SYNTHESIS AND ANTI-INFLAMMATORY ACTIVITY OF HYDRAZIDE DERIVATIVES OF 2-METHYLIDENE-1,4-DICARBOXYBUTANOIC ACID

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Hydrazide derivatives could exhibit various biological activities: antibacterial, tuberculostatic, antiviral, antidepressant, antihypertensive, antihistaminic, anti-inflammatory, analgesic and others. (1–3). CONN moiety is also found in powerful non-steroidal anti-inflammatory drugs like dipyrone (metamizole). In our previous research, we characterized hydrazide derivative of cis-1,2-cyclohexanediacrylic acid with strong anti-inflammatory and analgesic activity (2). Continuing the exploration of amidrazones chemistry [2–6] we have subjected compounds 1 and 2 to the reaction with itaconic anhydride aimed at the synthesis of novel hydrazide derivatives with suspected anti-inflammatory activity. Iaconic anhydride was chosen because of resemblance between itaconic acid and 2-methylpropyl group which is common among anti-inflammatory drugs (ibuprofen, naproxen, ketoprofen).

EXPERIMENTAL

Chemistry

Chemicals were purchased from Aldrich and used without further purification. Melting points were measured on a Mel TEMP 1002D apparatus and are uncorrected. The FT-IR spectra were recorded in KBr pellets on Perkin Elmer spectrometer in the range of 4000–400 cm⁻¹. The elemental analysis was carried out with a Vario Macro 11.45-0000 (Elementar Analysysteme GmbH, Germany). 1H NMR spectra were recorded on a Bruker Advance spectrometer in DMSO-⁶, with TMS as an internal standard. N⁰-substituted amidrazones 1, 2 were synthesized according to established procedure (7). All reactions were controlled by reversed-phase TLC chromatography.

2-Methylidene-4-oxo-4-{2-[(phenylamino)(pyridin-2-yl)methylene]hydrazinyl}butanoic acid (3)

A mixture of 2.12 g (0.01 mol) of N⁰-phenyl-(pyridin-2-yl)carbohydrazonamide 1 and 1.12 g (0.01 mol) of itaconic anhydride were dissolved in 100 mL of anhydrous diethyl ether prior to mixing them together (Scheme 1). The solution was vigorously stirred at ambient temperature for 4 h. The obtained precipitate of 3 was collected by filtration and washed with diethyl ether and methanol.

M.p. 135–136°C, yield 94%. 1H NMR (400 MHz, DMSO-δ6, δ, ppm): 3.20 (s, 1H, CHαiph), 3.67 (s, 1H, CHαiph), 5.70 (d, 1H, J = 13 Hz, =CH), 6.10 (d, 1H, J = 13 Hz, =CH), 6.5–8.52 (m, 9H, CH arom), 8.58 (s, 1H, NH), 9.88, 10.11 (s, 1H, NHCO), 12.46 (bs, 1H, COOH). FT-IR (KBr, cm⁻¹): 3449w; 3211m; 3061w; 2547m; 1700s; 1605s; 1522s; 1476s; 1434s; 1297s; 1210m; 1169m; 1000m; 986m; 777. Analysis: calc. for C₁₇H₁₆N₄O₃: C, 62.95; H, 4.97; N, 17.27%; found: C, 62.79; H, 4.68; N, 17.40%.

2-Methylidene-4-oxo-4-{2-[(phenylamino)(pyridin-4-yl)methylene]hydrazinyl}butanoic acid (4)
A mixture of 2.12 g (0.01 mol) of \( N \)-phenyl-(pyridin-4-yl)carbohydrazonamide 2 and itaconic anhydride (1.12 g, 0.01 mol) were dissolved in 100 mL anhydrous diethyl ether and left in ambient temperature for 2 days. The obtained precipitate of 4 was collected by filtration and washed with diethyl ether and methanol.

M.p. 136ñ137°C, yield 80%. \(^1\)H NMR (400 MHz, DMSO-\(d_6\), \( \delta \), ppm): 3.20 (s, 1H, CHaliph), 3.67 (s, CHaliph), 5.72 (d, 1H, \( J = 13 \) Hz, =CH), 6.15 (d, 1H, \( J = 13 \) Hz, =CH), 6.54ñ8.49 (m, 9H, CH arom), 8.59 (s, 1H, NH), 9.89, 10.12 (s, 1H, NHCO), 12.49 (bs, 1H, COOH). FT-IR (KBr, cm\(^{-1}\)) 3331m; 3248m; 3067m; 2920m; 2490m; 1655s; 1612s; 1553s; 1479s; 1381s; 1207m; 1148s; 1009m; 800m; 752s; 694m. Analysis: calc. for C\(_{17}\)H\(_{16}\)N\(_4\)O\(_3\): C, 62.95; H, 4.97; N, 17.27%; found: C, 62.85; H, 4.45; N 17.42%.

