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Phenolic 1,3-diketones attenuate lipopolysaccharide-induced inflammatory response by an alternative magnesium-mediated mechanism

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Phenolic 1,3-diketones attenuate lipopolysaccharide-induced inflammatory response by an alternative magnesium-mediated mechanism / Zusso, Morena; Mercanti, Giulia; Belluti, Federica; Di Martino, Rita Maria Concetta; Pagetta, Andrea; Marinelli, Carla; Brun, Paola; Ragazzi, Eugenio; Rita, Lo; Stifani, Stefano; Giusti, Pietro; Moro, Stefano. - In: BRITISH JOURNAL OF PHARMACOLOGY. - ISSN 0007-1188. - STAMPA. - 174:10(2017), pp. 1090-1103. [10.1111/bph.13746]

Availability:

This version is available at: <https://hdl.handle.net/11585/620348> since: 2018-02-07

Published:

DOI: <http://doi.org/10.1111/bph.13746>

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The final published version is available online at:
[\[https://doi.org/10.1111/bph.13746\]](https://doi.org/10.1111/bph.13746)

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Phenolic 1,3-diketones attenuate lipopolysaccharide-induced inflammatory response by an alternative magnesium-mediated mechanism

Morena Zusso¹, Giulia Mercanti¹, Federica Belluti², Rita Maria Concetta Di Martino², Carla Marinelli¹, Eugenio Ragazzi¹, Rita Lo³, Stefano Stifani³, Pietro Giusti^{1*}, Stefano Moro⁴

¹ Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Largo Meneghetti 2, 35131, Padua, Italy

² Department of Pharmacy and Biotechnology, Alma Mater Studiorum-University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

³ Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

⁴ Molecular Modeling Section, Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Via Marzolo 5, 35131, Padua, Italy

Running title:

Phenolic 1,3-diketones as anti-inflammatory agents

*Corresponding author:

Pietro Giusti, MD

Address: Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Largo E. Meneghetti 2, 35131 Padua, Italy

Tel.: +39 049 827 5076; fax: +39 049 827 5093

E-mail: pietro.giusti@unipd.it

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Abstract

Background and Purpose

TLR4 plays a key role in the induction of inflammatory responses both in peripheral organs and the CNS. Curcumin exerts anti-inflammatory functions by interfering with LPS-induced TLR4–myeloid differentiation protein-2 (MD-2) dimerization and suppressing pro-inflammatory mediator release. However, the inhibitory mechanism of curcumin remains to be defined.

Experimental Approach

Binding of bis-demethoxycurcumin (**GG6**) and its cyclized pyrazole analogue (**GG9**), which lacks the 1,3-dicarbonyl function, to TLR4–MD-2 was determined using molecular docking simulations. The effects of these compounds on cytokine release and NF- κ B activation were examined by ELISA and fluorescence staining in primary microglia. Their effects on interference with TLR4 dimerization were assessed by immunoprecipitation in Ba/F3 cells.

Key results

As for curcumin, both analogues bound to the hydrophobic region of the MD-2 pocket. However, only curcumin and **GG6**, both presenting the 1,3-diketone moiety, inhibited LPS-induced TLR4 dimerization, activation of NF- κ B and secretion of pro-inflammatory cytokines in primary cortical microglia. Consistent with the ability of 1,3-diketones to coordinate divalent metal ions, LPS stimulation in a low magnesium cell culture environment resulted in decreased pro-inflammatory cytokine release and NF- κ B activation in microglia and decreased TLR4–MD-2 dimerization in BaF3 cells. Curcumin and **GG6** also caused a significant reduction of NF- κ B activation and cytokine output in contrast to the pyrazole analogue **GG9**.

Conclusions and Implications

These results indicate that chemicals like 1,3-diketones, with a structural motif able to coordinate the magnesium ion, can modulate LPS-mediated TLR4–MD-2 signaling. Taken together, these studies identify a previously uncharacterized magnesium-mediated mechanism for LPS-induced inflammatory response.

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Abbreviations

DAPI, 4,6-diamidino-2-phenylindole

FCS, fetal calf serum

Iba1, ionized calcium binding adaptor molecule 1

MD-2, myeloid differentiation protein-2

MOE, Molecular operation environment

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PDB, Protein data bank

TLRs, Toll-like receptors

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Introduction

Neuroinflammation is a complex defense response of the CNS involved in tissue repair and induction of a protective immune response. However, if it becomes chronic it can be destructive to the brain, leading to neuronal cell damage and, ultimately, death associated with neurodegenerative and psychiatric disorders (Frank-Cannon *et al.*, 2009; Amor *et al.*, 2010). In neuroinflammation, cellular and molecular immune components such as microglia and astrocytes, cytokines, complement and pattern-recognition receptors are the critical players (Shastri *et al.*, 2013). Microglia, the major resident immune cells of the CNS, provide the first line of defense against invading pathogens or injury-related products (Hanisch and Kettenmann, 2007). Microglia-mediated defense mechanisms begin with the recognition of different pathogens through a limited number of pattern-recognition receptors, including Toll-like receptors (TLRs) which are transmembrane glycoprotein, expressed in a number of CNS cell types, including microglia (Bsibsi *et al.*, 2002; Kawai and Akira, 2010).

