European Journal of Medicinal Chemistry 45 (2010) 1059–1067

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Antioxidant and antiviral activities of silybin fatty acid conjugates

Radek Gažák [a], Kateřina Purchartová [a], Petr Marhol [a], Lucie Živná [a], Petr Sedmera [a], Kateřina Valentová [b], Nobuo Kato [c], Hiroyo Matsumura [c], Kunihiro Kaihatsu [c,**], Vladimír Křen [a,*]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague, Czech Republic
[b] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, CZ-775 15 Olomouc, Czech Republic
[c] The Institute of Scientific and Industrial Research, Department of Organic Fine Chemicals, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan

ARTICLE INFO

Article history:
Received 14 August 2009
Received in revised form
30 October 2009
Accepted 27 November 2009
Available online 6 December 2009

Keywords:
Silybin
Silibinin
Influenza virus
Lipophilic derivatives
Lipid peroxidation
Antiviral

ABSTRACT

Two selective acylation methods for silybin esterification with long-chain fatty acids were developed, yielding a series of silybin 7-*O*- and 23-*O*-acyl-derivatives of varying acyl chain lengths. These compounds were tested for their antioxidant (inhibition of lipid peroxidation and DPPH-scavenging) and anti-influenza virus activities. The acyl chain length is an important prerequisite for both biological activities, as they improved with increasing length of the acyl moiety.

1. Introduction

The flavonolignan silybin (**1**) is the major biologically active component of an extract from the seeds of the milk thistle (*Silybum marianum* (L.) Gaertn.) known as silymarin. Silybin is an active component in numerous phytopreparations used in the prevention and treatment of various liver diseases and as a protectant against a number of hepatotoxins and mycotoxins [1–3]. Natural silybin (**1**) is a nearly equimolar mixture of two diastereoisomers (Fig. 1) [4–7].

The cytoprotectivity of **1** is based on several mechanisms operating at various cell levels [8]. Silybin acts mainly as an effective radical scavenger (antilipoperoxidant), and also as an antioxidant [9]. The role of the individual hydroxyl groups of silybin and molecular mechanisms of its antiradical and antioxidant action have been explained recently [10,11]. The radical scavenging activity of **1** could be also partly involved in cell regulatory pathways based on reactive oxygen species (ROS) [12]. Detailed characterization of the role of the respective hydroxyl groups and moieties of the silybin molecule in these processes [11] has enabled the identification of suitable sites for derivatization of the silybin molecule without losing the biological activity of the resulting conjugates.

In the last decade, silybin has been in the spotlight due to its multiple beneficial activities [8,13,14] not directly related to its hepatoprotective and/or antioxidant (radical scavenging) activities. These mostly include anticancer and chemopreventive behavior. The prevention and treatment of prostate hyperplasia involving adenocarcinoma is a very recent and important silymarin application, which is also documented by current successful clinical tests (clinical phase II) [15].

The bioavailability and therapeutic efficacy of silybin is rather limited by its very low solubility in water (430 mg/L). A number of silybin water-soluble semisynthetic derivatives were designed to overcome this problem, *e.g.*, silybin bis-hemisuccinate (Legalon) [16], silybin-23-*O*-phosphate [17], silybin 23-*O*-β-glycosides [6], and silybinic acid [9]. However, modifications of silybin leading to an increase in its water-solubility usually led to an impairment of its antioxidant (antiradical) activity in the lipophilic milieu [9].

On the contrary, some lipophilic preparations of silybin, *e.g.*, its non-covalent complex with phosphatidyl choline – (Silipide, IdB 1016, Indena, IT) [18,19], possessed not only better bioavailability than silybin [20,21] but also exhibited higher antioxidant activities [22,23]. Moreover, Silipide also exhibits promising anticancer activities as was demonstrated by its significant inhibition of the

* Corresponding author. Tel.: +420 296 442 510; fax: +420 296 442 509.
** Corresponding author.
E-mail addresses: kunihiro@sanken.osaka-u.ac.jp (K. Kaihatsu), kren@biomed.cas.cz (V. Křen).

0223-5234/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.11.056

growth of human ovarian cancer xenografts [3]. Furthermore, silybin-containing liposomes composed of phospholipids and cholesterol were found to be much more effective hepatoprotectors in the murine model than silybin itself [24].

Such a positive effect of lipophilic formulations of silybin clearly demonstrates that the membrane-stabilizing function is one of the basic molecular mechanisms of silybin activity [25,26] and that silybin–phospholipid complexes are more effective than silybin. Nevertheless, current knowledge on the interactions of silybin with lipid bilayers is very poor. According to a study on the influence of silybin on liver microsomal membranes, it is believed that silybin molecules are incorporated into the hydrophobic–hydrophilic interface of membranes [27] and mainly interact with the polar head group region of the lipid bilayers, although some of the silybin molecules can partition into the hydrophobic region of the membrane [28].

The conjugation of the water-soluble antioxidant to a long-chain fatty acid has been demonstrated to improve the incorporation of antioxidants into liposomes (used as a model for cellular membranes) [29]. An improvement of the antioxidant activity in heterogeneous systems by lipophilization of the hydrophilic antioxidant has previously been observed: the antioxidant efficiency of retinyl ascorbate (an ester of lipophilic retinoic acid and the water-soluble antioxidant ascorbic acid) [30] as well as ferulates, dihydroferulates, caffeates, dihydrocaffeates and gallates were generally found to exhibit a larger antioxidative effect than the parent compounds [31–33]. Moreover, most of the previous studies proved that the antioxidant activity of these conjugates is dependent on the acyl (and/or alkyl) chain length and is generally better for derivatives with a longer aliphatic chain. The conjugation of epicatechin with fatty acids was also shown to change its biological activities, such as an increase in DNA polymerase inhibition and inhibition of angiogenesis [34].

There are several reports that describe the anti-hepatitis C virus (HCV) effect of medicinal herbs in clinical trials. Although the most preparations did not show positive effects on the clearance of serum HCV RNA or anti-HCV antibodies or on serum liver enzymes, silybin exhibited a significant effect, reducing serum aspartate aminotransferase and gamma-glutamyltranspeptidase activities [35,36]. The antiviral mechanism of silybin action is still unclear because its chemical and pharmacological properties may change when it is administered orally. However, silybin A, silybin B, isosilybin A, and isosilybin B elicited the strongest anti-NF-κB and anti-HCV activities of the standardized extracts of *Silybum marianum in vitro* [37]. Based on these findings, the antiviral effect of silybin derivatives may be ascribed to their antioxidative activities.

Peterhands et al. demonstrated that influenza virus infection induces oxidative stress and the production of ROS from phagocytes [38]. These agens may modify proteins and lipids, damage DNA, and alter mitochondrial membrane potential, producing apoptotic cytotoxicity. Effective delivery of silybin to the cellular membrane may increase its antiviral efficacy. Therefore, lipophilization of the silybin molecule by its conjugation to fatty acids can not only improve its antioxidant but also its anti-influenza virus activities.

The aim of the present study was to develop general method(s) for selective acylation of the silybin molecule with monocarboxylic acids and preliminary testing of these derivatives for their antioxidant and anti-influenza virus activities compared to **1**. Detailed knowledge of the effect of particular silybin OH groups on the antiradical mechanism [10,11] enabled us to rationally determine the most suitable sites for substitution. A further aim of this work was to evaluate the effect of the acyl chain length on the antioxidant and anti-influenza-virus activities of silybin and its lipoderivatives.

