data was analyzed and presented as fold change in expression over the GAPDH housekeeping gene of same sample.

Table 1. Primer Pairs for RT-PCR

mRNA	Primer sequence (5′-3′)
SOD	FP:GACTGAAGGCCTGCATGGATTC RP: CACATCGGCCACACCATCTTTG
CAT	FP:CTTCGACCCAAGCAACATGC RP:GATAATTGGGTCCCAGGCGATG
GAPDH	FP:GTCGGAGTCAACGGATTTGGTC RP:CTTCCCGTTCTCAGCCTTGAC

Data analysis

Representative values for various experiments were expressed as mean ± SEM of six replicates. Where applicable, data were analysed using analysis of variance followed by Duncan's multiple range test. Confidence exhibited at $p<0.05$ was considered statistically significant.

Results

Cytotoxicity study

Treating the cells with either glycine or kolaviron (300 µM glycine; 25 µg/ml kolaviron) didn't cause any significant effect on cell viability (data not shown). It has been reported that the reference value of glycine in plasma is 120-560 µM (Laposata, 2019) thus 300 µM was used to reflect normal plasma level.

Figure 1 shows the protective effect of glycine and kolaviron on LPS-induced cell death. It revealed that LPS caused significant reduction in cell viability as accessed via MTT assay. However, pre-treatment of the cells with glycine or kolaviron significantly reduced LPS-induced cell death (p<0.05). But pre-treatment with both glycine and kolaviron was better at reducing cell death than their individual effects (p<0.05).

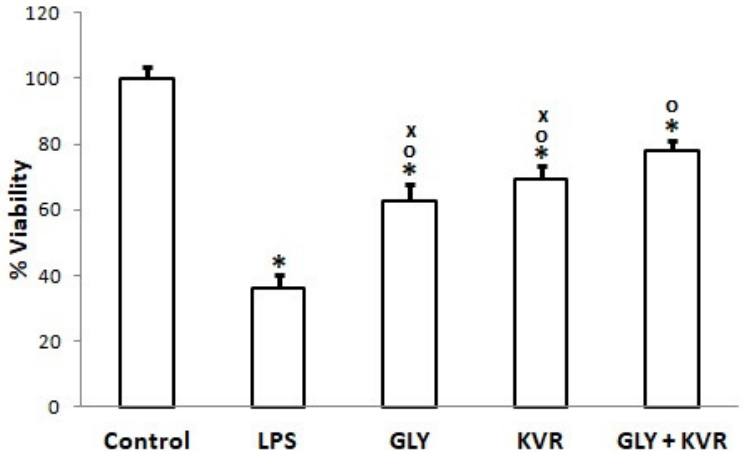

Figure 1. Effect of glycine and kolaviron on lipopolysacharide-induced cell death in U937 cells assessed by MTT assay

LPS, cells treated with 1 µg/ml Lipopolysaccaride only; GLY, cells supplemented with Glycine (300 µM) before exposure to Lipopolysaccharide (1 µg/ml); KVR, cells supplemented with Kolaviron (25 µg/ml before exposure to Lpopolysaccharide (1 µg/ml); GLY + KVR, Cells supplemented with both Glycine and Kolaviron (300 µM GLY + 25 µg/ml KVR) before exposure to Lipopolysaccharide. Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to control. *Significantly different from control; °Significantly different from LPS and ˣSignificantly different from GLY + KVR. p < 0.05.

Production of reactive oxygen species

As revealed in figure 2, treating the cells with LPS alone significantly produced reactive oxygen species (assessed via DCHF assay) when compared to untreated controls (p<0.05). However, incubating cells with glycine and kolaviron before treatment with LPS significantly reduced the production of ROS (p<0.05). But the combined pre-treatment of cells with glycine and kolaviron was better at reducing ROS production when compared to their separate effects (p<0.05)

Activation of macrophages

The activation of macrophages was evaluated by measuring the production of the pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and nitric oxide (NO). As shown in Figure 3, the production of the cytokines and NO was significant following treatment of the U937-derived macrophages with LPS (p<0.05). Pre-incubation with glycine and kolaviron significantly reduced the LPS-mediated inductions (p<0.05). Even though the pre-treatment with both glycine and kolaviron seem to produce greater reductions than their individual responses, the difference was only significant over glycine (p<0.05).