LPS, a well-characterized pathogen-associated molecular pattern present on the outer membrane of Gram-negative bacteria, is a potent activator of a variety of mammalian cell types (Ulevitch and Tobias, 1995). Among TLRs, TLR4 is the major LPS receptor and is a component of LPS signaling (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999; [Park](#) and [Lee](#), 2013). The cellular response to LPS requires specific interaction of LPS with the co-receptor myeloid differentiation protein-2 (MD-2), which is associated with the extracellular domain of TLR4 (Shimazu *et al.*, 1999; Nagai *et al.*, 2002; Park *et al.*, 2009). After LPS binding, TLR4–MD-2 complex dimerizes and a signal is then propagated by recruitment of intracellular signaling adaptor proteins leading to activation of transcription factor NF- κ B, which controls the final immune response through the synthesis of inflammatory molecules, such as cytokines (*e.g.*, TNF- α , IL-1 β and IL-6), chemokines, enzymes and reactive oxygen and nitrogen species (Bryant *et al.*, 2010). As a consequence, the binding of an antagonist to the extracellular domain would prevent receptor dimerization and, consequently, intracellular signaling activation.

Identification of molecules capable of inhibiting LPS-mediated TLR4–MD-2 complex activation and understanding how these molecules modulate TLR4–MD-2-mediated signaling represents an attractive strategy for attenuating the severity of various CNS inflammatory conditions. Recently, a number of small molecules have been identified as TLR4 antagonists by a variety of *in silico*, *in*

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vitro and *in vivo* assays. These natural and synthetic compounds, structurally unrelated to LPS, proved to directly bind to the MD-2 pocket, antagonize the cellular response to LPS and improve various inflammatory conditions (Park *et al.*, 2012). Among them, curcumin, the main bioactive component in the rhizome of the turmeric plant (*Curcuma longa*), is a safe and a highly pleiotropic molecule endowed with a wide range of beneficial activities, including anti-inflammatory, antitumor, anti-oxidative and cardiovascular protective effects (Hatcher *et al.*, 2008), interacting with diverse molecular targets (Gupta *et al.*, 2013). In particular, anti-inflammatory properties of curcumin are linked to numerous mechanisms (He *et al.*, 2015), involving also its ability to bind MD-2 (Gradišar *et al.*, 2007) and inhibit dimerization of the TLR4–MD-2 complex (Youn *et al.*, 2006), which is required for inflammatory intracellular signaling. However, the inhibitory mechanism of curcumin remains to be fully understood. Despite the fact that curcumin is an electrophilic thiol reactive compound, containing a bis- α , β -unsaturated 1,3-diketone moiety, which can react with cysteine residues through a Michael reaction, it binds MD-2 in proximity of Cys¹³³ residue, without the formation of a covalent adduct (Gradišar *et al.*, 2007).

In the present study, two derivatives strictly related to curcumin were synthesized to clarify the anti-inflammatory mechanism of curcumin at the molecular level. In particular, bis-demethoxycurcumin (**GG6**, 1E,4Z,6E-5-hydroxy-1,7-bis-4-hydroxyphenylhepta-1,4,6-trien-3-one) and its pyrazole-based analogue lacking the 1,3-dicarbonyl function [**GG9**, 4,4'-(1E,1'E)-(1H-pyrazole-3,5-diyl-bis-ethene-2,1-diyl)diphenol] were compared to curcumin in relation to their anti-inflammatory effects and binding mechanisms to the TLR4–MD-2 complex. We show that only 1,3 diketones suppress neuroinflammation and that the ability of the 1,3 diketone moiety to bind divalent cations, such as the magnesium ion, plays an important role in inhibiting LPS-induced TLR4–MD-2 complex activation and subsequent inflammatory response.

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Materials and methods

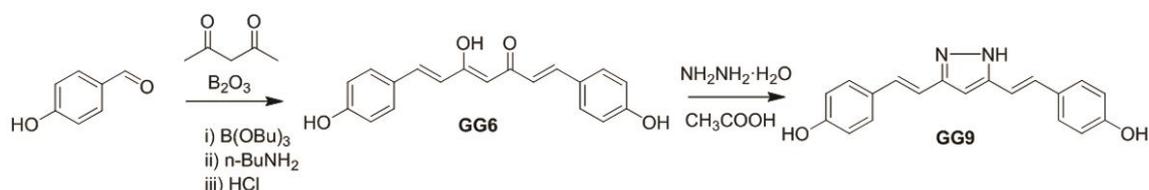
Reagents and materials

Unless otherwise specified, all reagents were from Sigma-Aldrich (Milan, Italy). Tissue culture media, antibiotics and fetal calf serum (FCS) were obtained from Invitrogen (San Giuliano Milanese, Italy). LPS (Ultra-Pure LPS-EB from *E. Coli*, 0111:B4 strain) was purchased from InvivoGen (InvivoGen Europe, Toulouse, France). The Ultra-Pure LPS-EB preparation used here only activates TLR4. Falcon tissue culture plasticware was purchased from BD Biosciences (SACCO srl, Cadorago (CO), Italy).