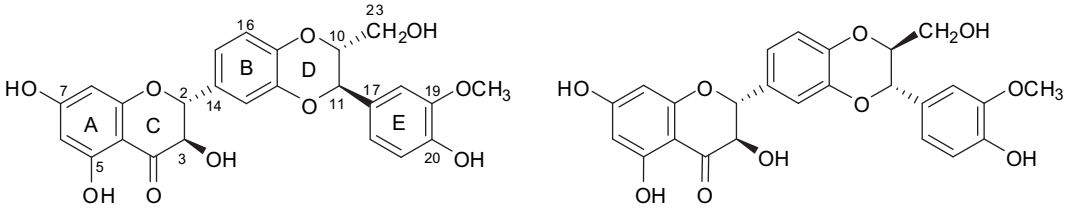
2. Results and discussion

2.1. Chemistry

According to our recent studies [10,11], the most suitable positions for silybin modification are 7-OH or 23-OH of **1**, as they are not responsible for its major antioxidant and antiradical action (23-OH) or can even act as a pro-oxidant moiety (7-OH). Although a selective enzymatic procedure for the acylation of silybin's primary alcoholic group (23-OH) was developed recently [39], producing reasonable yields of the corresponding esters of silybin with several short chain monocarboxylic acids, this procedure requires rather long reaction times, activation of the corresponding carboxylic acids (*e.g.*, vinyl esters) and provides rather low yields with long-chain acids. As an enzymatic approach leads exclusively to the esters at 23-OH of **1**, the development of new procedures for the selective esterification of other positions of **1** are required. Moreover, the development of a suitable general esterification method for the primary alcoholic group of **1** will be required to obtain sufficient yields of the corresponding esters of **1** with long-chain carboxylic acids.

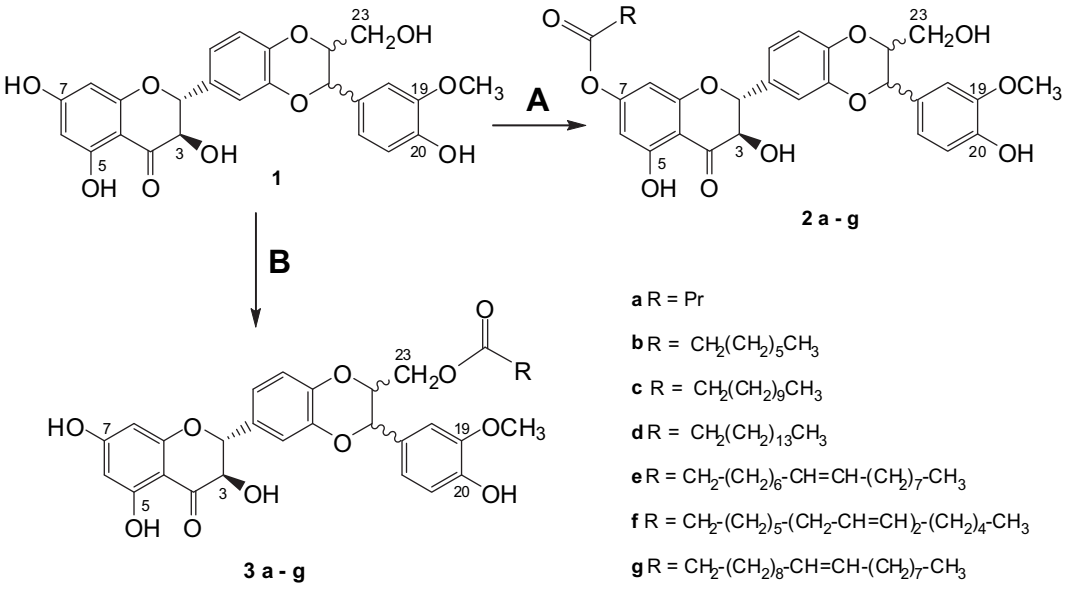
Initially, esterification reactions of silybin (**1**) using free carboxylic acids utilizing various well known activation reagents and procedures (1,1′-carbonyldiimidazole (CDI), *N*,*N*′-dicyclohexylcarbodiimid/4-dimethylaminopyridine (DCC/DMAP) – Steglich esterification, diethyl azodicarboxylate/triphenylphosphine (DEAD/Ph_3P) – Mitsunobu reaction) were tested, however, these attempts failed due to their low selectivity (DCC/DMAP) or did not work at all (CDI, Mitsunobu reaction). As a result, more reactive acylating reagents (namely acyl chlorides) were chosen.

Selective acylation of the 7-OH position of silybin was accomplished with the respective acyl chloride (1.5 equiv) in pyridine (Scheme 1). As the 7-OH of **1** represents its most acidic hydroxyl group, the achieved selectivity can be explained by an exclusive phenolate generation at this position caused by pyridine as a weak base. On the contrary, the reaction of **1** with acyl chloride in the presence of a Lewis acid ($BF_3 \cdot Et_2O$) led to the selective esterification of the 23-OH group of **1** (Scheme 1). Interestingly, the



silybin A (2*R*, 3*R*, 10*R*, 11*R*) silybin B (2*R*, 3*R*, 10*S*, 11*S*)

Fig. 1. Isomers of natural silybin (**1**).



Scheme 1. Reagents and conditions. A. Acyl chloride (1.5 equiv), Py, 0 °C, 1 h (29–45%); B. Acyl chloride (1 equiv), $BF_3 \cdot Et_2O$ (2.4 equiv), CH_2Cl_2/MeCN (1:1, v/v), 2 h at 0 °C, then 1 h at r.t. (28–32%).

formation of 23-*O*-acetylsilybin as a side product was observed in this reaction (less than 10%) despite no acetyl donor being present in the reaction mixture, suggesting that acetonitrile (used as a co-solvent) is able to act as a weak acetylation reagent under these conditions.

Although the yields of the corresponding esters were moderate, the reaction conditions were optimized to reduce side product formation (di- and tri-esters) enabling a convenient separation of the product from the unreacted **1** using a short and rapid column chromatography. Despite the complex nature of silybin (five hydroxyl groups) the fast reaction and selectivity make these methods a versatile approach for silybin esterification.

Structures of silybin esters were determined by NMR spectroscopy. All OH signals are visible in the ^{1}H NMR spectra of acyl silybins measured in DMSO, their experimental assignment was proved by HMBC. The absence of some of them (compared to the parent compound) provides the first hint of the acylation site. The downfield acylation shift is observable with H-3 or H-23 protons. The heteronuclear coupling between H-3 or H-23 and the acyl carbonyl is a direct proof of the acylation at these positions. An acylation at C-7 is manifested by chemical shift changes of C-6, C-7, C-8, and C-4a.

