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Bioorganic & Medicinal Chemistry 14 (2006) 3793–3810
Bioorganic & Medicinal Chemistry

New derivatives of silybin and 2,3-dehydrosilybin and their cytotoxic and P-glycoprotein modulatory activity

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Received 13 July 2005; revised 11 January 2006; accepted 17 January 2006
Available online 8 February 2006

Abstract—Large series of *O*-alkyl derivatives (methyl and benzyl) of silybin and 2,3-dehydrosilybin was prepared. Selective alkylation of the silybin molecule was systematically investigated. For the first time we present here, for example, preparation of 19-nor-2,3-dehydrosilybin. All prepared silybin/2,3-dehydrosilybin derivatives were tested for cytotoxicity on a panel of drugs sensitive against multidrug resistant cell lines and the ability to inhibit P-glycoprotein mediated efflux activity. We have identified effective and relatively non-cytotoxic inhibitors of P-gp derived from 2,3-dehydrosilybin. Some of them were more effective inhibitors at concentrations lower than a standard P-gp efflux inhibitor cyclosporin A. Another group of 2,3-dehydrosilybin derivatives also had better inhibitory effects on P-gp efflux but a cytotoxicity comparable with that of parent 2,3-dehydrosilybin. Structural requirements for improving inhibitory activity and reducing toxicity of 2,3-dehydrosilybin were established. Effect of E-ring substitution as well as an influence of the substituent size at the C-7-OH position of A-ring on P-gp-inhibitory activity was evaluated for the first time in this study.

1. Introduction

Silymarin obtained from the seeds of *Silybum marianum* (milk thistle) has been used since the time of ancient physicians and herbalists to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis, and jaundice, and to protect the liver against poisoning from chemical and environmental toxins, including snakebites, insect stings, mushroom poisoning, and alcohol. These are typical applications, for which silymarin, its main component silybin (**1**), and their preparations have been used and prescribed until now.[1,2]

Recently, however, silybin received attention due to its alternative beneficiary activities that are not directly bound to its hepatoprotective and/or cytoprotective actions.[3]

One of the most promising activities of this compound is its anticancer activity that results at least partially from its cytoprotective, antioxidant, and chemopreventing

Abbreviations: P-gp, P-glycoprotein; LRP, lung resistance-related protein; LNCaP, androgen dependent prostate cancer cell line; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; VEGR3, VEGF receptor 3; MDR, multidrug resistance; MRP1, multidrug resistance protein 1; DNR, daunorubicin; MTT assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide based cytotoxic assay; TCS, tumor cell survival; CyA, cyclosporin A; EC_{50}, half-maximal effective concentrations; I_{max}, maximum efflux inhibition; NBD, nucleotide-binding domain of P-glycoprotein; CEM, human T-lymphoblastic leukemia cell line CCRF-CEM; CEM-VCR, vincristine resistant CEM cells; CEM-DNR, daunorubicin resistant CEM cells; CEM-DNR 0.3A2, daunorubicin resistant subclone of CEM-DNR cell line; K562, human myeloid leukemia cell line; K-562-Tax, paclitaxel resistant K562 cells; L1210, mouse lymphocytic leukemia cell line; L1210-VCR, vincristine resistant L1210 cells; A549, human lung adenocarcinoma cell line; MCF7, human breast cancer cell line; NHL, normal human lymphocytes.

Keywords: Silymarin; Silybin; Dehydrosilybin; P-glycoprotein inhibition; Cancerostatic.

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doi:10.1016/j.bmc.2006.01.035

properties. Moreover silybin has the potential to be applied as an adjuvant in the anti-cancer chemotherapy since it diminishes carcinogenic potential of a number of chemicals.[4]

However, silybin also acts on the receptor level affecting various processes involved either in cancerogenesis and/or tumor proliferation. Modulations of various mitogenic, signaling, apoptotic, and cell-cycle regulators by silybin and silymarin were observed.[5–13] In a human prostate cancer, silymarin inhibits mitogenic signaling pathways and alters cell cycle regulators, leading to growth inhibition and death of advanced and androgen-independent prostate carcinoma cells.[14,15] Some steroid hormone-dependent tumors are also inhibited by silybin, since both the silymarin and silybin potentiated activity of antiandrogens in androgen dependent prostate cancer cell line LNCaP.[16,17]

Anticarcinogenic and anti-inflammatory effects of silymarin and silybin might be also related to the inhibition of the transcription factor NF-κB, which regulates and coordinates the expression of various genes involved in the inflammatory process, in cytoprotection and carcinogenesis. Silymarin was highly active in the inhibition of NF-κB factor activation by various signals.[18–21] Newly discovered effects of silymarin and silybin, for example, on the cell cycle regulation, apoptosis, nuclear and estrogenic receptors, and many recently reviewed by Křen and Walterová.[3]

Silybin was also investigated as cytoprotective agent and/or sensitizer for anticancer chemotherapy using classical cytostatics such as cisplatin or adriamycin without compromising antitumor activity.[22–24] On the contrary, potentiation of anticancer activity of those drugs by silybin was clearly demonstrated.[22]

One of the plausible explanations for synergistic effects of silybin and anti-cancer drugs is inhibition of efflux function of dominant multidrug resistance (MDR) transporter P-glycoprotein.[25] P-gp is a prominent member of ABC transporters protein family, that has a similar architecture plan comprising four major domains: two membrane bound domains, each with six transmembrane segments, and two cytosolic ATP binding motifs, commonly known as the Walker A and B domains, that bind and hydrolyze ATP (also known as the nucleotide-binding domains, or NBDs). Both ATP binding sites are essential for drug efflux. P-gp binds its substrates in the cytosolic membrane leaflet most likely via H-bond formation and moves them to the extracellular leaflet or to the extracellular aqueous environment at the expense of one to two molecules of ATP.

Modulation or inhibition of P-gp activity can be achieved by: (i) an inhibition of ATP binding, ATP hydrolysis or coupling of ATP hydrolysis to the translocation of substrates, (ii) inhibition of conformational changes required for drug extrusion via antibody binding to certain extracellular loops of the transporter, and (iii) non-competitive or competitive inhibition of P-gp achieved by direct interaction of a compound with one or more binding sites on P-gp.[26]

Besides ABC transport proteins there is another MDR mechanism represented for instance by the lung resistance-related protein (LRP). It is located intracellularly in vaults and transports toxic agents from the nucleus of neoplastic cells to the endosomal compartments.[27,28]

Silymarin increased daunomycin accumulation in P-gp-positive cells, but not in P-gp-negative cells, in a drug concentration and P-gp expression level-dependent manner.[29] Silymarin potentiated doxorubicin cytotoxicity in P-gp-positive cells, while it inhibited P-gp ATPase activity and azidopine photoaffinity labeling of P-gp, suggesting a direct interaction with P-gp substrate binding.[29] The 1.5 h accumulation of digoxin and vinblastine in Caco-2 cells was significantly increased by silymarin, and this effect was concentration dependent.[30] In the human prostate carcinoma DU145 cells, silybin potentiated doxorubicin-induced growth inhibition and apoptosis.[31] This P-gp inhibitory activity was pronounced in the 2,3-dehydrosilybin (**2**) derivatives carrying prenyl or geranyl substituents.[32] More recently, the capacity of silybin derivatives to chemosensitize drug-resistant cells with specific MDR transporter profile has been investigated.[33] In addition to P-gp, some flavonoids related to silybin were found to inhibit another important MDR-associated protein, the multidrug resistance protein 1 (MRP1).[33,34]

The aim of our study was to investigate in vitro anticancer and P-gp inhibitory activity of oxidized and selectively alkylated silybin derivatives in order to identify the potential anti-cancer and/or chemosensitizing compounds. Selective alkylation of the silybin hydroxyls was systematically investigated and a series of novel silybin and 2,3-dehydrosilybin derivatives were prepared (see Chart 1).

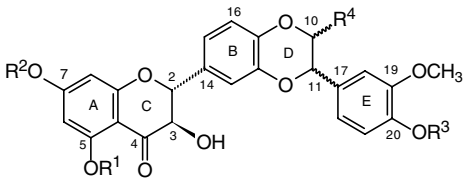
2. Results and discussion

2.1. Chemistry

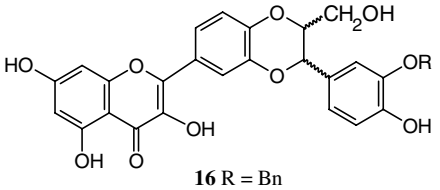
Silybinic acid (**3**) and 2,3-dehydrosilybinic acid (**4**) were prepared by CrO_3/H_5IO_6 oxidation of selectively acetylated derivatives of silybin (**1**) and 2,3-dehydrosilybin (**2**) in acetonitrile followed by deacetylation of the oxidized products.[35]

2.1.1. Preparation of monoacyl derivatives of silybin. Monoacylations of silybin (**1**) catalyzed by $BF_3{\cdot}Et_2O$ in CH_2Cl_2/CH_3CN led to 7-*O*-benzoylsilybin (**6**) and 23-*O*-pivaloylsilybin (**7**) (Scheme 1).

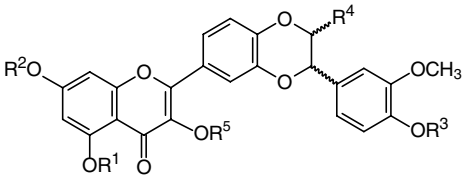
2.1.2. Preparation of silybin benzyl- and methyl ethers. Selective alkylation of the C-7 phenolic hydroxyl group of silybin was accomplished by refluxing **1** with benzyl bromide or methyl iodide in acetone in the presence of K_2CO_3; corresponding 7-*O*-benzylsilybin (**5**) or 7-*O*-methylsilybin (**10**) was formed. 20-*O*-Methylsilybin (**12**) was prepared by methylation of **1** with methyl io-



	R^1	R^2	R^3	R^4
1	H	H	H	CH_2OH
3	H	H	H	COOH
5	H	Bn	H	CH_2OH
6	H	Bz	H	CH_2OH
7	H	H	H	CH_2OPiv
10	H	Me	H	CH_2OH
12	H	H	Me	CH_2OH
19	H	Me	Me	CH_2OH
23	Me	Me	Me	CH_2OH

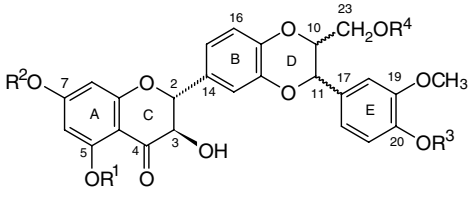


16 R = Bn
17 R = H



	R^1	R^2	R^3	R^4	R^5
2	H	H	H	CH_2OH	H
4	H	H	H	COOH	H
8	H	Bn	H	CH_2OH	H
9	H	H	H	CH_2OH	Me
11	H	Me	H	CH_2OH	H
13	H	H	Me	CH_2OH	H
18	H	Me	H	CH_2OH	Me
20	H	Me	Me	CH_2OH	H
21	H	H	Me	CH_2OH	Me
22	H	Me	Me	CH_2OH	Me
24	Me	Me	Me	CH_2OH	H

Chart 1.



1 $R^1 = R^2 = R^3 = R^4 = H$

a - g

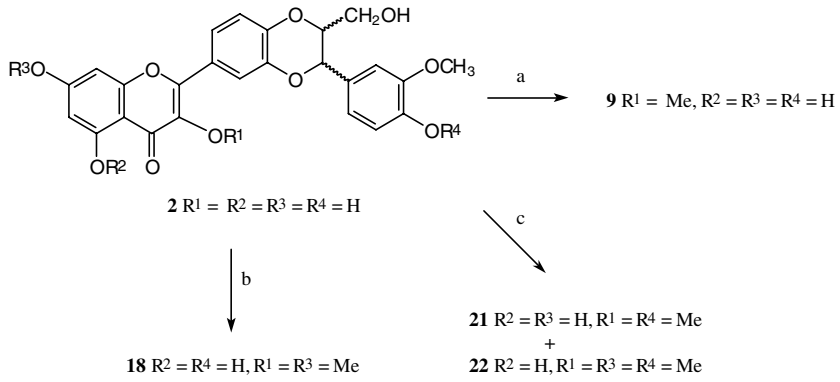
Compound (procedure)	R^1	R^2	R^3	R^4
5 (a)	H	Bn	H	H
6 (b)	H	Bz	H	H
7 (c)	H	H	H	Piv
10 (d)	H	Me	H	H
12 (e)	H	H	Me	H
19 (f)	H	Me	Me	H
23 (g)	Me	Me	Me	H

Scheme 1. Reagents and conditions: (a) BnBr, K_2CO_3, acetone, reflux, 3.5 h, 81%; (b) PhCOCl, $BF_3{\cdot}Et_2O$, CH_2Cl_2/CH_3CN (1:1, v/v), rt, 42 h, 90%; (c) $(CH_3)_3COCl$, $BF_3{\cdot}Et_2O$, CH_2Cl_2/CH_3CN (1:1, v/v), rt, 12 h, 60%; (d) MeI, K_2CO_3, acetone, reflux, 3.5 h, 53%; (e) MeI (1.2 equiv), NaH (3 equiv), DMF, rt, 2 h, 54%; (f) MeI (2 equiv), NaH (3 equiv), DMF, 1 h at 0 °C, then 1 h at rt, 21%; (g) dimethyl sulfate, K_2CO_3, acetone, reflux, 2 h, 56%.