Synthesis

GG6 and **GG9** analogues were synthesized according to previously reported procedures (Scheme 1). Spectroscopic data of these compounds were consistent with the chemical structures and were in good agreement with the literature data (Di Martino *et al.*, 2016; Ishida *et al.*, 2002). **GG6** was obtained according to the Pabon reaction (Pabon, 1964) in which 2,4-pentandione was firstly reacted with B_2O_3 and the obtained boric complex was then condensed with 4-hydroxybenzaldehyde in presence of *n*-tributylborate. Acidic hydrolysis allows to remove the boron complexation and to obtain the desired derivative. **GG6** was then reacted with hydrazine hydrate to obtain the pyrazole analogue **GG9**.

Scheme 1. General synthetic method for **GG6** and **GG9**.



Molecular modeling studies

Target structures

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The crystallographic structure of the human TLR4-human MD-2-E. coli LPS ternary complex was retrieved from the Protein Data Bank (PDB code: 3FXI) (Park *et al.*, 2009).

The assessment of crystallographic structure quality has been performed with the Structure Preparation tool of the Molecular Operation Environment program (MOE, version 2014.09; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2015). Critical structural issues (such as missing or poorly resolved atomic data, anomalous topological properties present in amino acids units as well as anomalous bonding patterns of non-amino acid units) were fixed when necessary. Hydrogen atoms were added and their appropriate protonation state fixed using the Protonate3D tool as implemented in the MOE program. To minimize contacts between hydrogen atoms, the structures were subjected to Amber99 force field (Cornell *et al.*, 1995) minimization until the *rms* of the conjugate gradient was $<0.1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ keeping the heavy atoms fixed at their crystallographic positions.

Molecular docking protocol

Curcumin and its analogues were built using the “Builder” module of MOE and each compound was docked into the presumptive binding sites (LPS binding site) using flexible MOE-Dock methodology. The purpose of MOE-Dock is to search for favorable binding configurations between a small, flexible ligand and a rigid macromolecular target. Searching is conducted within a user-specified 3D docking box, using the “tabù search” (Baxter *et al.*, 1998) protocol and the MMFF94 force field (Halgren, 1996). Charges for ligands were imported from the MOPAC program (Stewart, 1993) output files. MOE-Dock performs a user-specified number of independent docking runs (50 in the present case) and writes the resulting conformations and their energies to a molecular database file. The resulting ligand/protein complexes were subjected to MMFF94 all-atom energy minimization until the *rms* of conjugate gradient was $<0.1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$. GB/SA approximation (Wojciechowski and Lesyng, 2004) has been used to model the electrostatic contribution to the free energy of solvation in a continuum solvent model. The interaction energy values were calculated as the energy of the complex minus the energy of the ligand, minus the energy of the protein: $\Delta E_{\text{inter}} = E_{\text{(complex)}} - (E_{\text{(L)}} + E_{\text{(Protein)}})$.

Cell cultures

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Studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All animal-related procedures were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92), and were approved by the Institutional Animal Care and Use Committee. Primary microglial cells were isolated from mixed glial cell cultures prepared from the cerebral cortex of 1-day-old Sprague Dawley rat pups (CD strain), as previously described (Skaper *et al.*, 2012). Briefly, upon reaching confluence (7-10 days after isolation) microglia adhering to the astroglial monolayer were dislodged by shaking (200 r.p.m. for 1 h at 37°C), re-suspended in high-glucose DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 50 µg ml⁻¹ gentamicin, and plated on uncoated plastic wells at a density of 1.25 x 10⁵ cells cm⁻². Cells were allowed to adhere for 45 min and then washed to remove non-adhering cells. After a 24-h incubation period, the medium was replaced with serum-free medium or with a low magnesium solution containing 2.5 mM CaCl₂, 4.7 mM KCl, 1.2 mM KH₂PO₄, 118 mM NaCl, 25 mM NaHCO₃, 0.1 mM MgSO₄ and 11 mM glucose. Purity of the cultures was confirmed by immunocytochemistry using a primary polyclonal antibody against ionized calcium binding adaptor molecule 1 (Iba1, 1:800, Wako Chemicals USA Inc., Richmond, VA, USA). Ninety-seven percent of the cells were Iba1 immunopositive. Murine Ba/F3 cells, an IL-3 dependent pro-B cell line, stably expressing human TLR4-GFP, human TLR4-Flag, human MD-2-Flag and human CD14 were kindly provided by Dr. Kensuke Miyake (University of Tokyo, Tokyo, Japan). Cells were cultured in RPMI1640 medium, supplemented with 10% FCS, 100 µM 2-mercaptoethanol, recombinant murine IL-3 (~ 70 U ml⁻¹) (Saitoh *et al.*, 2004). Cells were maintained at 37°C in an atmosphere containing 5% CO₂.

