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Original Article

Investigating the Dual-Action Potential of (Z)-6-methoxy-2-(naphthalen-1-ylmethylene) Benzofuran-3(2H)-one (AU-23): A Novel Synthetic Aurone Derivative with Antibacterial and Anti-Inflammatory Activity

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ABSTRACT

Aim/Background: Aurone is a well-known, naturally occurring, minor flavonoid with significant biological properties. However, their low abundance in nature limits their application in the medical field. In this study, we investigated the antimicrobial and anti-inflammatory properties of a novel synthetic aurone, (*Z*)-6-methoxy-2-(naphthalen-1-ylmethylene) benzofuran-3(2*H*)-one (AU-23). **Materials and Methods:** AU-23 was synthesized via multistep synthesis reactions involving the oxidative cyclization of 2′-hydroxychalcones. AU-23 was tested for antibacterial, minimum inhibitory concentration and antibiofilm activity against several ATCC strains using agar well diffusion, broth-micro dilution and XTT assay methods respectively. **Results:** At various concentrations, it selectively inhibited the growth of four strains (*P. aeruginosa* ATCC 9027, methicillin-resistant *S. aureus* (MRSA) ATCC 33591, methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 43300). Moreover, it was observed that AU-23 had a bactericidal effect on sensitive strains of MSSA ATCC 25923 and *P. aeruginosa* ATCC 9027, as well as a bacteriostatic effect on MRSA ATCC 33591 and MRSA ATCC 43300. AU-23 also displayed effective antibiofilm activity against monomicrobial biofilms but not against polymicrobial biofilms. The findings of PCR study revealed that AU-23 downregulates the expression of pro-inflammatory cytokines and mediators (i.e., IL-6, IL-1β, iNOS and TNF-α), as well as crucial pattern recognition receptors (TLR4 and CD14) in LPS-stimulated RAW 264.7 cells. Molecular docking studies demonstrated that the ability of AU-23 to bind to the mouse TLR4/MD-2 complex was comparable to that of dexamethasone. **Conclusion:** the results of several studies conducted, suggests that AU-23 has the potential to function as an effective dual-action agent with antimicrobial and anti-inflammatory properties.

Keywords: Aurone, Antimicrobial activity, Antibiofilm activity, Anti-inflammatory activity, Docking study.

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INTRODUCTION

Bacterial infections, especially those caused by Multi-Drug Resistance pathogens (MDR), remain a significant health problem worldwide, accounting for one-third of all deaths.[1] MDR bacteria such as *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*) *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacter* spp. have recently been dubbed 'ESKAPE' pathogens, indicating their escape from antibacterial therapy.[2] The Infectious Diseases Society of America (IDSA) has identified MDR pathogens as one of the most problematic bacterial infection challenges, resulting in nearly 11.5 million life-threatening infections and over 1.5 million deaths annually.[3] Inflammation is a primary immune response to infection and, if prolonged, could be harmful and lead to various complications,

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including cancer, diabetes, inflammatory bowel disease and asthma.[4]

Bacterial Lipopolysaccharide (LPS) or endotoxin, a major inducer of the inflammatory response, is a component of the outer membrane of gram-negative bacteria that activates macrophage cells to release pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and IL-6, as well as pro-inflammatory mediators such as Nitric Oxide (NO) and Reactive Oxygen Species (ROS), *via* the activation of specific signaling through specific pattern recognition receptors such as Toll-Like Receptor 4 (TLR4) and a Cluster of Differentiation 14 (CD14).[5] Dysregulation of these inflammatory responses may result in extended inflammation, potentially leading to host tissue damage and chronic pathological inflammation.[6]

Aurones, 2-benzylidene-1-benzofuran-3(2*H*)-ones, which belong to the class of isomeric flavones, together with chalcones, flavanones and flavonols, are classified as minor flavonoids due to their very limited abundance in nature.[7] Unlike other flavonoids, aurones contain a benzofuranone ring connected to a phenyl ring by an exocyclic double bond, forming a thermodynamically more stable (*Z*) isomer.[8,9] Plants have designated aurones as phytoalexins in their defense mechanisms against various infections.[10] Although studies on the biological activities of aurones are still limited, these natural products and their synthetic analogues have proven to be promising bioactive compounds with a broad spectrum of activities, including antimicrobial and anti-inflammatory activities.[11] For instance, a synthetic aurone derivative, (*Z*)-2-(4-Benzyloxy-3-methoxy benzylidene)-4,6- dihydroxybenzofuran-3(2*H*)-one, exhibited high antibacterial activity against gram-positive bacteria.[12] Furthermore, (*Z*)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2*H*)-one is another synthetic aurone derivative that exhibits anti-inflammatory effects in both human (THP-1) macrophage-like cells (THP-1) and murine macrophage cells (RAW 264.7) macrophage-like cells and suppresses Lipopolysaccharide (LPS)-induced expression of inflammatory cytokines, including TNF-α, at the transcriptional level.[11] Recently, our research group discovered a novel aurone, (*Z*)-6-methoxy-2-(naphthalen-1-ylmethylene) benzofuran-3(2*H*)-one (AU-23), through intensive screening of the antimicrobial and anti-inflammatory properties of flavonoid-based compounds.[12] In the present study, we investigated the novel compound AU-23 to determine its biological mechanism and explore its potential applications in antimicrobial and anti-inflammatory activities. Our group is also conducting extensive research *in silico* to predict the molecular targets of AU-23, which will provide valuable insights for future drug development.

MATERIALS AND METHODS

The solvents, Dimethyl Sulfoxide (DMSO) and acetone were purchased from Sigma-Aldrich (Poland). Phosphate-Buffered Saline (PBS) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophe nyl)-2*H*-Tetrazolium-5-carboxanilide (XTT) were purchased from Carbosynth (UK), while menadione was purchased from Sigma-Aldrich (Germany). Vancomycin and gentamicin were purchased from Merck (Poland). Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) were purchased from Oxoid (UK). Penicillin-streptomycin solution, trypsin-EDTA and trypan-blue stain were purchased from Sigma Aldrich (Germany). PCR reagents, including RNA extraction, were purchased from Thermo Scientific (Waltham, MA, USA). The HiScript III 1st Strand cDNA Synthesis Kit was purchased from Vazyme (Nanjing, China)) and the CYBR Green Master Mix was purchased from PCR Biosystems (UK). DMEM with L-glutamine and fatal fetal bovine serum was purchased from Capricorn (Germany).

Bacterial Strains and Cell Lines

Four reference ATCC strains (MRSA33591 and MRSA43300) were obtained from a novel antibiotic laboratory (UKM), (MSSA25923 and *Pseudomonas aeruginosa* 9027) were purchased from American Type Tissue Collection (ATCC, Manassas, VA, USA). RAW264.7 macrophage cell line (ATCC TIB-71) was obtained from ATCC (Manassas, VA, USA).

Synthesis and characterization of 1-(2-Hydroxy-4-methoxyphenyl)-3-(naphthalen-1-yl) prop-2-en-1-one

An aqueous solution of KOH (40%, 15 mL) was added to a stirred solution of 2′-hydroxy-4′-methoxyacetophenone 1 (10 mmol) and 1-naphthaldehyde 2 (10 mmol) in absolute EtOH (30 mL). The mixture was sonicated for 1 hr and stirred at 25°C for another 12 hr. The dark red solution was acidified with 250 mL of 2 M HCl solution to give a yellow residue, which was then filtered and rinsed with distilled water. The residue was purified by flash chromatography using *n*-hexane/EtOAc (gradient 10-60% EtOAc) and recrystallized in absolute ethanol. The desired product was formed as a yellow solid with a yield of 42%; m.p. 145-146°C. ^{1}H-NMR (600 MHz, Chloroform-*d*) δ 13.50 (s, 1H), 8.09-8.00 (m, 2H), 7.87 (ddt, *J*=5.1, 8.6, 14.5 Hz, 4H), 7.80 (d, *J*=8.5 Hz, 1H), 7.69 (d, *J*=15.4 Hz, 1H), 7.58-7.49 (m, 2H), 6.51 (d, *J*=10.7 Hz, 1H), 6.49 (s, 1H), 3.87 (s, 3H). ^{13}C-NMR (151 MHz, Chloroform-*d*) δ 191.78, 166.76, 166.25, 144.48, 134.44, 133.38, 132.30, 131.26, 130.77, 128.77, 128.70, 127.82, 127.47, 126.82, 123.71, 120.44, 114.18, 107.79, 101.11, 55.61. ESI-HRMS: ($C_{20}H_{16}O_3$) calc. [M+H] 305.1179, found 305.1124.