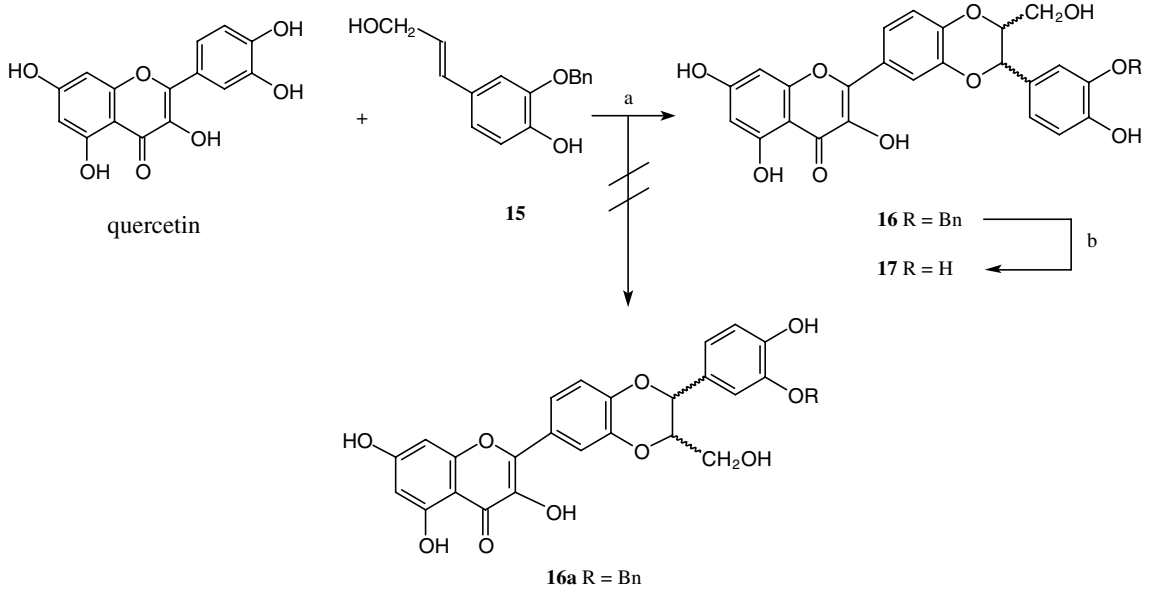
dide (1 equiv) in the presence of an excess of NaH in DMF at 0 °C. The reaction of **1** with 2 equiv of methyl iodide under the same conditions led to 7,20-di-*O*-methylsilybin (**19**). 5,7,20-Tri-*O*-methylsilybin (**23**) formation was achieved by the action of dimethyl sulfate and K_2CO_3 on silybin in boiling acetone. For all described alkylation methods of silybin, see Scheme 1.

2.1.3. Preparation of 2,3-dehydrosilybin methyl ethers by direct alkylation of 2. 3-*O*-Methyl-2,3-dehydrosilybin (**9**) was prepared analogously as 20-*O*-methylsilybin (**12**) by methylation of 2,3-dehydrosilybin (**2**) with methyl iodide (1 equiv; NaH, DMF, 0 °C). Selective formation of 3,7-di-*O*-methyl-2,3-dehydrosilybin (**18**) was observed in the reaction of **2** with dimethyl sulfate and K_2CO_3 in DMF at room temperature. The alkylation of **2** with methyl iodide and KOH in DMSO afforded 3,20-di-*O*-methyl-2,3-dehydrosilybin (**21**) and 3,7,20-tri-*O*-methyl-2,3-dehydrosilybin (**22**) (Scheme 2).

2.1.4. Preparation of 19-*O*-demethyl-2,3-dehydrosilybin. The preparation of 19-*O*-demethyl-2,3-dehydrosilybin (**17**) was based on the radical-coupling reaction of quercetin with (*E*)-3-*O*-benzyl-3,4-dihydroxy-cinnamyl alcohol (**15**) (Refs. 36,37) and followed catalytic hydrogenolysis of the benzyl group at C-19 of **16** (Scheme 3). Compound **15** can couple to quercetin in two possible ways, leading either to silybin- (**16**) or isosilybin-type (**16a**) products. Because of poor solubility of compound **17**, it was converted to its peracetate. ^1H and ^{13}C NMR spectra of this substance were experimentally (COSY, ROESY, TOCSY, HMQC, and gHMQC)



Scheme 2. Reagents and conditions: (a) MeI (1.1 equiv), NaH (1.2 equiv), DMF, 1.5 h at 0 °C, then 12 h at rt, 45%; (b) dimethyl sulfate, K_2CO_3, DMF, rt, 12 h, 32%; (c) MeI, KOH, DMSO, rt, 1 h, 25% of **21** and 24% of **22**.



Scheme 3. Reagents and conditions: (a) Ag_2CO_3 (1 equiv), benzene/acetone (2:1, v/v), reflux, 36 h, 10%; (b) H_2–Pd/C, THF, 12 h, 96%.

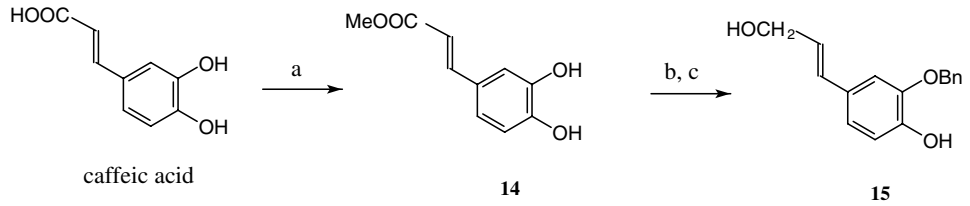
fully assigned. According to the coupling of H-11 to C-12a, observed by gHMQC, this compound belongs to the 2,3-dehydrosilybin series (the coupling of H-11 to C-16a would indicate an isosilybin analogue).

Published yields of an analogous reaction of taxifolin or luteolin with coniferyl alcohol are usually higher (40–70%). However, both the above flavonoids are considerably weaker radical scavengers than quercetin.[38] Indeed, we have observed the formation of side products, related to quercetin radical degradation (e.g., quercetin quinone). Thus, the main reaction is not radical coupling of quercetin with **15** but an elimination of radical species generated from quercetin leading to its degradation. As a proof of this speculation behavior of both starting compounds during reaction can serve, where quercetin is completely consumed, but **15** remains present in the reaction mixture (even after an addition of an excess of the fresh quercetin). Partial radical decomposition of the product formed could be another reason for its low yield (due to its rather high antiradical activity).

Moreover, Merlini and Zanarotti[39] reported the self-coupling reaction of some derivatives of coniferyl alcohol. Thus, the result of this radical-coupling reaction strongly depends on the reactivity of both starting compounds; the best yields are achieved when their reactivity is almost the same, which is not our case.

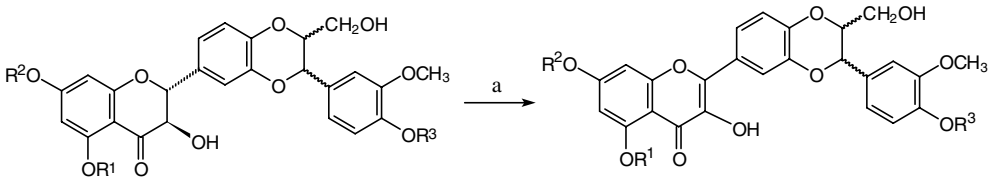
The starting (*E*)-3-*O*-benzyl-3,4-dihydroxycinnamyl alcohol (**15**) was prepared from the commercially available caffeic acid by its esterification leading to the methyl ester **14**, followed by the selective benzylation of the hydroxyl group at C-3 and finally the reduction of benzylated methyl ester to the respective alcohol **15** by $LiAlH_4$ (Scheme 4).

2.1.5. Oxidation of silybin derivatives to 2,3-dehydrosilybin derivatives. All silybin derivatives with free C-3 hydroxyl group can be oxidized to the corresponding 2,3-dehydrosilybin derivatives using the iodine in glacial acetic acid in the presence of potassium acetate[40] (Scheme 5)—7-*O*-benzyl-2,3-dehydrosilybin (**8**), 7-*O*-



caffeic acid **14** **15**

Scheme 4. Reagents and conditions: (a) MeOH, H_2SO_4, rt, 12 h, 98%; (b) BnCl (1.2 equiv), NaH (2.0 equiv), DMSO, rt, 1 h; (c) $LiAlH_4$, THF, 1 h at 0 °C, then 3 h at rt, 42%.



5 R^1 = R^3 = H, R^2 = Bn
10 R^1 = R^3 = H, R^2 = Me
12 R^1 = R^2 = H, R^3 = Me
19 R^1 = H, R^2 = R^3 = Me
23 R^1 = R^2 = R^3 = Me

8 R^1 = R^3 = H, R^2 = Bn
11 R^1 = R^3 = H, R^2 = Me
13 R^1 = R^2 = H, R^3 = Me
20 R^1 = H, R^2 = R^3 = Me
24 R^1 = R^3 = R^2 = Me

Scheme 5. Reagents and conditions: (a) I_2, glacial AcOH, anhydrous AcOK, reflux for 4 h, then EtOH/HCl, reflux for 1 h, 46–90%.

methyl-2,3-dehydrosilybin (**11**), 20-*O*-methyl-2,3-dehydrosilybin (**13**), 7,20-di-*O*-methyl-2,3-dehydrosilybin (**20**), and 5,7,20-tri-*O*-methyl-2,3-dehydrosilybin (**24**) were prepared by this way.

2.1.6. Structure determination. Since the starting natural silybin was a nearly equimolar mixture of the A and B isomers, some NMR signals were doubled (Tables 1 and 2). The correct proton signal multiplicity was therefore obtained from HOM2DJ and COSY. Fortunately, the chemical shift differences were in the ppb (part per billion) range so that common crosspeaks for both isomers were usually observed in the heterocorrelated experiments. This problem does not exist with 2,3-dehydrosilybins as the signals of both stereoisomers are undistinguishable. The unambiguous NMR signal assignment is essential for the structure elucidation. Therefore, the measurements were mostly performed in DMSO, in which the observation of OH signals is possible. The assignment of all hydroxyl groups represents an indirect way of the site of substitution determination: it provides the information on sites that are not affected. Some substituents (OCH_3 and OCH_2Ph) offer direct means of their localization: NOE to their neighbor protons (OCH_3), H,H-long range couplings (OCH_3, OCH_2Ph), $^3J_{C,H}$ (substituents' protons to the carbon at the site of substitution, Fig. 1) or even $^4J_{C,H}$ (AcO protons to the carbon at the site of substitution; seen in the gHMQC experiment only).

Except for acetates, the position of other acyls was deduced from the chemical shift changes in the assigned spectra. The HMBC experiment also resolves ambiguities arising during the assignment in **1** and **2** derivatives (C-2 vs C-3; H-6 vs H-8; H-13, H-15, H-16 vs H-18, H-21, H-22; C-12a vs C-16a; C-19 vs C-20; C-4 vs carbonyls in the **2**-series).

2.2. Biological studies

Analysis of the P-gp efflux activity in the presence or absence of silybin and 2,3-dehydrosilybin derivatives was based on flow cytometry measurement of content of the intracellular daunorubicin (DNR) (Fig. 2), a fluorescent substrate of P-gp pump.[41,42] Herein, we show that selected silybin derivatives effectively inhibited the P-gp mediated efflux of DNR from drug resistant and exclusively P-gp expressing K562-tax cells. The inhibition was concentration-dependent and displayed a classical sigmoidal dose–response curve (Fig. 3), which suggests a competitive inhibition with a constant concentration of DNR. The 50% effective drug concentration (EC_{50}) for silybin derivatives ranged from 0.1 to 20.1 μM, and thus in selected cases exceeded potency of standard P-gp inhibitor cyclosporin A (4.09 μM) (Fig. 3).

According to P-gp inhibitory activity of the compounds tested (Fig. 3), we can classify them into the following categories: (a) strong inhibitors: compounds **8**, **9**, **13**, **18**, **20**, and **22** (I_{max} > 40% and EC_{50} < 2 μM). Those are compounds, which reach at least 50% inhibition activity of CyA (79%) at concentration lower than half of the CyA EC_{50} value (4.09 μM). Compound **9** is included due to extremely high I_{max} (130%) at comparable EC_{50} as CyA; (b) medium inhibitors represented by the compounds **2**, **7**, and **21** (I_{max} > 40% and EC_{50} > 2 μM) and (c) the compounds without significant inhibitory activity (**1**, **5**, **11**, **17**, and **24**; I_{max} < 40%). Biological activity of compounds **3**, **4**, and **6** was difficult to analyze or interpret due to solubility problems.