Cell Viability Assay

Microglial cell viability was evaluated by a quantitative colorimetric method utilizing the metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). Briefly, cells were seeded at the density of 45 000 cells/well in 96-well plates and treated with test compounds. After 6h incubation, the medium was removed and the cells were incubated with MTT (0.18 mg ml⁻¹) for 4 h at 37°C. The formazan crystals were solubilized with DMSO. The plates were then read on a microplate reader (Victor2 Multilabel Counter, Wallac, Cambridge, MA, USA)

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using a test wavelength of 570 nm and a reference wavelength of 630 nm, and the results expressed as percentage viability relative to LPS-treated cultures.

Cytokine determination

Primary microglia were pre-treated for 1 h with increasing concentrations of test compounds and then stimulated with 100 ng ml⁻¹ Ultra-Pure LPS-EB for an additional 6 h. At the end of incubation, cell supernatants were collected and the levels of IL-1 β and TNF- α released by microglia into the culture medium were assayed using commercially available ELISA kits (Antigenix America, Huntington Station, NY, USA), according to the manufacturer's instructions. Cytokine concentrations (pg ml⁻¹) in the medium were determined by reference to standard curves obtained with known amounts of IL-1 β or TNF- α and the results are expressed as percentage relative to LPS-stimulated cultures.

Immunofluorescence

Microglial cells, grown on coverslips in 12-well plates, were pre-treated for 1h with 10 μ M curcumin, **GG6** or **GG9** and then stimulated with 100 ng ml⁻¹ Ultra-Pure LPS-EB for additional 90 min. Cells were fixed with 4% paraformaldehyde (pH 7.4, for 15 min at RT) and subsequently blocked with a blocking solution containing 5% normal goat serum and 0.1% Triton X-100 in PBS for 1 h (Zusso *et al.*, 2012). After blocking, cells were incubated with a mouse anti-p65 antibody (NF- κ B p65, 1:500; Santa Cruz Biotechnology Santa Cruz, CA, USA) followed by an Alexa Fluor 555-conjugated anti-mouse secondary antibody (1:1000; Invitrogen). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). The coverslips were mounted on microscope slides with Fluoromount-G mounting medium (Fisher Scientific, Milan, Italy) and fluorescent images for p65 translocation were captured with a confocal laser-scanning microscope (Zeiss LSM 800; Carl Zeiss AG, Oberkochen, Germany).

Immunoprecipitation and immunoblotting

Ba/F3 cells stably expressing human TLR4-GFP, human TLR4-Flag, human MD-2-Flag and human CD14 (80 x 10⁶ cells/condition) were pre-treated for 1h with curcumin, **GG6** or **GG9** (50 μ M) and then stimulated with 250 ng ml⁻¹ LPS for 30 min (Youn *et al.*, 2006). Protein extracts were prepared as described previously (Saitoh *et al.*, 2004). The samples were immunoprecipitated with mouse

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anti-GFP antibody (#NB600-597; Novus, Littleton, CO) for 16-18 h. The recovered immune complex was resolved using 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked with phosphate buffered saline containing 0.1% Triton X100 and 3% nonfat dry milk, and probed overnight with either mouse anti-Flag antibody (#F3165; Sigma-Aldrich, St. Louis, MO) or chicken anti-GFP antibody (#06-896; Millipore, Temecula, CA). Thereafter, the membrane was exposed to horseradish peroxidase-conjugated secondary antibody for 1 h and reactive bands were detected with ECL system (#RPN2106; GE Healthcare Amersham, Buckinghamshire, UK). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the results are expressed as percentage relative to LPS-stimulated cells.

Magnesium assessment

Magnesium assessment in DMEM and in the low magnesium solution was performed using an atomic absorbance spectrophotometer (Varian AA Duo 55 b/240, Agilent Technologies, Santa Clara, CA, USA) with a Ca/Mg lamp, air/acetylene flame and detection wavelength of 285.2 nm. Absorbance readings were obtained via integrated duplicate measurement and compared with a standard, calibrated curve.

Statistical analysis

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data were analyzed using the GraphPad software, version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as mean \pm SEM of at least 5 independent experiments in duplicate. As raw values varied between experiments, raw data have been normalized and expressed as percentage of respective control or LPS treatment. Differences between groups have been evaluated by using Student's *t* test for paired data. A *P* value of less than 0.05 was deemed statistically significant. Additional details are provided in the figure legends, where appropriate.

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Results

Effects of curcumin analogues on pro-inflammatory cytokine release by LPS-stimulated microglia