Synthesis and characterization of 6-Methoxy-2-(naphthalen-1-ylmethylene) benzofuran-3(2H)-one (AU-23)

To a solution of 3 mmol mercuric acetate ($Hg(OAc)_2$) in DMSO (10 mL), 1-(2-hydroxy-4-methoxyphenyl)-3-(naphthalen-1-yl) prop-2-en-1-one 3 (2 mmol) was added at 25°C. The mixture was then stirred at 160°C for 6 hr. The cooled reaction mixture was poured into ice-cold water and acidified with 100 mL of 2 M HCl solution. The solid residue was extracted with dichloromethane, dried over anhydrous magnesium sulfate and the solvent was evaporated. Finally, the product was purified by flash column chromatography using *n*-hexane/EtOAc (gradient 20-70% EtOAc) and recrystallised in absolute EtOH. The product was formed as a yellow solid, yielding 97%; m.p. 175-177°C. ^{1}H-NMR (600 MHz, Chloroform-d) δ 8.31 (s, 1H), 8.06 (d, J=8.6 Hz, 1H), 7.92 (d, J=7.3 Hz, 1H), 7.89 (d, J=8.6 Hz, 1H), 7.85 (d, J=8.1 Hz, 1H), 7.73 (d, J=8.5 Hz, 1H), 7.55-7.50 (m, 2H), 6.99 (s, 1H), 6.84 (s, 1H), 6.78 (d, J=8.5 Hz, 1H), 3.95 (s, 3H). 13C NMR (151 MHz, Chloroform-d) δ 182.98, 168.58, 167.46, 148.06, 133.61, 133.37, 131.95, 130.08, 128.68, 128.49, 127.73, 127.64, 127.33, 126.57, 125.88, 114.94, 112.23, 112.10, 96.74, 56.07. ESI-HRMS ($C_{20}H_{14}O_3$) calc. [M+H] 303.1023, found 303.1001.

Antimicrobial activities of aurone AU-23

Antimicrobial activity screening

The screening of antibacterial activity was conducted via agar well diffusion method.[13]A freshly prepared bacterial suspension solution adjusted to 1×10^6 CFU/mL was inoculated onto surface MHA plates using a sterile swab. The culture plates were then allowed to dry. A diameter of 6 mm was cut using a standard cork borer at the center of each seeded plate. 30 µg/disc of the aurone AU-23 was introduced into each well on the medium's surface. The plates were then incubated at 37°C overnight, after which the plates were observed and zones of inhibition were measured in millimetres. DMSO (10%) was used as the negative control, whereas both antibiotics, Vancomycin and Gentamicin sulfate were used as the positive control for gram-positive and gram-negative bacteria, respectively.

Investigation of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The broth micro-dilution method was used to determine the MIC in a sterile 96-well microtitre plate. This assay was performed in triplicate to ensure consistency, following the guidelines of the Clinical and Laboratory Standards Institute.[14] The concentration of synthesized aurone AU-23 used in this study against MRSA ATCC 33591, MRSA ATCC 43300, MSSA ATCC 25923 and *P. aeruginosa* ATCC 9027 ranged from 500 to 0.98 µg/mL, 125-0.24 µg/mL for vancomycin, 512-1 µg/mL for gentamicin. After adding sterile MBH, the synthesized compound was inserted into the wells at a concentration gradient. The diluted bacterial suspension was added to a final inoculum of 1×10^6 CFU/mL. The tested compounds in MHB were used as negative controls to ensure medium sterility. At the same time, the inoculum in MHB served as a positive control to ensure the adequacy of the broth for bacterial growth. To facilitate observation of bacterial growth in each well, 20 µL of 2,3, 5- Triphenyl Tetrazolium Chloride (TTC) at 2 mg/mL was added to each well after overnight incubation at 37°C. MBC was determined by culturing each clear well from a 96-well microtitre plate onto the MHA plate. The plates were then incubated at 37°C for 24 hr. The MBC value was determined as the lowest concentration of an antibacterial agent that showed no visible growth or killed 99.9% of the bacterial inoculum on agar plates.[15]

Time-kill assay

Bactericidal activity was defined as 99.9% killing of the final inoculum by ≥3-log^{10} decrease in CFU/mL. A time-kill assay was performed for four reference ATCC strains (MSSA 25923, MRSA 33400, MRSA 33591 and *P. aeruginosa* 9027) and aurone AU-23. After incubation in MHB at 37°C in a shaker incubator for 4-6 hr, a suspension of cell density (1×10^{-6} to 1×10^{-8} CFU/mL) of ATCC strains was prepared in MHB supplemented with this compound to a final concentration equal to 1×MIC, 2×MIC, 4×MIC and 8×MIC and incubated at 37°C with shaking (120-150 rpm). Bacterial suspensions without added compounds were used as untreated controls. At predetermined time intervals (0, 2, 4, 6, 8, 10, 12 and 24 hr), samples were collected, serially diluted in normal saline (10^{-1} to 10^{-6}) and then spotted (100 µL) onto MHA plates. After 24 hr of incubation, the colonies were enumerated. Assays were performed in triplicate for each pathogen. Data derived from the time-kill assays were analyzed using GraphPad Prism version 9. Log_{10} CFU/mL values of each pathogen were plotted versus time for the tested aurone AU-23.[16]

Evaluation of antibiofilm activity

Two ATCC bacterial strains were used in this assay, *S. aureus* (25923) and *P. aeruginosa* (9027) and their polymicrobial biofilms were formed by mixing equal ratios of both strains. 50 µL of each bacterial inoculum were added to a 96-well plate and incubated at 37°C overnight under static conditions. Following overnight incubation, 100 µL PBS was used to remove floating bacteria from the wells. Test wells containing adherent bacteria were treated with 100 µL of aurone AU-23 at different concentrations (125, 250 and 500 µg/mL). For the XTT assay, 4 mg of XTT was dissolved in 10 mL of pre-warmed PBS to prepare fresh XTT solutions. The solution was mixed with 100 mL menadione stock solution containing 55 mg of menadione in 100 mL acetone. The XTT solution (200 µL of XTT solution was added to each well of the plate and incubated for an additional 5 hr in the dark at 37°C. Next, 100 µL of the supernatant was transferred to a new 96-well plate and the absorbance was measured at 490 nm using an ELISA microplate reader.[17] Untreated cells were used as controls.

The percentage of biofilm formation was calculated using the following equation:

$$\text{Percentage inhibition of metabolic activity} = 1 - \left(\frac{\text{OD490 of cells treated with test agent}}{\text{OD490 of vehicle control}}\right) x\ 100$$

Anti-inflammatory Activity of Aurone AU-23

Cell culture

RAW264.7 macrophage cell line was cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin. The culture was incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere and the medium was changed every two days.

Cell viability assays

The RAW 264.7 macrophage cell line was adjusted to 3.5×10^4 cells/well and 100 µL aliquots were seeded into each well of a 96-well culture plate and incubated at 37°C in a CO_2 incubator until the cells reached 80% confluency. Synthetic aurone AU-23 at concentration of 200 µM to 0.78 µM was added to the wells, followed by incubation for 24 hr in a 5% CO_2 environment at 37°C. DMSO at a final concentration of 0.1 % was used as the negative control. Finally, MTT (20 µL) was added to each well containing the macrophages and incubated for another 4 hr. The absorbance of each sample was recorded at 570 nm wavelength using a microplate reader.[18]

Nitric Oxide (NO) assay

The RAW264.7 macrophage cell line (5×10^4 cells/mL) was stimulated with LPS at a concentration of 1 µg/mL, followed by treatment with AU-23 at concentrations of 6.25, 3.125 and 1.56 µM for 24 hr. After incubation, the cells were centrifuged and the supernatant was collected. Equal volumes of 50 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and the supernatant were added to a 96-well plate and incubated at room temperature for 15 min. Absorbance was measured at 540 nm using a microplate reader (Varioskan Flash). Nitrite concentrations were determined using a standard curve prepared by assaying serially diluted sodium nitrite.[19]

RNA isolation and qRT-PCR

Total RNA was extracted using a TRIzol reagent according to the manufacturer's protocol. Reverse transcription was performed to convert the RNA into cDNA (Vazyme, China). qRT-PCR amplification was performed using the SYBR Green master mix kit and an Eppendorf realplex[4] qPCR (Real-Time PCR Thermocycler) machine.[17] For IL-6, IL-1β, iNOS, TNF-α, CD14 and TLR4, experimentally validated all-in-one qPCR primers were purchased and validated from Gene Copoeia (Rockville, MD, USA). Data were collected and analyzed using the 2-ΔΔCT relative quantification method.[20] Values are presented as fold-changes relative to untreated cells. The primer sequences are listed in Table 1.

Reactive Oxygen Species (ROS) assay

RAW264.7 cells were treated with aurones at concentrations of 1.56 µM, 3.125 µM and 6.25 µM in the presence or absence of LPS (1 µg/mL) for 24 hr. After washing twice with cold PBS, the cells were stained with DHE at 5 µM for 30 min at 37°C, according to the manufacturer's instructions. The cells were collected, centrifuged and the pellet resuspended in PBS. Finally, fluorescence intensities were detected by LSR Fortessa™ CELL ANALYZER (Becton Dickinson and Company, BD Biosciences, San Jose, USA) flow cytometry.[21,22]

Molecular docking analysis

The crystal structures of the mouse TLR4/MD-2 complex, which binds to tetraacylated Lipid IVa (LP5), a precursor of *E. coli* LPS that acts as an agonist for certain mammalian species, were acquired from the Research Collaborator for Structural Bioinformatics Protein Data Bank (RCSB PDB: https://www.rcsb.org/) under PDB ID 3VQ2. The resolution of the structure was determined at 2.48 Å, revealing that five out of the six acyl chains of LPS are enclosed within the MD-2 cavity, whereas the sixth acyl chain is partially exposed on the surface of MD-2.[20] Moreover, this partially exposed acyl chain, along with the hydrophobic residues of MD-2, forms a secondary binding site for the hydrophobic patch on the C-terminal convex face of the horseshoe-shaped TLR4 structure, leading to the formation of the "m" shaped TLR4/MD-2/lipid IVa complex. Next, the UCSF

Table 1: Sequences of the primers used for PCR.

