Chemico-Biological Interactions 268 (2017) 13–23

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Antiproliferative activity and apoptosis induction by 3′,4′-dibenzyloxyflavonol on human leukemia cells

Mercedes Said [a], Ignacio Brouard [b], José Quintana [a], Francisco Estévez [a, *]

[a] Departamento de Bioquímica y Biología Molecular, Unidad Asociada al Consejo Superior de Investigaciones Científicas (CSIC), Instituto Universitario de Investigaciones Biomédicas y Sanitarias, Universidad de las Palmas de Gran Canaria, Spain
[b] Instituto de Productos Naturales y Agrobiología, CSIC, La Laguna, Tenerife, Spain

ARTICLE INFO

Article history:
Received 18 November 2016
Received in revised form
9 February 2017
Accepted 20 February 2017
Available online 22 February 2017

Keywords:
Apoptosis
Caspases
Cell cycle
Cytotoxicity
Flavonoids

ABSTRACT

In this study, we investigated the effects of synthetic 3′,4′-dibenzyloxyflavonol on viabilities of eight human tumor cells. It was cytotoxic against leukemia cells (HL-60, U-937, MOLT-3, K-562, NALM-6, Raji), with significant effects against P-glycoprotein-overexpressing K-562/ADR and Bcl-2-overexpressing U-937/Bcl-2 cells, but had no significant cytotoxic effects against quiescent or proliferating human peripheral blood mononuclear cells. The IC_{50} value for the leukemia HL-60 cells was 0.8 ± 0.1 μM. This indicates a 60–fold greater toxicity than the naturally occurring flavonol quercetin. Synthetic 3′,4′-dibenzyloxyflavonol induced S phase cell cycle arrest and was a potent apoptotic inducer in human leukemia cells. Cell death was (i) mediated by the activation and the cleavage of initiator and executioner caspases; (ii) prevented by the pan-caspase inhibitor z-VAD-fmk; (iii) associated with the release of cytochrome *c* and with the phosphorylation of members of the mitogen activated protein kinases including $p38^{MAPK}$, JNK/SAPK and ERK, and (iv) independent of the generation of reactive oxygen species. The synthetic 3′,4′-dibenzyloxyflavonol is a potent cytotoxic compound against several human leukemia cells and might be useful in the development of new strategies in the fight against cancer.

1. Introduction

Cancer has one of the highest worldwide mortality indices of any disease [1,2]. Interest in understanding the pathogenic process and the associated cellular mechanism has increased recent years [3]. Analyses of the worldwide incidence and mortality from 27 major cancers in 2012 indicated that leukemia accounted for some 352,000 new cases (2.5% of all new cancer cases) and for 265,000 deaths (3.2% of all deaths) [4]. Leukemia treatment includes chemotherapy, radiotherapy, immunotherapy and bone marrow transplantation. Despite the efforts and efficacy of these methods, leukemia mortality rates are still very high. Resistance to chemotherapy is one of the major limitations in the treatment. Natural products represent realistic options as potential cytotoxic compounds against cancer cells [5]. Flavonoids are polyphenols biosynthesized by the plants secondary metabolism and display a vast array of biological activities, including anticancer properties [6–9]. These compounds are present in the human diet because they are found in vegetables, flowers, fruits, herbs, nuts, seeds, spices, stems, as well as tea and red wine [10]. Epidemiological studies have shown that diets rich in flavonoids improve the quality of life of a patient with chronic diseases and cancer [11]. The well characterized flavonoid quercetin shows antitumor activity and this effect is mediated through apoptosis induction by modulating certain key intracellular signaling molecules and pathways [12,13]. Apoptosis can be executed by specific proteases, the caspases, which are activated by two main pathways [14,15]. The extrinsic pathway is initiated with the activation of the tumor necrosis factor receptor superfamily (TNF) or Fas receptor, involved in the recruitment and activation of the initiator caspase-8 or -10, which activate the effector caspases (−3, −6 and −7) [16]. The intrinsic pathway

Abbreviations: ERK, extracellular signal-regulated kinase; FL, 3′,4′-dibenzyloxyflavonol; H2-DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; IC50, 50% inhibition of cell growth; JNK/SAPK, c-jun N-terminal kinases / stress-activated protein kinases; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated extracellular kinases; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NAC, N-acetyl-l-cysteine; p38MAPK, p38 mitogen-activated protein kinases; ROS, reactive oxygen species.