Stimulation of microglia with TLR agonists, including LPS for TLR4, induces a pro-inflammatory process through the increased secretion of cytokines (Lehnardt, 2009). Inhibition of LPS-induced release of IL-1 β and TNF- α was initially used to examine the anti-inflammatory effects of the two curcumin analogues **GG6** and **GG9**. Cells were exposed to increasing concentrations (1-10 μ M) of curcumin (used as positive control), **GG6** or **GG9** for 1 h and then stimulated with LPS for 6 h to induce an inflammatory response. Supernatants were collected and subjected to ELISA to measure IL-1 β and TNF- α concentration. In unstimulated cells low or undetectable amounts of IL-1 β and TNF- α were released and these basal levels remained unchanged after treatment with the three compounds (data not shown). Treatment with LPS induced a marked release of IL-1 β and TNF- α (taken as 100%; dashed lines in Figure 1A and B) that was significantly suppressed by curcumin, as we previously showed (Mercanti *et al.*, 2014), and its 1,3-diketone analogue **GG6**. Specifically, LPS-induced release of IL-1 β was reduced to about 50% of LPS level by 10 μ M of both curcumin and **GG6** ($42.3 \pm 4.6\%$ and $55.7 \pm 10.3\%$, respectively) (Figure 1A). TNF- α release was also significantly inhibited by 10 μ M curcumin and **GG6** ($52.4 \pm 9.0\%$ and $63.9 \pm 2.6\%$, respectively) (Figure 1B), demonstrating that **GG6** analogue had effects similar to those of the parent molecule. In contrast, **GG9**, the pyrazole-based analogue, failed to inhibit the LPS-induced release of both IL-1 β and TNF- α (Figure 1A and B), indicating that 1,3-diketone moiety is crucial for the anti-inflammatory activity. Furthermore, the three compounds did not affect cell viability at the concentrations tested (1-10 μ M) (Figure 1C), indicating that the decrease in IL-1 β and TNF- α release induced by curcumin and **GG6** did not result from cytotoxicity.

Effects of curcumin analogues on NF- κ B activation in LPS-stimulated microglia

In LPS-induced inflammatory response, activation of the transcription factor NF- κ B through the translocation of its p65 subunit from the cytosol to the nucleus is considered a key event that controls the expression of a large number of cytokines and other immune response genes (Ghosh and Hayden, 2008). To investigate whether the studied compounds reduced the LPS-induced pro-inflammatory cytokine release by preventing NF- κ B activation, the subcellular distribution of p65 subunit was studied by immunofluorescent staining in unstimulated and LPS-stimulated microglia.

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As expected, in unstimulated control cells, p65 subunit was mainly distributed in the cytoplasm with only minor nuclear staining detected (Figure 2A). This distribution remained unchanged after treatment with curcumin, **GG6** and **GG9** (10 μ M) (Figure 2B-D). Stimulation with LPS for 90 min increased translocation of p65 into the nucleus, indicative of NF- κ B activation (Figure 2E). Pre-treatment with curcumin and **GG6** strongly attenuated LPS-induced nuclear localization of p65 (Figure 2F and G). Conversely, pre-treatment with **GG9** analogue did not show this effect (Figure 2H), confirming the structure-activity relationship observed in inhibition of cytokine release.

Effects of curcumin analogues on TLR4 dimerization

We next investigated whether curcumin analogues could inhibit TLR4 dimerization, one of the initial steps involved in activating TLR4-mediated signaling pathway. For these studies, we used Ba/F3 cells stably transfected with human TLR4-GFP, TLR4-Flag, MD-2-Flag and CD14, as previously described (Saitoh *et al.*, 2004). Cells were pre-incubated with curcumin, **GG6**, **GG9** or vehicle (untreated control) for 1h prior to LPS stimulation for 30 min. To detect TLR4 dimerization, TLR4-GFP was immunoprecipitated and co-precipitation of TLR4-Flag was probed with anti-Flag antibody (Figure 3). A fraction of Flag-tagged TLR4 was detected after GFP immunoprecipitation in the absence of LPS stimulation, but receptor dimerization strongly increased in response to LPS stimulation (Figure 3A and B). As previously demonstrated in this cell line (Youn *et al.*, 2006), curcumin inhibited LPS-induced dimerization and, interestingly, the 1,3-diketone derivative **GG6** also markedly blocked this effect. In agreement with the effects on inhibition of cytokine release and NF- κ B activation, the pyrazole analogue **GG9** did not affect dimerization of TLR4, confirming a key role for the 1,3-diketone moiety in the first step of the inflammatory cascade.

The binding mode of curcumin analogues to MD-2

To explain the observed structure-activity relationship, which showed an anti-inflammatory effect of curcumin and its 1,3-diketone derivative, molecular docking simulations were carried out to characterize the putative binding mode of curcumin analogues to the LPS binding site of MD-2. In accord with previous studies (Wang *et al.*, 2015), molecular docking showed that curcumin, like the two analogues analyzed in the present study, could be accommodated into the large hydrophobic binding pocket of MD-2 occupying a relevant portion of the LPS binding site (Figure 4) and assuming different binding modes. Among all generated binding modes of each ligand, the

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energetically more stable were those that showed important interactions to MD-2, such as hydrogen bonding, engaged with residues Arg90, Glu92, and Tyr102. These binding modes are well conserved in both curcumin analogues. In fact, as shown in Figure 5, curcumin (panel A), **GG6** (panel B) and **GG9** (panel C) analogues are sited into the LPS binding site of MD-2 guaranteeing the same cavity's occupancy and the same interaction networks.

Interestingly, as shown in the crystallographic structure coded by PDB as 3FXI, in proximity to entrance of the LPS binding site of MD-2, a metal ion was found at the interface between MD-2-LPS complex to TLR4, either directly or indirectly through water molecules (Park *et al.*, 2009). As suggested in the original crystallographic study, this is probably a magnesium ion, whose role was at that time suggested to be dispensable for LPS binding and receptor dimerization (Park *et al.*, 2009). New molecular docking simulations were performed by using a sampling grid larger enough to include the metal ion. As expected, the presence of 1,3-diketone moiety, as well as its enolate form, allows the appearance of an alternative binding mode close to magnesium ion (Figure 6). On the contrary, pyrazole analogue did not show this peculiarity, confirming its inability to coordinate metal ions.