Gene name	Forward Primer (5′-3′ end)	Reverse Primer (5′-3′ end)
B-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
IL-6	GCCTTCTTGGGACTGATGCT	GACAGGTCTGTTGGGAGTGG
IL-1β	AACTCAACTGTGAAATGCCACC	CATCAGGACAGCCCAGGTC
iNOS	AGACCTCAACAGAGCCCTCA	TCGAAGGTGAGCTGAACGAG
TNF-α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT
TLR4	GCAAAGTCCCTGATGACATTCC	CATGCCTTGTCTTCAATTGTT
CD14	CCTCCAAGTTTTAGCGCTGC	CAGCATCCCGCAGTGAATTG

Chimera 1.14 package (University of California, SF, USA) was employed to select protein chains, eliminate water molecules and ligands and rectify any artefacts.[23] The structure was subsequently refined using Discovery Studio (DS) 3.1 (Accelrys, Inc., San Diego, CA, USA), which involves adding missing atoms, filling gaps in loop regions, adjusting sidechain conformations, removing alternate conformations, standardizing atom names and protonating titratable residues.

Molecular docking simulations were conducted using CDOCKER method protocols within the receptor-ligand interaction section of DS 3.1.[24] The active binding sites for the mouse TLR4/MD-2 complex were identified as the binding positions for LP5, with a radius of 15.0 Å. During the docking process, multiple conformations of each ligand were generated through high-temperature molecular dynamics simulations, involving heating the ligands to 700 K and cooling them back to 300 K. Subsequently, the ligands underwent refinement using simulated annealing and full force minimisation while the receptor remained fixed. The generated ligand conformations were clustered based on their binding interactions with proteins to identify favourable binding modes. The conformation with the lowest CDOCKER interaction energy, which assesses the binding affinity and stability, was chosen as the best predicted binding pose. The 3D structures of synthesized aurone AU-23 and dexamethasone were constructed using ChemDraw Professional 15.0 (Perkin Elmer Inc., Waltham, MA, USA) and imported into DS 3.1. Parameters for isomer generation, tautomerism and ionization changes were disabled and the ligand was prepared by standardizing charges, adding hydrogens, enumerating ionization states, ionizing functional groups, removing duplicates and optimizing the CHARMm force field in DS 3.1.[25]

The validation process involved redocking the original ligands and calculating the Root Mean Square Deviation (RMSD) of the redocked ligands from the original conformation until most poses exhibited an RMSD ≤2.0 Å.[26] It is essential to acknowledge that the quality and accuracy of the protein structures and parameters significantly influence the outcomes of molecular docking simulations, necessitating adherence to a rigorous docking protocol.

Statistical analysis

Statistical data analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All analyses were performed using GraphPad Prism (version 9.0). The significance level was set at $p<0.05$.

RESULTS

In our previous study, 2'-hydroxychalcones were synthesized *via* the Claisen-Schmidt condensation reaction and successfully characterized using spectroscopic tools. The synthesis of compound 3 was successful when KOH, which serves as a base, was used for coupling 2′-hydroxy-4′-methoxyacetophenone (1) with commercially available 1-naphthaldehyde (2) *via* the Claisen-Schmidt condensation reaction. Overall, the reaction afforded 3 in a moderate yield (42%). We observed that the product yield significantly increased to 92% when the reaction was optimized under ultrasound irradiation at 40ºC.[27] The desired product of the aurone derivative, AU-23, was prepared *via* oxidative cyclization by refluxing 3 with $Hg(OAc)_2$ in DMSO (Figure 1).[28] Oxidative cyclization of 3 using $Hg(OAC)_2$ is highly effective and efficient for synthesizing AU-23aurone (4 derivative AU-23). The reaction is simple, selective and affords the desired product in high yields. Indeed, using $Hg(OAC)_2$ as a catalyst enables precise control over reaction conditions, improving reaction efficiency and product purity.[29] The final products were purified by flash chromatography and recrystallized in absolute ethanol to afford the corresponding AU-23 in high yield (97%). Moreover, Nuclear Magnetic Resonance (NMR) and high-resolution mass spectrometry (ESI-HRMS) elucidated all the synthesis products. All the spectra were consistent with the assigned structures of the synthesized compounds. The NMR spectra provided evidence of the reaction progression by comparing the characteristic hydroxyl and α, β-unsaturated proton signals found in the intermediate (compound 3) and the aurone product (AU-23). NMR showed that the hydroxyl and α, β-unsaturated proton signal of compound 3 resonated at δ_H 13.50 ppm, while these signals were eliminated in the final aurone product. Additionally, the appearance of a β-unsaturated singlet proton signal at δ_H 6.99 ppm indicated the loss of a neighbouring proton Hα.

Antibacterial activity of Aurones AU-23

Primary screening with agar well diffusion method

Primary screening of AU-23 was performed to test its antibacterial activity against the reference ATCC strains. The compound exhibited significant antimicrobial activity against MRSA 43300, MRSA 33591, MSSA 25923 and *P. aeruginosa* 9027,

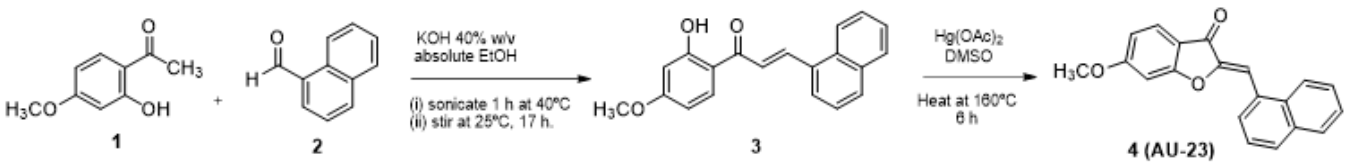

Figure 1: Synthesis scheme of AU-23 derivative.

as shown in Figure 2. The most potent antibacterial activity of AU-23 (30 µg/disc) was observed against gram-positive bacteria; (A) MRSA 43300 with an Inhibition Zone (IZ) of 18.33±0.4 mm (1), followed by (B) MSSA 35923 (14.67±0.6 mm) (1), (C) MRSA 33591 (13.50±0.3 mm) (1), compared to the positive control (vancomycin 30 µg/disc) (3). Additionally, the compound demonstrated antimicrobial activity against gram-negative bacteria, particularly (D) *P. aeruginosa* 9027, displaying an inhibition zone of 12.33±0.4 mm (1), in comparison to gentamycin (10 µg/disc) (3) and negative control (10% of DMSO) (2).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

For a quantitative assessment, the antimicrobial effectiveness of the synthetic aurone AU-23 was evaluated against both gram-positive and gram-negative bacteria using MIC and MBC assays. The MIC represents the lowest compound concentration necessary to inhibit bacterial growth. At the same time, the MBC denotes the lowest concentration required to eradicate bacteria with either a 100% or 99.9% reduction in viability. Notably, the MIC of AU-23 was lower for MRSA 43300 and MSSA 25923 than for MRSA 33591 and *P. aeruginosa* 9027. Specifically, the MIC and MBC values of AU-23 against MSSA 25923 were determined to be 15.63 µg/mL and 31.25 µg/mL, respectively. Interestingly, the MIC and MBC values for MRSA 43300 were found to be equal (31.25 µg/mL). On the other hand, the MIC values for MRSA 33591 and *P. aeruginosa* 9027 were 62.5 µg/mL, while their corresponding MBC values were twice their respective MIC values, as presented in Table 2.

Time-Kill Assay (TKA) of AU-23

The time-kill assay was conducted using the broth micro-dilution method following the guidelines set by CLSI. The purpose of this assay was to assess the dynamic interaction between the novel compound AU-23 and selected ATCC bacterial strains at various concentrations, namely 1×MIC, 2×MIC, 4×MIC and 8×MIC for each strain. The kinetic profile of AU-23, as depicted in Figure 3, revealed variations in its activity against different strains. Notably, it demonstrated a bactericidal effect on *P. aeruginosa* 9027, with a reduction in viable bacterial cells of ≥3 log10 compared to the initial inoculum after 4-10 hr of exposure at 2×MIC, 4×MIC and 8×MIC. Conversely, AU-23 exhibited a bacteriostatic effect on MRSA 43300 and MRSA 33591 without any noticeable bactericidal impact, as indicated by a reduction of CFU (colony-forming units) ≤3 log10. However, MSSA 25923 exhibited the highest sensitivity to the tested compound. Although a certain level of bactericidal effect was observed at 2×MIC, a more potent effect was observed at 4×MIC and 8×MIC after 4-6 hr of treatment.

