New model system for testing effects of flavonoids on doxorubicin-related formation of hydroxyl radicals

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Doxorubicin belongs to anthracycline cytotoxic drugs and it is widely used as a major therapeutic agent in the treatment of various types of tumors. However, its therapeutic use is limited by the development of myelosuppression and cardiotoxicity after a specific cumulative dose is reached. The aim of this study was to investigate the effect of flavonoids, either natural or synthetic on doxorubicin-mediated formation of oxidative stress implicated in doxorubicin toxicity. Doxorubicin caused a concentration-dependent increase in the formation of hydroxyl radicals in minipig liver microsomes used as an in-vitro model system. When bacterial membranes heterologously expressing human NADPH cytochrome-P450 oxidoreductase were incubated with doxorubicin, formation of the superoxide radical under aerobic conditions and the doxorubicin–semiquinone radical under anaerobic conditions was detected. Forty different flavonoids were tested for their potency to prevent NADPH-induced or Fe^{2+}-induced peroxidation of lipids in the microsomal system. According to the results, seven flavonoids were selected for evaluation of their potency to inhibit doxorubicin-dependent formation of hydroxyl radicals assessed by electron spin resonance. Myricetin, fisetin, and kaempferol were found to produce a significant protective effect against hydroxyl radicals in the minipig liver microsomal system. In conclusion, this study shows the use of a novel cost-effective in-vitro model system for preselection of antioxidants for testing of their protective effects against toxicity of anthracyclines and potentially other oxidative stress-inducing chemicals. ***Anti-Cancer Drugs*** **22:176–184 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Anthracyclines belong to the most effective and widely used cytotoxic agents in the treatment of solid tumors [1]. The leading anthracycline, doxorubicin, is used predominantly in the therapy of breast cancer and hematological malignancies [2,3]. The main mechanisms of the anticancer effects of doxorubicin include DNA intercalation and strand breakage and the inhibition of topoisomerase II [4,5]. The dose-limiting side effect of doxorubicin is the development of acute and chronic cardiotoxicity leading to arrhythmia and congestive heart failure [6,7].

A number of mechanisms have been proposed to contribute to the development of doxorubicin toxicity including alterations of iron homeostasis, the production of reactive oxygen species (ROS) or apoptosis [6,8,9]. The clinical manifestation is most likely a result of complex interactions of all of these processes. However, most investigators agree that doxorubicin-mediated production of ROS may be the initiating reaction, especially in the acute phase of cardiotoxicity [6]. Doxorubicin-induced ROS can damage cellular macromolecules, that is, mitochondrial DNA [10], initiate lipid peroxidation of cellular membranes, or modify iron homeostasis by releasing iron from ferritin [6].

Flavonoids are the most abundant polyphenolic components of the human diet present in fruits, vegetables, and plant-derived beverages such as tea and red wine. Flavonoids possess a variety of biochemical and pharmacological activities such as antioxidant, antiviral, anticarcinogenic, and anti-inflammatory effects [11]. The antioxidant properties of flavonoids and their ability to chelate free iron could also reduce the cardiotoxicity of doxorubicin. Fourteen natural flavonoids were tested in the earlier described system of untreated rat liver microsomes for their potency to inhibit peroxidation of lipids and hydroxyl-radical formation induced by four experimental settings [12]. Fisetin, kaempferol, morin, quercetin, and eriodictyol were very efficient antioxidants against doxorubicin—NADPH-mediated oxidative stress. Interestingly, myricetin strongly stimulated peroxidation of lipids in Fe^{3+}-doxorubicin system [12].

The aim of this study was to search for potentially effective inhibitors of doxorubicin-related toxicity. According to a pilot study, the effect of 40 natural and

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synthetic flavonoids with antioxidant properties [12] on lipid peroxidation in two experimental settings and the doxorubicin-mediated ROS formation were studied using the previously established model system consisting of minipig liver microsomes [13]. Bacterial membranes heterologously expressing recombinant human NADPH cytochrome P450 oxidoreductase (NPR, EC 1.6.2.4) were used for study of formation of superoxide radical and doxorubicin–semiquinone radical.