Effect of a low magnesium environment on inflammatory response

To examine the effect of magnesium on the inflammatory response, microglial cells cultured in a low magnesium environment (0.1 ± 0.05 mM, determined by atomic absorbance spectroscopy) were exposed to curcumin, **GG6** or **GG9** for 1 h and then stimulated with LPS for 6 h. Cell viability as well as low basal levels of IL-1 β and TNF- α were not significantly affected by a low extracellular magnesium concentration (Figure 7A-C). Under these experimental conditions, however, LPS-induced release of both cytokines was significantly decreased compared to control conditions (DMEM; magnesium concentration, 0.9 ± 0.1 mM). Specifically, IL-1 β and TNF- α released in the supernatant were reduced to $25.0 \pm 9.5\%$ and $37.2 \pm 7.2\%$, respectively (Figure 7D and E, white bars). Furthermore, treatment with curcumin and **GG6** caused a remarkable concentration-dependent reduction in cytokine output, whereas **GG9** was unable to inhibit IL-1 β and TNF- α release (Figure 7D and E, gray bars). Furthermore, the reduced release of IL-1 β and TNF- α induced by a low magnesium environment was well correlated with a decreased NF- κ B activation in microglia (Figure 8A and B) and a suppression of TLR4 dimerization in Ba/F3 cells (Figure 8C).

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Discussion

Curcumin possesses well-known anti-inflammatory activities, and numerous studies suggest that MD-2 may be the direct target of curcumin in its inhibition of LPS signaling (Youn *et al.*, 2006; Gradišar *et al.*, 2007; He *et al.*, 2015). However, the exact interaction between curcumin and MD-2 is not completely understood. In the last decades, several curcumin analogues have been synthesized with the aim to learn more about curcumin mechanism of action and to establish structure-activity relationships. Although the 1,3-diketone moiety in the curcumin structure has been considered responsible for its chemical instability (Oliveira *et al.*, 2015) and some mono-carbonyl analogues of curcumin displayed anti-inflammatory effects *in vitro* and *in vivo* (Zhang *et al.*, 2014; Zhang *et al.*, 2015), this structural motif seems to be important for inhibition of LPS-mediated TLR4–MD-2 activation/dimerization (Youn *et al.*, 2006). Here, we approached the question of this chemical moiety's involvement in the microglial anti-inflammatory response by using two curcumin derivatives: bis-demethoxycurcumin (**GG6**) and its cyclized pyrazole analogue (**GG9**), lacking the 1,3-dicarbonyl function. Non-cytotoxic concentrations of curcumin and **GG6** suppressed, in a similar manner, LPS-induced inflammatory response in primary cortical microglia. Previous studies have suggested that prevention of inflammatory cascade by curcumin may be linked to its ability to inhibit LPS-induced dimerization of TLR4 (Youn *et al.*, 2006). Here we showed that also **GG6**, sharing with curcumin the 1,3-diketone moiety, suppressed this effect, whereas the pyrazole-based analogue **GG9** was unable to inhibit LPS-induced pro-inflammatory response in microglia and TLR4 dimerization. Collectively, these results show the fundamental role of 1,3 diketone moiety for the anti-inflammatory effects of curcumin and **GG6**, by directly suppressing LPS-induced initiation of receptor signaling and provide us important structural information, that prompted us to characterize the binding mode of the two curcumin analogues to the LPS binding site on MD-2. Molecular modeling studies showed that, as for curcumin (Wang *et al.*, 2015), both analogues could bind the hydrophobic region of the MD-2 pocket, leading to a stable complex via interactions with Arg90, Glu92 and Tyr102 residues, all of which are conserved between human, mouse and rat (Shimazu *et al.*, 1999; Akashi *et al.*, 2000; Winnall *et al.*, 2011). Considering the size of the LPS binding cavity, it is also not surprising that these compounds may assume different poses inside this binding site. Based on the comparison, it is not possible to explain the remarkably decrease in the observed anti-inflammatory activity of the pyrazole analogue **GG9**, compared to 1,3-diketone analogue **GG6**.