2.2. Antioxidant activity

The role of the individual hydroxyl groups of silybin on its antiradical action was recently determined [10,11]. These studies not only defined the positions, which react with radicals directly (20-OH of **1**), but also evaluated the effect of other OH groups substitutions on the antioxidant activity of **1**. Despite substantial amounts of useful knowledge being obtained, the effect of substitution was only studied with the methyl group. Such an approach enables the SAR-study of similar compounds but cannot evaluate the effect of the substitution in terms of the change in physicochemical properties, *e.g.* their lipophilicity. As the lipid peroxidation of biomembranes caused by free radicals is one of the most serious pathological events in organisms, most studies have been aimed at evaluating the inhibitory activity of antioxidants against this process. Lipophilicity is a crucial factor, which influences the inhibition activity of antioxidants against lipid peroxidation. Therefore, a series of 7-*O*- and 23-*O*-acyl-derivatives was synthesized with the aim of determining which of these positions on silybin is more suitable for the substitution and to evaluate the effect of the length of aliphatic acyl chain upon its lipid peroxidation inhibitory activity. Although the inhibition of lipid peroxidation represents an assay that better reflects the ability of antioxidants to protect against oxidative damage in biological systems, the radical-scavenging activities of acyl-derivatives against DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals were also measured for comparison with other previously published data on silybin. Esters of silybin with unsaturated acids were also prepared, however due to their enormous susceptibility to auto-oxidation observed during their structural analysis, these compounds were excluded from biological testing.

The results obtained (Table 1) clearly demonstrate that the length of the acyl chain is decisive for lipid peroxidation inhibitory activity. Surprisingly, acyl derivatives with short acyl chains (C_4–C_8) are inferior inhibitors than the parent silybin. On the other hand, longer acyl derivatives (C_{12} and mainly C_{16}) are better inhibitors. This observation is consistent with the behavior of 2,2,5,7,8-pentamethyl-6-chromanol (an analogue of α-tocopherol having only a methyl instead of the phytyl group) and α-tocopherol – both compounds exert essentially the same antioxidant activity in an organic solution, but α-tocopherol possesses a higher activity against lipid peroxidation in the membranes and low density lipoproteins due to higher mobility within and between membranes and particles [40–42]. The esterification of silybin by short chain acids probably improves its interaction with biomembranes compared with silybin itself, however, it simultaneously reduces its mobility and hinder its ability for self-stabilization by dimerization [11,43]. This ability is probably restored with longer chain esters, which, due to their higher lipophilicity and better mobility within membranes, exhibited a higher inhibitory activity in both types of silybin substitutions.

As far as the site of silybin acylation is concerned, the more suitable position for its substitution is the 7-OH group, mainly with long-chain fatty acids. A plausible explanation of this behavior probably lies in the recently described [11] pro-oxidative activity of

Table 1
Antioxidant properties of silybin and its fatty acid esters (LPX – inhibition of lipid peroxidation).

	Antiradical activity DPPH[a]	Antioxidant activity LPX[b]
	IC_{50} (mmol/L)	IC_{50} (μmol/L)
Silybin (**1**)	2.1 ± 0.1	58.1 ± 3.6
7-*O*-Butanoylsilybin (**2a**)	2.3 ± 0.1	100.6 ± 29.4
7-*O*-Octanoylsilybin (**2b**)	4.0 ± 0.2	116.7 ± 36.0
7-*O*-Dodecanoylsilybin (**2c**)	3.0 ± 0.2	44.7 ± 0.5
7-*O*-Palmitoylsilybin (**2d**)	3.6 ± 0.3	28.9 ± 1.0
23-*O*-Butanoylsilybin (**3a**)	3.4 ± 0.6	91.7 ± 5.2
23-*O*-Octanoylsilybin (**3b**)	3.2 ± 0.4	61.1 ± 5.9
23-*O*-Dodecanoylsilybin (**3c**)	2.8 ± 0.8	50.5 ± 4.5
23-*O*-Palmitoylsilybin (**3d**)	3.2 ± 0.6	51.9 ± 3.8

Data are expressed as mean values from three measurements in three independent experiments and standard deviations.
[a] DPPH – 1,1-diphenyl-2-picrylhydrazyl radical scavenging; IC_{50} (μM) – the concentration of the tested compound required to reduce the absorbance of DPPH by 50%.
[b] LPX – Inhibition of microsomal lipid peroxidation–the activity was calculated as the concentration of the tested compound inhibiting the color reaction with thiobarbiturate by 50% (IC_{50}).

Table 2
Cytotoxicity and anti-influenza virus A/PR8/34/(H1N1) activity of silybin and its fatty acid esters.

	Cytotoxicity CC_{50}[a] (μmol/L)	Antiviral activity EC_{50}[b] (μmol/L)	Selectivity SI[c]
Silybin (**1**)	>800	ND	–
7-*O*-Butanoylsilybin (**2a**)	300	14	21.4
7-*O*-Octanoylsilybin (**2b**)	240	6	40.0
7-*O*-Dodecanoylsilybin (**2c**)	>560	6	>93.3
7-*O*-Palmitoylsilybin (**2d**)	ND[d]	ND[d]	–
23-*O*-Butanoylsilybin (**3a**)	300	24	12.5
23-*O*-Octanoylsilybin (**3b**)	30	18	1.7
23-*O*-Dodecanoylsilybin (**3c**)	120	3	40.0
23-*O*-Palmitoylsilybin (**3d**)	>480	3	>160

[a] CC_{50} represents the concentration of compound required to reduce cell viability by 50% relative to the control well without test compound.
[b] EC_{50} represents the concentration of compound required to reduce plaque number by 50% relative to the control well without test compound.
[c] SI (Selectivity index) is the ratio of CC_{50} to EC_{50}.
[d] CC_{50} and EC_{50} of **2d** were not determined due to its low water solubility.

this moiety. Accordingly, substitution of the 7-OH can switch off this undesirable activity (as far as antioxidant activity is concerned) and in long-chain acyl-derivatives leads to more effective inhibitors compared to the corresponding 23-*O*-acyl-derivatives. Nevertheless, the impact of esterification of both silybin positions on the spatial interactions with lipid bilayers can play an important role in the resulting antioxidant activity of the conjugate during lipid oxidation in heterogeneous systems [31].

Our results on the DPPH-scavenging activity for 7-*O*-acyl-derivatives (Table 1) are partially consistent with our recent observations with methyl-derivatives of silybin, where 7-OH methylation slightly lowered the DPPH-scavenging activity of the corresponding derivative in comparison with silybin [11]. However, 7-OH methylation led to a very low decrease in scavenging activity compared to acylation. The effect of silybin 23-OH acylation was much more striking, since 23-OH was considered to be ineffective for radical scavenging. Nevertheless, a similar decrease in capacity of lipophilic conjugates of water-soluble antioxidants to scavenge DPPH radicals in a homogenous system (EtOH or MeOH solution) was observed in most of the previous studies, *e.g.* for esters of dihydrocaffeic acid [44] and gallic acid esters [31,33]. Additionally,

Table 3
^{1}H NMR data (400 MHz, d_6-DMSO, 30 °C) of 7-*O*-acyl silybins **2a-d.**

