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Structure prerequisite for antioxidant activity of silybin in different biochemical systems *in vitro*

Zs. Varga[a,*], I. Seres[a], E. Nagy[a], L. Ujhelyi[a], G. Balla[b], J. Balla[a], S. Antus[c]

[a]*First Department of Medicine, Division of Nephrology, Hungary*
[b]*Department of Neonatology, Health and Medical Science Center, Hungary*
[c]*Department of Organic Chemistry, University of Debrecen. H-4012. Debrecen, Nagyerdei krt. 98. Pob.19. Hungary*

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Abstract

Structural analogues (flavanone: **2**–**4** and flavone: **5** and **6**, respectively) of silybin (**1a**) were synthesized and tested for inhibitory activity on $O_2^{\bullet -}$ release and PKC translocation in PMA-stimulated neutrophils as well as xanthine oxidase activity in order to identify the molecular structures responsible for the antioxidant property of silybin. Concerning the prevention of hem-mediated oxidative modification of LDL by silybin, the hydroxyl radical scavenging activity of its structural analogues was also determined. We demonstrated that the basic skeleton of **1a** (**4**) is responsible for its inhibitory activity on $O_2^{\bullet -}$ release in PMA-stimulated neutrophils via inhibition of PKC translocation, since introduction of a double bound and hydroxyl groups at C-5 and C-7 position (**5** and **6**) did not result in further increase in inhibition of $O_2^{\bullet -}$ release. It has been shown that the presence of the phenolic hydroxyl group at C-5 and C-7 of **1a** is essential for the inhibition of xanthine oxidase activity. Moreover, introduction of a double bond into the C-ring of **2** and **3**, resulting in flavone derivatives (**5** and **6**), markedly enhanced the antioxidant effect in all the tested systems. Finally, silybin (**1a**) and its flavon derivatives (**5** and **6**) directly scavenged hydroxyl radicals as well. On the basis of these results it might be concluded that different moiety of silybin is responsible for inhibition of overproduction of $O_2^{\bullet -}$ in stimulated neutrophils, xanthine oxidase activity, and for prevention of hem-mediated oxidative modification of LDL.

Keywords: Silybin; Oxidative stress; Antioxidants; Protein kinase C; Xanthine oxidase; Low density lipoprotein

Introduction

Recently, it has been demonstrated that several groups of phenolic compounds including flavanolignans, such as silybin, isosilybin, silydianin and silychristin display very potent antioxidant and anticancer activity (Zi et al., 1998; Afaq et al., 2002; Dhanalakshmi et al., 2002; Singh et al., 2002, 2003; Gallo et al., 2003; Agarwal et al., 2003), and prevents hepatocytes from ethanol induced damage (van Pelt

Abbreviations: PKC, protein kinase C; NADPH, nicotinamide adenine dinucleotide phosphate; PMA, phorbol myristate acetate; LDL, low density lipoprotein; Ca, calcium; PS, phosphatidylserine; DAG, diacyglycerol; HBSS, Hanks' balanced salt solution; PMSF, phenylmethylsulphonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*, *N*, *N*′, *N*′-tetraacetic acid; AHP, 2-amino-4-hydroxypteridine

*Corresponding author. Tel.: 36 52 411 717/5833; fax: 36 52 414 951.
*E-mail address:*vargazs@ibel.dote.hu (Zs. Varga).

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et al., 2003). The pharmacological activities of flavonoids are assumed to derive mainly from the inhibition of certain enzymes involved in the formation of reactive oxygen species (ROS). In fact, many flavonoids have been reported to be potent inhibitors of ROS production enzymes such as xanthine oxidase (Cos et al., 1998; Sheu et al., 1998). Furthermore, flavonoids inhibit superoxide anion formation in stimulated neutrophils (Limasset et al., 1993; Lu et al., 2001; Selloum et al., 2001; Varga et al., 2001), and inhibit Cytochrome P-450 enzyme activity (Beckmann-Knopp et al., 2000) or scavenge superoxide anions (Furuno et al., 2002).

We have previously demonstrated that silybin (**1a**) inhibits the release of superoxide anion ($O_2^{\bullet -}$) in phorbol-myristate-acetate (PMA)-stimulated neutrophils and modification of its lipophilicity significantly alters its action (Varga et al., 2001). It was also demonstrated that silybin (**1a**) inhibits protein kinase C (PKC) translocation and NADPH oxidase activity in PMA-stimulated neutrophils in a concentration dependent manner, and an increase in lipid solubility by methylation of the phenolic hydroxyl groups of **1a** (**1a**→**1b**) improves its antioxidant ability presumably via enhanced penetration through the membrane lipid bilayer (Varga et al., 2004).

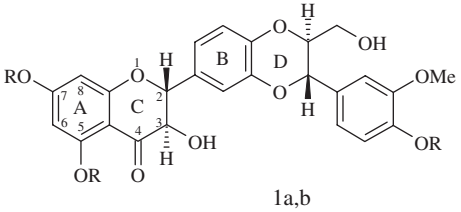
Flavonoids have been recently classified into several classes according to their ability to inhibit xanthine oxidase activity and scavenge superoxide anion formation (Cos et al., 1998; Furuno et al., 2002). In order to rank silybin (**1a**) in this classification and get further insights in the structure prerequisite of its antioxidant activity we set our sights on the study of **1a** and its related compounds (**2–6**) regarding their effect on xanthine oxidase activity, free radical (e.g. superoxide anion and hydroxyl radicals) scavenging ability and prevention of hem-mediated modification of low density lipoprotein (LDL). Considering the fact that $O_2^{\bullet -}$ production involves the activation of PKC in a wide variety of cells including neutrophils (Gopalakrishna and Jaken, 2000), and silybin (**1a**) has been found to be its potent inhibitor (Varga et al., 2004), we determined the PKC activity in PMA stimulated neutrophils exposed to silybin's analogues (**2–6**).

Materials and methods

Compounds

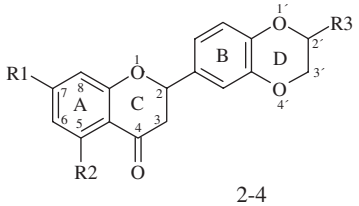
Silybin (**1a**) was purchased from Sigma (Sigma Co, St. Louis, MO, USA). Flavanone (**2–4**) and flavone (**5** and **6**) derivatives were prepared as described previously (Czompa et al., 2000). Structures of these compounds are presented in Fig. 1.

Flavonoid stock solutions and diluted working solutions were prepared in DMSO except for the case when hydroxyl radical scavenging activity was studied (see below). From these solutions, 5 μl was added to the samples to achieve the required concentrations. The



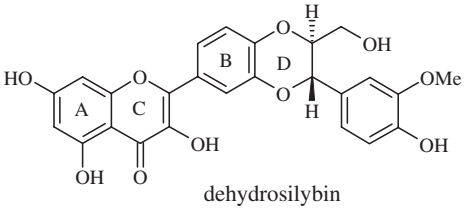
1a,b

	R
a	H
b	Me

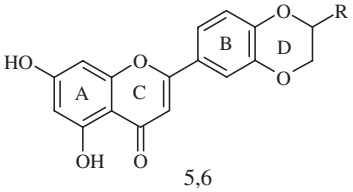


2-4

	R1	R2	R3
2	OH	OH	CH_2OH
3	OH	OH	H
4	H	H	H



dehydrosilybin



5,6

	R
5	CH_2OH
6	H

Fig. 1. Structures of tested molecules.