Food Chemistry 175 (2015) 494–499

Contents lists available at ScienceDirect

Food Chemistry

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

Synthetic analogues of flavonoids with improved activity against platelet activation and aggregation as novel prototypes of food supplements

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ARTICLE INFO

Article history:
Received 7 August 2014
Received in revised form 8 November 2014
Accepted 3 December 2014
Available online 16 December 2014

Chemical compounds studied in this article:
Quercetin, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (PubChem CID: 5280343)
Apigenin, 5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one (PubChem CID: 5280443)
2-Phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives, **1–4** (Chart 1)
2,3-Diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives, **5–7** (Chart 1)

Keywords:
Quercetin
Apigenin
Platelet aggregation
P-selectin expression
2-Phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones
2,3-Diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones

ABSTRACT

We investigated the ability of quercetin and apigenin to modulate platelet activation and aggregation, and compared the observed efficacy with that displayed by their synthetic analogues 2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, **1–4**, and 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, **5–7**. Platelet aggregation was explored through a spectrophotometric assay on platelet-rich plasma (PRP) treated with the thromboxane A_2 mimetic U46619, collagen and thrombin in presence/absence of various bioisosteres of flavonoids (12.5–25–50–100 μM). The platelet density, (mean platelet component, MPC), was measured by the Advia 120 Hematology System as a marker surrogate of platelet activation. The induced P-selectin expression, which reflects platelet degranulation/activation, was quantified by flow cytometry on PRP.

Our synthetic compounds modulated significantly both platelet activation and aggregation, thus turning out to be more effective than the analogues quercetin and apigenin when tested at a concentration fully consistent with their use *in vivo*. Accordingly, they might be used as food supplements to increase the efficacy of natural flavonoids.

1. Introduction

At the site of vascular injury, the first step in the formation of hemostatic plugs is the accumulation of platelets, which play a key role in preventing blood loss after injury (Davì & Patrono, 2007). Nevertheless, platelets cause the formation of pathogenic thrombi in patients with atherothrombotic disease, who experience acute coronary syndromes, ischemic stroke/transient ischemic attack and peripheral artery disease (Freynhofer, Bruno, Wojta, & Huber, 2012). The formation of thrombi begins with the rupture of an atherosclerotic plaque and contact between the thrombogenic matrix and platelets. This in turn leads to platelet adhesion, activation, aggregation and the subsequent formation of a thrombus and its micro-embolization (Badimon, Storey, & Vilahur, 2011; Davì & Patrono, 2007). Multiple pathways, including those stimulated by thrombin, thromboxane A_2 (TxA_2) and collagen, can activate platelets. Each of these agonists stimulates a distinct platelet activation pathway leading to platelet-mediated

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http://dx.doi.org/10.1016/j.foodchem.2014.12.005
0308-8146/

thromboembolic events. The prominent role of platelets in these events is highlighted by the proven clinical benefits of acute and chronic antiplatelet therapy on atherothrombotic diseases. Despite the documented advantages and widespread use of antiplatelet agents, the long-term morbidity and mortality rates associated with the atherothrombotic disease remain significant, and risk of bleeding is considerable (Freynhofer et al., 2012; Palacio, Hart, Pearce, & Benavente, 2012). Epidemiologic and intervention studies, focusing on diet regimens enriched with foods of plant origin, clearly demonstrate that dietary components may influence hemostasis, altering predisposition to atherothrombotic diseases.

Actually, a number of dietary flavonoids identified in foods of plant origin have shown *ex vivo* inhibition of platelet function (Janssen et al., 1998; Pearson et al., 2002; Ryu et al., 2013; Wright, Spencer, Lovegrove, & Gibbins, 2013). Many studies have related flavonoid structure, such as number and substitution of hydroxyl groups, degree of unsaturation or glycosylation status, to diverse mechanisms of inhibition of platelet signaling (Guerrero et al., 2007; Guglielmone, Agnese, Núñez Montoya, & Cabrera, 2005; Hubbard et al., 2003). The flavonoid quercetin inhibits collagen-stimulated whole platelet tyrosine phosphorylation and intracellular mobilization of calcium and various events in signaling generated by the collagen receptor GPVI (Hubbard et al., 2003). Certain flavonoids may behave as TxA_2 antagonists (Guerrero et al., 2007; Guglielmone et al., 2005) but also may interfere in cell-signaling events downstream of TxA_2 receptor (Guerrero et al., 2007) or prevent an increase in actin polymerization and platelet shape change (Pastore, Funaki, Janmey, & Bucki, 2005).

Despite being endowed with intriguing activities on both platelet activation and aggregation, all the investigated flavonoids possess a significant drawback, as their effects are found at high, non-physiological concentrations, seldom reached in the circulation. Moreover, their low solubility and stability, coupled with unfavorable pharmacokinetic properties and the ability to modulate additional and unrelated molecular targets, limit their exploitability either as food supplements or even as drug candidates (Galati & O'Brien, 2004; Gao & Hu, 2010). However, they represent an excellent and logical source of inspiration for medicinal chemists, who may design synthetic analogues to achieve safer and more effective compounds.