Table 1. ^{1}H NMR data (399.89 MHz, 30 °C) of silybin derivatives

Proton	**5**[a]	**6**[a]	**7**[a]	**10**[b]	**12**[b]	**19**[b]	**23**[b]
2	5.002 d (11.8)	5.090 d (12.2)	4.984 d (11.9)	5.136 d (11.4)	5.080d (11.3)	5.139 d (11.4)	5.046 d (11.1)
3	4.549 d (11.9)	4.632 d (12.2)	4.543 d (11.9)	4.668 dd (11.4, 5.4)	4.605 dd (11.3, 6.2)	4.671 dd (11.4, 6.3)	4.381 dd (11.1, 4.8)
	4.536 d (11.9)	4.619 d (12.2)	4.526 d (11.9)	4.655 dd (11.4, 5.4)	4.596 dd (11.3, 6.2)	4.660 dd (11.4, 6.3)	4.369 dd (11.1, 4.8)
6	6.199 d (2.2) 6.201 d (2.2)	6.516 d (2.1)	6.035 d (2.2) 6.033 d (2.2)	6.188 d (2.2)	5.915 d (2.1)	6.120 d (2.3)	6.223 d (2.3)
8	6.115 d (2.2) 6.127 d (2.2)	6.461 d (2.1) 6.447 d (2.1)	5.967 d (2.2) 5.958 d (2.2)	6.100 d (2.2) 6.094 d (2.2)	5.873 d (2.1) 5.868 d (2.1)	6.099 d (2.3) 6.093 d (2.3)	6.177 d (2.3) 6.171 d (2.3)
10	4.064 m	4.062 ddd (8.3, 4.1, 2.6)	4.236 ddd (8.1, 4.1, 3.0)	4.176 ddd (7.9, 4.7, 2.5)	4.204 ddd (7.8, 4.0, 2.5)	4.206 ddd (7.7, 4.6, 2.5)	4.199 ddd (7.8, 4.8, 2.6)
	4.049 m	4.057 ddd (8.3, 4.1, 2.6)	4.227 ddd (8.1, 4.1, 3.0)	4.166 ddd (7.9, 4.7, 2.5)	4.189 ddd (7.8, 4.0, 2.5)	4.196 ddd (7.7, 4.6, 2.5)	4.190 ddd (7.8, 4.8, 2.6)
11	4.962 d (8.3)	4.969 d (8.3)	4.894 d (8.1) 4.887 d (8.1)	4.915 d (7.9)	4.967 d (7.8)	4.974 d (7.7)	4.969 d (7.8)
13	7.210 d (1.8) 7.188 d (1.9)	7.217 d (2.0) 7.199 d (2.0)	7.183 d (2.0) 7.170 d (2.0)	7.099 d (1.8) 7.092 d (1.8)	7.093 d (2.1) 7.087 d (2.1)	7.111 d (2.2) 7.105 d (2.2)	7.081 d (1.9) 7.072 d (1.9)
15	7.098 dd (8.3, 1.9) 7.079 dd (8.3, 1.8)	7.111 dd (8.3, 2.0) 7.097 dd (8.3, 2.0)	7.080 dd (8.5, 2.0) 7.069 dd (8.5, 2.0)	7.028 dd (8.3, 1.8)	7.011 dd (8.3, 2.1)	7.035 dd (8.3, 2.2) 7.031 dd (8.3, 2.2)	7.017 dd (8.3, 1.9) 7.010 dd (8.3, 1.9)
16	7.058 d (8.3) 7.053 d (8.3)	7.066 d (8.3) 7.059 d (8.3)	7.031 d (8.3) 7.023 d (8.3)	6.977 d (8.3) 6.974 d (8.3)	6.984 d (8.3)	6.983 d (8.3) 6.980 d (8.3)	6.971 d (8.3) 6.967 d (8.3)
18	6.942 d (1.8)	6.939 d (1.4)	6.878 d (2.0) 6.873 d (2.0)	7.014 d (2.0)	7.051 m	7.056 m	7.058 d (2.0) 7.056 d (2.0)
21	6.971 m	6.961 m	6.950 d (7.7) 6.948 d (7.7)	6.803 d (8.1)	6.977 d (8.3) 6.973 d (8.3)	7.007 d (8.1)	6.983 d (8.1)
22	6.946 m	6.961 m	6.893 dd (7.7, 2.0) 6.891 dd (7.7, 2.0)	6.866 dd (8.1, 2.0)	7.021 dd (8.3, 2.1)	6.987 dd (8.1, 2.0)	7.004 dd (8.1, 2.0)
23d	3.811 dd (12.5, 2.8) 3.809 dd (12.5, 2.8)	3.815 dd (12.2, 2.6)	4.395 dd (12.3, 3.0) 4.383 dd (12.3, 3.0)	3.546 ddd (12.2, 3.4, 2.5)	3.550 dm (11.7)	3.554 ddd (12.3, 5.1, 2.5)	3.550 ddd (12.3, 5.2, 2.6)
23u	3.569 dd (12.5, 4.0)	3.579 dd (12.2, 4.1) 3.565 dd (12.2, 4.1)	3.934 dd (12.3, 4.1) 3.920 dd (12.3, 4.1)	3.351 ddd (12.2, 4.7, 3.4)	3.340 m	3.351 ddd (12.3, 5.7, 4.6)	3.347 ddd (12.3, 5.2, 4.8)
5-OMe				— —	— —	— —	3.798 s 3.795 s
7-OMe				3.788 s 3.787 s	— —	3.789 s 3.787 s	3.798 s —
19-OMe	3.962 s	3.926 s	3.907 s	3.780 s	3.774 s	3.776 s	3.774 s
20-OMe				— —	3.767 s 3.765 s	3.770 s 3.768 s	3.768 s 3.765 s
3-OH				5.890 d (5.4)	5.798 d (6.2)	5.854 d (6.3)	5.322 d (4.8)
5-OH	11.171 s 11.166 s	11.106 s 11.109 s	11.197 s	11.841 s	11.875 s	11.844 s	—
7-OH				—	—	—	—
20-OH	5.782 s	5.740 s	5.749 s	9.122 s	—	—	—
23-OH				4.934 t (3.4)	—	4.954 dd (5.7, 5.1)	4.950 dd (5.2, 5.2)

[a] $CDCl_3$.

[b] DMSO-d_6. Additional signals—**5**: 5.081 (2H, m, $-CH_2-$), 7.386 (5H, m, Ph); **6**: 7.523 (2H, m, 2× *meta*-Ph), 7.663 (1H, m, *para*-Ph), 8.166 (2H, 2× *ortho*-Ph); **7**: 1.221 (9H, s, CMe_3), 1.223 (18H, s, 2× CMe_3).

Table 2. ^{13}C NMR data (100.55 MHz, 30 °C) of silybin derivatives

Carbon	**5**[a]	**6**[a]	**7**[a]	**10**[b]	**12**[b]	**19**[b]	**23**[b]
2	83.07 83.09	83.22 83.18	83.05 83.02	82.68 82.72	82.63 82.59	82.71 82.67	82.18
3	72.32 72.40	72.73 72.65	72.34 72.30	71.57 71.51	71.54 71.48	71.58 71.52	72.55 72.48
4	195.89 195.90	197.42 197.40	195.75 195.74	198.30	197.73 197.71	198.33	190.13
4a	100.98	104.14	100.83 100.82	101.40	100.52	101.40	103.60
5	163.61 163.62	162.89 162.87	163.84 163.83	163.03	163.37	163.04	161.70
6	96.41	103.77	97.13 97.11	94.95	96.16	94.96	93.00
7	167.92	159.58	165.62 165.61	167.61	166.94	167.61	165.74
8	95.38 95.40	102.29	95.96 95.94	93.87	95.11	93.87	93.63
8a	162.90	162.42 162.40	163.17 163.16	162.41	162.54 162.52	162.41	163.76
10	78.34 78.37	78.39 78.36	76.05 76.02	78.14	78.12 78.09	78.05	78.07
11	76.39 76.44	76.43 76.39	76.63	75.85	75.75	75.70	75.70
12a	143.94 143.97	143.98 143.96	143.78	143.28 143.26	143.23 143.21	143.69 143.66	143.18 143.15
13	116.46 116.61	116.64 116.52	116.54 116.40	116.65 116.58	116.67 116.58	116.67 116.61	116.53 116.43
14	129.46 129.49	129.02	129.33 129.32	129.94 129.89	130.18 130.14	129.99 129.94	130.29 130.24
15	121.15	121.12 120.92	121.25 121.08	121.33 121.17	121.42 121.27	121.39 121.25	121.20 121.01
16	117.20 117.32	117.35 117.25	117.46 117.36	116.37 116.32	111.74	116.40 116.36	116.39 116.34
16a	144.14 144.17	144.27 144.24	144.13 144.12	143.71 143.68	143.69 143.67	143.19 143.18	143.56 143.53
17	127.86	127.84	127.53 127.52	127.53	129.10	129.08	129.11
18	109.56	109.61 109.58	109.49	111.82 111.76	111.34 111.29	111.31 111.26	111.30 111.26
19	146.97	146.98 146.97	147.05 147.04	147.67	149.22	149.19	149.18
20	146.48	146.49	146.64	147.07	148.88	148.84	148.85
21	114.67 114.68	114.69 114.68	114.80 114.79	115.36	116.44 116.39	111.70	111.70
22	120.83 120.89	120.84	120.86	120.54	120.34	120.28	120.29
23	61.70	61.91	62.71	60.21	60.21	60.17	60.18
5-OMe	—	—	—	—	—	—	55.99
7-OMe	—	—	—	55.97	—	55.98	55.79
19-OMe	56.08 56.07	56.08 56.07	56.04 56.03	55.75 —	55.68 —	55.64 —	55.64 —
20-OMe	—	—	—	—	55.63	55.59	55.59

[a] $CDCl_3$.

[b] DMSO-d_6. Additional signals—**5**: 70.48 (–CH_2–), 127.43 (2× *ortho*-Ph), 128.40 (*para*-Ph), 128.74 (2× *meta*-Ph), 135.56 (*ipso*-Ph); **6**: 128.70 (2× *meta*-Ph), 130.32 (2× *ortho*-Ph), 134.06 (*para*-Ph), 163.88 (C═O); **7**: 27.18 (3× Me), 38.97 (C), 178.25 (*piv*-CO).

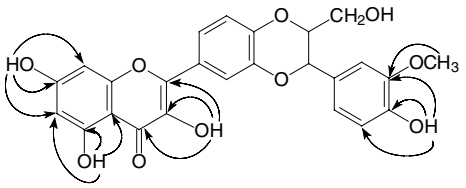


Figure 1. Diagnostic heteronuclear couplings in **2** observed by HMBC.

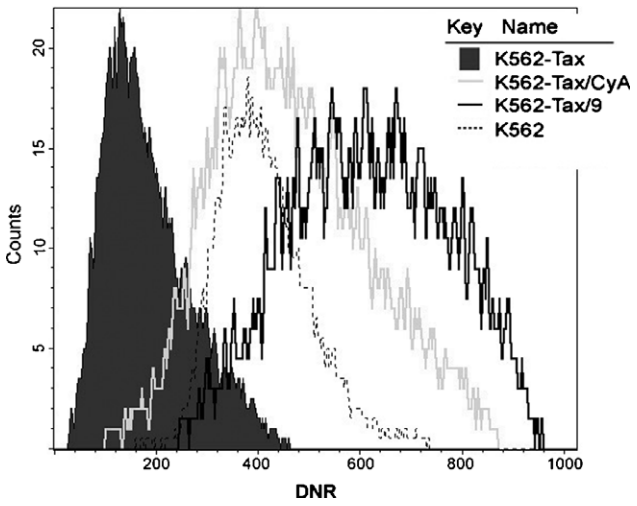


Figure 2. The effects of CyA (6.25 μM) and 2,3-dehydrosilybin derivative 9 (6.25 μM) on inhibition of P-gp function measured as daunorubicin efflux in K562-Tax cells. Note increased accumulation of daunorubicin in CyA/**9** treated cells in comparison with cells with no added inhibitor. Maternal K562 cells are included for illustration.

A comparison of the P-gp inhibitors at one given concentration enables to define the order of their inhibition activity. Thus, the sequence of the activities for good inhibitors is **22** > **13** > **8** > **20** ≈ **18** (at the arbitrary reference concentration 1 μM). Although the compound **9** is a strong inhibitor (I_{max} = 130%) it exhibited a biological activity at higher EC_{50} value (4.8 μM), so it cannot be compared with the rest of the compounds at the reference concentration. The order of inhibitory activity of the second group of compounds was determined as **21** > **2** > **7** (at the arbitrary reference concentration 1 μM).

A relatively high intrinsic cytotoxicity at doses close to P-gp inhibitory activity of many effective MDR cell modulators is a major obstacle of their therapeutic use. Results of the in vitro cytotoxicity measurements using the MTT assay (Table 5) showed that the drug concentration lethal to 50% of the cells (TCS_{50}) ranged from 2.44 to 250 μM in our compounds. Preferential activity against tumor cell lines compared to resting normal human lymphocytes was demonstrated, which is an important observation with potential impact for further development (Table 5).