Proton	**2a**	**2b**	**2c**	**2d**
2	5.234 d (11.6)	5.232 d (11.6)	5.229 d (11.6)	5.225 d (11.6)
3	4.777 dd (11.6,6.0)	4.776 dd (11.6,6.3)	4.772 dd (11.6,6.3)	4.768 dd (11.6,6.4)
	4.768 dd (11.6,6.0)	4.765 dd (11.6,6.3)	4.761 dd (11.6,6.3)	4.758 dd (11.6,6.4)
6	6.358 d (2.1)	6.349 d (2.0)	6.346 d (2.1)	6.342 d (2.1)
8	6.317 d (2.1)	6.308 d (2.0)	6.305 d (2.1)	6.301 d (2.1)
	6.311 d (2.1)	6.303 d (2.0)	6.299 d (2.1)	6.295 d (2.1)
10	4.181 m	4.180 ddd (7.8,4.6,1.7)	4.176 ddd (7.8,4.7,2.4)	4.172 ddd (7.9,4.9,2.5)
		4.173 ddd (7.8,4.6,1.7)	4.171 ddd (7.8,4.7,2.4)	4.167 ddd (7.9,4.9,2.5)
11	4.920 d (7.8)	4.918 d (7.8)	4.919 d (7.8)	4.911 d (7.9)
13	7.114 d (2.0)	7.113 d (2.0)	7.111 d (1.8)	7.105 d (2.1)
	7.109 d (2.0)	7.107 d (2.0)	7.105 d (1.8)	7.099 d (2.1)
15	7.039 dd (8.0,2.0)	7.037 dd (8.2,2.0)	7.036 dd (8.3,1.8)	7.031 dd (8.3,2.1)
	7.036 dd (8.0,2.0)	7.034 dd (8.3,2.0)	7.032 dd (8.3,1.8)	7.028 dd (8.3,2.1)
16	6.986 d (8.0)	6.985 d (8.2)	6.984 d (8.3)	6.980 d (8.3)
	6.983 d (8.0)	6.983 d (8.3)	6.981 d (8.3)	6.978 d (8.3)
18	7.021 d (1.7)	7.021 d (2.0)	7.016 d (2.0)	7.013 d (2.0)
	7.017 d (1.7)	7.017 d (2.0)		7.009 d (2.0)
21	6.807 d (8.1)	6.807 d (8.1)	6.806 d (8.1)	6.800 d (8.2)
		6.805 d (8.1)	6.805 d (8.1)	
22	6.871 dd (8.1,1.7)	6.870 dd (8.1,2.0)	6.869 dd (8.1,2.0)	6.863 dd (8.2,2.0)
		6.869 dd (8.1,2.0)		
23 d	3.551 dm (11.7)	3.550 ddd (12.3,4.5,1.7)	3.551 ddd (12.2,4.4,2.5)	3.543 ddd (12.2,5.3,2.5)
23 u	3.352 m	3.356 ddd (12.3,4.6,4.5)	3.355 ddd (12.2,4.7,4.4)	3.348 ddd (12.2,5.3,4.9)
19-OMe	3.782 s	3.782 s	3.782 s	3.776 s
			3.781 s	
3-OH	5.940 d (6.0)	5.930 d (6.3)	5.932 d (6.3)	5.937 d (6.4)
5-OH	11.660 s	11.664 s	11.669 s	11.890 s
7-OH	–	–	–	–
20-OH	9.116 s	9.114 s	9.109 s	9.128 s
23-OH	4.924 t (4.5)	4.924 t (4.5)	4.923 t (4.4)	4.953 t (5.3)

Additional signals – **2a**: 2.541 (t, 2 H, J = 7.2 Hz, 2 × H-2′), 1.636 (m, 2 H, 2 × H-3′), 0.947 (t, 3 H, J = 7.4 Hz, 3 × H-3′); **2b**: 2.551 (t, 2 H, J = 7.4 Hz, 2 × H-2′), 1.610 (m, 2 H, 2 × H-3′), 0.862 (t, 3 H, J = 6.8 Hz, 3 × H-8′); **2c**: 2.546 (t, 2 H, J = 7.3 Hz, 2 × H-2′), 1.605 (m, 2 H, 2 × H-3′), 0.851 (t, 3 H, J = 6.8 Hz, 3 × H-12′); **2d**: 2.544 (t, 2 H, J = 7.3 Hz, 2 × H-2′), 1.600 (m, 2 H, 2 × H-3′), 0.847 (t, 3 H, J = 7.0 Hz, 3 × H-16′).

these studies demonstrated that the DPPH-scavenging ability in a homogenous system is independent of the aliphatic chain length and usually lower than that of the parent non-conjugated antioxidant.

2.3. Cytotoxicities of silybin and its acyl-derivatives

The cytotoxic effects of silybin and its 7-*O*- and 23-*O*-acyl-derivatives to Madin-Darby Canine Kidney (MDCK) cells, given as CC_{50}, were evaluated by cell viability assay. As shown in Table 2, silybin (**1**) did not display an apparent cytotoxic effect even at a concentration of 800 μM. However, the cytotoxicities of 7-*O*- and 23-*O*-acyl-derivatives increased as a function of acyl length. Notably, the 7-*O*-octanoyl (**2b**), 23-*O*-octanoyl (**3b**), and 23-*O*-dodecanoyl (**3c**) silybin derivatives exhibited relatively high cytotoxicities (Table 2). This may be due to their increased cell membrane permeabilities. A similar phenomenon was also reported in the case of acyl derivatives of epigallocatechin-3-*O*-gallate [45]. On the other hand, 7-*O*-dodecanoyl (**2c**), 7-*O*-palmitoyl (**2d**), and 23-*O*-palmitoyl (**3d**) esters exhibited considerably lower cytotoxicities, probably due to their poor water solubilities.

Table 4
^{13}C NMR data (100 MHz, d_6-DMSO, 30 °C).