Materials and methods

Chemicals

Doxorubicin hydrochloride, solvents, quercetin, fisetin, myricetin, kaempferol, morin, β-NADP, glucose-6-phosphate, 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl were purchased from Sigma-Aldrich (Prague, Czech Republic). DMPO was purified before use as recommended by Bruker BioSpin GmbH (Rheinstetten/Karlsruhe, Germany) and stored at −20°C until use. Glucose-6-phosphate dehydrogenase was from Boehringer (Mannheim, Germany). Apigenin, apigenin 7-*O*-glucuronyl methyl ester, asebotin, baicalein, 5,7-dihydroxy-2′,8-dimethoxyflavanone, caffeic acid, catechin, epicatechin, eriodictyol, galic acid, 7-hydroxy-2′,5,8-trimethoxyflavanone, kaempferol 3-*O*-glucoside, lupinifolin, luteolin, luteolin 7-*O*-glucuronide, naringenin, phloridzin (dihydrochalcone), quercetin 3-*O*-glucoside, rivularin, rutin, wogonin, and 4′-hydroxywogonin were a generous gift from Yune-Fang Ueng (National Research Institute of Chinese Medicine, Taipei, Taiwan, Republic of China). Synthetic flavonoid derivatives, A12, A55B, AB-2DE, FBB-14, CB284, CB285, CB287, CB436, kaempferid, MH-11, ML-30, ML-50 (4-hydroxy-6-methoxyaurone), and ND-285 were prepared according to Boumendjel *et al.* [14]. The chemical structures of the examined flavonoids are listed in Fig. 1.

Animals

Minipig male castrates (Brno, white variety of Goettingen minipig; Research Institute of Veterinary Medicine, Brno, Czech Republic) weighing 22–31 kg, aged 6 months were used. The animals ($N = 2$) were fed with standard diet for pigs (A1) mixed with bread (0.5 kg each day) and essentially no cytochrome P450 induction was done during this experiment. The animals were killed by a single shot and exsanguinated, liver samples were taken from the medial lobes and stored at −80°C until preparation of microsomes. The microsomes were prepared by differential centrifugation [15] and stored in aliquots at −80°C until use. The total cytochrome P450 (P450, EC 1.14.14.1) content was measured by spectrophotometry according to Omura and Sato [16] and protein was determined using the bicinchoninic acid method (Pierce, Rockford, Illinois, USA).

Preparation of bacterial membranes

Escherichia coli DH5-α cells (Invitrogen, Paisley, UK) were transformed by the pCW′hNPR plasmid carrying the coding sequence of human NPR, as described elsewhere [17]. Transformed cells were cultivated in the expression medium and the membrane fraction was isolated according to the protocols described earlier [18]. The NPR content was assessed by spectrophotometry [19].

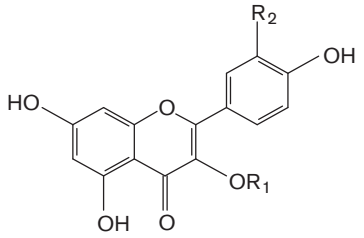
Incubations

Samples were prepared on ice in gas-tight amber vials. Minipig microsomes (0.5 mg of microsomal protein/ml) or bacterial membranes containing heterologously expressed NPR (100 pmol NPR/ml) were incubated with various concentrations of doxorubicin (5, 10, 15, 30, and 50 μmol/l) in the presence or absence of an NADPH-generating system (50 mmol/l $MgCl_2$, 25 mmol/l glucoso-6-phosphate, 2.5 mmol/l $NADP^+$, and 3.5 U/ml glucoso-6-phosphate dehydrogenase) in potassium phosphate buffer (50 mmol/l), pH 7.4 at 37°C for 10 min in a total volume of 300 μl without shaking. The reaction mixtures for the assessment of lipid peroxidation were incubated in glass test tubes under air in a shaking water bath in the dark for 60 min at 37°C. The final concentrations in 500 μl of total volume were: 66 mmol/l phosphate buffer, pH 7.4, 1 mg of microsomal protein/ml and the NADPH-generating system (as described above). In the system with iron-induced peroxidation of lipids, the same incubation mixture was used with the exception that 1 mmol/l phosphate buffer, pH 7.4 and 50 μmol/l $FeSO_4$, freshly prepared before use, were used instead of the NADPH-generating system. The tested flavonoids were added as a methanolic solution with 0, 2, 4, 10, 20, 50, and 100 μmol/l of final concentration of flavonoids whereas the final concentration of methanol did not exceed 1%. The formation of thiobarbituric acid-reactive substances was quantified by malondialdehyde standards [20]. This method has been shown to be quite efficient, simple, and quick, and a good correlation with other methods for evaluation of lipid peroxidation [21]. The effect of selected flavonoids was then tested in the samples containing 30 μmol/l doxorubicin. The tested flavonoids were added as a methanolic solution (30, 50, and 100 μmol/l) whereas the final concentration of methanol did not exceed 1%. Control samples contained only methanol instead of the flavonoid. Each sample contained 400 mmol/l DMPO as a spin trap. Incubations for the assessment of the formation of doxorubicin–semiquinone radical were performed in the absence of DMPO under an argon atmosphere (anaerobic conditions).