Tested compounds can be further categorized based on their intrinsic cytotoxicity (Table 5) into two basic groups: (i) The compounds toxic for both the tumor cell lines and also for the normal human lymphocytes (**2**, **5**, **8**, **9**, **11**, and **18**) and (ii) the compounds selectively toxic to tumor cells but not for the normal human lymphocytes (**13**, **17**, **20**, **21**, **22**, and **24**). Interestingly, the compound **22** displayed a substantially higher cytotoxicity in MDR cells compared to drug sensitive counterparts, with the only exception of Cem-VCR cell line for which was compound **22** rather non-toxic (Table 5). Interestingly, drug resistant subline CEM-VCR is the most LRP expressing cell line in our collection[43] and this may suggest that LRP mediated mechanisms of MDR participate in resistance to compound **22**. Finally, no significant cytotoxicity was found for silybin itself.

Concluding biological part of experimentation, we have identified new silybin based substances as effective P-gp inhibitors with no or low cytotoxic capacity against normal human lymphocytes (**7**, **13**, **20**, **21**, and **22**). Importantly, all these compounds (except **7**) are highly potent inhibitors at lower doses than the lowest TCS_{50} value on the most sensitive cell line. In contrast, the compounds **2**, **8**, **9**, and **18** are effective inhibitors of P-gp efflux but they also exhibit significant intrinsic cytotoxicity against all tested cell lines including normal human lymphocytes.

In accordance with reported findings,[25,44] our results demonstrate the importance of double bond in the positions C-2 and C-3 of C ring (**1** vs **2** or **5** vs **8**) for the P-gp-inhibitory activity of flavonoids. Further important structural motifs for this activity in simple flavones are as follows: the presence of free C-3-OH group of ring C and C-5-OH group of ring A in close proximity to the carbonyl group in the position C-4 of ring C was found responsible for high binding affinity to mouse P-gp NBDs (Ref. 45), while free C-7-OH group did not produce any effect. On the contrary, we demonstrated that the substitution of the position C-3-OH of 2,3-dehydrosilybin (**2**) by methyl group led in almost all cases (**9**, **18**, **21**, and **22**) to a significant improvement of in vitro anti-cancer activity and capacity to block functional activity of P-gp (compound **21** exhibits a slightly lower activity than **2** but at 3.5 times lower concentration). A substitution of C-3-OH position does not reduce cytotoxicity of these compounds (**2** vs **9**) but the methylation of C-20-OH decreases cytotoxicity to a great extent (**9** vs **21**), while the blocking of C-3-OH together with C-7-OH by methyl groups does not show this effect (**9** vs **18**).

Effects of A-ring substitution in the compound **2** (mainly its C-6 and C-8 positions) to the binding affinity to P-gp transporters were extensively studied in recent years. An introduction of either geranyl- or prenyl-group to the positions C-6 or C-8 of **2** led to significant increase in binding affinity toward recombinant NBD2 of mammalian MDR cancer cells.[25] The same authors showed that geranylation exhibits higher effect on the binding affinity than introduction of a smaller prenyl group and that the substitution of C-8 position had stronger effect on the binding to the NBD2 than a substitution of the C-6 one. Similar effect on binding affinity to NBD of *Leishmania tropica* P-gp-like transporter was also demonstrated but the prenyl group was more influential than

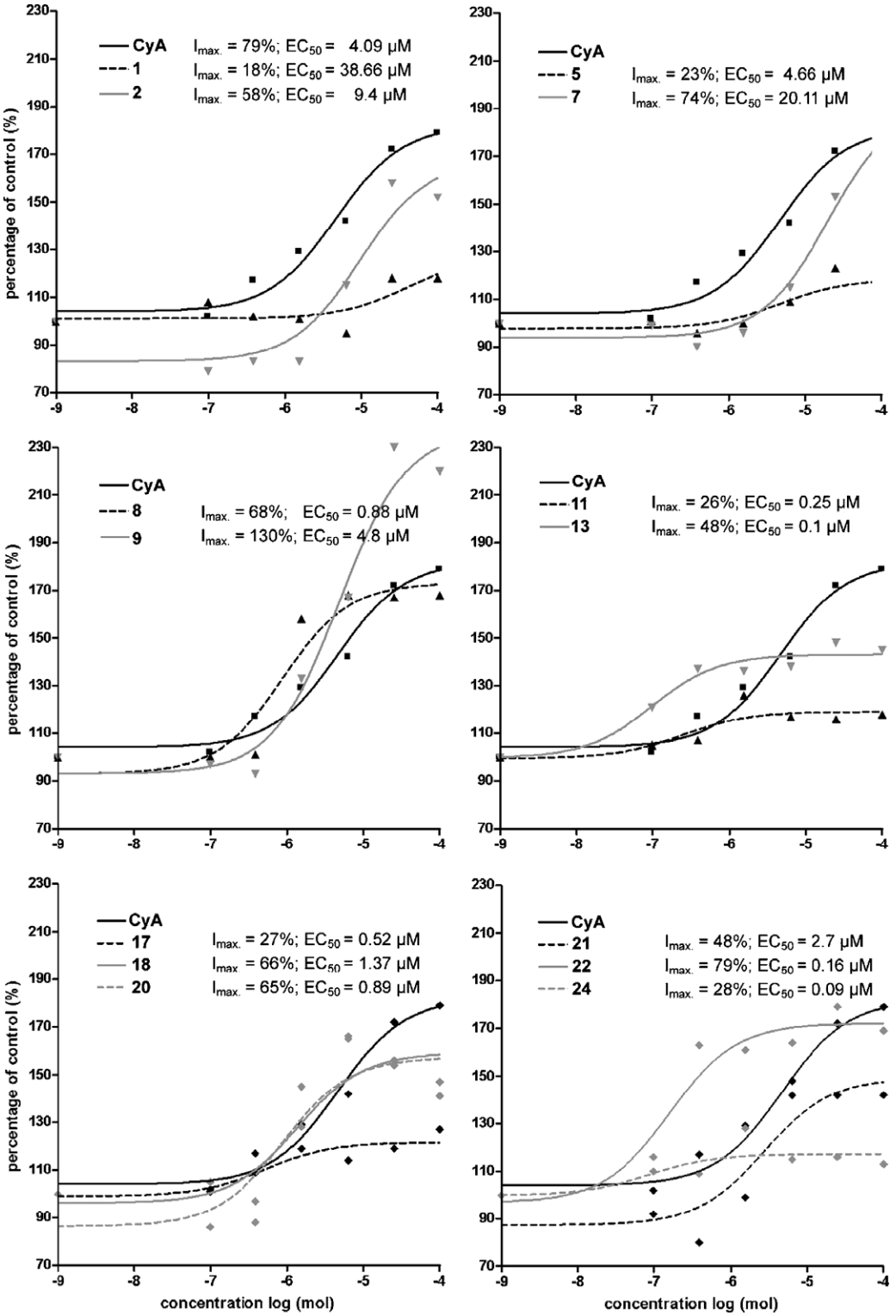


Figure 3. Inhibition of P-gp mediated daunorubicin efflux in K562-Tax cells by silybin/2,3-dehydrosilybin derivatives compared to reference inhibitor cyclosporin A. Data are expressed as the percentage of relative fluorescence intensity in untreated cells. The average number of cells per analysis was 5000.

the geranyl group in this case. (Ref. 44) However, an explanation of higher binding affinity of these compounds compared to **2** consists in a potentiation of their interaction with hydrophobic steroid-interacting region vicinal to the ATP-binding site of cytosolic NBD due to the presence of hydrophobic residue at the A-ring.[45] Flavonoids inhibit a number of ATP-utilizing enzymes by competitive interaction at their ATP site[46] but the

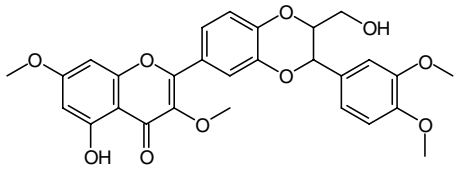
prenylation (or geranylation) of their A-ring significantly increases their selectivity to P-gp-transporter, which results in the lowering of their cytotoxicity.[44]

Based on these findings we investigated the effect of 2,3-dehydrosilybin alkylation in the position C-7-OH, which is between two positions critical for the activity (C-6 and C-8). Results obtained were somewhat controversial: introduction of methyl group led to diminished P-gp inhibition (**2** vs **11**), while a substitution by the benzyl group increased the activity (**2** vs **8**). The cytotoxicity of 7-*O* alkyl derivatives was comparable with the cytotoxicity of **2** but it was reduced by additional substitution of C-20-OH (**8**, **11**, and **18**—toxic vs **20**, **22**, and **24**—non-toxic).

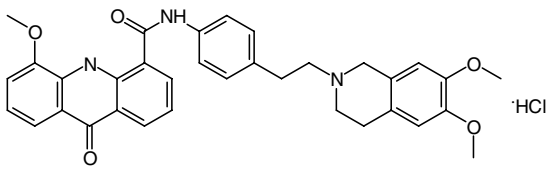
These results indicate that free C-7-OH position is critical for the binding to ATP-site of the NBD: its blocking even by a small methyl group in **11** deteriorates this affinity (lower inhibitory activity and similar cytotoxicity compared to those of **2**), while introduction of a spatially bulkier benzyl group in **8**, which can probably reach the hydrophobic binding site (similarly as C-6 and C-8 alkylated derivatives), leads to increasing of the inhibitory activity of **2**. Nevertheless, an improvement of inhibitory activity of 7-*O* methyl derivatives was observed in the cases when the second methyl group was present in the structure (**11** vs **18** and **20**). Additional increase in the activity was achieved in the case of 3,7,20-tri-*O*-methyl derivative **22**, which was the best inhibitor from our tested series.

This effect can be well explained by comparing the structure of 2,3-dehydrosilybin (**2**) with that of another P-gp modulator GF 120918 that is at present already under clinical testing.[47,48] Rather high structural resemblance of this compound with **2** that is further potentiated by its methylation in positions 3, 7, and 20 well correlates with the favourable effect of these modifications. The very best fit gives our best dehydrosilybin derivative **22**—Chart 2 (for molecular modeling of the compounds involved, see also Supplementary material to this paper).

Interestingly, benzylation of C-7-OH of **1** (yielding **5**) changed the toxicity of this non-toxic compound to the level of highly toxic **2** but the effect on P-gp inhibition activity was less remarkable (approximately the same value of I_{max} but at 8 times lower concentration than **1**).



22



GF 120918

Chart 2.

We have also observed the effect of C-5-OH substitution: P-gp inhibitory activity of **24** was markedly lowered compared to compound **20** (which is consistent with the observation of Conseil et al.[45]) while the influence on the cytotoxicity was not so significant.

Some flavone and flavanon derivatives containing *N*-benzylpiperazine side chain were prepared by Ferté et al.[49] In agreement with our results, this study confirmed that the replacement of the C-7-OH position of simple flavones by methoxylated *N*-benzylpiperazine groups strongly improves their P-gp-inhibitory activity, while the substitution of C-5-OH leads to the loss of this ability.

The effect of the E ring substitution was noted in our study as well. Selective methylation of C-20-OH group of **2** (yielding **13**) was connected with a significant increase of P-gp inhibition and a decrease of cytotoxicity for normal human lymphocytes. Indeed, almost all derivatives of **2** with 20-OMe (**13**, **20**, **21**, and **22**) showed better P-gp inhibitory activity together with lower cytotoxicity for normal human lymphocytes compared to the parent compound **2**. Maitrejean et al.[25] demonstrated significance of monolignol unit for strengthening of binding affinity of flavonoids to recombinant cytosolic NBD2 (nucleotide-binding domain) of P-gp. We proved that substitution of monolignol unit (ring E of **2**) by its methylation further improves this binding affinity but also decreases the intrinsic cytotoxicity of new derivatives. Additionally, we showed that the presence of methoxyl group at the C-19 position of **2** is also essential for P-gp inhibitory activity (**2** vs **17**).

Effect of bulky pivaloyl group at C-23-OH position of **1** on its P-gp inhibitory activity was rather surprising: compound **7** showed four times higher activity than **1** at approximately two times lower concentration.

This improvement of silybin activity can be explained by an increase of its lipophilicity. The above-mentioned effect of monolignol unit, together with importance of its substitution may suggest the presence of a new hydrophobic binding region on the NBD. However, steric proximity of bulky lipophilic pivaloyl group to the E ring can be another reason for its improved P-gp inhibitory activity due to a reinforcement of this hydrophobic interaction. Interestingly, the compound **7** is cytotoxic for most of cancer cell lines but not for non-malignant human lymphocytes.

The P-gp inhibitory activity also correlates with lipophilicity of the compounds quite well, however, with some exceptions, for example, 2,3-dehydrosilybin (**2**) versus its trimethylderivative **24**. Zanden et al. have recently demonstrated that the inhibition activity on P-gp homologous MRP-associated proteins depends rath-

er on the planarity of their molecules (represented here by the dihedral angle between the B and C rings) and the number of hydroxyl and methoxyl groups than on lipophilicity of the flavonoid derivatives.[50] They also confirmed that the methoxyl group instead of free hydroxyl group improves the inhibition activity.