C	1	2a	2b	2c	2d	3a	3b	3c	3d
2	82.65	82.81	82.79	82.80	82.77	82.52	82.52	82.51	82.52
	82.62	82.76	82.75	82.76	82.57	82.48	82.47	82.47	82.47
3	71.56	71.81	71.80	71.81	71.81	71.52	71.53	71.53	71.54
	71.49	71.75	71.74	71.75	71.52	71.45	71.45	71.46	71.47
4	197.76	199.51	199.49	199.50	199.47	197.72	197.69	197.71	197.65
	197.74	199.49	199.47	199.48		197.69	197.66	197.68	197.61
4a	100.55	104.81	104.80	104.79	104.80	100.50	100.48	100.49	100.45
5	163.38	161.99	161.98	161.98	163.34	163.35	163.35	163.34	163.36
6	96.16	102.94	102.91	102.90	102.92	96.13	96.14	96.12	96.15
7	166.90	158.09	158.09	158.09	158.10	166.90	166.96	166.88	167.01
8	95.11	101.68	101.65	101.63	101.66	95.08	95.09	95.07	95.11
8a	162.55	161.86	161.85	161.85	161.98	162.49	162.49	162.48	162.48
	162.54	161.85	161.84	161.83	161.84	162.48	162.47	162.47	
10	78.23	78.17	78.17	78.19	78.19	75.05	75.05	75.05	75.06
	78.20			78.17		75.03		75.03	
11	75.93	75.89	75.89	75.88	75.90	75.99	75.97	75.96	75.96
12a	143.33	143.36	143.32	143.33	143.50	143.13	143.13	143.12	143.25
	143.32	143.33		143.31	143.32	143.10	143.09	143.08	143.22
13	116.67	116.71	116.70	116.69	116.64	116.79	116.81	116.80	116.80
	116.58	116.75	116.63	116.62		116.72	116.69	116.68	116.67
14	130.15	129.72	129.71	129.70	129.68	130.57	130.57	130.56	130.57
	130.11	129.68	129.67	129.67		130.53	130.54	130.53	130.54
15	121.37	121.44	121.42	121.41	121.31	121.57	121.57	121.55	121.54
	121.22	121.32	121.28	121.27	121.30	121.41	121.37	121.33	121.32
16	116.42	116.52	116.42	116.41	116.38	116.46	116.44	116.43	116.44
	116.37	116.38		116.37		116.41	116.38	116.37	116.37
16a	143.73	143.83	143.82	143.82	143.80	143.25	143.25	143.24	143.21
	143.70	143.80	143.80	143.80	143.67	143.24	143.23	143.22	143.09
17	127.59	127.53	127.51	127.51	127.54	126.63	126.63	126.63	126.63
		127.51						126.62	
18	111.86	111.83	111.79	111.78	111.80	111.80	111.78	111.77	111.78
	111.81	111.75	111.73	111.73		111.75	111.74	111.72	111.73
19	147.73	147.69	147.67	147.68	147.69	147.81	147.83	147.82	147.83
	147.72	147.68		147.67		147.82	147.82	147.81	147.82
20	147.71	147.09	147.07	147.08	147.16	147.39	147.39	147.39	147.40
	147.05	147.08		147.06	147.07	147.38		147.38	
21	114.44	115.38	115.36	115.36	115.38	115.48	115.46	115.44	115.45
	115.43					115.47	115.44		
22	120.59	120.56	120.55	120.53	120.56	120.68	120.67	120.65	120.65
						120.66	120.64	120.63	
23	60.28	60.22	60.21	60.21	60.23	62.42	62.43	62.43	62.42
OMe	55.81	55.75	55.74	55.74	55.74	55.74	55.74	55.73	55.74
1′		170.76	170.89	170.90	170.91	172.46	172.60	172.59	172.60
2′		35.33	33.50	33.50	33.52	35.12	33.26	33.25	33.27
3′		17.17	24.18	24.17	24.17	17.87	24.40	24.39	24.40
4′		13.31	28.28	28.31	28.30	13.40	28.36	28.44	28.44
5′			28.32				28.40		
6′			31.09				31.14		
7′			22.03				22.05		
8′			13.93				13.94		
9′									
10′				31.30				31.32	
11′				22.10				22.11	
12′				13.94				13.96	
13									
14′					31.31				
15′					22.11				
16′					13.96				13.96

Additional signals – **2c**: 28.99 (2 C), 28.28, 28.71, 28.64; **2d**: 29.04, 28.96, 28.84, 28.72, 28.63; **3c**: 28.01 (2 C), 28.92, 28.72, 28.70. **3d**: 29.08, 29.07, 29.04, 29.01, 28.91, 28.73.

Table 5
^{1}H NMR data (400 MHz, d_6-DMSO, 30 °C) of 23-*O*-acyl silybins **3a-d**.

Proton	3a	3b	3c	3d
2	5.095 d (11.2)	5.093 d (11.2)	5.093 d (11.0)	5.087 d (11.2)
3	4.606 dd (11.2,5.2)	4.602 m	4.594 m	4.595 dd (11.2,6.3)
	4.596 dd (11.2,5.2)			4.582 dd (11.2,6.3)
6	5.920 d (2.1)	5.915 m	5.918 m	5.915 d (2.1)
8	5.879 d (2.1)	5.871 m	5.874 m	5.871 d (2.1)
	5.874 d (2.1)			5.864 d (2.1)
10	4.520 ddd (7.9,4.9,2.8)	4.514 m	4.513 m	4.510 ddd (7.9,5.0,2.9)
	4.510 ddd (7.9,4.9,2.8)			4.505 ddd (7.9,5.0,2.9)
11	4.932 d (7.9)	4.929 d (7.7)	4.929 d (7.8)	4.924 d (7.9)
13	7.119 d (1.9)	7.115 m	7.110 d (2.1)	7.112 d (1.9)
	7.112 d (1.9)			7.100 d (1.9)
15	7.039 dd (8.3,1.9)	7.035 m	7.032 m	7.031 dd (8.3,1.9)
	7.035 dd (8.3,1.9)			7.022 dd (8.3,1.9)
16	6.992 d (8.3)	6.974 d (8.2)	6.981 d (8.0)	6.978 d (8.3)
	6.989 d (8.3)			6.973 d (8.3)
18	7.029 d (1.9)	7.024 m	7.022 m	7.020 d (2.0)
21	6.810 d (8.2)	6.805 d (7.9)	6.804 d (7.9)	6799 d (8,1)
22	6.869 dd (8.2,1.9)	6.863 m	6.862 m	6.857 dd (8.2,2.0)
23d	4.144 dd (12.4,2.8)	4.141 dd (12.2,2.5)	4.138 dd (12.1,2.5)	4.136 dd (12.3,2.9)
				4.132 dd (12.3,2.9)
23 u	3.949 dd (12.4,4.9)	3.935 dd (12.2,4.6)	3.936 dd (12.1,4.5)	3.929 dd (12.3,5.0)
19-OMe	3.780 s	3.778 s	3.777 s	3.774 s
	3.778 s			3.771 s
3-OH	5.790 d (5.2)	5.787 br s	5.788 d (5.7)	5.794 d (6.3)
5-OH	11.873 s	11.873 s	11.872 s	11.870 s
7-OH	10.791 s	10.826 s	10.809 s	10.810 br s
20-OH	9.177 s	9.181 s	9.171 s	9.182 s
23-OH	–	–	–	–

Additional signals – **3a**: 2.273 (m, 2 H, 2 × H-2′), 1.529 (m, 2 H, 2 × H-3′), 0.877 (t, 3 H, J = 7.4 Hz, 3 × H-4′); **3b**: 2.303 (m, 2 H, 2 × H-2′), 1.501 (m, 2 H, 2 × H-3′), 0.851 (t, 3 H, J = 6.8 Hz, 3 × H-8′); **3c**: 2.302 (m, 2 H, 2 × H-2′), 1.502 (m, 2 H, 2 × H-3′), 0.848 (t, 3 H, J = 6.2 Hz, 3 × H-12′); **3d**: 2.298 (m, 2 H, 2 × H-2′), 1.945 (m, 2 H, 2 × H-3′), 1.240 (m, 2 H, 2 × H-4′), 0.843 (t, 3 H, J = 7.0 Hz, 3 × H-16′).

2.4. Anti-influenza virus activities of silybin and its acyl-derivatives

The anti-influenza virus activities of silybin and its 7-*O*- and 23-*O*-acyl-derivatives were studied by plaque formation assay in an MDCK monolayer. Silybin did not exhibit an antiviral effect (Table 2). In contrast, 7-*O*- and 23-*O*-acyl silybin derivatives showed potent antiviral activities (Table 2). An exception is 7-*O*-palmitoylsilybin (**2d**) because it suffers from poor water solubility in the cell culture medium. The antiviral effect of 7-*O*- and 23-*O*-acyl silybin derivatives was also increased as a function of acyl length (Table 2). Based on their cytotoxicities, we expected that the introduction of octanoyl or dodecanoyl groups to silybin should increase their cell permeability and increase their antiviral activity. In fact, 7-*O*-octanoyl (**2b**), 7-*O*-dodecanoyl (**2c**), 23-*O*-dodecanoyl (**3c**), and 23-*O*-palmitoyl (**3d**) silybin derivatives displayed potent antiviral effects and high selectivity index values (Table 2). These silybin derivatives could probably inhibit ROS generated by influenza virus infection and inhibited the virus-induced cytopathic effect.