The activity of AU-23 against bacterial biofilm

The XTT assay measured the metabolic activity of living bacterial cells within a biofilm community. Reducing XTT generates a formazan compound, which can be quantified using colourimetric analysis. The XTT assay consists of three components: XTT, menadione and Phosphate-Buffered Saline (PBS). It is important

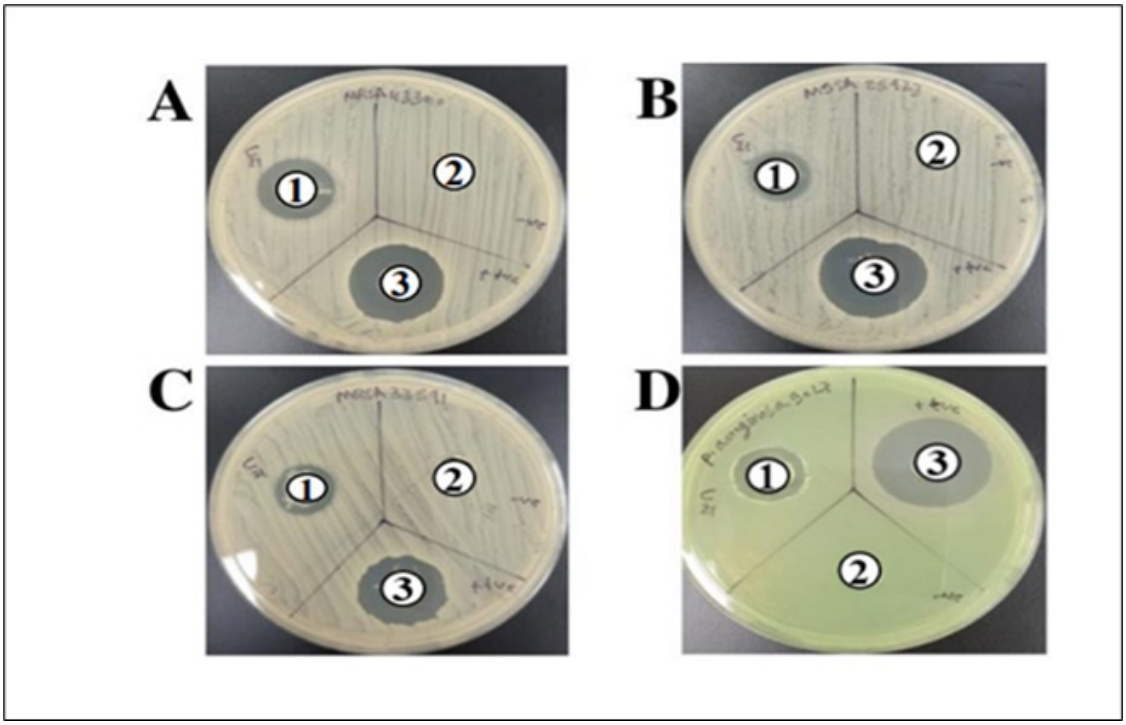

Figure 2: Antibacterial activity of AU-23 against (A) MRSA 43300, (B) MSSA 25923, (C) MRSA 33591 and (D) *P. aeruginosa* 9027.

Table 2: **The MIC and MBC values of AU-23 (µg/mL) against ATCC strains.**

Compounds	ATCC Bacterial Strains							
	MSSA25923		MRSA43300		MRSA35591		*P. aeruginosa 9027*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Aurone	15.63	31.25	31.25	31.25	62.50	125	62.50	125
Vancomycin	0.24	0.49	0.49	0.49	0.98	0.98	-	-
Gentamicin	-	-	-	-	-	-	4	8

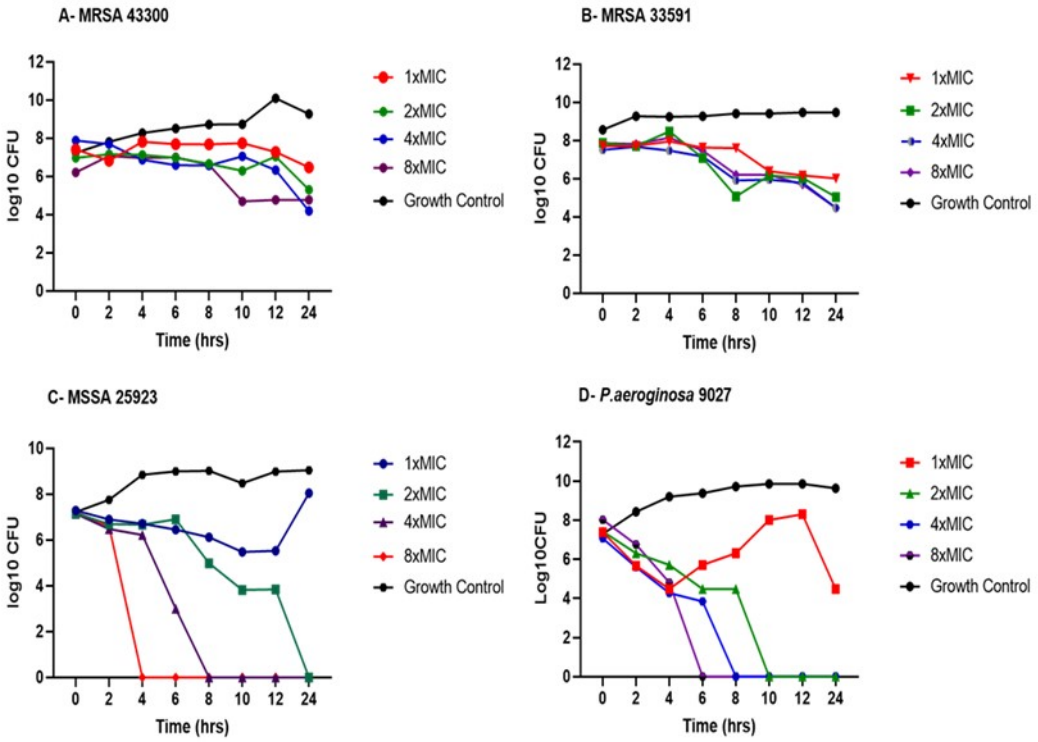

Figure 3: Kinetic Time-Kill Assay of AU-23 Against Four ATCC Strains.

to note that XTT reduction depends on cell activity rather than cell mass.[30] When the bacterial strains (MSSA 25923 and *P. aeruginosa* 9027) were grown in a biofilm mode, AU-23 was a suitable biofilm inhibitor. It reduces metabolically active cells efficiently in a dose-dependent manner. This antibiofilm effect was significantly higher against both ATCC strains, MSSA 25923 and *P. aeruginosa* 9027, when grown in mono-microbial biofilm mode (separately) with an inhibition rate of (64% and 59%) at MIC of 500 µg/mL, respectively, in comparison with the untreated control group. Furthermore, when the two strains were co-cultured together (polymicrobial biofilm mode), the tested compound maintained its antibiofilm activity but at a weaker range (18-49%) compared to the untreated control group (Figure 4).

Anti-inflammatory Properties of AU-23

Cell viability

The effect of AU-23 on cell viability was evaluated to determine the appropriate concentration for further anti-inflammatory studies. RAW264.7 cells were exposed to a wide range of concentrations of the compound (ranging from 0 µM to 200 µM) at different time intervals (24, 48 and 72 hr). The results obtained from the MTT assay revealed that AU-23 at concentrations of 25, 50, 100 and 200 µM exhibited toxicity, with cell viability below 80% compared to the untreated control group. However, when cells were exposed to AU-23 at a concentration of 6.25 µM or lower (Figure 5), we observed higher cell viability after 24, 48 and 72 hr compared with the untreated control group. These findings suggest that a lower concentration of AU-23, specifically up to 6.25 µM, is suitable for continued investigations focused on its anti-inflammatory properties.

Nitric Oxide (NO) inhibition

NO is a crucial inflammatory indicator that participates in various molecular and biological pathways and involves multiple physiological and pathophysiological processes.[31] Although NO neurological benefits function and defense mechanisms, excessive NO production has been associated with several