**Blood samples and cell culture**

The whole blood was obtained from healthy volunteers (blood donors, median age 30 years, range 20ñ35). The study was undertaken according to the Helsinki Declaration with approval from the ethical committee of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz, Poland. Venous blood samples (9 mL) have been drawn into tubes containing heparin (Medlab). Whole blood was diluted 1:5 with RPMI 1640 (PAA) in sterile, non-adherent, polypropylene tubes (Falcon, Becton Dickinson). Thereafter, specimens (1 mL of diluted whole blood per specimen) were incubated for 24 h, at 37°C and 5% CO\(_2\), with examined compound and/or LPS also without stimulation. The compounds 3, 4 were initially dissolved in DMSO (Sigma). Final concentrations of compounds in the culture were 0.1 µg/mL, 1 µg/mL, 10 µg/mL and the
concentration for LPS was 1 µg/mL. Control cultures (with LPS or without stimulation) contained DMSO in the same concentrations as in respective concentrations of the compounds.

Cytokine assay

The assay was performed as previously described (9, 10). Cytokine IL-6 and TNF-α concentrations in cell culture supernatants were estimated following 24 h of culture of whole blood (1 mL), using ELISA (Becton Dickinson) according to the manufacturer’s procedure.

Determination of cell toxicity

Apoptosis was analyzed by using the annexin V-FITC apoptosis detection kit (Becton Dickinson), after 24 h stimulation of peripheral blood mononuclear cells (PBMCs) by the studied compounds. Briefly, PBMCs were isolated from heparinized whole blood by density gradient centrifugation (LSM...
Obtained PBMCs were subjected to 24-h culture with studied compounds. Thereafter, cells were centrifuged (400 ◊ g) for 5 min at 4°C. The supernatant was removed and 195 µL of binding buffer and 5 µL of annexin V-FITC were added. The cells were incubated for 15 min in dark, at RT, and then centrifuged. Following supernatant removal, 190 µL of binding buffer and 10 µL of PI (propidium iodide) were added to the cell pellet. Then, the cells were incubated for 5 min in dark, at RT. Thereafter, the cells were analyzed in FACScan flow cytometer (Becton Dickinson). Flow cytometry acquisition and analysis were performed on at least 10000 acquired events. The obtained cytometric data were analyzed using FlowJo version 7.6.1 software (Tree Star).

RESULTS AND DISCUSSION

In this study, two new hydrazide derivatives have been synthesized with good yields. The structures of the newly synthesized compounds were confirmed by 1H NMR and IR spectrometry and elemental analyses. In the IR spectra of 3 and 4, characteristic absorption bands at around 3331-3494, 3211 – 3248, 3061 – 3067, 1692 – 1700, 1476 – 1479 cm⁻¹ regions were observed, confirming the presence of OH, NH, CH₃amn, C=O, C=N groups, respectively. In 1H NMR spectra for compounds 3 and 4, single signals of protons of carboxylic group at δ = 12.4 ppm were found. Single signals at δ = 8.5 were assigned for amide protons of the amidrazone groups and signals in the range δ = 9.88 – 10.11 ppm for hydrazide (=N-NH-CO) protons. In the range δ = 6.5–8.5, nine protons of aromatic substituents were observed. The signals in the range 3.2 – 3.67 ppm were attributed to aliphatic protons and two doublets in the range δ = 6.1 – 5.72 ppm to C=CH₂ group. The elemental analysis and spectral data were found in agreement with the assigned molecular structures of 3 and 4. The position of =CH₂ group was discussed in our recent work (8).

The toxicity of compounds 3 and 4 was determined by flow cytometry in the 24 h cultures of human peripheral blood mononuclear cells (Fig. 1 and 2). PBMC stimulated in the highest dose resulting in about 84% of viable cells that is similar to control cells.

Compounds 3 and 4 at concentration of 0.1, 1.0 and 10 µg/mL were tested for their ability to affect LPS-inducible tumor necrosis factor α (TNF-α) and IL-6 production in human whole blood cell cultures. IL-6 and TNF-α are pro-inflammatory cytokines – mediators of inflammatory development [11].

The results (Fig. 3) shown that the inhibition of TNF-α production in LPS-stimulated whole blood cultures for both tested compounds was strongest in concentration 10 mg/mL; derivative 3 lowered it by 53% and 4 by 23%. Both compounds in concentration of 1 mg/mL and 4 in concentration 0.1 mg/mL inhibited TNF-α production slightly (by about 10%). The TNF-α production was not stimulated by derivatives 3 and 4 in examined concentration range.
The results shown in Figure 4 indicated that compound 3 did not influence on IL-6 production in LPS-stimulated whole blood cultures, however, this cytokine production is elevated by compound 4 by about 10–20%. The level of IL-6 production is also increased in cells treated only by 4. On the other hand, the IL-6 production is reduced in cultures stimulated by 3 by about 10–35%.

Compound 3 exhibited anti-inflammatory effect in concentration of 10 mg/mL by reducing TNF-α production in LPS-stimulated cells by 53%. Compound 4 decreased TNF-α production in LPS-stimulated cells in the highest dose by 23%, but it also elevated the synthesis of IL-6, which is disadvantageous (11). Derivative 3 at this concentration was not toxic for peripheral blood mononuclear cells, which is promising for future studies.

REFERENCES