However, the design of pharmacologically more active structures also requires reasonable solubility of the compounds and this aspect must be taken into account since flavonolignans and especially 2,3-dehydrosilybin (**2**) suffer from low solubility in both lipophilic and hydrophilic solvents. In those cases, the appropriate substitution of selected positions with OH groups seems to improve at least solubility in lipophilic environment.

2.3. Conclusion

We have found a new group of non-toxic inhibitors of P-gp, based on *O*-alkylated derivatives of 2,3-dehydrosilybin (**13**, **20**, **21**, and **22**). These compounds are very effective inhibitors at lower doses than the lowest TCS_{50} value. The compounds **2**, **8**, **9**, and **18** are effective inhibitors of P-gp efflux but they exhibit high intrinsic cytotoxicity to all tested cell lines. On the other hand, derivatives with inhibitory activity lower than that of parent **2** were also identified (**11**, **17**, and **24**).

SAR study of these derivatives disclosed several structural requirements important for improving P-gp inhibitory activity of parent 2,3-dehydrosilybin (**2**):

(a) Methylation of C-20-OH position (the most important modification).
(b) Methylation of C-3-OH position.
(c) Presence of free C-5-OH position.
(d) introduction of spatially bulkier benzyl group at the C-7-OH position.

The factors with deteriorative effect on P-gp inhibitory activity of **2** were also defined:

(a) Selective methylation of C-7-OH position only (without no other substituted position)
(b) Absence of C-19 methoxyl group.
(c) Methylation of C-5-OH position.

In addition, impact of hydroxyl group substitution on a reduction of cytotoxicity of **2** was evaluated. The methylation of C-20-OH group was the most influential, while the effects of further substitutions were less important.

Compounds with markedly higher cytotoxicity (mainly for cancer cells) than **2** were found as well (**8**, **11**, **20**, and **24**). It seems substitution of C-7-OH (either by methyl or benzyl group) or C-5-OH leads to increase in cytotoxicity of 2,3-dehydrosilybin derivatives in cancer cell lines.

Interestingly, C-7-OH benzylation of silybin substantially increases its general cytotoxicity.

3. Experimental

3.1. General methods

The reactions were monitored by TLC on silica gel F_{254} (Merck) and the spots were visualized by UV light and by charring with 5% H_2SO_4 in ethanol.

NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz for ^1H, 100.55 MHz for ^{13}C) in $CDCl_3$ or DMSO-d_6 (see text) at 30 °C. Chemical shifts were referenced to the residual solvent signal (δ_H 7.265, δ_C 77.00; δ_H 2.50, δ_C 39.60). Digital resolution used justified reporting the proton and carbon chemical shifts to three and two decimal places, respectively. All 2D NMR experiments (HOM2DJ, gCOSY, TOCSY, HMQC, and HMBC) were performed using standard manufacturers' software. The sequence for 1D-TOCSY experiments was obtained through Varian User Library, while the sequence gHMBC was obtained from Varian Application Laboratory in Darmstadt (D).

Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, FRG) with BE geometry. Samples dissolved in methanol:water (2:1, v/v) were continuously infused through a stainless capillary held at 3.3 kV into Finnigan ESI source via linear syringe pump at a flow rate of 40 μL/min.

3.2. Chemistry

Silybin (natural, mixture of A + B diastereomers—in all following synthetic sections) (**1**) was kindly donated by the IVAX Co., Opava, Czech Republic. 2,3-Dehydrosilybin (**2**) was prepared as described previously.[35] Silybinic acid (**3**) and 2,3-dehydrosilybinic acid (**4**) were prepared by CrO_3/H_5IO_6 oxidation of selectively acetylated derivatives of silybin/2,3-dehydrosilybin in acetonitrile with following deacetylation of oxidized products.[35]

3.2.1. 7-*O*-Benzylsilybin (5). K_2CO_3 (210 mg; 1.520 mmol) and benzyl bromide (50 μL, 0.421 mmol) were added to the solution of silybin (**1**, 105 mg, 0.218 mmol) in dry acetone (30 mL). The mixture was stirred and refluxed for 3.5 h under argon. The reaction was quenched by addition of concd HCl (1 mL) and after short stirring the reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (2× 30 mL). The combined organic layers were dried (Na_2SO_4) and evaporated; the residue afforded after flash chromatography (chloroform/acetone/toluene/formic acid 85:10:5:1) the title compound **5** (101 mg, 81%) as a white amorphous solid. MS-ESI (*m*/*z*) 573 $[M+H]^+$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.2.2. 7-*O*-Benzoylsilybin (6). Benzoyl chloride (0.2 mL, 1.721 mmol) and $BF_3{\cdot}Et_2O$ (0.1 mL, 50% solution in diethyl ether) were added to a solution of **1** (198 mg, 0.410 mmol) in dichloromethane/acetonitrile (90 mL, 1:1 v/v) at 0 °C and the mixture was stirred for 42 h at room temperature. The reaction mixture was then dilut-

ed with saturated aqueous solution of $NaHCO_3$ and extracted with ethyl acetate (2× 30 mL). The organic layers were combined, dried (Na_2SO_4), and evaporated; the residue afforded after flash chromatography (ethyl acetate/petroleum ether 1:1) the title compound **6** (220 mg, 90%) as a white amorphous solid. MS-ESI (*m*/*z*) 587 $[M+H]^+$. For ^1H and ^{13}C NMR data, see Tables 1 and 2.

3.2.3. 23-*O*-Pivaloylsilybin (7). Pivaloyl chloride (0.6 mL, 4.872 mmol) and $BF_3{\cdot}Et_2O$ (0.6 mL, 50% solution in diethyl ether) were added to a cooled (0 °C) solution of **1** (530 mg, 1.042 mmol) in dichloromethane/acetonitrile (90 mL, 1:1, v/v). After 1 h of stirring at 0 °C, the mixture was warmed up to the room temperature and stirred for another 12 h. Then it was diluted with water and extracted with dichloromethane (2× 50 mL). Organic layers were combined, dried (Na_2SO_4), and evaporated. Flash chromatography (chloroform/acetone/formic acid, 9:1:0.1) afforded **7** (350 mg, 60%) as a white amorphous solid. MS-ESI (*m*/*z*): 567 $[M+H]^+$. For ^1H and ^{13}C NMR data, see Tables 1 and 2.

3.2.4. 7-*O*-Benzyl-2,3-dehydrosilybin (8). Iodine solution (250 mg I_2 dissolved in 15 mL of glacial acetic acid, 0.984 mmol) was added dropwise within 30 min to a stirred solution of **5** (250 mg, 0.437 mmol) and anhydrous potassium acetate (1.5 g, 15.306 mmol) in glacial acetic acid (15 mL). The reaction mixture was refluxed under stirring for 4 h. After cooling to room temperature and diluting with 150 mL of ice-cold water, the excess iodine was removed by addition of solid $Na_2S_2O_3$ (until the bleaching of the solution). Fine yellow precipitate was filtered off, washed with water to neutral pH and after drying it was dissolved in ethyl acetate. The solvent was evaporated, the evaporated residue was re-dissolved in CH_2Cl_2 (10 mL), and 50 mL of ethanolic HCl (42 mL EtOH + 8 mL concd HCl) was added. The resulting mixture was refluxed under stirring for 1 h to cleave acetates formed during the oxidation in acetic acid. The mixture was then diluted in 150 mL of water and extracted with ethyl acetate (2× 75 mL). The organic layer was dried (Na_2SO_4) and evaporated.

The crude product afforded after flash chromatography (chloroform/acetone/toluene/formic acid, 85:5:10:1) the title compound (**8**) (210 mg, 84%) as a yellow amorphous solid. MS-ESI (*m*/*z*): 571 $[M+H]^+$. ^1H NMR (DMSO-d_6): 3.377 (1H, ddd, J = 12.2, 5.9, 4.6 Hz, H-23u), 3.580 (1H,ddd, J = 12.2, 4.8, 2.5 Hz, H-23d), 3.792 (3H, s, OCH_3), 4.279 (1H, ddd, J = 7.8, 4.6, 2.5 Hz, H-10), 4.971 (1H, d, J = 7.8 Hz, H-11), 4.979 (1H, dd, J = 5.9, 4.8 Hz, 23-OH), 5.212 (2H, s, –CH_2–), 6.421 (1H, d, J = 2.2 Hz, H-6), 6.819 (1H, d, J = 8.1 Hz, H-21), 6.890 (1H, d, J = 2.0 Hz, H-8), 6.894 (1H, dd, J = 8.1, 2.0 Hz, H-22), 7.050 (1H, d, J = 2.0 Hz, H-18), 7.129 (1H, m, H-16), 7.348 (1H, m, H-*para*), 7.409 (2H, m, 2× H-*meta*), 7.465 (2H, m, 2× H-*ortho*), 9.141 (1H, s, 20-OH), 9.673 (1H, s, 3-OH), 12.386 (1H, s, 5-OH). ^{13}C NMR (DMSO-d_6): 55.79 (OCH_3), 60.14 (C-23), 70.01 (CH_2), 75.92 (C-11), 78.61 (C-10), 93.02 (C-8), 98.13 (C-6), 104.26 (C-4a), 111.84 (C-18), 115.39 (C-21), 116.24 (C-13), 116.86 (C-16), 120.61 (C-22), 121.42 (C-15), 123.70 (C-14), 127.28 (C-17), 127.85 (2× C-*ortho*), 128.13 (C-*para*), 128.54 (2× C-*meta*), 136.19 (C-*ipso*), 136.71 (C-3), 143.48 (C-12a), 145.21 (C-16a), 146.24 (C-2), 147.15 (C-20), 147.11 (C-19), 156.11 (C-8a), 160.42 (C-5), 164.06 (C-7), 176.18 (C-4).

3.2.5. 3-*O*-Methyl-2,3-dehydrosilybin (9). Methyl iodide (0.034 mL, 0.550 mmol) was added to a cooled (0 °C) mixture of **2** (240 mg, 0.50 mmol) and NaH (47 mg; 60% w/w in mineral oil, 0.596 mmol) in dry DMF (2.5 mL) and the reaction mixture was stirred for 1.5 h at 0 °C and then 12 h at room temperature. The reaction was quenched by addition of concd HCl (0.2 mL) and 20 mL of water. The yellow precipitate was filtered off, dried, and dissolved in ethyl acetate. After evaporation, flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave the title compound (**9**) (110 mg, 45%) as a yellow amorphous solid. MS-ESI (*m*/*z*): 495 $[M+H]^+$. For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.2.6. 7-*O*-Methylsilybin (10). K_2CO_3 (210 mg; 1.520 mmol) and MeI (0.1 mL, 1.606 mmol) were added to a stirred mixture of **1** (530 mg, 1.099 mmol) in dry acetone (45 mL) and the reaction mixture was refluxed for 3.5 h under argon. The reaction was quenched by the addition of concd HCl (1 mL) and after short stirring the reaction mixture was diluted with water (150 mL) and extracted with ethyl acetate (2× 50 mL). The organic layer was dried (Na_2SO_4) and evaporated.

The evaporated residue afforded after flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) the title compound (**10**) (290 mg, 53%) as a white amorphous solid. MS-ESI (*m*/*z*): 497 $[M + H]^+$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.2.7. 7-*O*-Methyl-2,3-dehydrosilybin (11). Compound **10** (200 mg, 0.403 mmol) and anhydrous potassium acetate (1.2 g, 12.245 mmol) were dissolved in glacial acetic acid (12 mL). To a stirred mixture, iodine solution (200 mg I_2 dissolved in 12 mL of glacial acetic acid, 0.787 mmol) was dropwise added within 30 min. The reaction and workup were performed analogously as for 7-*O*-benzyl-2,3-dehydrosilybin (**8**). The evaporated residue afforded after flash chromatography (chloroform/acetone/toluene/formic acid, 85:5:10:1) the title compound (**11**) (180 mg, 90%) as a yellow amorphous solid. MS-ESI (*m*/*z*): 495 $[M+H]^+$. For ^1H and ^{13}C NMR data, see Tables 3 and 4.