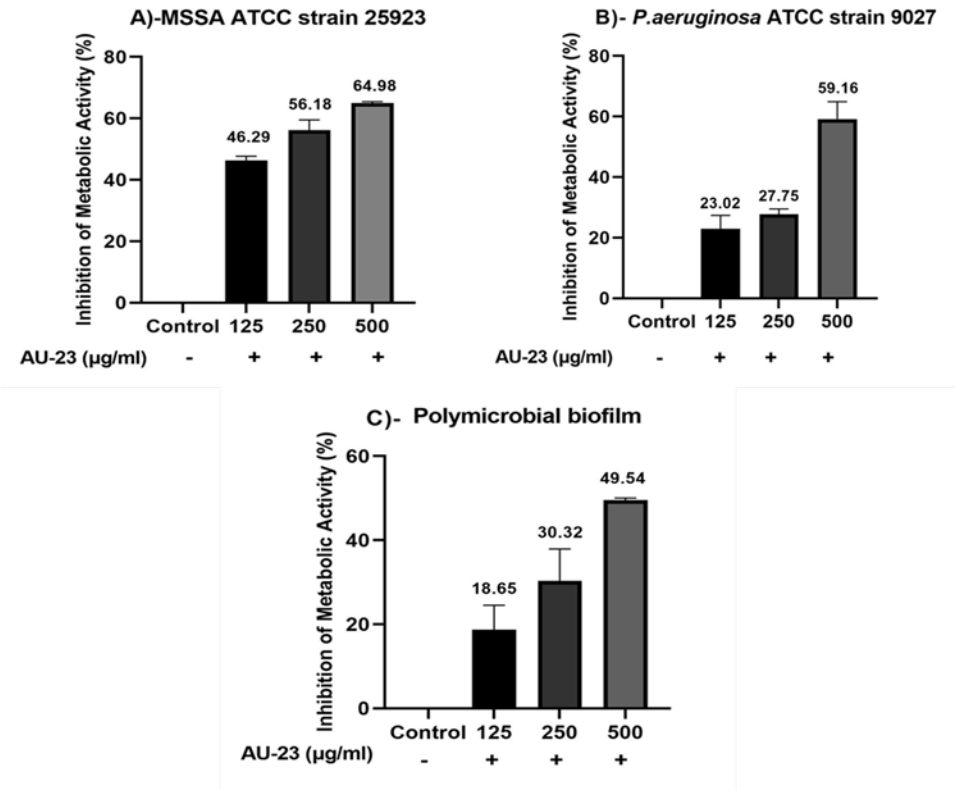

Figure 4: The XTT assay shows the percentage of inhibition of the metabolic activity of AU-23 against biofilm formation; A) monomicrobial biofilm MSSA ATCC 25923; B) monomicrobial biofilm *P. aeruginosa* ATCC 9027; and C) Polymicrobial biofilm (both strains).

diseases, particularly inflammatory diseases.[32] It is well-known that when stimulated with LPS, macrophages produce massive amounts of NO. Based on the results, LPS treatment alone leads to a significant release of NO production. However, pre-treated LPS-induced RAW264.7 cells with compound aurone AU-23 show a substantial decrease in NO release compared to the untreated control group (Figure 6). AU-23 showed dose-dependent inhibition of NO release. The NO levels are expressed as means values±SEM of the three independent experiments (n=3). #: significantly different compared to the untreated group. * p<0.05; ** p≤0.001; p≤0.0001 **: p<0.01, as compared to the LPS-induced group. Notably, AU-23 at a concentration of 6.25 µM, along with the positive control drug dexamethasone (10 µM), exhibited a significant decrease in NO production (p≤0.0001), surpassing the effects observed with concentrations of 3.125 µM and 1.56 µM (p≤0.05). These findings highlight the potential of AU-23, particularly at a concentration of 6.25 µM, in effectively mitigating NO production in LPS-induced RAW264.7 cells, suggesting its potential anti-inflammatory properties.

The effect of AU-23 on LPS-stimulated pro-inflammatory cytokine gene expression (IL-6, IL-1β, iNOS, TNF-α) via (CD14 and TLR4)

Quantitative Reverse Real-Time Polymerase Chain Reaction (qRT-PCR) was conducted on LPS-stimulated RAW264.7 cells pre-treated with AU-23 at varying concentrations (3.125 µM and 6.25 µM) to determine its inhibitory effect on the pro-inflammatory cytokines, mediators (IL-6, IL-1β, iNOS, TNF-α), cell surface receptor CD14 and co-receptor TLR4. β-actin was used as a housekeeping gene to ensure reverse-transcripted cDNA quality. The results revealed that pre-treated with AU-23 at concentrations of 3.125 µM and 6.25 µM significantly downregulated the expression of all pro-inflammatory cytokines and mediators, as well as the cell surface receptor CD14 and co-receptor TLR4, in a concentration-dependent manner compared to the untreated group (Figure 7). The results related the gene expression levels were normalized with β-actin. Data are shown as means±SEM (n =3). *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, as compared to the untreated LPS-stimulated cell. These findings suggest that AU-23 has the potential to inhibit the expression of pro-inflammatory factors and modulate the activity of specific pattern recognition receptors, such as TLR4 and CD14, in LPS-induced RAW264.7 cells.

The effects of AU-23 on LPS-stimulated intracellular ROS in RAW264.7 macrophage cells

Several studies reported that inflammation is closely related to oxidative stress, in which intracellular Reactive Oxygen Species (ROS) levels increase.[33] Stimulation of macrophages with bacterial LPS has been shown to induce elevated ROS production, which is

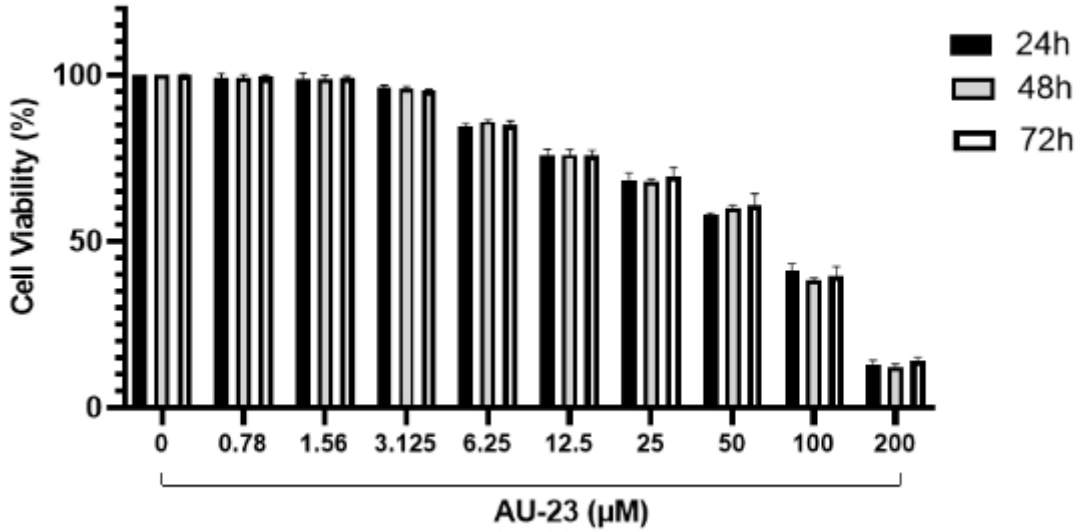

Figure 5: The cytotoxic effect of AU-23 on RAW264.7 cells. Data are presented as mean±SEM from three independent experiments.

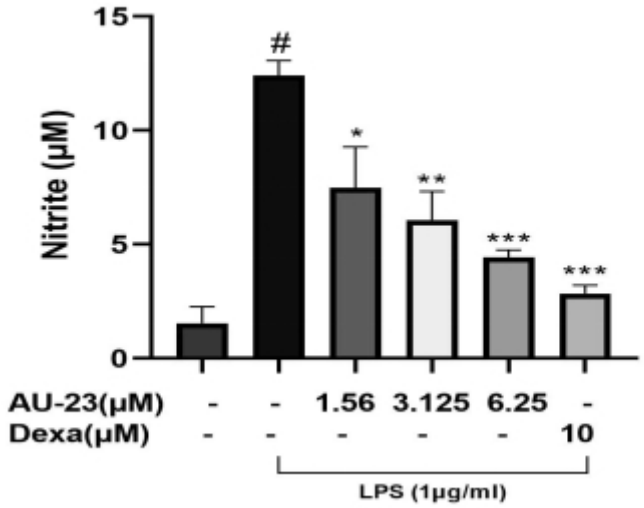

Figure 6: The inhibitory effects of AU-23 on NO release in the cell-free culture supernatant of LPS-stimulated RAW 264.7 cells.

a critical factor in developing various inflammatory diseases. In our study, LPS at a 1 μg/mL concentration effectively stimulated ROS production, as depicted in Figure 8. Data are shown as means±SEM (n=2). #: significantly different compared to the untreated cells group, **: p<0.01 as compared to the untreated LPS-induced group. However, pre-treated LPS-induced RAW264.7 cells with AU-23 at range concentrations (1.56 μM, 3.125 μM, 6.25 μM) and dexamethasone (10 μM) exhibited significant inhibition of LPS-induced ROS production (p<0.01) compared to the untreated group. These findings highlight the potential of AU-23 and the positive control drug dexamethasone in effectively attenuating ROS production induced by LPS in RAW264.7 cells.