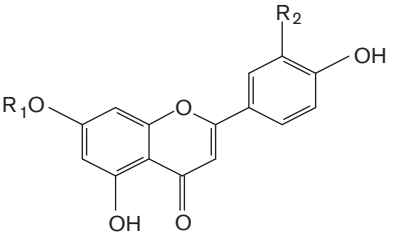
Electron spin resonance measurements

After incubation, the samples were transferred to a flat quartz cell and the first-derivative spectra were recorded in a Bruker Elexsys E540 spectrometer (Bruker BioSpin GmbH) for 5 min. Thirty scans were accumulated to produce the spectrum. Experimental settings were as

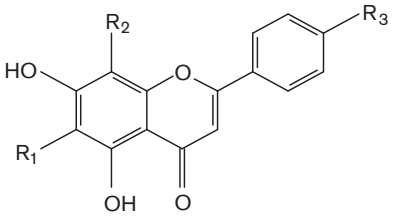
Fig. 1



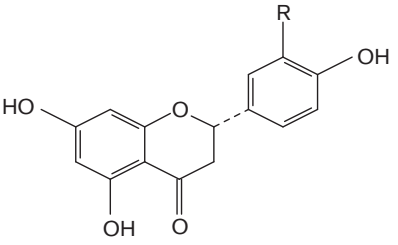
Kaempferol: R_1=R_2=H
Kaempferol 3-*O*-glucoside:
R_1=β-D-glucosyl, R_2=H
Quercetin: R_1=H, R_2=OH
Quercetin 3-*O*-glucoside:
R_1=β-D-glucosyl, R_2=OH



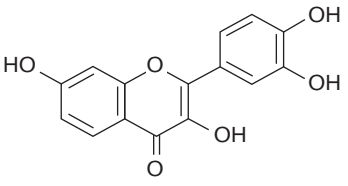
Apigenin: R_1=R_2=H
Apigenin 7-*O*-glucuronyl methyl ester:
R_1=β-D-glucuronyl methyl ester, R_2=H
Luteolin: R_1=H, R_2=OH
Luteolin 7-*O*-glucuronide:
R_1=β-D-glucuronyl, R_2=OH



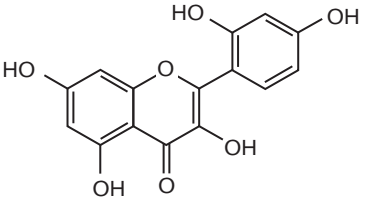
Baicalein: R_1=OH, R_2=R_3=H
Wogonin: R_1=R_3=H, R_2=OCH_3
4′-hydroxywogonin: R_1=H, R_2=OCH_3, R_3=OH