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As previously reported, a metal ion, probably Mg^{2+} , is present at the interface between the MD-2–LPS complex and TLR4. However, its presence was considered superfluous because EDTA treatment was unable to block the binding of LPS and receptor dimerization (Park *et al.*, 2009). It is known that 1,3-diketones efficiently coordinate divalent metal ions (including copper, nickel, cobalt, zinc and magnesium) (Vyas *et al.*, 2013). In principle, the presence of a metal ion, like Mg^{2+} , can also affect docking simulations facilitating ligand movement from inside the LPS binding cavity of MD-2 closer to the metal coordination sphere, where possible in terms of stability of the complex. Molecular docking simulations including Mg^{2+} confirmed that only the presence of a 1,3-diketone moiety allowed the appearance of poses close to the metal ion, confirming the inability of the pyrazole analogue **GG9** to coordinate metal ions. This computational evidence led us to hypothesize that the greater anti-inflammatory activity of 1,3-diketone derivatives is associated with their ability to coordinate Mg^{2+} that could, in turn, affect proper assembly of the TLR4–MD-2–LPS ternary complex. Exposing microglial cells to a low magnesium environment resulted in a significant decrease in LPS-induced IL-1 β and TNF- α release and NF- κ B activation, compared to normal magnesium conditions. Moreover, curcumin and **GG6** markedly reduced cytokine output, in contrast to **GG9**, confirming the capability of the 1,3 diketone moiety to coordinate divalent cations, such as magnesium, which may play a critical role in anti-inflammatory responses. These findings contrast with previous studies, conducted both in rodent and human cells, which suggested a link between magnesium deficiency and inflammation and, conversely, showed that elevated magnesium limited the inflammatory response by inhibiting NF- κ B activation and pro-inflammatory cytokine production (Lin *et al.*, 2010; Lee *et al.*, 2011; Sugimoto *et al.*, 2012; Gao *et al.*, 2013). This discrepancy may reflect differences in experimental conditions, such as the length of exposure to a low magnesium environment or LPS concentration. Sugimoto and colleagues showed a decreased cytokine production induced by magnesium supplementation using a low LPS concentration (50 pg ml⁻¹) that was unable to activate all cells. In contrast, high LPS concentrations, similar to those used here, abrogated the magnesium effect (Sugimoto *et al.*, 2012).

The mechanisms by which magnesium exerts its effect on inflammatory responses are poorly understood. Magnesium, a known antagonist of some calcium ion channels (Sonna *et al.*, 1996), may counteract the toxic consequences of excessive calcium entry into immune cells, including microglia (Lin *et al.*, 2010; Gao *et al.*, 2013). Other potential cellular mechanisms concerning the relationship between magnesium status and inflammation have been considered, such as ion effects

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on NMDA receptors, release of substance P and activation of NF- κ B (Mazur *et al.*, 2007). Furthermore, the possibility that magnesium functions via an intra- or extracellular mechanism has been debated [Mazur *et al.*, 2007; Gao *et al.*, 2013). Here, we found a previously unrecognized direct link between a low magnesium environment and LPS-mediated TLR4 activation, by showing a complete inhibition of TLR4–MD-2 complex dimerization in LPS-stimulated Ba/F3 cells exposed to a low Mg²⁺ concentration. These results provide evidence for a new and alternative mechanism for the LPS-mediated activation of TLR4, suggesting the possibility that chemicals with a structural motif able to coordinate Mg²⁺, like 1,3-diketones, can modulate LPS-mediated TLR4–MD-2 signaling, an important component of immune responses in the nervous system (Kigerl *et al.*, 2014). At the same time, one needs to keep in mind that neuroinflammation and microglial activation may also involve activation of multiple TLRs, by exogenous and endogenous ligands, in a context-dependent manner (Li *et al.*, 2016). Furthermore, the TLR4–MD-2 complex and its dimerization process may not be the exclusive targets through which curcumin and its 1,3-diketone analogue may modulate neuroinflammation. Even so, the mechanism proposed in this study may provide useful information for the design and development of novel agents targeting the MD-2 protein, applied to neuropathologies in which prolonged TLR4–MD-2 activation is known to exacerbate disease conditions.

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Author contributions

M.Z., S.M. and P.G. designed research; M.Z., G.M., C.M., F.B., R.D.M., R.L. and S.M. performed research; M.Z., S.S., E.R., S.M. and P.G. analyzed data; M.Z. and S.M. wrote the paper; all authors read and approved the final manuscript.

Acknowledgements

This work was financially supported by a grant “Progetto di Ateneo”, University of Padua, Italy (CPDA144389/14 to M.Z.); by a PRIN (20103W4779) Project Grant from MIUR, Italy; and by the Canadian Institute of Health Research (grants MOP-123270 and MOP-123500 to S.S.). The computational work coordinated by S.M. was supported with financial support from the University of Padua, Italy. We thank Dr. Carla Argentini for technical assistance, Dr. Chiara Calore for assistance with atomic absorption spectroscopy, Dr. Andrea Pagetta for excellent support with confocal microscopy and for his help in preparing Figure 2 and 8, and Dr. Miyake for providing us with Ba/F3 cells. MMS Lab is also very grateful to Chemical Computing Group for the scientific and technical partnership. Finally, the authors would like to thank Dr. Stephen D. Skaper for critical reading of the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. Effect of curcumin, **GG6** and **GG9** on cytokine release from LPS-stimulated cortical microglia and cell viability analysis. Microglia were subcultured for 24 h in 10% serum-containing medium, which was replaced with serum-free medium before pre-treatment with curcumin, **GG6** and **GG9** (1-10 μM) for 1 h and further stimulation with 100 ng ml^{-1} LPS for 6 h. Supernatants were collected and analyzed for (A) IL-1 β and (B) TNF- α release. (C) At the end of incubation, cell viability was also determined by MTT assay. Results are expressed as percentage of cytokine release and cell viability relative to LPS-stimulated microglia. Data are means \pm SEM (n = 5). * P < 0.05 and ** P < 0.01 versus LPS stimulation (dashed line), Student's t test for paired data.