Molecular Docking Studies of the TLR4/MD2 Complex with AU-23

Reflecting the experimental result, we investigated the potential of AU-23 to bind to a TLR4 and a molecular docking simulation

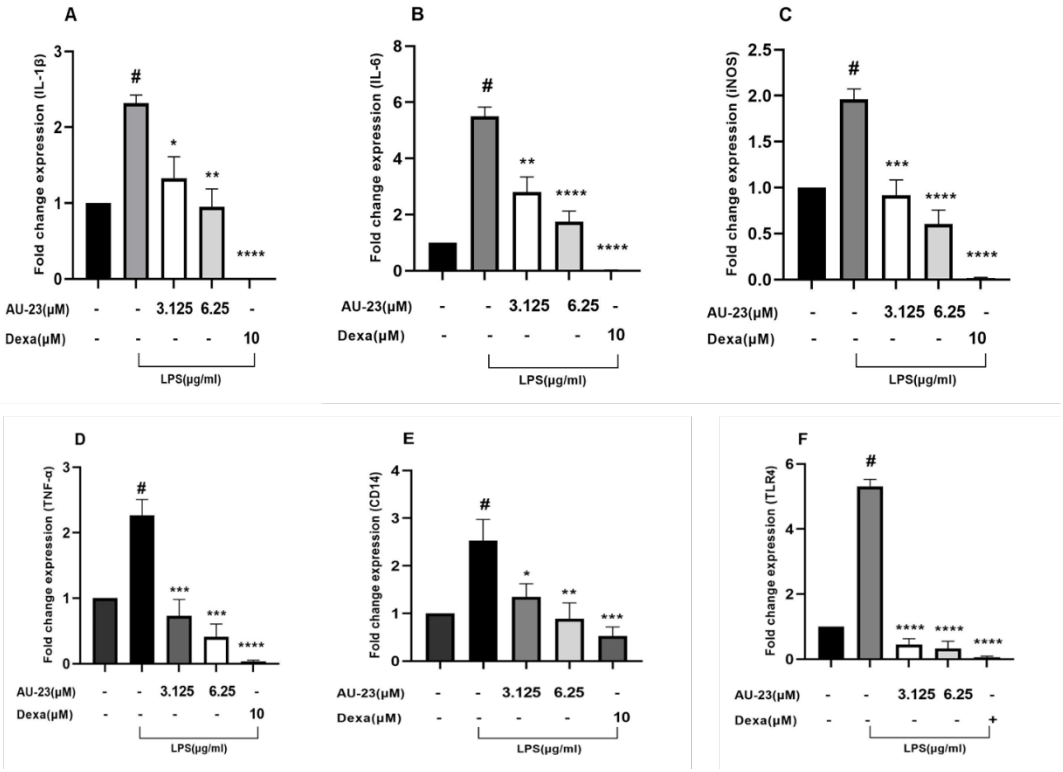

Figure 7: Effects of AU-23 on LPS-stimulated gene expression level of IL-1β (A), IL-6 (B), iNOS (C), TNF-α (D), TLR4 (E) and CD14 (F) in RAW264.7 cells.

was performed to determine its binding affinity to the murine TLR4/MD2 complex (PDB: 3VQ2, ligand: LP5). This study compared the CDOCKER interaction energy values and binding poses of AU-23 with those of dexamethasone, a well-established TLR4/MD2 inhibitor.[34] The CDOCKER method employing a CHARMm-based docking tool with a rigid receptor using Discovery Studio (DS) 3.1 was utilized for the calculations. Both the AU-23 and dexamethasone docking complexes were recognized within the hydrophobic pocket of MD2. The docking results' reliability and accuracy depended on the method's validation. Therefore, this study validated the CDOCKER method for molecular docking using the 3VQ2 crystal structure. To determine the accuracy of the docking procedure, we compared the position of the native ligand LP5 before docking with its work after docking. The Root-Mean-Square Deviation (RMSD) between the two poses was calculated and determined to be 1.9 Å (Figure 9), which is below the commonly accepted threshold of 2.0 Å.[35] This result suggests that the CDOCKER method is appropriate for the molecular docking of the 3VQ2 crystal structure.

The docking results indicate that AU-23 and dexamethasone have a similar attraction to MD2, as evidenced by their comparable CDOCKER interaction energies of -20.96 and -25.51 kcal/mol, respectively. This suggests that AU-23 has a potential affinity for the murine TLR4/MD2 complex, comparable to that of dexamethasone, which is known to have anti-inflammatory properties. Docking analysis further revealed that AU-23 establishes hydrophobic contacts with various labelled sidechains and residues within the MD2 pocket, particularly with Phe121. This interaction resembled the binding pattern observed with the antagonistic ligand eritoran.[35] Additionally, the naphthalene moiety of aurone AU-23 was involved in π-π stacking, further enhancing its hydrophobic interactions with MD-2 residues (Figure 10).

Based on these findings, it can be inferred that AU-23 has the potential to interact with the murine TLR4/MD2 complex and may function as a TLR4/MD2 inhibitor. This suggests that aurone AU-23 could have implications for developing novel anti-inflammatory therapies, potentially targeting the TLR4/MD2 signaling pathway. Although computational studies have shown promising progress, it is crucial to confirm the binding of aurone AU-23 to the murine TLR4/MD2 complex protein through experimental validation.

DISCUSSION

Aurones, classified as secondary metabolites, are part of the polyphenol family. More specifically, they are the lower structural counterparts of the best-known flavones.[35] Natural and synthetic analogues exhibit various biological activities, including antibacterial, anti-inflammatory, anticancer, antiviral and antioxidant properties.[9]

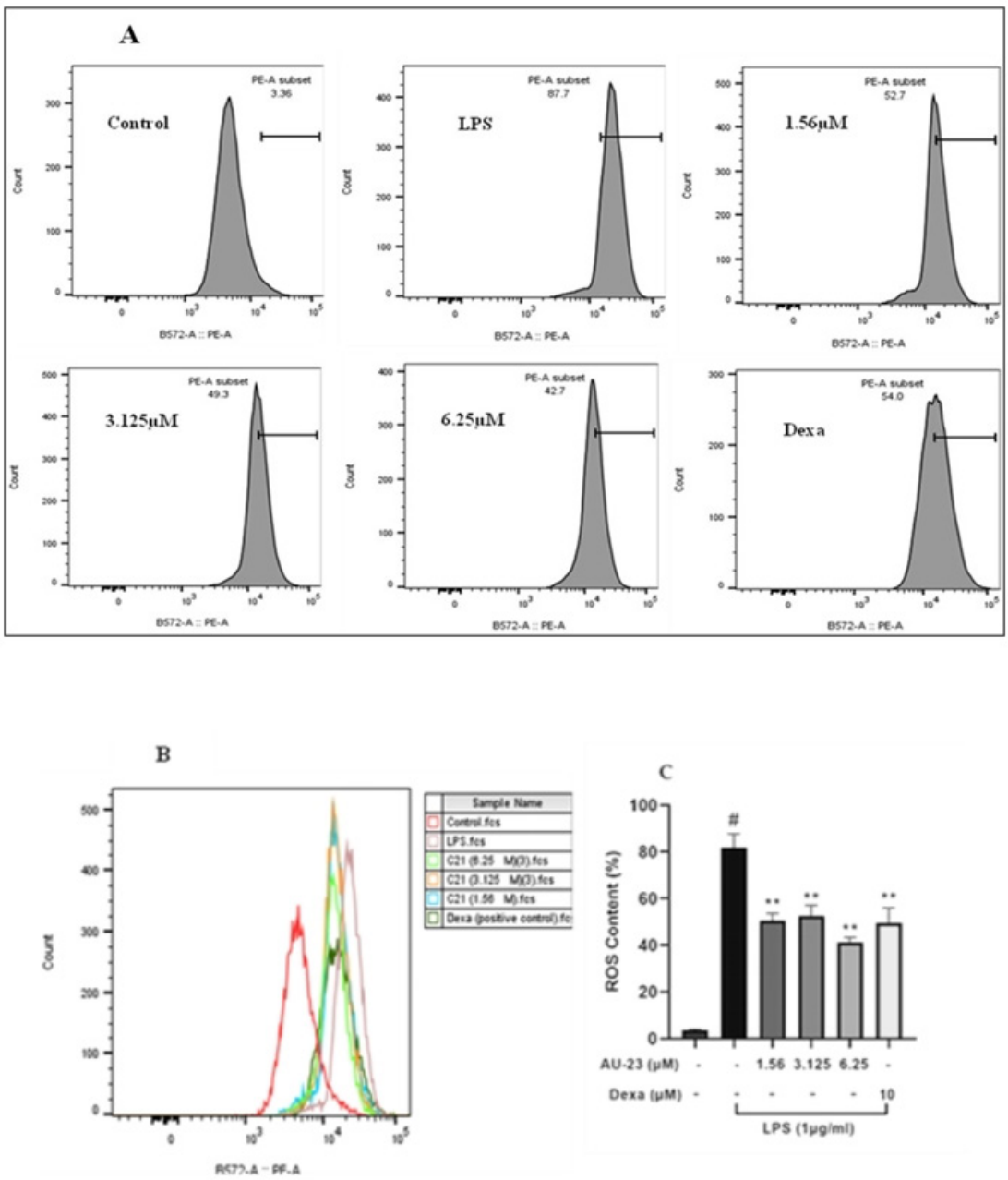

Figure 8: The effect of AU-23 on ROS production in RAW264.7 cells after being stimulated with LPS for 24 hr. (A) and (B) The graphs present the differences between all groups. (C) The ROS content was expressed as the percentage of cells with bright fluorescence.