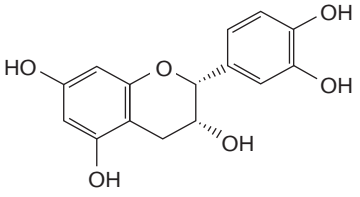
Eriodictyol: R=OH
Naringenin: R=H



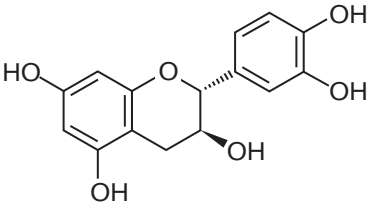
Fisetin



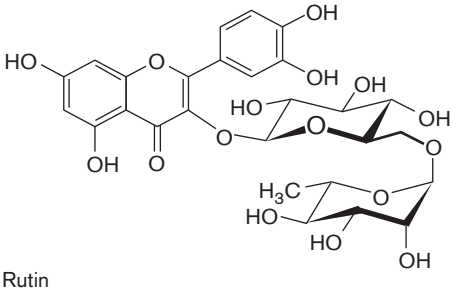
Morin



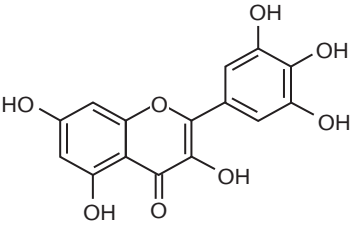
Epicatechin



Catechin

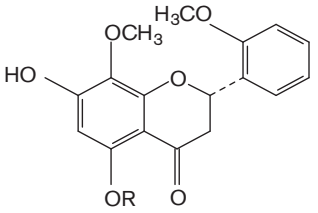


Rutin

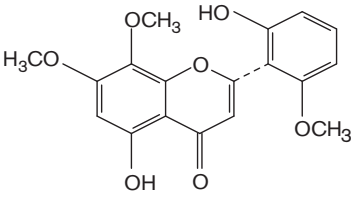


Myricetin

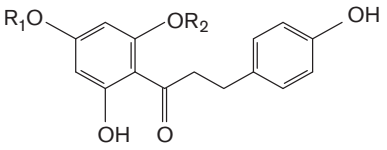
Fig. 1 (*continued*)



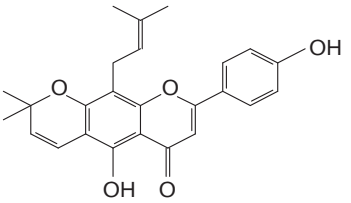
5,7-dihydroxy-2′,8-dimethoxyflavanone: R=H
7-hydroxy-2′,5,8-trimethoxyflavanone: R=CH_3



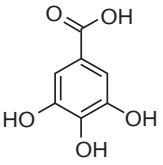
2′,5-dihydroxy-6′,7,8-trimethoxyflavone
(Rivularin)



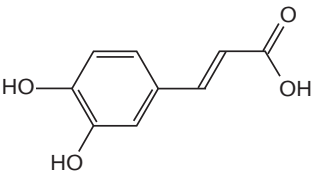
Asebotin: R_1=CH_3, R_2=β-D-glucosyl
Phloridzin: R_1=H, R_2=β-D-glucosyl



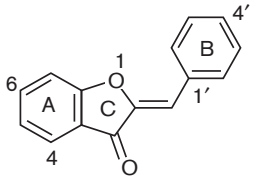
Lupinifolin



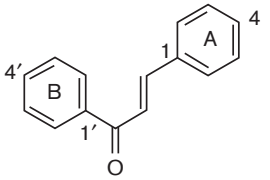
Gallic acid



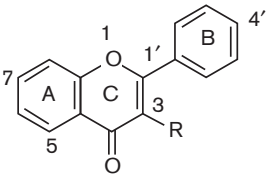
Caffeic acid



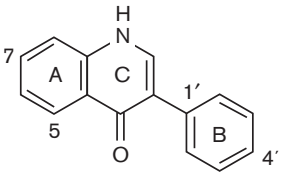
Aurones
A-55B: 4-OH; 6-OMe; 7-I; 4′-CN
CB-284: 4,6-OMe; 4′-Br
CB-285: 4,6-OMe; 4′-Cl
CB-287: 4,6,3′,4′,6′-OMe
ML-30: 4-OH; 6-OMe; 4′-CH_2CH_3
ML-50: 4-OH; 6-OMe;
ND-285: 4-OH; 6-OMe; 4′-Cl