3. Conclusion

Two selective acylation methods for silybin esterification with monocarboxylic acids were developed, yielding a series of silybin 7-*O*- and 23-*O*-acyl-derivatives of different acyl chain lengths. The results of experiments focusing on the inhibition by the tested esters of the lipid peroxidation of microsomes induced by *tert*-butylhydroperoxide proved that the length of the acyl chain of the corresponding ester plays an important role in this activity. However, the site of silybin acylation was found to be less significant in achieving a high inhibitory activity. The palmitates of silybin were found to be the best inhibitors of lipid peroxidation from our tested series; a more pronounced effect was connected with 7-OH esterification than with 23-OH. This observation is in agreement with the previously suggested pro-oxidant function of the 7-OH of silybin [11]. However, the rather contradictory results of silybin esters in the DPPH assay, together with the absence of precise information on the interaction of these lipophilic derivatives with biomembranes and their behavior in solution (possibility of micelles formation) calls for further studies of these promising semisynthetic lipophilic derivatives of silybin.

According to the cytotoxic effect of silybin esters, the octanoyl ester seemed to have the highest affinity to cells. However, dodecanoyl and palmitoyl esters exhibited more potent antiviral effects. This can be explained by both their antioxidant properties and cell membrane permeabilities. The antiviral mechanisms of silybin derivatives remain unclear, however, our synthetic study is showing the way to the new leads in the preparation of anti-influenza drugs, which is a very topical issue at present (*e.g.*, swine flu outbreak).

4. Experimental protocols

4.1. General methods

Silybin (mixture of A and B, ca 1:1) was kindly provided by Dr. L. Cvak (TAPI Galena, IVAX Pharmaceuticals, Opava, CZ). Acyl chlorides (octanoyl chloride, dodecanoyl chloride) were prepared by reacting the corresponding carboxylic acid with an excess of oxalyl chloride and used immediately after preparation without further purification. All other chemicals were purchased from Sigma–Aldrich and used without further purification. The reactions were monitored by TLC on F_{254} silica gel (Merck) and the spots were visualized with UV light and by charring with 5% H_2SO_4 in ethanol.

^{1}H and ^{13}C NMR spectra were recorded at 30 °C in DMSO on Varian Inova 400 or Bruker Avance III 400 NMR spectrometer (400

and 100 MHz, respectively). The residual solvent signal (δ_H 2.500, δ_C 39.60) served as an internal reference. All 2D NMR experiments were performed using the standard manufacturer's software (ChemPack 3.2 or TopSpin 2.1).

HRMS – measurements were performed on a commercial APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and a Dual II ESI/MALDI ion source (Bruker Daltonics, Billerica, USA) using MALDI ion source, positive-ion mode. The interpretation of mass spectra was done using the software package DataAnalysis version 3.4 (Bruker Daltonics, Billerica, USA). Positive-ion electrospray ionization (ESI) mass spectra were recorded on a LC^{Q}DECA spectrometer (ThermoQuest, San Jose, USA). HPLC-analyses were carried out on a Spectra Physics analytical system (San Jose, USA) comprised of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/VIS detector. The columns employed were a EC NUCLEOSIL 100-5 C18 AB, 125 × 3 mm (Macherey–Nagel, DE) or Chromolite Speed ROD, RP-18e, 50 × 4.6 mm monolithic column (Merck, DE).

4.2. General method A – preparation of silybin 7-O-acyl derivatives

The corresponding acyl chloride (0.311 mmol, 1.5 equiv) was added to a cooled solution (0 °C) of silybin (**1**; 100 mg, 0.207 mmol) in dry pyridine (7 mL) and the reaction mixture stirred at 0 °C for 1 h. The reaction was stopped by diluting the reaction mixture with ice-cold HCl (50 mL, 5% solution in water, v/v) and after brief stirring the solution extracted with ethyl acetate (3 × 30 mL), the organic phase washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

Flash chromatography on silica gel (chloroform/acetone/formic acid 95:5:1) yielded the corresponding 7-*O*-acyl-silybin (**2a**-**g**).

4.2.1. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-7-butanoyloxy-3,5-dihydroxy-4H-1-benzopyran-4-one (**2a**)

Compound **2a** was prepared according to general method A as a white amorphous solid (45%). HR-MS (MALDI) calcd for $C_{29}H_{28}O_{11}$ (M^+): 552.1632, found 552.1622. For ^1H and ^{13}C NMR data see Tables 3 and 4.

4.2.2. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-octanoyloxy-4H-1-benzopyran-4-one (**2b**)

Compound **2b** was prepared according to general method A as a white amorphous solid (41%). HR-MS (MALDI) calcd for $C_{33}H_{36}O_{11}$ (M^+): 608.2258, found 608.2253. For ^1H and ^{13}C NMR data see Tables 3 and 4.

4.2.3. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-dodecanoyloxy-4H-1-benzopyran-4-one (**2c**)

Compound **2c** was prepared according to general method A as a white amorphous solid (32%). HR-MS (MALDI) calcd for $C_{37}H_{44}O_{11}$ (M^+): 664.2884, found 664.2880. For ^1H and ^{13}C NMR data see Tables 3 and 4.

4.2.4. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-hexadecanoyloxy-4H-1-benzopyran-4-one (**2d**)

Compound **2d** was prepared according to general method A as a white amorphous solid (36%). HR-MS (MALDI) calcd for $C_{41}H_{52}O_{11}$ (M^+): 720.3510, found 720.3509. For ^1H and ^{13}C NMR data see Tables 3 and 4.

4.2.5. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-(cis-9-octadecenoyl)-oxy-4H-1-benzopyran-4-one (**2e**)

Compound **2e** was prepared according to general method A as a yellowish amorphous solid (34%). LRMS (ESI): *m*/*z* 769.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

4.2.6. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-(cis,cis-9,12-octadecadienoyl)-oxy-4H-1-benzopyran-4-one (**2f**)

Compound **2f** was prepared according to general method A as a yellowish amorphous solid (29%). LRMS (ESI): *m*/*z* 767.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

4.2.7. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5-dihydroxy-7-(cis-11-eicosenoyl)-oxy-4H-1-benzopyran-4-one (**2g**)

Compound **2g** was prepared according to general method A as a yellowish amorphous solid (29%). LRMS (ESI): *m*/*z* 797.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

4.3. General method B – preparation of silybin 23-O-acyl derivatives

Silybin (**1**;100 mg; 0.207 mmol) was dissolved in a CH_3CN/CH_2Cl_2 mixture (1:1, 10 mL, v/v). Acyl chloride (0.207 mmol, 1 equiv) and BF_3•Et_2O (0.062 mL, 0.248 mmol, 50% solution in diethyl ether; v/v) were added and the mixture was stirred for 2 h at 0 °C; then additional $BF_3 \cdot Et_2O$ (0.062 mL, 0.248 mmol, 50% solution in diethyl ether, v/v) was added and the stirring continued for 1 h at room temperature. The mixture was then diluted with a saturated ice-cold solution of $NaHCO_3$ (15 mL) and briefly stirred (approx. 10 min). The solution was extracted with ethyl acetate (2 × 15 mL), the organic phase washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

Flash chromatography on silica gel (chloroform/acetone/formic acid 95:5:1) yielded the corresponding 23-*O*-acyl-silybin (**3a-g**).