* Corresponding author. Departamento de Bioquímica y Biología Molecular, Universidad de Las Palmas de Gran Canaria, Paseo Blas Cabrera Felipe s/n, Las Palmas de Gran Canaria 35016, Spain.
E-mail address: francisco.estevez@ulpgc.es (F. Estévez).

http://dx.doi.org/10.1016/j.cbi.2017.02.010
0009-2797/

involves permeabilization of the mitochondrial outer membrane by the activation of the pro-apoptotic proteins Bax and Bak of the Bcl-2 family proteins [17]. This induces cytochrome *c* release in the cytosol which associates with Apaf-1 (apoptotic protease-activating factor 1) in a multimeric complex called the apoptosome. This activates the initiator caspase (caspase-9), which in turn activates the effector caspases (−3, −6 and −7) [18].

The results from several studies suggest that flavonoids with a hydroxy group at carbon 3 are potential anticancer agents [19] and that the presence of 3′-hydroxy-4′-methoxy groups on the B ring enhances cytotoxicity [20]. The justification for investigating quercetin derivatives is provided by the fact that this compound has been described to display antitumor activity [21]. Since the flavonoids are phenolic compounds and therefore prone to oxidation to quinones, we carried out the protection of the 3′,4′-dihydroxy group to block the potential oxidation and to generate a more chemically stable compound. It was hypothesized that the introduction of two benzyloxy groups at positions 3′,4′ in the B ring might improve the cytotoxicity against cancer cell lines due to an increase in lipid solubility that would facilitate cell penetration. Here, we synthesized the flavonoid 3′,4′-dibenzyloxyflavonol and studied i) its potential cytotoxic effects against a panel of human leukemia cells and ii) the signal transduction pathways involved in the mechanism of action.

2. Materials and methods

2.1. Reagents

Compound used as starting material and reagents were obtained from Aldrich Chemical Co. or other chemical companies and utilized without further purification. Nuclear Magnetic Resonance (NMR): ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 spectrometer with standard pulse sequences operating at 400 in ^{1}H and 100 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent. Chemical shifts (δ) are given in ppm relative to the residual solvent signals, and coupling constants (J) are reported in hertz. HRESIMS was performed with a LCT Premier XE Micromass Waters spectrometer in the positive-ionization mode (Waters Corporation). IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Analytical thin layer chromatography (TLC) was performed using silica gel 60 (230–400 mesh) aluminum sheets. All commercially available chemicals were used without further purification.

The inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (z-VAD-fmk), benzyloxycarbonyl-Asp(OMe)-Glu (O–Me)-Val-Asp(O–Me) fluoromethyl ketone (z-DEVD-fmk), benzyloxycarbonyl-Ile-Glu-Thr-Asp(OMe) fluoromethyl ketone (z-IETD-fmk), benzyloxycarbonyl-Leu-Glu-His-Asp(OMe) fluoromethyl ketone (z-LEHD-fmk), PD98059, U0126, SP600125 and SB203580 were purchased from Sigma (Saint Louis, MO, USA). The caspase inhibitor VEID-CHO was from Calbiochem (Darmstadt, Germany). Acrylamide, bisacrylamide, ammonium persulfate and *N*,*N*, *N*′,*N*′-tetramethylethylenediamine were from Bio-Rad (Hercules, CA, USA). Antibodies for caspase-3, caspase-7, caspase-8 and caspase-9, were purchased from Stressgen-ENZO (Victoria, British Columbia, Canada). Anti-caspase-6 and anti-caspase-4 monoclonal antibodies were from Medical & Biological Laboratories (Nagoya, Japan). Anti-Phospho JNK/SAPK (T183/Y185), anti-p44/42 MAP Kinase, anti-Phospho-p44/42 MAP Kinase (T202/Y204), anti-$p38^{MAPK}$ and a phosphorylated form (T180/Y182) of $p38^{MAPK}$ antibodies were purchased from New England BioLabs (Cell Signaling Technology, Beverly, MA, USA). Monoclonal antibodies for anti-α-tubulin and anti-β-Actin (clone AC-74) were purchased from Sigma (Saint Louis, MO, USA). Polyclonal anti-human Bax and Bid antibodies and monoclonal anti-cytochrome *c* antibody were from BD Pharmingen (San Diego, CA, USA). Secondary antibodies were from GE Healthcare Bio-Sciences AB (Little Chalfont, UK). PVDF membranes were from Millipore (Temecula, CA). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

2.1.1. General procedure for the synthesis of 3′,4′-dibenzyloxyflavonol

3′,4′-Dibenzyloxyflavonol was obtained using a combination of a Claisen-Schmidt condensation of a 2-hydroxyacetophenone and a benzaldehyde followed by a cyclization, based on established protocols [22,23]. The synthesis of 3′,4′-dibenzyloxyflavonol [2-(3,4-Dibenzyloxyphenyl)-3-hydroxy-4H-chromen-4-one] was performed as following.