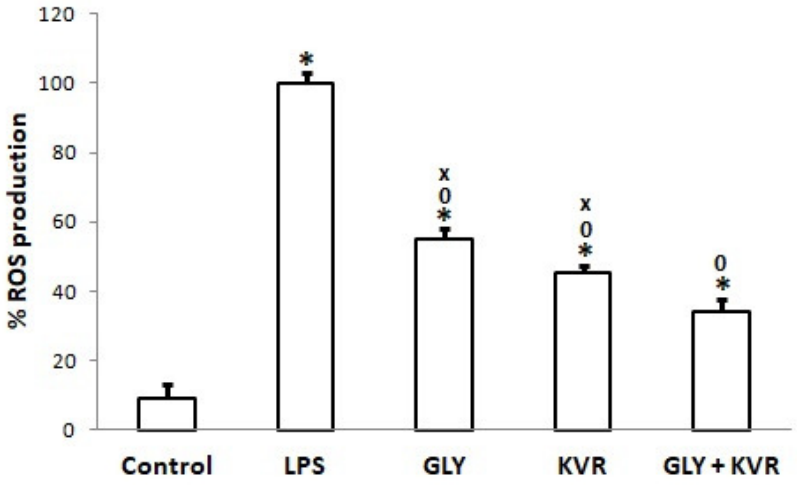

Figure 2. Effect of glycine and kolaviron on lipopolysacharide (LPS) -induced production of ROS in U937 cells

LPS, cells treated with 1 µg/ml Lipopolysaccaride only; GLY, cells supplemented with Glycine (300 µM) before exposure to Lipopolysaccharide (1 µg/ml); KVR, cells supplemented with Kolaviron (25 µg/ml before exposure to Lipopolysaccharide (1 µg/ml); GLY + KVR, Cells supplemented with both Glycine and Kolaviron (300 µM GLY + 25 µg/ml KVR) before exposure to Lipopolysaccharide. Each bar represents mean ± S.E.M of six replicates expressed as % production in comparison to LPS. *Significantly different from control; °Significantly different from LPS and ˣSignificantly different from GLY + KVR. $p < 0.05$.

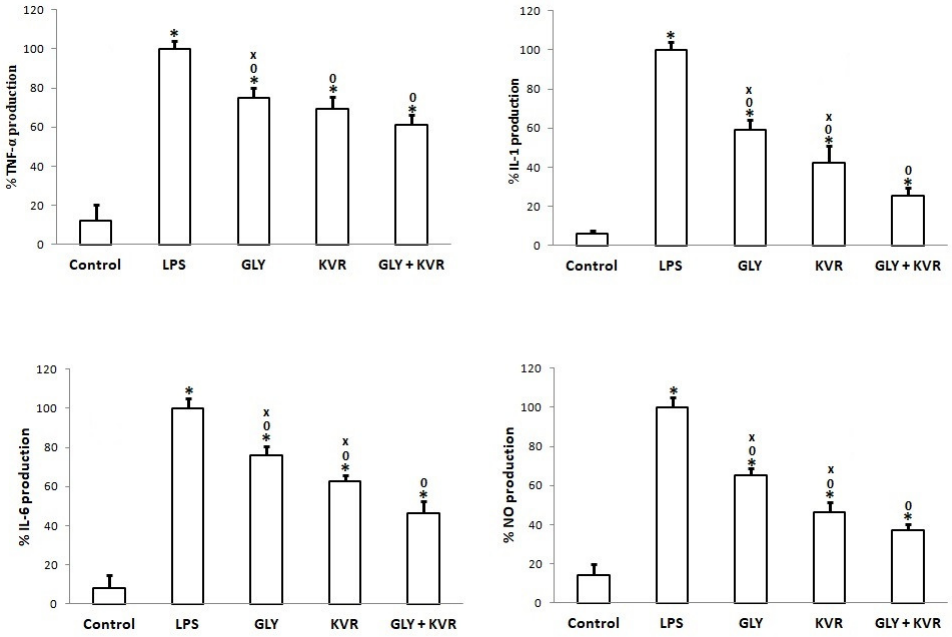

Figure 3. Effect of glycine and kolaviron on lipopolysacharide (LPS)-induced production of cytokines and nitric oxide (NO) in U937-derived macrophages