4.3.1. 2-[2,3-dihydro-2-(butanoyloxymethyl)-3-(4-hydroxy-3-methoxyphenyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3a**)

Compound **3a** was prepared according to general method B as a white amorphous solid (28%). HR-MS (MALDI) calcd for $C_{29}H_{28}O_{11}$ (M^+): 552.1632, found 552.1615. For ^1H and ^{13}C NMR data see Tables 4 and 5.

4.3.2. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(octanoyloxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3b**)

Compound **3b** was prepared according to general method B as a white amorphous solid (30%). HR-MS (MALDI) calcd for $C_{33}H_{36}O_{11}$ (M^+): 608.2258, found 608.2241. For ^1H and ^{13}C NMR data see Tables 4 and 5.

4.3.3. 2-[2,3-dihydro-2-(dodecanoyloxymethyl)-3-(4-hydroxy-3-methoxyphenyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3c**)

Compound **3c** was prepared according to general method B as a white amorphous solid (30%). HR-MS (MALDI) calcd for $C_{37}H_{44}O_{11}$ (M^+): 664.2884, found 664.2879. For ^1H and ^{13}C NMR data see Tables 4 and 5.

*4.3.4. 2-[2,3-dihydro-2-(hexadecanoyloxymethyl)-3-(4-hydroxy-3-methoxyphenyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3d**)*

Compound **3d** was prepared according to general method B as a white amorphous solid (32%). HR-MS (MALDI) calcd for $C_{41}H_{52}O_{11}$ (M^+): 720.3510, found 720.3511. For ^1H and ^{13}C NMR data see Tables 4 and 5.

*4.3.5. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-((cis-9-octadecenoyl)-oxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3e**)*

Compound **3e** was prepared according to general method B as a yellowish amorphous solid (32%). LRMS (ESI): *m/z* 769.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

*4.3.6. 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-((cis,cis-9,12-octadecadienoyl)-oxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3f**)*

Compound **3f** was prepared according to general method B as a yellowish amorphous solid (29%). LRMS (ESI): *m/z* 767.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

*4.3.7. 2-[2,3-dihydro-2-((cis-11-eicosenoyl)-oxymethyl)-3-(4-hydroxy-3-methoxyphenyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (**3g**)*

Compound **3g** was prepared according to general method B as a yellowish amorphous solid (27%). LRMS (ESI): *m/z* 797.4 ($M + Na^+$). For ^1H and ^{13}C NMR data see Supplementary data.

4.4. Antioxidant activity

4.4.1. DPPH (1,1-diphenyl-2-picrylhydrazyl radical) scavenging

The absorbance change in DPPH (stable radical) was measured in the reaction mixture containing a solution of the compound tested (65 μL, 0–20.0 mM, DMSO/MeOH 1:9, v/v) and 65 μL of DPPH (0.1 mM, DMSO/MeOH 1:9, v/v) at 540 nm for 10 min [46]. The antiradical activity of the compound tested is expressed as the concentration required to reduce the absorbance by 50% (IC_{50}).

4.4.2. Inhibition of microsomal lipid peroxidation

Microsomes were isolated from rat liver of the strain Wistar as described previously [47] and resuspended in 50 mM Tris-HCl buffer with 100 mM KCl and 0.1 mM EDTA (pH 7.4). The protein concentration in the microsomal suspension was determined using the Bradford method. This suspension (400 μL, 0.625 mg protein/mL) was then mixed with the compounds tested (50 μL, 0–100 μM in DMSO) and incubated in the presence of *tert*-butylhydroperoxide (50 μL, 10 mM in DMSO) at 37 °C for 1 h. The products of lipid peroxidation were determined *via* a standard reaction with thiobarbituric acid: addition of ice-cold mixture (0.7 mL) of thiobarbituric acid (26 mM) and trichloroacetic acid (918 mM), heating (90 °C for 15 min), cooling, separation by centrifugation (10 min, 10 000 rpm, 4 °C) and absorbance measurement at 535 nm. The activity was calculated as the concentration of the tested compound that inhibited the color reaction with thiobarbiturate (without the tested compound) by 50 % (IC_{50}).

4.5. Cytotoxicity

4.5.1. Evaluation of cytotoxicities of silybin derivatives on Madin-Darby Canine Kidney (MDCK) cells

MDCK cells were seeded into 96-well culture plate at 1.5×10^4 cells/well in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum and left for 4–5 h. Afterwards the cells were washed twice with Dulbecco's Phosphate-Buffered Salines (D-PBS); then the Opti-MEM I reduced serum medium (Gibco BRL) with 0.2% dimethyl sulfoxide containing respective silybin derivative sample at various concentrations was added to each well and the cell cultures were incubated for 2 h. Then the cells were washed twice with D-PBS, and maintained in D-MEM containing 0.2% BSA for 24 h at 37 °C in 5% CO_2. The cytotoxicity of each silybin derivatives was evaluated by methyltetrazolium (MTT) reduction assay according to manufacture's protocol (Promega).

4.6. Antiviral activity

4.6.1. Evaluation of direct antiviral inhibitory effects of silybin derivatives on influenza A/PR8/34 (H1N1)

Each silybin derivative was mixed with the influenza virus in Opti-MEM I reduced serum medium containing 0.2% DMSO. After being incubated for 30 min at room temperature, the mixed solution was applied to a confluent monolayer of MDCK cells in a 6-well plate (MOI = 2.5×10^{-4}) and incubated for 1 h at room temperature. Then, after the solution was removed from each well, the cell sheets were washed with D-PBS. After that, overlay medium (DMEM containing 0.8% Oxoid agar No.1, 6.0×10^{-4}% trypsin, and 0.2% BSA) was added to each well. After incubating for approx. 2 days at 37 °C in 5% CO_2, the cell sheets were fixed with 5% glutaraldehyde and stained with methylene blue solution. The plaques formed in each well were counted and the inhibitory effects of each sample on virus infection were evaluated.

Acknowledgements

This work was supported by grant KJB400200701 from the Grant Agency of the Academy of Sciences of Czech Republic and by institutional research concept AV0Z50200510 and grants OC08049, LC06010 and MSM6198959216 from the Ministry of Education, Youth and Sports of the Czech Republic.

Supplementary data

Dose response curves for cytotoxicities and antiviral activities of the tested compounds, supporting analytical data (NMR-spectra and HPLC) of the compounds **2a-g** and **3a-g**, and structural characterization of the compounds **2e-g** and **3e-g** are available as supplementary data. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2009.11.056.