Figure 2. Effect of curcumin, **GG6** and **GG9** on nuclear translocation of NF- κB p65 in unstimulated and LPS-stimulated cortical microglia. Microglia were subcultured for 24 h in 10% serum-containing medium, which was replaced with serum-free medium before stimulation with curcumin (Curc), **GG6** and **GG9** (10 μM) \pm 100 ng ml^{-1} lipopolysaccharide (LPS). Cells were further subjected to immunofluorescence staining for NF- κB p65. DAPI counterstaining is shown in the images in the left column and in the merged images in the right column. Experiments were performed 5 times and representative confocal images showing subcellular localization of p65 in unstimulated and LPS-stimulated microglia are shown in (A-D) and (E-F), respectively. Scale bar, 10 μm .

Figure 3. Effect of curcumin, **GG6** and **GG9** on LPS-induced TLR4 dimerization. (A) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag and CD14 were pre-treated with curcumin (Curc), **GG6** or **GG9** (50 μM) for 1 h and then stimulated with 250 ng ml^{-1} LPS for 30 min. Cell lysates were then immunoprecipitated with mouse anti-GFP antibody and immunoblotted with mouse anti-Flag (upper) or chicken anti-GFP (lower) antibody. Experiments were performed at least 5 times and representative results are shown. (B) Histograms showing the mean OD of the detected TLR4-Flag (left) and TLR4-GFP (right) bands, relative to LPS-stimulated cells. Data are means \pm SEM (n = 5). * P < 0.05 and ** P < 0.01 versus LPS stimulation, Student's t test for paired data. IP, immunoprecipitation; IB, immunoblot; Veh, vehicle.

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Figure 4. Overall structure of the human TLR4-MD2 complex derived by the crystallographic structure 3FXI. All atoms are voluntarily not showed. *Bottom on the left:* TLR4 (coloured in orange) and MD-2 (coloured in magenta) are represented showing their secondary structure. *Top on the right:* the LPS binding cavity of MD-2 is zoomed and shown as Connolly surface. Hydrophobic regions of the Connolly surface are coloured in green, polar in magenta and the exposed to the solvent in red. Mg²⁺ ion is represented by CPK and is coloured in bronze.

Figure 5. Molecular docking analysis of the energetically most stable binding poses between (A) curcumin, (B) **GG6**, (C) **GG9** and MD-2. As described in Figure 4, the LPS binding cavity of MD-2 is zoomed and shown as Connolly surface. Hydrophobic regions of the Connolly surface are coloured in green, polar in magenta and the exposed to the solvent in red. Mg²⁺ ion is represented by CPK and is coloured in bronze.

Figure 6. Molecular docking analysis of the two alternative binding modes between **GG6** and MD-2. As described in Figure 4, the LPS binding cavity of MD-2 is zoomed and shown as Connolly surface. Hydrophobic regions of the Connolly surface are coloured in green, polar in magenta and the exposed to the solvent in red. Mg²⁺ ion is represented by CPK and is coloured in bronze.

Figure 7. Effect of curcumin, **GG6** and **GG9** in a low magnesium environment on cytokine release from LPS-stimulated cortical microglia. Microglia were subcultured for 24 h in 10% serum-containing medium, which was replaced with a solution containing a low magnesium concentration before pre-treatment with curcumin, **GG6** or **GG9** (1-10 µM) for 1 h and further stimulation with 100 ng ml⁻¹ LPS for 6 h. (A) Microglial cell viability was determined by MTT assay after exposing cells to a low magnesium environment. Supernatants were collected and analyzed for (B and D) IL-1β and (C and E) TNF-α release. Results are expressed as percentage of cell viability and cytokine release relative to control (DMEM) or LPS-stimulated microglia. Data are means ± SEM (n = 5). **P* < 0.05 and ***P* < 0.01 versus LPS stimulation (dashed line), Student's *t* test for paired data.

Figure 8. Effect of a low magnesium environment on NF-κB p65 nuclear translocation and TLR4 dimerization. (A-B) Microglial cells, exposed to a low magnesium environment, were stimulated with 100 ng ml⁻¹ LPS for 90 min and further subjected to immunofluorescence staining for NF-κB

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p65. DAPI counterstaining is shown in the images in the left column and in the merged images in the right column. Experiments were performed at least 5 times and representative confocal images showing subcellular localization of p65 in unstimulated and LPS-stimulated microglia are shown in (A) and (B), respectively. Arrowheads point to examples of p65 nuclear localization, while arrows point to examples of cytoplasmic distribution. Scale bar, 10 μm . (C) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag and CD14, exposed to a low magnesium environment, were stimulated with 250 ng ml^{-1} LPS for 30 min. Cell lysates were then immunoprecipitated with mouse anti-GFP antibody and immunoblotted with mouse anti-Flag (upper) or chicken anti-GFP (lower) antibody. Experiments were performed 5 times and representative results are shown. IP, immunoprecipitation; IB, immunoblot; Veh, vehicle.

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