LPS, transformed cells treated with 1 µg/ml Lipopolysaccaride only; GLY, transformed cells supplemented with Glycine (300 µM) before exposure to Lipopolysaccharide (1 µg/ml); KVR, transformed cells supplemented with Kolaviron (25 µg/ml before exposure to Lipopolysaccharide (1 µg/ml); GLY + KVR, transformed cells supplemented with both Glycine and Kolaviron (300 µM GLY + 25 µg/ml KVR) before exposure to Lipopolysaccharide. Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to LPS. *Significantly different from control; °Significantly different from LPS and ˣSignificantly different from GLY + KVR. $p < 0.05$.

Effect on antioxidant enzyme expression

The effect of glycine and kolaviron on LPS-induced enzyme expression was investigated via RT-PCR as described. As shown in Figure 4, LPS significantly reduced the expression of superoxide dismutase and catalase, however glycine and kolaviron enhanced gene expression closer to control level (Figure 4). The pre-incubation with both glycine and kolaviron did not cause any significant change in expression when compared to their separate responses (p>0.05).

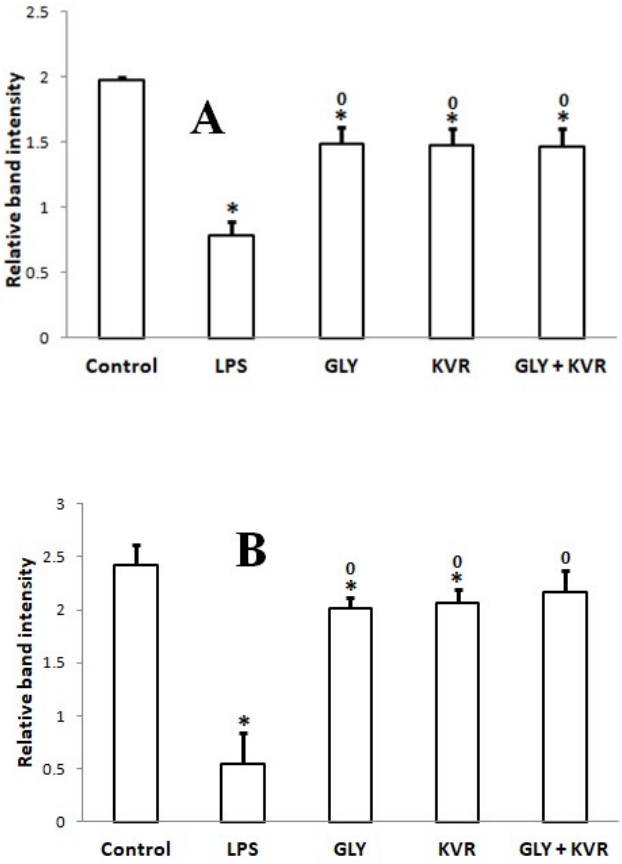

Figure 4. Expression of superoxide dismutase (SOD) and Catalase (CAT) in U937-derived macrophages (a) Ratios of intensities of (A) SOD and (B) CAT over housekeeping gene GAPDH for RT-PCR of mRNA isolated from U937-derived Macrophages. LPS, transformed cells treated with 1 µg/ml Lipopolysaccaride only; GLY, transformed cells Supplemented with Glycine (300 µM) before exposure to Lipopolysaccharide (1 µg/ml); KVR, transformed cells supplemented with Kolaviron (25 µg/ml before exposure to Lipopolysaccharide (1 µg/ml); GLY + KVR, transformed cells supplemented with both Glycine and Kolaviron (300 µM GLY + 25 µg/ml KVR) before exposure to Lipopolysaccharide. Each bar represents mean ± S.E.M of six replicates expressed as % Viability in comparison to control. *Significantly different from control; °Significantly different from LPS. p < 0.05.

Discussion

The effect of LPS on cell systems range from cytotoxicity to inflammation thus it is used as an effective model for the study of bacterial infections (Dou *et al*., 2017; Huang and Hu, 2017). Although inflammation is a defense mechanism cells employ to fight injury, it is associated with some chronic diseases if the response is deregulated (Huang and Hu, 2017; Ryu *et al*., 2018).