References

[1] P. Morazzoni, E. Bombardelli, Fitoterapia 66 (1995) 3–42.
[2] K. Flora, M. Hahn, H. Rosen, K. Benner, Am. J. Gastroenterol. 93 (1998) 139–143.
[3] D. Gallo, S. Giacomelli, C. Ferlini, G. Raspaglio, P. Apollonio, S. Prisley, A. Riva, P. Morazzoni, E. Bombardelli, G. Scambia, Eur. J. Cancer 39 (2003) 2403–2410.
[4] A. Pelter, R. Hänsel, Tetrahedron Lett. 25 (1968) 2911–2916.
[5] H. Wagner, L. Hörhammer, R. Münster, Arzneim. Forsch. 18 (1968) 688–695.
[6] V. Křen, J. Kubisch, P. Sedmera, P. Halada, V. Přikrylová, A. Jegorov, L. Cvak, R. Gebhardt, J. Ulrichová, V. Šimánek, J. Chem. Soc. Perkin Trans. 1 (1997) 2467–2474.
[7] D.Y.-W. Lee, Y. Liu, J. Nat. Prod. 66 (2003) 1171–1174.
[8] R. Gažák, D. Walterová, V. Křen, Curr. Med. Chem. 14 (2007) 315–338.
[9] R. Gažák, A. Svobodová, J. Psotová, P. Sedmera, V. Přikrylová, D. Walterová, V. Křen, Bioorg. Med. Chem. 12 (2004) 5677–5687.
[10] P. Trouillas, P. Marsal, A. Svobodová, J. Vostálová, R. Gažák, J. Hrbáč, P. Sedmera, V. Křen, R. Lazzaroni, J.-L. Duroux, D. Walterová, J. Phys. Chem. A 112 (2008) 1054–1063.
[11] R. Gažák, P. Sedmera, M. Vrbacký, J. Vostálová, Z. Drahota, P. Marhol, D. Walterová, V. Křen, Free Radic. Biol. Med. 46 (2009) 745–758.
[12] R.J. Williams, J.P.E. Spencer, C. Rice-Evans, Free Radic. Biol. Med. 36 (2004) 838–849.
[13] M.C. Comelli, U. Mengs, C. Schneider, M. Prosdocimi, Integr. Cancer Ther. 6 (2007) 120–129.

[14] G. Deep, R. Agarwal, Integr. Cancer Ther. 6 (2007) 130–145.
[15] R.P. Singh, R. Agarwal, Mol. Carcinogen. 45 (2006) 436–442.
[16] R. Braatz, K. Gurler, G. Bergish, G. Halbach, H. Soicke, K. Schmidt, Czech. Patent 273 610, 1985; Chem. Abstr. 105, (1985) P127476b.
[17] G. Pifferi, R. Pace, M. Conti, Il Farmaco 49 (1994) 75–76.
[18] M. Conti, S. Malandrino, M.J. Magistretti, Jpn. J. Pharmacol. 60 (1992) 315–321.
[19] G. Buzzelli, S. Moscarella, A. Gusti, A. Duchini, C. Marena, M. Lampertico, Int. J. Clin. Pharmacol. Ther. Toxicol. 31 (1993) 456–460.
[20] P. Morazzoni, M.J. Magistretti, C. Giachetti, G. Zanolo, Eur. J. Drug Metab. Pharmokinet. 17 (1992) 39–44.
[21] N. Barzaghi, F. Crema, G. Gatti, G. Pifferi, E. Perucca, Eur. J. Drug Metab. Pharmokinet. 15 (1990) 333–338.
[22] R. Carini, A. Comoglio, E. Albano, G. Poli, Biochem. Pharmacol. 43 (1992) 2111–2115.
[23] A. Comoglio, A. Tomasi, S. Malandrino, G. Poli, E. Albano, Biochem. Pharmacol. 50 (1995) 1313–1316.
[24] H. Maheshwari, R. Agarwal, C. Patil, O.P. Katare, Arzneim. Forsch. 53 (2003) 420–427.
[25] A. Valenzuela, A. Garrido, Biol. Res. 27 (1994) 105–112.
[26] P. Letteron, G. Labbe, C. Degott, A. Berson, B. Fromenty, M. Delaforge, D. Larrey, D. Pessayre, Biochem. Pharmacol. 39 (1990) 2027–2034.
[27] T. Parasassi, A. Martellucci, F. Conti, B. Messina, Cell Biochem. Funct. 2 (1984) 85–88.
[28] O. Wesołowska, B. Łania-Pietrzak, M. Kuzdzał, K. Stańczak, D. Mosiądz, P. Dobryszycki, A. Ozyhar, M. Komorowska, A.B. Hendrich, K. Michalak, Acta Pharmacol. Sin. 28 (2007) 296–306.
[29] T. Nakayama, K. Ono, K. Hashimoto, Biosci. Biotechnol. Biochem. 62 (1998) 1005–1007.
[30] K. Abdulmajed, C. McGuigan, C.M. Heard, Free Radic. Res. 39 (2005) 491–498.
[31] H. Kikuzaki, M. Hisamoto, K. Hirose, K. Akiyama, H. Taniguchi, J. Agric. Food Chem. 50 (2002) 2161–2168.
[32] Z. Lu, G. Nie, P.S. Belton, H. Tang, B. Zhao, Neurochem. Int. 48 (2006) 263–274.
[33] Ch.S. Hunneche, M.N. Lund, L.H. Skibsted, J. Nielsen, J. Agric. Food Chem. 56 (2008) 9258–9268.
[34] K. Matsubara, A. Saito, A. Tanaka, N. Nakajima, R. Akagi, M. Mori, Y. Mizushina, Life Sci. 80 (2007) 1578–1585.
[35] J.P. Liu, E. Manheimer, K. Tsutani, C. Gluud, Cochrane Database Syst. Rev. 4 (2001) CD003183.
[36] J.P. Liu, E. Manheimer, K. Tsutani, C. Gluud, Am. J. Gastroenterol. 98 (2003) 538–544.
[37] S.J. Polyak, C. Morishima, M.C. Shuhart, C.C. Wang, Y. Liu, D.Y. Lee, Gastroenterology 132 (2007) 1925–1936.
[38] E. Peterhans, Biochem. Biophys. Res. Commun. 91 (1979) 383–392.
[39] E. Theodosiou, M.H. Katsoura, H. Loutrari, K. Purchartová, V. Křen, F.N. Kolisis, H. Stamatis, Biocat. Biotrans. 27 (2009) 161–169.
[40] N. Gotoh, N. Noguchi, J. Tsuchiya, K. Morita, H. Sakai, H. Shimasaki, E. Niki, Free Radic. Res. 24 (1996) 123–134.
[41] E. Niki, N. Noguchi, Acc. Chem. Res. 37 (2004) 45–51.
[42] V.W. Bowry, K.U. Ingold, Acc. Chem. Res. 32 (1999) 27–34.
[43] R. Gažák, P. Sedmera, M. Marzorati, S. Riva, V. Křen, J. Mol. Catal. B, Enzym. 50 (2008) 87–92.
[44] F.A. Silva, F. Borges, C. Guimares, J.L. Lima, C. Matos, S. Reis, J. Agric. Food Chem. 48 (2000) 2122–2126.
[45] S. Mori, S. Miyake, T. Kobe, T. Nakaya, S.D. Fuller, N. Kato, K. Kaihatsu, Bioorg. Med. Chem. Lett. 18 (2008) 4249–4252.
[46] C. Deby, G. Margotteaux, C.R. Seances Soc. Biol. Fil. 164 (1970) 2675–2681.
[47] H. Haraguchi, T. Saito, N. Okamura, A. Yagi, Planta Med. 61 (1995) 333–336.