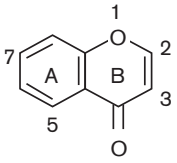
Chalcone
FBB-14: 4-I; 2′,4′,6′-OH



Flavone (R=H)
CB-436: 3-OMe; 5,7-OH; 4′-Br
Flavonol (R=OH)
AB-2DE: 5,7-OH; 4′-I
Kaempferid: 5,7-OH; 4′-OMe



Azaisoflavone
A-12: 3-Phenyl; 5,7-OH



Chromone
MH-11: 2-CO-N⬡N′CH_3; 5-OH

Chemical structures of all tested flavonoids.

follows: microwave frequency (9.76 GHz); field centered at 3480 G; sweep width (200 G); resolution (1024 points); time constant (1.28 ms); modulation frequency (100 kHz); modulation amplitude (1 G); receiver gain (60 dB); conversion time (10.24 ms); microwave power output (25 mW). The second integral value of the spectra was compared with the second integral of the spectrum of freshly prepared 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl solution obtained under identical conditions on the same day. The concentration of hydroxyl radicals was calculated from calibration with the precise concentration of the 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl solution being determined spectrophotometrically as recommended by Bruker Biospin GmbH.

Statistics

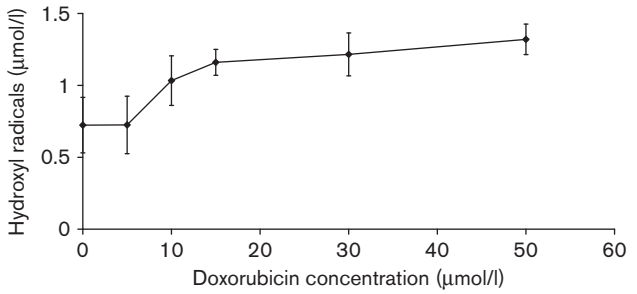
All results are expressed as the average of the three measurements; means ± standard deviation. Electron spin resonance (ESR) measurements were taken during several independent measurements. The Student's *t*-test was used for the analysis of differences between groups.

Results

Doxorubicin-mediated formation of reactive oxygen species and semiquinone radical

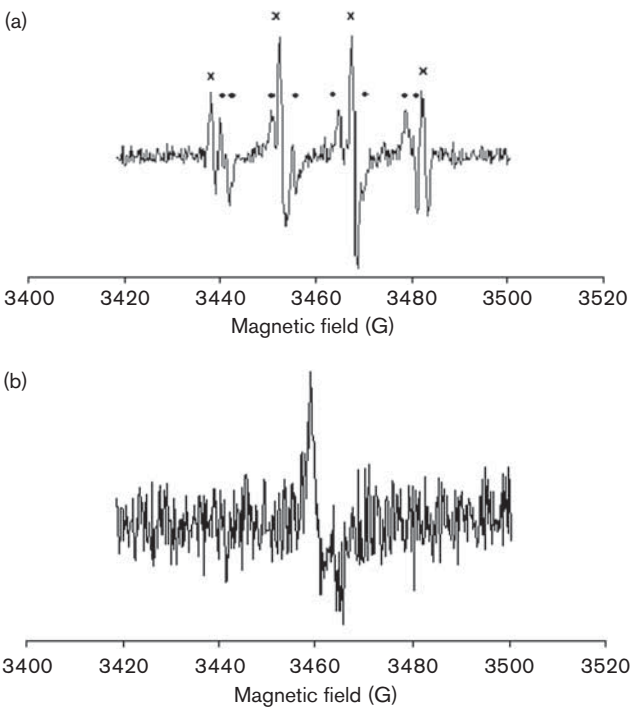
The ESR spectra obtained after incubation of minipig liver microsomes with the NADPH-generating system and doxorubicin in the presence of DMPO showed a typical quartet pattern centered at $g = 2.004$ with the intensity ratio of 1 : 2 : 2 : 1 and hyperfine splitting of $a_N = a_H = 14.71$ G, which belongs to the DMPO adduct of hydroxyl radicals (DMPO/OH) [22]. In incubations of minipig liver microsomes with increasing concentrations of doxorubicin in the presence of the NADPH-generating system, the formation of hydroxyl radicals followed a concentration-dependent manner (Fig. 2). No formation of hydroxyl radicals was observed in the same experimental setting in the absence of the NADPH-generating system (results not shown). For experiments with flavonoids a doxorubicin concentration (30 μmol/l) showing the plateau-like effect was chosen. Neither superoxide radicals, the precursor of hydroxyl radicals, nor doxorubicin–semiquinone radicals were detected under these conditions. Therefore, an additional model system was established to confirm that ESR is capable of detecting these radicals. When bacterial membranes with heterologously expressed NPR were used as a model system, another spectrum apart from hydroxyl radicals was detected under aerobic conditions at 30 μmol/l doxorubicin (Fig. 3a). This spectrum centered at $g = 2.014$ with hyperfine splitting constants $a_N = 14.4$ G, $a_H(\beta) = 11.8$ G, and $a_H(\gamma) = 1.72$ G belongs to the DMPO adduct of superoxide radicals [23]. The doxorubicin–semiquinone radical was detected in this system under anaerobic conditions just at 300 μmol/l doxorubicin (Fig. 3b). In the samples of NPR membranes incubated with the NADPH-generating system in the absence of doxorubicin a pure spectrum of hydroxyl radicals was observed.