3.2.8. 20-*O*-Methylsilybin (12). MeI (0.160 mL, 2.579 mmol) was added to a solution of **1** (1.032 g, 2.139 mmol) and NaH (257 mg; 60% w/w in mineral oil, 6.417 mmol) in DMF (15 mL) and the mixture was stirred for 2 h at room temperature. The reaction was quenched by the addition of concd HCl (1 mL) and then the mixture was diluted with water (75 mL). The white precipitate was filtered off, dried, and dissolved in ethyl acetate. After evaporation, flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave the

Table 3. ^{1}H NMR data (399.89 MHz, DMSO-d_6, 30 °C) of 2,3-dehydrosilybin derivatives

Proton	**9**	**11**	**13**	**18**	**20**	**21**	**22**	**24**
6	6.192 d (2.1)	6.336 d (2.2)	6.183 d (2.0)	6.356 d (2.2)	6.345 d (2.2)	6.195 d (2.1)	6.368 d (2.2)	6.462 d (2.2)
8	6.474 d (2.1)	6.784 d (2.2)	6.444 d (2.0)	6.785 d (2.2)	6.795 d (2.2)	6.445 d (2.1)	6.800 d (2.2)	6.866 d (2.2)
10	4.309 ddd (7.9, 4.6, 2.4)	4.276 ddd (7.8, 4.7, 2.5)	4.301 ddd (7.8, 4.5, 2.6)	4.316 ddd (8.0, 4.5, 2.5)	4.308 ddd (7.9, 4.4, 2.4)	4.333 ddd (7.9, 4.6, 2.5)	4.350 ddd (7.9, 4.6, 2.4)	4.293 ddd (7.9, 4.6, 2.5)
11	4.970 d (7.9)	4.967 d (7.8)	5.025 d (7.8)	4.975 d (8.0)	5.029 d (7.9)	5.027 d (7.9)	5.037 d (7.9)	5.025 d (7.9)
13	7.617 d (2.2)	7.816 d (2.2)	7.778 d (2.2)	7.688 d (2.2)	7.828 d (2.2)	7.626 d (2.2)	7.699 d (2.2)	5.778 d (2.2)
15	7.639 dd (8.5, 2.2)	7.810 dd (9.2, 2.2)	7.754 dd (8.6, 2.2)	7.699 dd (8.5, 2.2)	7.819 dd (8.7, 2.2)	7.643 dd (8.4, 2.2)	7.708 dd (9.0, 2.2)	7.790 dd (9.2, 2.2)
16	7.136 d (8.5)	7.122 d (9.2)	7.121 d (8.6)	7.146 d (8.5)	7.133 d (8.7)	7.138 d (8.4)	7.159 d (9.0)	7.118 d (9.2)
18	7.051 d (1.9)	7.047 d (2.0)	7.087 d (1.8)	7.055 d (2.0)	7.089 d (1.7)	7.094 d (1.8)	7.096 d (1.8)	7.088 d (1.8)
21	6.824 d (8.2)	6.818 d (8.1)	6.998 d (8.3)	6.824 d (8.1)	7.000 d (8.3)	7.002 d (8.3)	7.003 d (8.3)	6.998 d (8.3)
22	6.896 dd (8.2, 1.9)	6.892 dd (8.1, 2.0)	7.028 dd (8.3, 1.8)	6.898 dd (8.1, 2.0)	7.030 dd (8.3, 1.7)	7.033 dd (8.3, 1.8)	7.036 dd (8.3, 1.8)	7.029 dd (8.3, 1.8)
23	3.580 dd (12.4, 2.4)	3.577 ddd (12.3, 4.4, 2.5)	3.581 dd (12.4, 2.6)	3.586 ddd (12.2, 5.0, 2.5)	3.585 dd (12.5, 5.1, 2.4)	3.591 dd (12.3, 2.5)	3.593 ddd (12.4, 4.8, 2.4)	3.581 ddd (12.3, 5.1, 2.5)
	3.374 dd (12.4, 4.6)	3.376 ddd (12.3, 4.7, 4.4)	3.371 dd (12.4, 4.5)	3.378 ddd (12.2, 5.0, 4.5)	3.375 ddd (12.5, 5.1, 4.4)	3.375 dd (12.3, 4.6)	3.378 ddd (12.4, 4.8, 4.6)	3.372 ddd (12.3, 5.8, 4.6)
3-OMe	3.800 s			3.825 s		3.804 s	3.828 s	
5-OMe				—				3.875 s
7-OMe		3.845 s		3.844 s	3.850 s		3.850 s	3.857 s
19-OMe	3.792 s	3.792 s	3.783 s	3.793 s	3.783 s	3.785 s	3.786 s	3.782 s
20-OMe			3.783 s	—	3.783 s	3.785 s	3.782 s	3.782 s
3-OH		9.650 s		—	9.662 s			8.918 s
5-OH	12.604 s	12.379 s	12.396 s	12.571 s	12.381 s	12.605 s	12.572 s	
7-OH	9.211 s			—				
20-OH	8.306 s	9.148 s		9.170 s				
23-OH	—	4.989 t (4.4)		4.993 t (5.0)			5.008 t (4.8)	4.987 dd (5.8, 5.1)

Table 4. ^{13}C NMR data (100.55 MHz, DMSO-d_6, 30 °C) of 2,3-dehydrosilybin derivatives

Carbon	9	11	13	18	20	21	22	24
2	154.63	146.19	145.64	154.92	146.16	154.63	154.92	141.29
3	138.21	136.69	136.38	138.45	136.72	138.21	138.46	138.06
4	177.93	176.19	175.99	178.14	176.21	177.94	178.14	171.09
4a	104.20	104.13	102.96	105.31	104.13	104.28	105.31	106.31
5	161.26	160.38	160.70	160.92	160.37	161.26	160.91	160.14
6	98.79	97.55	98.41	97.87	97.54	98.71	97.86	95.73
7	164.72	165.08	164.58	165.27	165.08	164.46	165.27	163.79
8	93.91	92.14	93.64	92.46	92.15	93.86	92.48	92.92
8a	156.49	156.20	156.30	156.37	156.19	156.45	156.37	158.10
10	78.61	78.61	78.46	78.63	78.50	78.51	78.52	78.41
11	75.97	75.92	75.75	75.96	75.76	75.80	75.79	75.76
12a	143.58	143.48	143.31	146.15	143.39	143.48	143.53	144.29
13	116.74	116.25	116.22	116.78	116.24	116.74	116.79	115.56
14	122.77	123.73	123.88	122.63	123.77	122.78	122.66	124.12
15	121.96	121.42	121.28	122.06	121.46	122.00	122.10	120.76
16	117.07	116.85	116.87	117.07	116.88	117.06	117.09	116.83
16a	146.01	145.19	144.98	143.63	145.15	145.97	146.11	143.43
17	127.17	127.29	128.84	127.14	128.82	128.71	128.68	128.89
18	111.96	111.84	111.32	111.94	111.31	111.44	111.41	111.30
19	147.74	147.72	148.88	147.71	148.87	148.89	148.86	148.56
20	147.23	147.15	149.24	147.22	149.26	149.32	149.30	149.24
21	115.43	115.40	111.71	115.41	111.69	111.75	111.72	111.68
22	120.72	120.62	120.36	120.69	120.36	120.44	120.44	120.35
23	60.13	60.15	60.09	60.10	60.10	60.07	60.05	60.12
3-OMe	59.81			59.78		59.78	59.78	
5-OMe				—				56.22
7-OMe		56.05		56.10	56.05		56.10	55.98
19-OMe	55.81	55.80	55.65	55.80	55.66	55.61	55.67	55.66
20-OMe			55.59	—	55.59	55.67	55.59	55.591

title compound (**12**) (560 mg, 54%) as a white amorphous solid. MS-ESI (*m*/*z*): 497 $[M+H]^+$. For ^1H and ^{13}C NMR data, see Tables 1 and 2.

3.2.9. 20-*O*-Methyl-2,3-dehydrosilybin (13). Iodine solution (200 mg I_2 dissolved in 12 mL of glacial acetic acid, 0.787 mmol) was dropwise added within 30 min to a mixture of the compound **12** (200 mg, 0.403 mmol) and anhydrous potassium acetate (1.2 g, 12.245 mmol) in glacial acetic acid (12 mL). The reaction and workup were performed analogously as for **8**. The crude product afforded after flash chromatography (chloroform/acetone/toluene/formic acid, 85:5:10:1) the title compound **13** (176 mg, 88%) as a yellow amorphous solid. MS-ESI (*m*/*z*): 495 $[M+H]^+$. For ^1H and ^{13}C NMR data, see Tables 3 and 4.

3.2.10. Caffeic acid methyl ester (14). Caffeic acid (2.5 g, 13.873 mmol) was dissolved in dry methanol (150 mL) and H_2SO_4 (2.5 mL, 96%, v/v) was added. Reaction mixture was stirred at room temperature for 12 h. Reaction mixture was then evaporated to approximately 50 mL, treated by ice-cold saturated solution of $NaHCO_3$ (150 mL) and after short stirring it was extracted with ethyl acetate (3× 75 mL). Organic layers were combined, dried (Na_2SO_4) and evaporated to afford pure **14** (2.65 g, 98%). MS-ESI (*m*/*z*): 195 $[M+H]^+$. ^1H NMR (DMSO-d_6, 30 °C): 3.686 (3H, s, OCH3), 6.264 (1H, d, J = 15.9 Hz, H-2), 6.760 (1H, d, J = 8.2 Hz, H-5′), 6.995 (1H, dd, J = 8.2, 2.1 Hz, H-6′), 7.049 (1H, d, J = 2.1 Hz, H-2′), 7.481 (1H, d, J = 15.9 Hz, H-3), 9.329 (2H, br s, 3′-OH + 4′-OH); ^{13}C NMR (DMSO-d_6, 30 °C): 51.17 (OMe), 113.74 (C-2), 114.81 (C-2′), 115.76 (C-5′), 121.37 (C-6′), 125.52 (C-1′), 145.13 (C-3), 145.59 (C-3′), 148.43 (C-1).

3.2.11. (*E*)-3-*O*-Benzyl-3,4-dihydroxycinnamyl alcohol (15). NaH (206 mg, 5.167 mmol, 60% w/w in mineral oil) was dissolved at 0 °C in DMSO (8 mL) and the solution was stirred for 5 min under argon. Methyl ester **14** (500 mg, 2.575 mmol) was added and the mixture was stirred additional 5 min followed by benzyl chloride (0.357 mL, 3.088 mmol) addition and stirring for 1 h at room temperature. The reaction was quenched by addition of concd HCl (1 mL) and 50 mL of water. Aqueous solution was extracted with CH_2Cl_2 (2× 50 mL), organic layers were combined, dried (Na_2SO_4) and evaporated; the residue was re-dissolved in dry THF (30 mL), the solution was cooled to 0 °C and $LiAlH_4$ (500 mg, 13.193 mmol) was added portionwise. Reaction mixture was stirred 1 h at 0 °C and then 3 h at room temperature. The excess of $LiAlH_4$ was destroyed under cooling by addition of water (3 mL). Celite (2 g) was added to the resulting solution and after short stirring the insoluble impurities were filtered off. The filter cake was thoroughly washed with ethyl acetate and then with acetone. All filtrates were combined and evaporated to a yellowish syrup. Flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave **15** (280 mg, 42%) as a yellowish oil. MS-ESI (*m*/*z*): 257 $[M + H]^+$. ^1H NMR (DMSO-d_6, 30 °C): 4.281 (2H, dd, J = 6.0, 1.5 Hz, 2 × H-1), 5.114 (2H, s, –CH_2O–), 5.795 (1H, br

s, 4′-OH), 6.186 (1H, dt, J = 15.8, 6.0 Hz, H-2), 6.514 (1H, dt, J = 15.8, 1.5 Hz, H-3), 6.892 (1H, d, J = 8.2 Hz, H-5′), 6.925 (1H, dd, J = 8.2, 1.7 Hz, H-6′), 6.999 (1H, d, J = 1.7 Hz, H-2′), 7.373 (1 H, m, *para*-Ph), 7.417 (2H, m, 2× *meta*-Ph), 7.429 (2H, m, 2× *ortho*-Ph); ^{13}C NMR (DMSO-d_6, 30 °C): 63.77 (C-1), 71.21 (–CH_2O–), 110.06 (C-2′), 114.76 (C-5′), 120.63 (C-6′), 126.24 (C-2), 127.83 (2× C-*ortho*), 128.45 (C-*para*), 128.75 (2× C-*meta*), 129.26 (C-1′), 131.26 (C-3), 136.23 (C-*ipso*), 145.88 (C-4′), 145.92 (C-3′).