Extensive documentation supports the fact that natural flavonoids possess antimicrobial properties. However, the potential use of synthetic flavonoids as antibacterial agents and anti-inflammatory agents has received less attention.[11] The current study investigated the antimicrobial activity of a novel synthetic aurone compound, AU-23. The results demonstrated that AU-23 exhibited inhibitory effects against four reference ATCC bacterial strains commonly associated with wound infection. The diameter of the zone inhibition obtained with AU-23 was lower in all four strains tested compared to the positive controls. The outcomes were comparable and similar to the report of Bandgar *et al.* (2010), who synthesized 2,2-bisaminomethylated aurone analogues, which showed moderate to good zones of inhibition against *E. coli, S. aureus, B. subtilis, K. pneumoniae* and *P. vulgaris* compared to the standard drug.[35]

The (MIC and MBC) of the novel AU-23 were estimated to determine the bacteriostatic (inhibit) or bactericidal (kill) activity. The significance of this investigation should not be regarded as a presumption that a bactericidal drug is superior to a bacteriostatic drug. It is important to mention that this compound could be bacteriostatic for one strain or species and

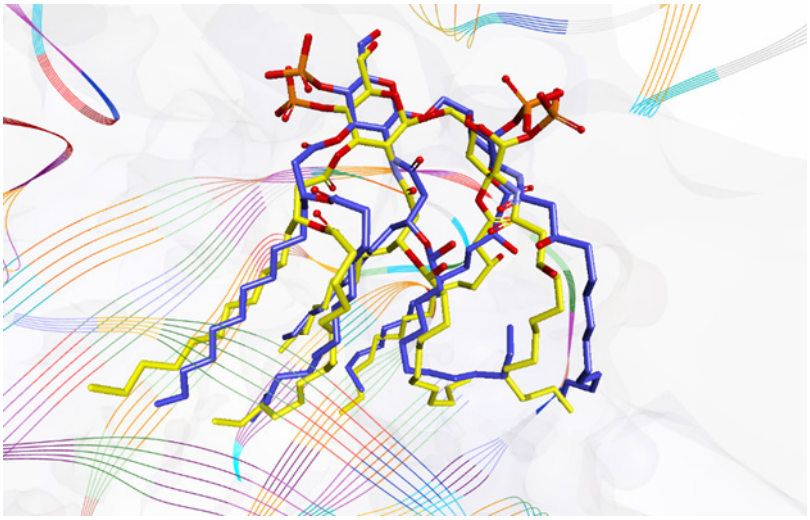

Figure 9: Superposition of the redocked conformation of tetraacylated Lipid IVa (LP5) over the co-crystal LP5 retrieved from the protein crystal structure. The original LP5 was yellow and the redocked MK-591 was purple.

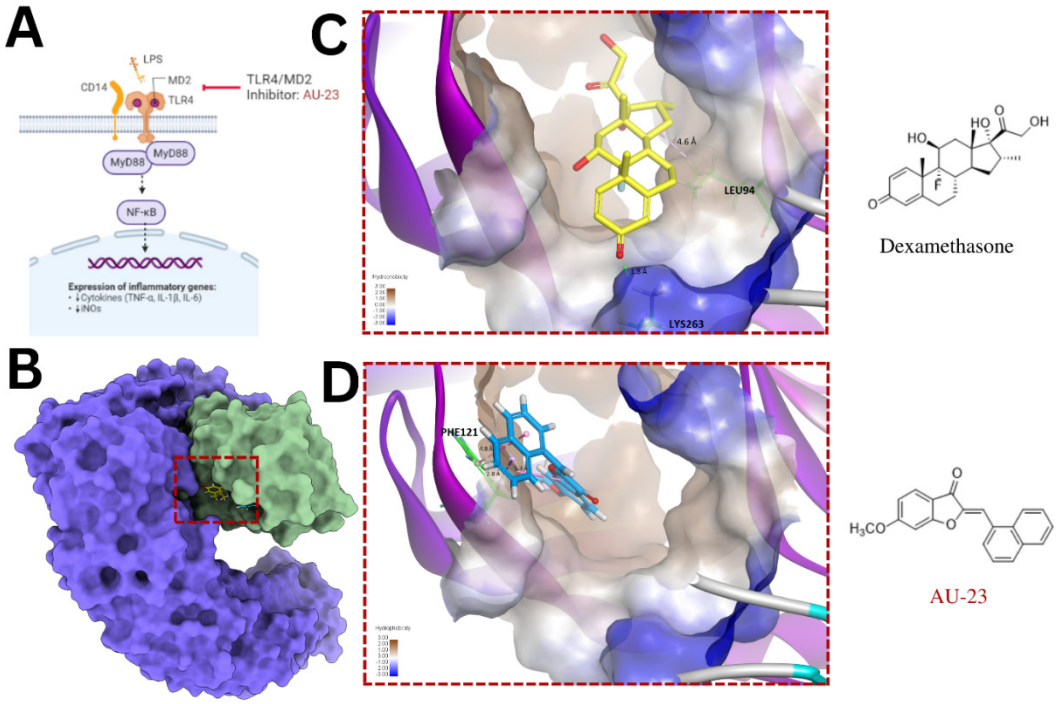

Figure 10: (A) Illustration of the inhibitory effects of aurone AU-23 in LPS-stimulated murine macrophages. (B) Superimposition of docking poses of dexamethasone and aurone AU-23. Plausible binding interactions of (C) dexamethasone and (D) aurone AU-23 with the murine TLR4/MD2 complex (PDB: 3VQ2).

may be bactericidal (killing) for other bacterial strains.[37] However, some studies suggested that treating bacterial infections with bactericidal drugs is desirable to bacteriostatic drugs.[38] On the other hand, concentration-dependent drugs were preferred to time-dependent drugs.[37] The difference between MBC and MIC values *in vitro* was well explained. The compound is considered bactericidal if the MBC value is ≤4 times compared to the MIC value. On the other hand, if the MBC value is ≥4 times the MIC value, the compound is considered bacteriostatic.[38] Based on the previously mentioned description, the results from this study revealed that the MIC of this promising compound against MSSA 25923, MRSA 43300, MRSA 33591 and *P. aeruginosa* 9027 was 15.63, 31.25, 62.5 and 62.5 µg/mL, respectively. The MBC values of AU-23 against MSSA 25923 and MRSA 43300 were 31.25 µg/mL and 125 µg/mL against the two other ATCC strains (MRSA 33591 and *P. aeruginosa* 9027). The present study aligned with, which found the screened aurone analogue compounds to have good antibacterial activity against gram-positive and

gram-negative bacteria. The MIC values reported were similar to the value reported in this study (25-50 µg/mL) versus (15.63-62.5 µg/mL), respectively.[39]

Furthermore, numerous studies also confirmed the promising antibacterial activity of several synthetic flavonoids.[10,40] A recent study reported that the newly synthesized quinolone-based aurone analogues were promising leads and had remarkable antibacterial effects compared to standard medications.[39] The antibacterial activity of these compounds was against gram-positive bacteria, namely (*S. aureus* and *B. subtilis*) and gram-negative bacteria, specifically, (*E. coli, P. aeruginosa, V. cholerae*) with MIC values ranging between 12.5-500 µg/mL). This activity was related to the minor alteration in the peripheral substitutions on rings A, B and C that may significantly impact the antimicrobial activity of the aurone analogues. Tiwari and co-researchers reported that methoxy-substituted ferrocenyl aurones exhibited significant antibacterial activity against both gram-positive bacteria (*Staphylococci*) and gram-negative bacteria (*E. coli*) with MIC values ranging from 2-32 mg/mL.[41]

The time-killing analyses have been performed to give a qualitative report about the time required for the antibacterial drug to inhibit the development or kill the bacteria at an exact concentration.[42] Besides the MIC and MBC results, AU-23 was preferred for time-killing analysis; 1×MIC, 2×MIC, 4×MIC, 8×MIC and growth control were administered to the four ATCC strains as per CLSI guidelines. An antibacterial drug is bactericidal if it inhibits the initial bacterial inoculum by $3\log_{10}$ CFU/mL or higher. In this study, AU-23 showed a decline in bacterial growth and bacteriostatic effect against MRSA 43300 and MRSA 33591 and a decrease in the viable cells of $\leq 3 \log_{10}$ was recorded after 8 hr of exposure to the AU-23 at 8 ×MIC in comparison to the initial inoculum. In contrast, AU-23 at 8×MIC, 4×MIC and 2×MIC levels showed bactericidal activity against MSSA 25923 after 4, 8 and 24 hr of AU-23 addition. Contrary to this, AU-23 at 1×MIC level showed temporary bacteriostatic activity after 10 and 12 hr of exposure. Then, the bacterial cell regrew again at a higher 3log. AU-23 at 8×MIC, 4×MIC and 2×MIC levels showed bactericidal activity against *P. aeruginosa* 9027 after 6, 8 and 10 hr of AU-23 addition. In contrast, AU-23 at 1×MIC level showed temporary bacteriostatic activity after 4 hr of compound exposure; after that, the bacterial cell regrew again<4log after 12 hr of compound addition and then the bacterial growth declined again after 12-24 hr. Hence, a higher rate of time-killing was reported in both sensitive ATCC strains (MSSA 25923 and *P. aeruginosa* 9027) while showing a bacteriostatic effect against two other MDR strains (MRSA43300 and MRSA33591).

