European Journal of Medicinal Chemistry 86 (2014) 153–164

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis of chlorinated flavonoids with anti-inflammatory and pro-apoptotic activities in human neutrophils

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ARTICLE INFO

Article history:
Received 5 May 2014
Received in revised form
8 August 2014
Accepted 9 August 2014
Available online 14 August 2014

Keywords:
Neutrophils
Reactive species
Apoptosis
Caspase 3
Flavonoids

ABSTRACT

Neutrophils are considered the central cells of acute inflammation. Flavonoids have been suggested as therapeutic agents to avoid damages induced by inflammatory processes. It is well known the reactivity of flavonoids with hypochlorous acid produced by neutrophils, to form stable mono and dichlorinated products. In this study, we synthesized novel chlorinated flavonoids and investigated their effect in neutrophils' oxidative burst and in its lifespan, in comparison with the parent non-chlorinated flavonoids. The obtained results demonstrate that chlorinated flavonoids were more efficient than their parent compounds in modulating neutrophils' oxidative burst in phorbol myristate acetate-activated neutrophils. Some of the tested flavonoids drive neutrophil apoptosis in a caspase 3-dependent fashion. The present data showed that 8-chloro-3′,4′,5,7-tetrahydroxyflavone (**4a**) constitute an alternative anti-inflammatory therapy, due to the proven ability to suppress mechanisms engaged at the onset and progression of inflammation.

1. Introduction

Inflammation is an orchestrated biological process, induced by microbial infection or tissue injury, characterized by redness, oedema, fever, pain, and loss of function [1]. Inflammation is increasingly found to be involved in the development of several chronic diseases such as arteriosclerosis, obesity, diabetes, neurodegenerative diseases and even cancer. Among them, cardiovascular diseases and cancer are main causes of mortality in many countries [1]. A major trigger of inflammation is the recognition of microbes by specific receptors of the innate immune system, which play a crucial role in the induction of early signals, initiating and establishing the inflammatory setting [2,3]. Neutrophils are considered to be the central cells of acute inflammation [4]. Accordingly, in the event of an inflammatory process, it is observed an increase in the number, lifespan, mobility, tissue influx ability, and phagocytic capacity of neutrophils [5,6].

One of the most important mechanisms used by neutrophils to protect the organism against the invader is the production of an array of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5]. However, the sustained overproduction of reactive species or the impairment of antioxidant defenses, may result in a pro-oxidant status of the cells known as oxidative stress, leading to detrimental effects to the host, namely alterations on the normal function of lipids, proteins or DNA [4,5,7,8]. In addition, an abnormal, ineffective or absent resolution of inflammation leads to tissue irreversible damages. Despite the lifespan of neutrophils of only a few hours, under physiological conditions, in inflammatory environment their survival is promoted by delaying apoptosis, a form of cell death. Therefore, novel strategies of anti-inflammatory therapy that manipulate neutrophils activity namely the production of reactive species or their lifespan will be useful in both acute and chronic inflammation [9]. In this respect, the antioxidant and anti-inflammatory properties of flavonoids present in a wide variety of plants, may represent valid alternatives in the treatment of chronic inflammatory processes. Flavonoids occur mainly in fruit, vegetables, nuts, seeds, flowers, and bark and are generally present in plants as glycosides [10,11]. They are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, isoflavones, flavonols, flavanonols, flavanols and flavanones. It was already reported the ability of flavonoids to modulate

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http://dx.doi.org/10.1016/j.ejmech.2014.08.035
0223-5234/

neutrophils' oxidative burst [12]. However, despite the known reactivity of flavonoids with HOCl to form stable mono and dichlorinated products [13], there are no reports in literature about the effect of chlorinated metabolites of flavonoids on human neutrophils oxidative burst. Taking into account the lack of information about flavonoids ability to alter neutrophils' apoptosis, it is also of paramount importance to investigate their effect on neutrophils lifespan. As such, it is our purpose to understand if flavonoids metabolites may have biological and chemical properties distinct from their parent compounds. Thus, in this study, we synthesized novel chlorinated flavonoids and investigated their effect in neutrophils' oxidative burst and in its lifespan, in comparison with the parent non-chlorinated flavonoids.

Table 1 shows the chemical structure of the studied flavonoids.

2. Materials and methods

2.1. Materials

The following reagents were purchased from Sigma–Aldrich Co. LLC (St. Louis, USA): phorbol-12-myristate-13-acetate (PMA), *N*-acetyl-3,7-dihydroxyphenoxazine (amplex red), peroxidase from horseradish (HRP), histopaque 1077, histopaque 1119, Dulbecco's Phosphate Buffer saline, without calcium chloride and magnesium chloride (PBS), trypan blue solution 0.4%, dimethylsulfoxide (DMSO), trizma, superoxide dismutase (SOD), catalase, D-(+)-glucose, RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin, luteolin (**6**), and quercetin (**7**). 4-Aminobenzoyl hydrazide (ABAH) was purchased from Calbiochem (San Diego, CA, USA). 2-[6-(4′-Amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) was purchased from Invitrogen, Life Technologies Ltd (Paisley, UK). Luminol was purchased from Fluka Chemie GmbH (Steinheim, Germany). Hemacolor® was obtained from Merck (Darmstadt, Germany). Annexin-V-FLUOS Staining Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Z-DEVD-FMK was obtained from BD Pharmingen™. All chemicals and solvents used in the synthesis procedures were obtained from commercial sources and used as received or dried by standard procedures.