3.2.12. 19-*O*-Demethyl-19-*O*-benzyl-2,3-dehydrosilybin (16). The alcohol **15** (420 mg, 1.641 mmol) and quercetin·2H_2O (555 mg, 1.641 mmol) were dissolved in the mixture of benzene (100 mL) and acetone (50 mL). Resulting mixture was stirred for 10 min at 60 °C and then Ag_2CO_3 (451 mg, 1.640 mmol) was added. Reaction mixture was stirred under reflux for 30 h, then left to cool to a room temperature and filtered through the Celite pad. The filter cake was thoroughly washed with acetone. After evaporation of combined filtrates, flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave **16** (90 mg, 10%) as a yellow amorphous solid. MS-ESI (m/z): 557 $[M+H]^+$. ^{1}H NMR (DMSO-d_6): 3.333 (1H, m, H-23), 3.548 (1H, dm, J = 12.4 Hz, H-23d), 4.238 (1H, ddd, J = 7.8, 4.6, 2.6 Hz, H-10), 4.948 (1H, d, J = 7.8 Hz, H-11), 4.989 (1H, t, J = 4.6 Hz, 23-OH), 5.115 (2H, s, –CH_2–), 6.196 (1H, d, J = 2.1 Hz, H-6), 6.459 (1H, d, J = 2.1 Hz, H-8), 6.851 (1H, d, J = 8.3 Hz, H-21), 6.906 (1H, dd, J = 8.3, 1.9 Hz, H-22), 7.116 (1H, m, H-16), 7.145 (1H, d, J = 1.9 Hz, H-18), 7.317 (1H, m, H-*para*), 7.383 (2H, m, 2× H-*meta*), 7.493 (2H, m, 2× H-*ortho*), 7.762 (2H, m, H-15, H-13), 9.222 (1H, s, 20-OH), 9.554 (1H, s, 3-OH), 10.811 (1H, s, 7-OH), 12.410 (1H, s, 5-OH). ^{13}C NMR (DMSO-d_6): 60.08 (C-23), 70.18 (CH_2), 75.78 (C-11), 78.53 (C-10), 93.59 (C-8), 98.31 (C-6), 103.13 (C-4a), 113.99 (C-18), 115.75 (C-21), 116.22 (C-23), 116.86 (C-16), 121.09 (C-22), 121.27 (C-15), 123.82 (C-14), 127.23 (C-17), 127.75 (C-*para*), 127.89 (2× C-*ortho*), 128.29 (2× C-*meta*), 136.37 (C-3), 137.23 (C-*ipso*), 143.38 (C-16a), 145.03 (C-12a), 145.79 (C-2), 146.59 (C-19), 147.54 (C-20), 156.28 (C-8a), 160.74 (C-5), 164.12 (C-7), 176.05 (C-4).

3.2.13. 19-*O*-Demethyl-2,3-dehydrosilybin (17). The compound **16** (80 mg, 0.144 mmol) was dissolved in THF (10 mL), Pd/C (40 mg, 10% w/w) was added and the reaction mixture was stirred for 12 h at room temperature under hydrogen. The mixture was filtered through Celite pad, which was washed by acetone (2× 10 mL). Evaporation of the combined filtrates gave pure **17** (64 mg, 96%) as a yellow amorphous solid.

The structural proof was performed on its hexaacetate (obtained after standard Ac_2O/Py peracetylation procedure). MS-ESI (m/z): 467 $[M+H]^+$. ^{1}H NMR ($CDCl_3$): 2.063 (3H, s, 23-Ac), 2.308 (3H, s, 20-Ac), 2.313 (3H, s, 19-Ac), 2.337 (3H, s, 7-Ac), 2.347 (3H, s, 3-Ac), 2.430 (3H, s, 5-Ac), 4.030 (1H, dd, J = 12.4, 4.1 Hz, H-23u), 4.300 (1H, ddd, J = 7.9, 4.1, 3.6 Hz, H-10), 4.442 (1H, dd, J = 12.4, 3.6 Hz, H-23d), 5.016 (1H, d, J = 7.9 Hz, H-11), 6.857 (1H, d, J = 2.2 Hz, H-6), 7.079 (1H, d, J = 8.6 Hz, H-16), 7.299 (3H, m, H-18, H-21, H-22), 7.318 (1H, d, J = 2.2 Hz, H-8), 7.459 (1H, dd, J = 8.6, 2.2 Hz, H-15), 7.511 (1H, d, J = 2.2 Hz, H-13). ^{13}C NMR ($CDCl_3$): 20.54 (23-Ac), 20.59 (9-Ac, 10-Ac), 20.65 (7-Ac), 21.00 (3-Ac), 21.11 (5-Ac), 62.25 (C-23), 75.81 (C-10, C-11), 108.87 (C-8), 113.64 (C-6), 114.73 (C-4a), 117.40 (C-13), 117.56 (C-16), 122.37 (C-15), 122.63 (C-18), 122.99 (C-14), 124.02 (C-21), 125.38 (C-22), 123.52 (C-3), 133.95 (C-17), 142.59 (C-20), 142.91 (C-19), 143.33 (C-12a), 145.64 (C-16a), 150.37 (C-5), 154.11 (C-7), 154.66 (C-2), 156.84 (C-8a), 167.75 (3-CO), 167.79 (7-CO), 167.83 (9-CO), 167.86 (10-CO), 169.21 (5-CO), 170.09 (C-4), 170.25 (23-CO).

3.2.14. 3,7-Di-*O*-methyl-2,3-dehydrosilybin (18). K_2CO_3 (345 mg, 2.500 mmol) and dimethyl sulfate (0.178 mL, 1.870 mmol) were added to a solution of **2** (300 mg, 0.624 mmol) in dry DMF (5 mL) and the reaction mixture was stirred for 12 h at room temperature under argon. The reaction mixture was poured into 75 mL of solution of concd HCl in water (1/14, v/v). The yellow precipitate was filtered off, washed with water, dried, and dissolved in ethyl acetate. After flash chromatography (chloroform/acetone/toluene/formic acid, 85:5:10:1), the title compound **18** was obtained as a yellow amorphous solid (100 mg, 32%). MS-ESI (m/z): 509 $[M+H]^+$. For ^{1}H and ^{13}C NMR data, see Tables 3 and 4.

3.2.15. 7,20-Di-*O*-methylsilybin (19). MeI (0.129 mL, 2.071 mmol) was added to a solution of **1** (500 mg, 1.036 mmol) and NaH (124 mg; 60% w/w in mineral oil, 3.100 mmol) in DMF (7 mL), the mixture was stirred under argon for 1 h at 0 °C and then 1 h at room temperature. The reaction was quenched by addition of HCl (2 mL) and water (50 mL). The white precipitate was filtered off, dried and dissolved in ethyl acetate. After evaporation, flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave **19** (110 mg, 21%) as a white amorphous solid. MS-ESI (m/z): 511 $[M+H]^+$. For ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

3.2.16. 7,20-Di-*O*-methyl-2,3-dehydrosilybin (20). Iodine solution (200 mg I_2 dissolved in 12 mL of glacial acetic acid, 0.787 mmol) was added dropwise within 30 min to a solution of the compound **19** (200 mg, 0.403 mmol) and anhydrous potassium acetate (1.2 g, 12.245 mmol) in glacial acetic acid (12 mL). The reaction and workup were performed analogously as for **8**. The crude product afforded after flash chromatography (chloroform/acetone/toluene/formic acid, 85:5:10:1) the title compound **20** (162 mg, 81%) as a yellow amorphous solid. MS-ESI (m/z): 509 $[M+H]^+$. For ^{1}H and ^{13}C NMR data, see Tables 3 and 4.

3.2.17. 3,20-Di-*O*-methyl-2,3-dehydrosilybin (21) and 3,7,20-tri-*O*-methyl-2,3-dehydrosilybin (22). Powdered KOH (360 mg, 6.429 mmol) was stirred for 5 min in DMSO (4 mL). 2,3-Dehydrosilybin (**2**, 300 mg, 0.624 mmol) in 4 mL of DMSO was added and the mixture was stirred for 10 min. MeI (0.200 mL, 3.210 mmol) was added and the reaction mixture was stirred for 1 h

at room temperature. The reaction was quenched by addition of concd HCl (1 mL) and diluted in water (50 mL). The yellow precipitate was filtered off, dried, and dissolved in ethyl acetate. After evaporation, flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) gave **21** (80 mg, 25%) and **22** (80 mg, 24%) as yellow amorphous solids. MS-ESI (*m*/*z*): 509 $[M+H]^+$ and 523 $[M+H]^+$, respectively. For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.2.18. 5,7,20-Tri-*O*-methylsilybin (23). K_2CO_3 (960 mg; 6.638 mmol) and dimethyl sulfate (0.474 mL, 4.979 mmol) were added to a solution of **1** (400 mg, 0.829 mmol) in dry acetone (30 mL) and the mixture was stirred and refluxed for 2 h under argon. The reaction was quenched by addition of concd HCl (2 mL) and water (100 mL). The mixture was extracted with ethyl acetate (2× 50 mL). The organic layer was dried (Na_2SO_4), and evaporated to afford after flash chromatography (chloroform/acetone/toluene/formic acid, 85:10:5:1) the title compound **23** as a white amorphous solid (245 mg, 56%). MS-ESI (*m*/*z*): 525 $[M+H]^+$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.2.19. 5,7,20-Tri-*O*-methyl-2,3-dehydrosilybin (24). Iodine solution (120 mg I_2 dissolved in 15 mL of glacial acetic acid, 0.472 mmol) was dropwise added within 30 min to a solution of **23** (120 mg, 0.229 mmol) and anhydrous potassium acetate (700 mg, 7.143 mmol) in glacial acetic acid (15 mL). The reaction and workup were performed analogously as for **8**. The crude product gave after flash chromatography (chloroform/acetone/formic acid, 90:10:1) the title compound **24** (55 mg, 46%) as a yellow amorphous solid. MS-ESI (*m*/*z*): 523 $[M+H]^+$. For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.3. Biological studies

3.3.1. Cell cultures. Cell lines CCRF-CEM (CEM, human T-lymphoblastic leukemia), K562 (human myeloid leukemia), L1210 (mouse lymphocytic leukemia), L1210-VCR (vincristine resistant mouse lymphocytic leukemia), A549 (human lung adenocarcinoma), and MCF7 (human breast cancer) were purchased from the American Tissue Culture Collection (ATTC, Manassas, VA, USA). The drug resistant sublines of CEM and K562 cells were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicin (Cem-DNR and clone Cem-DNR 0.3A2), vincristine (Cem-VCR) or paclitaxel (K562-Tax), respectively.[43] The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

Phenotype characteristics of the drug resistant cell lines were previously published[43] and are as follows: CEM-DNR ($P\text{-gp}^+$, MRP^-, and LRP^+), CEM-DNR 0.3A2 ($P\text{-gp}^+$, MRP^-, and LRP^-), CEM-VCR ($P\text{-gp}^+$, MRP^-, and LRP^+), and K562-Tax ($P\text{-gp}^+$, MRP^-, and LRP^-) (Table 5).

3.3.2. Cytotoxic MTT assay. Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30,000 cells/well based on cell growth characteristic). Cells were added by pipette (80 μL) into 96-well microtiter plates. Inoculates were allowed a preincubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Fourfold dilutions, in 20 μL aliquots, of tested compounds were added to the microtiter plate wells. All test compound concentrations (250–0.2441 μM) were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period, formazan produced was dissolved by the addition of 100 μL/well of 10% aqueous SDS (pH 5.5), followed by a further incubation at 37 °C overnight. The absorbance (*A*) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumor cell survival (TCS) was calculated using the following equation: $TCS = (A_{\text{drug-exposed}}/\text{mean } A_{\text{control}}) \times 100\%$. The TCS_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

3.3.3. Modulation of P-gp efflux function. Paclitaxel resistant derivative of human myeloid leukemia cell line K562, K562-Tax cells previously shown to overexpress exclusively functional P-gp and not another MDR proteins were used to investigate P-gp modulatory activity of silybin/2,3-dehydrosilybin derivatives.[43] Cells were cultured in RPMI 1640 medium supplemented with sodium bicarbonate and FCS 10% only. Following an overnight incubation, 10^6 of the cells were seeded in six-well plates with or without the reference P-gp inhibitor (cyclosporin A, CyA) or tested derivatives for 1 h and labeled with P-gp fluorescent substrate daunorubicin (DNR, 1 μM) for another 1 h. Both the CyA and tested compounds were tested in wide concentration range (100–0.0977 μM) in fourfold dilutions and six concentrations. Following this period, the cells were washed in PBS and re-suspended in the complete medium with an identical concentration of cyclosporin A or tested drugs but no DNR. Treated cellular suspensions were allowed to culture for 1 h at 37 °C in order to pump out the DNR by functional P-gp. Finally, K562-Tax myeloblasts were put on ice until the red fluorescence data of DNR accumulation were collected through a 585/42 bandpass filter, using a linear amplification (FL-2 height) on the flow cytometer. The ratio of the mean DNR fluorescence of treated versus untreated cells was calculated in order to evaluate the capacity of reference inhibitor cyclosporin A or tested substances to block the functional activity of P-gp.