Fig. 2



The formation of hydroxyl radicals by doxorubicin in minipig liver microsomes. Samples containing various concentrations of doxorubicin (5, 10, 15, 30, and 50 μmol/l) were incubated with minipig liver microsomes and NADPH-generating system as described in Materials and methods. Difference between the control sample without doxorubicin and samples with 15, 30, and 50-μmol/l doxorubicin was significant ($P < 0.05$) by Student's *t*-test.

Fig. 3



Electron spin resonance (ESR) spectra of radicals formed by doxorubicin in model system containing human NADPH cytochrome P450 oxidoreductase (NPR). (a) Spectrum of hydroxyl (x) and superoxide radicals (●) in bacterial membranes containing NPR incubated with 30-μmol/l doxorubicin in the presence of NADPH-generating system under aerobic conditions. (b) Spectrum of the doxorubicin–semiquinone radical in the NPR membranes incubated with 300 μmol/l doxorubicin in the presence of the NADPH-generating system under anaerobic conditions.

Effects of natural and synthetic flavonoids against peroxidation of lipids

Forty different flavonoids (Fig. 1) were tested for their potency to prevent peroxidation of lipids in two experimental settings *in vitro*. The NADPH-generating system or the Fe^{2+} system was used for induction of lipid peroxidation and half-maximal inhibitory concentrations (IC_{50}) of different flavonoids were evaluated (Table 1). Of all the flavonoids tested, eriodictyol, quercetin, kaempferol, fisetin, myricetin, morin, and 4-hydroxy-6-methoxyaurone have shown a considerably higher effect in both experimental settings. These flavonoids were selected for further testing of their potency to influence doxorubicin-induced oxidative stress. Baicalein, apigenin 7-*O*-glucuronyl methyl ester, and quercetin 3-*O*-glucoside also proved to be highly effective (Table 1) but due to the lack of sufficient material these substances were not tested further.

Table 1 Effect of flavonoids on peroxidation of lipids in two experimental settings

Flavonoids	IC_{50} with Fe^{2+} (μmol/l)	IC_{50} with NADPH (μmol/l)
Apigenin	40	5
Apigenin 7-*O*-glucuronyl methyl ester	7	2
Asebotin	40	8
Baicalein	6.4	0.7
5,7-Dihydroxy-2′,8-dimethoxyflavanone	No effect	10–15
Caffeic acid	4.5	>30
Catechin	3	22.2
Epicatechin	1.6	15.9
Eriodictyol	1.5	1.2
Fisetin	5.5	6.9
Galic acid	6.7	>40
7-Hydroxy-2′,5,8-trimethoxyflavanone	No effect	7
Kaempferol	5.5	2
Kaempferol 3-*O*-glucoside	53	No effect
Lupinifolin	10	34
Luteolin	17	3.5
Luteolin 7-*O*-glucuronide	12	1.3
Morin	7.5	8.8
Myricetin	4.2	7.5
Naringenin	>30	>40
Phloridzin (dihydrochalcone)	14	9
Quercetin	1.5	2.5–6
Quercetin 3-*O*-glucoside	6	2.5
Rivularin	No effect	0.9
Rutin	No effect	>30
Wogonin	25	4
4′-hydroxywogonin	13–15	0.5
A-12	45	3
A-55B	50	0
AB-2DE	No effect	No effect
CB-284	No effect	No effect
CB-285	No effect	No effect
CB-287	No effect	No effect
CB-436	No effect	No effect
FBB-14	70	1
Kaempferid	60	2.5
MH-11	No effect	No effect
ML-30	No effect	No effect
ML-50 (4-hydroxy-6-methoxyaurone)	15	0.5
ND-285	No effect	No effect