2.2. General measurements

Melting points were measured in a Reichert Thermovar apparatus fitted with a microscope and are uncorrected. NMR spectra were recorded on a Bruker Avance 300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C), in $CDCl_3$ as solvent if not stated otherwise. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz; the internal standard was TMS. Unequivocal ^{13}C assignments were made with the aid of 2D gHSQC and gHMBC (delays for one-bond and long-range $J_{C/H}$ couplings were optimised for 145 and 7 Hz, respectively) experiments. Positive-ion ESI mass spectra were acquired with a Q-TOF 2 instrument [dilution of 1 μL of the sample in chloroform solution (ca. 10^{-5} M) in 200 μL of 0.1% trifluoroacetic acid/methanol solution]. Nitrogen was used as nebuliser gas and argon as collision gas. The needle voltage was set at 3000 V, with the ion source at 80 °C and the desolvation temperature at 150 °C. The cone voltage was 30 V. High-resolution mass spectra analyses were performed on a Bruker MicrOTOF spectrometer (University of Vigo). Elemental analyses were obtained with a LECO 630-200-200 CHNS analyser (University of Aveiro). Preparative thin-layer chromatography was performed with Merck silica gel (60 DGF_{254}). Column chromatography was performed with Merck silica gel (60, 70–230 mesh). All chemicals and solvents used were obtained from commercial sources and used as received or dried by standard procedures.

2.3. Synthesis of 2′-hydroxyacetophenones ***1a–d***

2.3.1. Synthesis of 2′-hydroxy-4′,6′-dimethoxyacetophenone (***1a***)

2′-Hydroxy-4′,6′-dimethoxyacetophenone (**1a**) was prepared according to a procedure previously described in the literature [14].

2.3.2. Synthesis of chloro-2′-hydroxyacetophenones ***1b–d***

To a stirred solution of 2′-hydroxy-4′,6′-dimethoxyacetophenone (**1a**) (150 mg, 0.76 mmol) in THF (30 mL) it was added *N*-chlorosuccinimide (NCS) (0.11 g, 0.84 mmol, for **1b**,**d** or 0.21 g, 1.61 mmol, for **1c**). The solution was refluxed, under nitrogen atmosphere, for 24 h. The reaction mixture was filtered, evaporated to dryness and purified by preparative thin-layer chromatography using a (1:1) mixture of light petroleum:dichloromethane (for **1b**,**d**) or dichloromethane (for **1c**) as eluent. The obtained oily residues were precipitated in ethanol:water giving chloro-2′-hydroxy-4′,6′-dimethoxyacetophenones **1b–d** in moderate yields: **1b** (62 mg, 35%) and **1d** (70 mg, 39%) as light yellow solids and **1c** (100 mg, 49%) as a white solid.

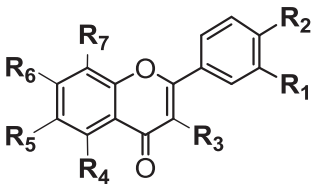
2.3.3. 3′-Chloro-2′-hydroxy-4′,6′-dimethoxyacetophenone (***1b***)

Mp 191–192 °C. ^{1}H NMR: δ 2.62 (s, 3H, 2-C*H*$_3$), 3.93 (s, 3H, 4′-OC*H*$_3$), 3.96 (s, 3H, 6′-OC*H*$_3$), 6.00 (s, 1H, H-5′), 14.45 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR: δ 33.1 (2-*C*H$_3$), 55.7 (4′-OCH$_3$), 56.2 (6′-OCH$_3$), 86.5 (C-5′), 101.8 (C-3′), 106.2 (C-1′), 161.0 (C-6′), 161.6 (C-4′), 161.7 (C-2′), 203.4 (C=O) ppm. MS (ESI$^+$) m/z (rel. int.): 231 ($[C_{10}H_{11}^{35}ClO_4+H]^+$, 67), 233 ($[C_{10}H_{11}^{37}ClO_4+H]^+$, 24), 253 ($[C_{10}H_{11}^{35}ClO_4+Na]^+$, 92), 255 ($[C_{10}H_{11}^{37}ClO_4+Na]^+$, 27). Anal. Calcd for $C_{10}H_{11}ClO_4$ (230.64): C 52.07, H 4.81; found: C 52.13, H 4.79%.

2.3.4. 3′,5-Dichloro-2′-hydroxy-4′,6′-dimethoxyacetophenone (***1c***)

Mp 103–104 °C. ^{1}H NMR: δ 2.76 (s, 3H, 2-C*H*$_3$), 3.95 (s, 3H, 6′-OC*H*$_3$), 3.98 (s, 3H, 4′-OC*H*$_3$), 13.69 (s, 1H, 2′-O*H*) ppm. ^{13}C NMR:

Table 1
Chemical structure of the studied flavonoids.

Compound		R_1	R_2	R_3	R_4	R_5	R_6	R_7
4a		OH	OH	H	OH	H	OH	**Cl**
4b		OH	OH	H	OH	**Cl**	OH	**Cl**
4c		OH	OH	**Cl**	OH	H	OH	H
4d		OH	OH	**Cl**	OH	H	OH	**Cl**
5 Flavone		H	H	H	H	H	H	H
6 Luteolin		OH	OH	H	OH	H	OH	H
7 Quercetin		OH	OH	OH	OH	H	OH	H