Next, Biofilm-producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit antibiotic resistance through various methods like restricted penetration of antibiotics into biofilms, decreased growth rate and expression of resistance genes.[43] Gram-positive and Gram-negative bacteria both can form biofilms. Bacteria frequently involved comprise *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus mirabilis.*[44] In the antibiofilm activity part, AU-23 demonstrated good antibiofilm activity against MSSA 25923 and *P. aeruginosa* 9027 when cultured separately as monomicrobial biofilms at a concentration of 500 µg/mL, with inhibition rates of 64% and 59%, respectively. However, when the strains were co-cultured to form a polymicrobial biofilm, AU-23 exhibited a slight decrease in metabolic activity, as measured by the XTT assay, even at high concentrations (500 µg/mL). This finding is consistent with previous studies showing higher effective concentrations of antibacterial agents required against polymicrobial biofilms than planktonic growth.[16] This study showed that the novel synthesized AU-23 could inhibit the bacterial growth of four referenced ATCC strains. In addition, these active aurones showed bactericidal effects against all strains at their MIC values within 24 hr.[41] Moreover, the extracellular polymeric substances provide an additional layer of defence against the activity of antibiotics and the host's immune system. Therefore, synthetic compounds' relatively diminished effectiveness against biofilm formation should not be considered a significant drawback.

Inflammation is a natural response of the human body to harmful stimuli or pathogens. However, excessive or prolonged inflammation can lead to various diseases, including autoimmune disorders and chronic inflammation.[21] Therefore, it is crucial to effectively control the timing and progression of inflammation for proper healing. This study investigated the anti-inflammatory properties of aurone derivative AU-23 in RAW264.7 cells stimulated by LPS. The effects of this compound were evaluated in terms of cell viability, NO production, expression of pro-inflammatory genes associated with toll-like receptors TLR4 and CD14 and generation of ROS. The novel synthesized compound AU-23 demonstrated no toxicity to RAW264.7 cells at concentrations below 12.5 µM, with enhanced cell viability observed at doses ranging from 0.87 µM to 6.25 µM. Furthermore, subsequent assays revealed that AU-23 did not exert cytotoxic effects on LPS-induced RAW264.7 cells while effectively inhibiting the production of pro-inflammatory cytokines, mediators, NO and ROS.

Indeed, macrophages, acting as both responders and regulators of the inflammatory and immune responses, play a crucial role in the inflammatory process.[18] When stimulated, macrophages release pro-inflammatory cytokines and mediators such as TNF-α, IL-6, IL-1β, iNOS, TLR4 and CD14.[45,46] TNF-α, a critical inflammatory mediator primarily produced by macrophages, is implicated in inflammatory diseases leading to hypersensitivity reactions associated with chronic inflammation.[47] Dysregulation of TNF-α is a significant aspect of the pathophysiology of chronic

inflammatory diseases and pointing TNF-α with anti-TNF-α agents is an excellent strategy for managing chronic inflammatory disorders.[48] IL-6 is an important cytokine released by T cells and macrophages secrete IL-6 to induce immune responses during infection and after trauma, such as burns or other tissue damage that causes inflammation. It has pro- and anti-inflammatory properties. Its overproduction could lead to various illnesses, such as inflammatory bowel disease and rheumatoid arthritis.[49] Therefore, controlling and regulating the IL-6 expression is thought to be an essential therapeutic option to manage these diseases.

Moreover, IL-1β is another multi-functional pro-inflammatory cytokine secreted in response to microbial infection and its dysregulation is implicated in autoimmune disease, vascular illness and multiple sclerosis.[50] As a result, regulating IL-1β expression could be an excellent therapeutic point to control these diseases. Meanwhile, all these mentioned cytokines and mediators to be released from the macrophage cells require the cooperation of TLR4 with CD14 to recognize and bind to microbial elements such as LPS. Therefore, regulating these pattern recognition receptors' function could be critical for controlling and managing the immune system response.[51] In the present study, we found that the novel synthetic AU-23 not only has antimicrobial properties but also down-regulates the expression of TNF-α, IL-1β and IL-6 as well as TLR4 and CD14 in RAW 264.7 cells after being induced with LPS. These findings suggest that this compound could inhibit the release of pro-inflammatory factors stimulated by LPS, resulting in defensive and anti-inflammatory properties. Another study by Wang and coworkers (2017) explored the anti-inflammatory activity of a series of dihydropyranoaurone derivatives and found their potential to suppress NO production and iNOS expression in LPS-induced RAW264.7 cells.[52] These findings shed light on the structure-activity relationship of aurone derivatives.

NO is released by macrophage cells in response to bacterial LPS, serving as an essential inflammatory regulator and oxidative mediator that protects the host from bacterial pathogens.[53] However, NO overproduction is associated with chronic inflammatory diseases.[45] We evaluated the suppressive effect of the synthetic aurone on NO production in LPS-induced RAW264.7 cells and found that pre-treatment with aurone AU-23 significantly decreased NO levels. Additionally, aurone AU-23 inhibited the excessive expression of inducible Nitric Oxide Synthase (iNOS), a vital influencer of NO production, at lower concentrations than dexamethasone (positive control). These results highlight the potential of aurone in inhibiting iNOS and reducing the production of the inflammatory mediator NO.

In comparison to a study conducted by Park and coworkers (2017), where they designed and synthesized several aurone derivatives and assessed their impact on NO and iNOS in LPS-induced RAW264.7 cells, it was observed that aurone exhibited significantly decreased iNOS levels.[11] This indicates that aurone effectively inhibits the activity of inducible Nitric Oxide Synthase (iNOS), consequently reducing the production of the inflammatory mediator NO. These outcomes are attributed to substituting and altering functional groups within the aurone molecule.

Contrarily, ROS are commonly recognized as intracellular molecules that function as pro-inflammatory signaling molecules and possess the capacity to regulate the immune response.[54] However, at elevated intracellular concentrations, ROS can lead to cellular injury and apoptosis.[55] Therefore, in the current study, the impact of a newly developed synthetic compound, AU-23, on the generation of ROS in LPS-stimulated RAW264.7 cells was examined. Remarkably, the administration of AU-23 resulted in a substantial dose-dependent reduction in ROS production. These findings indicate that AU-23 can effectively diminish the excessive generation of ROS induced by LPS in RAW264.7 cells. Consequently, this compound may promise to mitigate cellular damage and suppress inflammation.

CONCLUSION

In conclusion, a novel synthetic aurone AU-23 exhibiting antibacterial and anti-inflammatory activity was designed and synthesized. From the antimicrobial activity results of the tested compound, AU-23 showed a potential bactericidal effect against sensitive ATCC bacterial strains (*P. aeruginosa* and MSSA 25923) and a bacteriostatic effect against MDRs ATCC strains (MRSA43300 and MRSA33591). In the case of the anti-inflammatory activity results, AU-23 inhibited LPS stimulated inflammatory response in RAW264.7 cells by reducing NO production, downregulated the expression of IL-6, IL-1β, iNOS, TNF-α, *via* (CD14 and TLR4) pathway and reduced LPS-induced high levels of intracellular ROS content. Overall, AU-23 is a promising therapeutic option and a novel synthetic agent for treating bacterial infection and inflammation.

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CONFLICT OF INTEREST

The author and all co-authors whose names are listed immediately below, Rabia Mrehil Elsalamia, Waqas Ahmad, Mahani Mahadiaa, Masriana Hassan, Noraziah Mohamad Zin, Anwar Salm Kalifa Kafo, Chin Chai Yee, Kamal Rullah, Najwa Mohamad, Mohammad Javed Qureshi, Yaman Walid Kassab, declare that they have no conflicts of interest in connection with

this document and the material described is not in the process of being published nor is it intended for publication elsewhere.

FUNDING

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ETHICAL APPROVAL

The approval from the institutional ethics committee of CUCMS Animal care and Use Committee (CACUC) was obtained prior to the commencement of the study (Clearance number CACUC/2023/1.

ABBREVIATIONS

ATCC: American Type Culture Collection; **CD14:** Cluster differentiation 14; **CFU:** Colony forming Unit; **COX-2:** Cyclooxygenase-2; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl sulfoxide; **IDSA:** Infectious Diseases Society of America; **IL-1β:** Interleukin-1β; **IL-6:** Interleukin-6; **iNOS:** Inducible nitric oxide synthase; **LPS:** Lipopolysaccharides; **MD-2:** Myeloid differentiation-2; **MRSA:** Methicillin-resistant *Staphylococcus aureus*; **MSSA:** Methicillin-susceptible *Staphylococcus aureus*; **NF-kB:** Nuclear Factor Kappa B; **NO:** Nitric oxide.

SUMMARY

The Novel synthetic AU-23 showed potent antibacterial activity against four reference ATCC strains, MRSA 43300, MRSA 33591, MSSA 25923 and *P.aeruginosa* 9027, associated with wound infection. Moreover, pretreatment of murine macrophage RAW264.7 cells with AU-23 for 24 hr reduced LPS-activated Nitric Oxide (NO) production. Next, AU-23 exhibited prominent inhibitory effects on the RAW264.7 cells' expression of iNOS, IL-1β, IL-6 and TNF-α via down-regulating their mRNA expression through specific pattern recognition receptors TLR4 and co-receptor CD14 and NF-κB signalling pathway and reduced LPS-induced high levels of intracellular ROS content.

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