3.3.4. Statistical analysis. With exception of kinetic parameters, all values are expressed as means and standard deviations (SD). Kinetic and statistical analyses were calculated using PRISM 4 (GraphPad Software Inc., San Diego, CA). Kinetic values, for example, half-maximal effective concentrations (EC_{50}) and maximum efflux inhibition (I_{max}), were estimated from the

Table 5. Cytotoxic activity of silybin/2,3-dehydrosilybin derivatives on a panel of drug sensitive versus drug resistant cell lines[a] compared to normal human lymphocytes

Compound	Cytotoxicity (TCS_{50}[b,c], μM)										
	CEM	Cem-VCR	Cem-DNR	Cem-DNR 0.3A2	K 562	K 562-Tax	L1210	L1210-VCR	MCF7	A 549	Normal human lymphocytes
1	155 [3.871]	>250 [0]	>250 [0]	>250 [0]	136.5 [12.23]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	214.83 [16.555]	156.5 [18.75]
2	17.28 [0.445]	28.89 [1.164]	6.18 [1.863]	16.49 [1.309]	10.99 [2.711]	34.73 [13.372]	>250 [0]	31.09 [18.246]	20.1 [2.047]	8.46 [1.072]	22.45 [1.8]
3	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]
4	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]	>250 [0]
5	7.83 [0.929]	13.01 [3.211]	16.09 [2.751]	26.2 [4.874]	8.203 [0.056]	16.65 [0.76]	>250 [0]	24.2 [6.437]	18.02 [1.139]	13.41 [1.711]	16.37 [0.7656]
6	26.1 [2.05]	41.5 [1.545]	57.23 [8.504]	88.74 [7.405]	34.92 [2.159]	52.77 [25.466]	>250 [0]	160.31 [2.424]	42.51 [10.07]	71.22 [13.605]	158.6 [7.838]
7	22.55 [1.849]	86.27 [10.591]	68.25 [4.876]	78.23 [7.069]	17.07 [0.841]	28.07 [3.089]	>250 [0]	105.33 [24.372]	93.86 [3.953]	34.79 [2.612]	>250 [0]
8	2.83 [0.05]	11.79 [1.2]	9.22 [0.39]	7.93 [1.74]	3.3 [1.25]	4.93 [0.83]	4.82 [2.64]	2.56 [0.1]	9.64 [0.89]	4.24 [1.4]	12.50 [2.12]
9	12.19 [1.89]	24.40 [9.33]	11.77 [0.5]	11.55 [0.82]	13.23 [1.66]	10.52 [1.09]	29.68 [4.71]	27.14 [14.95]	14.68 [0.82]	11.42 [1.04]	49.77 [2.88]
11	8.34 [0.47]	10.34 [1.47]	9.09 [0, 52]	5.57 [1.73]	8.25 [1.44]	3.38 [0.26]	8.46 [1.1]	8.07 [4.93]	10.21 [0.55]	8.75 [2]	16.66 [7.03]
13	9.29 [1.43]	11.54 [1.45]	10.05 [0.39]	10.38 [0.29]	9.85 [0.54]	9.64 [0.57]	11.41 [1.61]	10.46 [0.56]	12.69 [1.44]	9.56 [0.71]	204.99 [53.08]
17	11.81 [0.96]	201.56 [55.84]	42.83 [2.45]	42.24 [1.2]	12.88 [0.95]	35.91 [1.5]	43.73 [2.5]	32.62 [13.64]	48.85 [4.29]	11.67 [1.45]	>250 [0]
18	10.29 [0.78]	11.45 [0.94]	10.04 [0.55]	9.03 [2.08]	8.96 [0.36]	7.89 [0.57]	9.17 [0.64]	8.87 [0.69]	11.59 [0.58]	10.08 [2.93]	68.31 [34.56]
20	3.24 [0.43]	4.84 [2.17]	27.37 [3.5]	3.02 [0.6]	3.6 [0.4]	2.81 [0.07]	4.42 [0.89]	2.44 [0.13]	8.63 [2.72]	6.49 [4.63]	>250 [0]
21	10.73 [1.07]	8.92 [2.84]	8.45 [1.04]	7.54 [2.54]	10.61 [1.41]	3.47 [0.73]	9.48 [1.79]	6.62 [3.7]	10.05 [0.54]	9.02 [2.88]	242.95 [14.1]
22	130.57 [5.2]	203.75 [32.37]	19.95 [4.51]	19.18 [11.67]	116.84 [7.89]	75.14 [16.02]	58.7 [2.82]	27.58 [7.14]	109.27 [15.77]	182.17 [76.31]	>250 [0]
24	3.06 [0.32]	4.58 [2.67]	2.44 [0.12]	3.45 [0.43]	4.48 [0.66]	2.56 [0.19]	3.03 [0.39]	6.54 [3.86]	11.26 [2.95]	3.3 [0.24]	187.42 [29.65]

[a] CEM—human T-lymphoblastic leukemia cell line CCRF-CEM; CEM-VCR vincristine resistant CEM cells; CEM-DNR—daunorubicin resistant CEM cells; CEM-DNR 0.3A2—daunorubicin resistant subclone of CEM-DNR cell line; K562—human myeloid leukemia cell line; K562-Tax—paclitaxel resistant K562 cells; L1210—mouse lymphocytic leukemia cell line; L1210-VCR—vincristine resistant L1210 cells; A549—human lung adenocarcinoma cell line; MCF7—human breast cancer cell line.

[b] Data are expressed as mean values from six measurements in three independent experiments and standard deviations in brackets [SD].

[c] Maximum/minimum tested concentrations were 250/0.2441 μM, respectively. The TCS_{50} values indicate the drug concentration lethal to 50% of the cells.

non-linear regression equation with the method of least squares and the EC_{50} value is the concentration at which half-maximal efflux was achieved.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (Grant No. 303/02/1097), the Czech Ministry of Education (Research concepts No. MSM 6198959216, MSM 0021620835, and AV0Z50200510) and KONTAKT project No. 7-2006-16. Authors are indebted to Drs. K. Hofbauerová and V. Kopecký Jr., Institute of Physics, Charles University, Prague, for calculation of molecular models of some compounds involved in this study.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.01.035.

References and notes

1. Flora, K.; Hahn, M.; Rosen, H.; Benner, K. *Am. J. Gastroenterol.* **1998**, *93*, 139.
2. de Groot, H.; Rauen, M. *Fundam. Clin. Pharmacol.* **1998**, *12*, 249.
3. Křen, V.; Walterová, D. *Biomed Papers* **2005**, *149*, 29.
4. Dorai, T.; Aggarwal, B. B. *Cancer Lett.* **2004**, *215*, 129.
5. Ahmad, N.; Gali, H.; Javed, S.; Agarwal, R. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 294.
6. Zi, X.; Mukhtar, H.; Agarwal, R. *Biochem. Biophys. Res. Commun.* **1997**, *239*, 334.
7. Zi, X.; Feyes, D. K.; Agarwal, R. *Clin. Cancer Res.* **1998**, *4*, 1055.
8. Zi, X.; Agarwal, R. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 528.
9. Zi, X.; Zhang, J.; Agarwal, R.; Pollak, M. *Cancer Res.* **2000**, *60*, 5617.
10. Singh, R. P.; Tyangi, A. K.; Zhao, J.; Agarwal, R. *Carcinogenesis* **2002**, *23*, 499.
11. Singh, R. P.; Agarwal, R. *Antioxid. Redox Signal.* **2002**, *4*, 655.
12. Kang, S. N.; Lee, M. H.; Kim, K.-M.; Cho, D.; Kim, T. S. *Biochem. Pharmacol.* **2001**, *61*, 1487.
13. Jiang, C.; Agarwal, R.; Lu, J. X. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 371.
14. Zi, X.; Agarwal, R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7490.
15. Bhatia, N.; Agarwal, R. *The Prostate* **2001**, *46*, 98.
16. Zhu, W.; Zhang, J.-S.; Young, Y. F. *Carcinogenesis* **2001**, *22*, 1399.
17. Thelen, P.; Jarry, H.; Ringert, R.-H.; Wuttke, W. *Planta Med.* **2004**, *70*, 397.
18. Manna, S. K.; Mukhopadhyay, A.; Van, N. T.; Aggarwal, B. B. *J. Immunol.* **1999**, *163*, 6800.
19. Saliou, C.; Valacchi, G.; Rimbach, G. *Methods Enzymol.* **2001**, *335*, 380.
20. Dhanalakshmi, S.; Singh, R. P.; Agarwal, C.; Agarwal, R. *Oncogene* **2002**, *21*, 1759.
21. Yoo, H. Y.; Jung, S. N.; Hwang, Y. S.; Park, J. S.; Kim, M. H.; Jeong, M.; Ahn, S. J.; Ahn, B. W.; Shin, B. A.; Park, R. K.; Jung, Y. D. *Int. J. Mol. Med.* **2004**, *13*, 81.
22. Giacomelli, S.; Gallo, D.; Apollonio, P.; Ferlini, C.; Distefano, M.; Morazzoni, P.; Riva, A.; Bombardelli, E.; Mancuso, S.; Scambia, G. *Life Sci.* **2002**, *70*, 1147.
23. Soose, M. *Eur. J. Protistol.* **1994**, *30*, 394.
24. Bokemeyer, C.; Fels, L. M.; Dunn, T.; Voigt, W.; Gaedeke, J.; Schmol, H.-J.; Stolte, H.; Lentzen, H. *Br. J. Cancer* **1996**, *74*, 2036.
25. Maitrejean, M.; Comte, G.; Barron, D.; El Kirat, K.; Gwenaelle, C.; Di Pietro, A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 157.
26. Seelig, A.; Gatlik-Landwojtowicz, E. *Mini-Rev. Med. Chem.* **2005**, *5*, 135.
27. Kickhoefer, V. A.; Rajavel, K. S.; Scheffer, G. L.; Dalton, W. S.; Scheper, A. J.; Rome, L. H. *J. Biol. Chem.* **1998**, *273*, 8971.
28. Kong, L. B.; Siva, A. C.; Rome, L. A.; Stewart, P. H. *Structure* **1999**, *7*, 371.
29. Zhang, S. H.; Morris, M. E. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1258.
30. Zhang, S. Z.; Morris, M. E. *Pharm. Res.* **2003**, *20*, 1184.
31. Tyagi, A. K.; Singh, R. P.; Agarwal, C.; Chan, D. C.; Agarwal, R. *Clin. Cancer Res.* **2002**, *8*, 3512.
32. Rodriguez, R. J.; Miranda, C. L.; Stevens, J. F.; Deinzer, M. L.; Buhler, D. R. *Food Chem. Toxicol.* **2001**, *39*, 437.
33. Trompier, D.; Baubichon-Cortay, H.; Chang, X. B.; Maitrejean, M.; Barron, D.; Riordon, J. R.; Di Pietro, A. *Cell. Mol. Life Sci.* **2003**, *60*, 2164.
34. Leslie, E. M.; Mao, Q.; Oleschuk, C. J.; Deeley, R. G.; Cole, S. P. C. *Mol. Pharmacol.* **2001**, *59*, 171.
35. Gažák, R.; Svobodová, A.; Psotová, J.; Sedmera, P.; Přikrylová, V.; Walterová, D.; Křen, V. *Bioorg. Med. Chem.* **2004**, *12*, 5677.
36. Antus, S.; Baitz-Gács, E.; Bauer, R.; Gottsegen, Á.; Seligmann, O.; Wagner, H. *Liebigs Ann. Chem.* **1989**, 1147.
37. Guz, N. R.; Stermitz, F. R. *J. Nat. Prod.* **2000**, *63*, 1140.
38. Pietta, P. G. *J. Nat. Prod.* **2000**, *63*, 1035.
39. Merlini, L.; Zanarotti, A. *Tetrahedron Lett.* **1975**, *16*, 3621.
40. Pelter, A.; Hänsel, R. *Chem. Ber.* **1975**, *108*, 790.
41. Jiang, X.; Macey, M. G.; Collins, P.-W.; Newland, A. C. *Br. J. Hematol.* **1994**, *86*, 547.
42. Lee, J. S.; Scala, S.; Matsumoto, Y.; Dickstein, B.; Robey, R.; Zhan, Z.; Altenberg, G.; Bates, S. E. *J. Cell Biochem.* **1997**, *65*, 513.
43. Nosková, V.; Džubák, P.; Kuzmina, G.; Ludková, A.; Stehlík, D.; Trojanec, R.; Janošťáková, A.; Kořínková, G.; Mihál, V.; Hajdúch, M. *Neoplasma* **2002**, *49*, 418.
44. Pérez-Victoria, J. M.; Pérez-Victoria, J. F.; Conseil, G.; Maitrejean, M.; Comte, G.; Barron, D.; Di Pietro, A.; Castanys, S.; Gamarro, F. *Antimicrob. Agents Chemother.* **2001**, *45*, 439.
45. Conseil, G.; Baubichon-Cortay, H.; Dayan, G.; Jault, J. M.; Barron, D.; Di Pietro, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9831.
46. Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. *Pharmacol. Rev.* **2000**, *52*, 673.
47. Hyafil, F.; Vergely, C.; Du Vignaud, P.; Grand-Perret, T. *Cancer Res.* **1993**, *53*, 4595.
48. Planting, A. S. T.; Sonneveld, P.; Gaast, A.; Sparreboom, A.; Burg, M. E. L.; Luyten, G. P. M.; Leeuw, K.; Boer-Dennert, M.; Wissel, P. S.; Jewell, R. C.; Paul, E. M.; Purvis, N. B., Jr.; Verweij, J. *Cancer Chemother. Pharmacol.* **2005**, *55*, 91.
49. Ferté, J.; Kühnel, J. M.; Chapuis, G.; Rolland, Y.; Lewin, G.; Schwaller, M. A. *J. Med. Chem.* **1999**, *42*, 478.
50. Zanden, J. J.; Wortelboer, H. M.; Bijlsma, S.; Punt, A.; Usta, M.; Bladeren, P. J.; Rietjens, I. M. C. M.; Cnubben, N. H. P. *Biochem. Pharmacol.* **2005**, *69*, 699.