In the current experiment, LPS caused significant cytotoxicity which was reduced by glycine and kolaviron. Both glycine and kolaviron have been reported to be cytoprotective in various models. Mechanisms through which glycine protects cells include the inhibition of mitochondrial permeability transition, blocking of death channels, interaction of ionophores etc (Nishimura and Lemasters, 2001; Ruiz-Maena *et al*., 2004; Weinberg *et al*., 2016). Flavonoids (such as kolaviron) protect cells by preventing DNA damage and upregulation of detoxifying enzymes and the modulation of intracellular signalling pathways (Okoko, 2018).

It has been reported that LPS-induced generation of ROS plays a role in the pathogenesis of sepsis and inflammation (Liu *et al*., 2019; Yu and Tan, 2019; Wang *et al*., 2020), thus we hypothesized that LPS-mediated cell death could be linked to the generation of ROS.

It revealed that LPS caused significant production of ROS that was significantly reduced by glycine and kolaviron both separately, and in combination (Figure 2). Oxidative stress is caused by the imbalance between the production of ROS and biological antioxidant systems. A major consequence is the modification of molecules such as DNA, lipids and proteins which could cause cell death (Lee and Cho, 2009).

Glycine, the simplest amino acid, is an intricate part of protein structure with primary pharmacological activities. It has been found to be cytoprotective via the reduction of ROS produced due to xenobiotics though the exact mechanism is not clear (Shafiekhani *et al*., 2019; Wang *et al*., 2019).

Flavonoids suppress the production of reactive oxygen species via direct and indirect modes. They neutralize ROS and also induce the up-regulation of glutathione, γ-glutamylcysteine ligase, glutathione S-transferase and NAD(P)H:quinone oxidoreductase in different cell systems (Chow *et al*., 2005; Chen *et al*., 2006; Angeloni *et al*., 2007).

Pro-inflammatory cytokines are released by macrophages in response to infections, injury and stress as part of the innate immune response (Aldrich and Sevick-Muraca, 2013). During the inflammatory process, cytokines are primary mediators of the immune response but overproduction causes serious damage to host cells through the activation of cytotoxic pathways (Lee and Cho, 2009).

Macrophage activation by bacterial LPS is an important event involved in inflammation and inflammation-related disorders and is characterized by the up-regulation of cytokines and production of nitric oxide (Speranza *et al*., 2010; Franceschelli *et al*., 2014). In this study, LPS stimulated macrophages to produce IL-1, IL-6, TNF-α and nitric oxide (NO). Macrophages play a critical role in the initiation, maintenance and resolution of infection. However, persistent activation could be cytotoxic as a consequence of the overproduction of these intracellular mediators thus the reduction of macrophage-induced cytotoxicity could be a therapeutic strategy for inflammatory disorders (Liu *et al*., 2012). The release of NO could be a consequence of oxidative stress. Oxidative stress increases the intracellular level of inducible nitric oxide synthase (iNOS) which catalyses the conversion of arginine to NO. This could facilitate the generation of the peroxynitrite which is a powerful oxidant (Modlinger *et al*., 2004; Förstermann *et al*., 2017).

This study revealed that both glycine and KVR reduced LPS-mediated production of the cytokines and NO thus they could be considered as therapeutic agents for inflammatory disorders. It has been reported that glycine activates a ligand-gated chloride channel which hyperpolarizes the membrane which leads to the blocking of cytokine synthesis (Wheeler *et al*., 1999).

The downregulation of the synthesis of the antioxidant enzymes, SOD and CAT, by LPS also correlates with the production of reactive oxygen species which has been reported (Tang *et al*., 2018; Ye *et al*., 2019; Zhang *et al*., 2019). Pre-treatment of the U937-derived macrophages with glycine and kolaviron reduced the LPS-mediated suppression of antioxidant enzyme expression. This corroborates the indirect antioxidant nature of glycine and kolaviron.

Conclusions

Both glycine and kolaviron are good at reducing LPS-induced U937 cell death and activation of U937-derived macrophages. Kolaviron seems to be better than glycine at protecting cells but could depend on the concentrations used. In most of the experiments, the use of glycine and kolaviron together gave better protection than their separate responses but there was no indication if it was additive or synergistic. The experiment reveals that glycine and kolaviron could protect against diseases due to macrophage activation thus could be exploited pharmacologically.

Authors' Contributions

Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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