Conditions for incubations were described in Materials and methods.

The effect of flavonoids on doxorubicin-mediated formation of hydroxyl radicals in minipig liver microsomes

On the basis of experiments with lipid peroxidation, natural flavonoids–eriodictyol, fisetin, kaempferol, morin, myricetin and quercetin– and synthetic aurone 4-hydroxy-6-methoxyaurone were selected as the most effective inhibitors for further testing. Selected flavonoids were tested for their potency to inhibit doxorubicin-induced oxidative stress in the ESR system described above. Three of the tested compounds (myricetin, fisetin, and kaempferol) were found to have a significant inhibitory effect at the highest tested concentration (100 μmol/l, Table 2). No clear concentration-dependent trend was observed.

Discussion

We investigated possible interactions of natural and synthetic flavonoids with doxorubicin-mediated formation of hydroxyl radicals as an important mechanism influencing the therapeutic effect and toxicity of doxorubicin. The major goal of this study was to identify potentially effective inhibitors of mechanisms leading to doxorubicin toxicity and to establish a convenient screening model system.

The chronic phase of doxorubicin toxicity is probably mediated by metabolic conversion of doxorubicin to doxorubicinol [24] by cytoplasmic NADPH-dependent aldose (AKR1B1; EC 1.1.1.21), aldehyde (AKR1A1; EC 1.1.1.2), and carbonyl (CBR1; EC 1.1.1.184) reductases. The main mechanism of the doxorubicinol toxicity is its interaction with iron and subsequent formation of ROS affecting biomacromolecules [6]. In contrast, doxorubicin can also be reduced to its corresponding semiquinone radical by accepting one electron. This reaction may be catalyzed by a number of intracellular enzymes as cytosolic xanthine oxidase (EC 1.17.3.2), microsomal, NPR or mitochondrial NADH dehydrogenase (EC 1.6.99.3) [6]. Semiquinone can further activate oxygen to form ROS such as the superoxide radical and hydrogen peroxide, which in turn in the presence of iron produces very reactive hydroxyl radicals [11].

On account of the limited availability of human biological material we tested doxorubicin-mediated ROS formation using minipig liver microsomes as a model system. Minipig liver microsomes have been shown earlier to contain major biotransformation enzymes in amounts and activities comparable with the humans [25,26]. Toxic effects of model substances with the quinone motif (benzoquinone, hydroquinone, catechol, and doxorubicin) have been followed in terms of their ability to destroy cytochrome P450 and induce the formation of oxidative stress and subsequent peroxidation of lipids in our earlier studies [27–29] and doxorubicin has been shown to significantly destroy P450 both in the presence and absence of NADPH [13].

Table 2 The influence of flavonoids on the formation of hydroxyl radicals by doxorubicin

	The concentration of hydroxyl radicals (μmol/l) formed after incubations with the specified concentrations of flavonoids		
	30 μmol/l	50 μmol/l	100 μmol/l
Control samples	1.33 ± 0.07 (100%)	1.29 ± 0.18 (100%)	1.17 ± 0.15 (100%)
Fisetin	1.28 ± 0.19 (96%)	1.22 ± 0.01 (95%)	0.79 ± 0.02* (68%)
Quercetin	1.50 ± 0.18 (113%)	1.35 ± 0.23 (105%)	1.14 ± 0.18 (97%)
Myricetin	1.18 ± 0.08 (89%)	1.10 ± 0.08 (85%)	0.64 ± 0.06* (55%)
Kaempferol	1.30 ± 0.01 (98%)	1.18 ± 0.27 (91%)	0.79 ± 0.10* (68%)
Morin	1.41 ± 0.12 (106%)	1.18 ± 0.16 (91%)	1.01 ± 0.19 (86%)
Eriodictyol	1.39 ± 0.06 (104%)	1.52 ± 0.18 (118%)	1.32 ± 0.12 (113%)
4-hydroxy-6-methoxyaurone	1.49 ± 0.10 (112%)	1.27 ± 0.08 (98%)	1.12 ± 0.24 (96%)