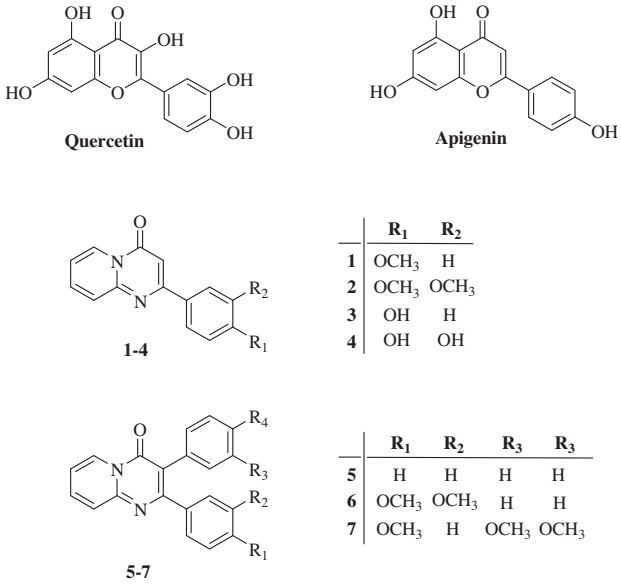
Prompted by these consideration and deepening our recent studies on bioisosteres of flavonoids (Del Turco et al., 2014; La Motta et al., 2007), we assumed that compounds bearing the pyrido[1,2-*a*]pyrimidin-4-one heterocyclic core would exhibit the same inhibitory efficacy against platelet aggregation and activation as that of natural compounds like quercetin and apigenin.

Accordingly, we embarked in the functional evaluation of suitably substituted 2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, **1–4** (Chart 1) and 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, **5–7** (Chart 1), whose ability to modulate platelet activation and aggregation has been verified through *ex vivo* assays, performed on platelet-rich plasma samples.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) except where specified. 2-Phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives **1–4** and 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones **5–7** (Chart 1) were synthesized by Stefania Sartini and Concettina La Motta at the Department of Pharmacy of the University of Pisa, Italy, following previously reported procedures (Del Turco et al., 2014; La Motta et al., 2007, 2013).



	R_1	R_2
1	OCH_3	H
2	OCH_3	OCH_3
3	OH	H
4	OH	OH

	R_1	R_2	R_3	R_3
5	H	H	H	H
6	OCH_3	OCH_3	H	H
7	OCH_3	H	OCH_3	OCH_3

Chart 1. Quercetin, apigenin, and pyrido[1,2-*a*]pyrimidin-4-one analogues, **1–7**.

Stock solutions of apigenin, quercetin and test compounds were dissolved in sterile dimethyl sulfoxide (DMSO), and stored at −80 °C at a maximum solubility of 50 mM. Since the final concentration of DMSO in the culture medium never exceeded 0.2% (v/v), DMSO (0.2%) alone served as control and did not show any effect on cell viability (data not shown) or cell function.

2.2. Platelet isolation

We obtained whole blood from 10 healthy adult, Italian volunteers (range 20–35 years, with a male/female ratio of 5/5) who had not taken medications or anti-platelet drugs for the preceding 3 weeks. To participate in this study, the volunteer blood donors gave their informed consent, according to the ethical standards of the committee of our institution. To reduce artefactual platelet activation, blood samples were drawn from an antecubital vein using a 19-gauge needle, and the first 2.5 ml were discarded. All blood samples were collected in Vacutainer tubes (Becton Dickinson, San Jose, CA, USA) containing 3.8% trisodium-citrate solution as anticoagulant.

Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood (175*g*, 15 min, 25 °C) and a platelet count was performed with the automated cell analyzer ADVIA® 2120 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Platelet-poor plasma (PPP) was obtained by a second centrifugation of the remaining blood (1000*g*, 10 min, 25 °C). PRP was adjusted to a platelet count of 300×10^9 platelets/l by diluting in PPP.

2.3. Platelet aggregation

To assess platelet aggregation in 96-well plates, a modified light transmission method was used (Armstrong et al., 2008). PRP (300×10^9 platelets/l) was treated with increasing concentrations (12.5–25–50–100 μM) of our compounds, apigenin, quercetin or DMSO alone (0.2%) before each challenge. The stimulation occurred with the following agonists: collagen (5 μg/ml), U46619 (1 μM) or Thrombin (1 U/ml). These agonists were chosen since together they represent the range of platelet agonists most widely used for *in vitro* platelet testing (Cattaneo et al., 2009).

In a 96-well plate, (100 μl) of PRP alone or PRP treated as above described were added with a multi-channel pipette to the agonist