2.1.2. 3,4-Dibenzyloxy-2'-hydroxy-chalcone (**3**)

A mixture of the acetophenone **1** (5–10 mmol, 1 equiv) and the corresponding aldehyde **2** (1 equiv) in EtOH (20–40 ml) was stirred at room temperature. Then, a 50% aqueous solution of NaOH (2–4 ml) was added. The reaction mixture was stirred at room temperature until aldehyde consumption. After that, HCl (10%) was added until neutrality. The solid was filtered and crystallized from MeOH. The chalcone was isolated as an orange solid, mp 104–105 °C (70%). IR (KBr, cm^{-1}) ν_{max}: 3062, 3032, 2867, 1685, 1637, 1578, 1560, 1508, 1488, 1453, 1440, 1379, 1339, 1313, 1258, 1201, 1175, 1156, 1136, 1025, 980, 844.^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 12.92$ (s, 1H); 7.89 (dd, 1H, $J = 8.1$, 1.5 Hz); 7.84 (d, 1H, $J = 15.3$ Hz); 7.53–7.47 (m, 6H); 7.45–7.33 (m, 7H); 7.27 (d, 1H, $J = 6.3$ Hz); 7.27–7.23 (m, 1H); 7.04 (d, 1H, $J = 8.4$ Hz); 6.98 (d, 1H, $J = 8.2$ Hz); 6.96 (ddd, 1H, $J = 8.1$, 7.0, 1.0 Hz); 5.25 (s, 4H)· ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 193.9$, 163.9, 152.3, 149.5, 145.8, 137.3, 137.0, 136.6, 129.9, 129.0, 129.0, 128.9, 128.4, 127.8, 127.6, 124.3, 120.5, 119.1, 119.0, 118.5, 115.0, 114.6, 72.0, 71.3. HRMS (ESI-FT-ICR) *m/z*: 459.1575 $[M+Na]^+$; calcd. for $C_{29}H_{24}O_4Na$: 459.1572.

2.1.3. 2-(3,4-Dibenzyloxyphenyl)-3-hydroxy-4H-chromen-4-one (**4**)

A solution of 2-hydroxychalcone **3** (2–4 mmol) in 3.0 M KOH in MeOH (20–30 ml) was cooled at 0 °C. An aqueous solution of H_2O_2 (30%) (5–8 ml) was added to the chalcone solution. The resulting mixture was stirred at room temperature, until the starting material was totally consumed (as evidenced by TLC). The reaction mixture was cooled in an ice bath and distilled water (20–40 ml) was added. HCl (2 M) was added until pH 2 and the precipitate was filtered and washed with distilled water and extracted with EtOAc. The organic layer was washed with brine until neutrality and dried with $MgSO_4$ anhydrous. The solvent was evaporated in vacuo and the flavonol was crystallized from MeOH. The flavonol was isolated as a light yellow solid, mp 144–145 °C (62%). IR (KBr, cm^{-1}) ν_{max}: 3314, 3032, 2922, 2855, 1611, 1560, 1510, 1482, 1470, 1435, 1410, 1382, 1335, 1270, 1204, 1143, 1123, 1110, 1018, 857, 808.^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 8.25$ (dd, 1H, J = 8.0, 1.3 Hz); 7.96 (d, 1H, $J = 2.0$ Hz); 7.86 (dd, 1H, $J = 8.6$, 2.0 Hz); 7.68 (ddd, 1H, $J = 8.4$, 7.2, 1.4 Hz); 7.56–7.50 (m, 5H); 7.48–7.33 (m, 7H); 7.08 (s, 1H); 7.07 (d, 1H, $J = 8.6$ Hz); 5.29 (s, 2H); 5.27 (s, 2H)· ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 173.6$, 155.6, 151.1, 148.9, 145.3, 138.2, 137.4, 137.1, 133.7, 129.0, 128.9, 128.3, 128.3, 127.9, 127.6, 125.7, 124.8, 124.5122.4, 121.0, 118.5, 114.9, 114.5, 71.9, 71.3. HRMS (ESI-FT-ICR) *m/z*: 473.1365 $[M+Na]^+$; calcd. for $C_{29}H_{22}O_3Na$: 473.1365.

2.2. Cell culture and cytotoxicity assays

HL-60, U-937, MOLT-3, K-562 and NALM-6 cells were from DSMZ (German Collection of Microoganisms and Cell Cultures,