Flavonoids at 30, 50, and 100 μmol/l concentrations were incubated with mini-pig liver microsomes, the NADPH-generating system and, 30 μmol/l doxorubicin as described in Materials and methods. In control samples without flavonoid, corresponding amount of solvent (methanol) was added. Data are presented as means ± standard deviation ($n = 3$); the percentage in parentheses represents the ratio of the value to the control sample.
*$P < 0.05$ for Student's *t*-test compared with corresponding control sample.

The production of hydroxyl radicals by doxorubicin observed in this study is in agreement with the proposed mechanism of acute doxorubicin toxicity, which includes one-electron reduction of doxorubicin to its semiquinone, subsequent activation of oxygen and the production of ROS [6,10,11]. In our study, doxorubicin–semiquinone radical was detected just at extremely high 300 μmol/l doxorubicin concentration in NPR-expressing bacterial membranes under anaerobic conditions (mimicking the hypoxic tumor cell environment) but not in minipig liver microsomes under aerobic conditions (normoxic 'normal cell' environment). The failure to detect the doxorubicin–semiquinone radical under aerobic conditions may be caused by technical conditions of the measurement (it was not possible to keep samples in an argon atmosphere during the sample transfer to ESR cuvette and during spectra measurement). However, doxorubicin–semiquinone formation was proven indirectly by detecting the formation of superoxide radicals in samples of NPR-expressing recombinant bacterial membranes with 30 μmol/l doxorubicin and the NADPH-generating system. It was thus experimentally shown that NPR (also present in minipig liver microsomes) is capable of reducing doxorubicin and initiating the whole cascade of oxygen activation and ROS formation. As the ESR spectrum of the DMPO adduct of superoxide radicals is quite complex and overlaps with the spectrum belonging to hydroxyl radicals, we decided to use only hydroxyl radicals for quantitative analysis.

We tested 40 natural and synthetic flavonoids for their potency to prevent peroxidation of lipids in two experimental settings *in vitro* (NADPH-generating system or Fe^{2+} system) validated in our earlier report [12]. Peroxidation of lipids is biologically relevant to doxorubicin-induced oxidative stress as increased levels of ROS can initiate lipid peroxidation. Iron and other transient metals participate in ROS formation and peroxidation of the lipids. The hydroxyl radical is very reactive (rate constant for reaction of hydroxyl radicals with linoleate is 8×10^9 mol/l^{-1} s^{-1}, [30]). On the basis of IC_{50} of the tested flavonoids, eriodictyol, fisetin, kaempferol, myricetin, morin, quercetin, and 4-hydroxy-6-methoxyaurone were selected for ESR study in the system with doxorubicin-induced oxidative stress. These molecules belong to three different subclasses of flavonoids: five flavonols, one flavanone, and one aurone. Myricetin, fisetin, and kaempferol at 100 μmol/l concentration significantly inhibited the doxorubicin-mediated formation of hydroxyl radicals in this study. However, no clear concentration-dependent trend was observed and IC_{50} could not be determined. Although the plasma concentrations of these derivatives have not yet been fully characterized, the mean human plasma concentration of quercetin from high-vegetable diet was approximately 0.05 μmol/l [31]. Olthof *et al.* [32] estimated that the mean plasma concentration of quercetin after ingestion of quercetin-3-glucoside and quercetin-4′-glucoside by human volunteers was 5.0 ± 1.0 and 4.5 ± 0.7 μmol/l, respectively. Maximum plasma concentration of kaempferol 6 h after endive consumption was 0.10 ± 0.01 μmol/l [33]. It should be mentioned that minipig liver microsomes treated with 30 μmol/l doxorubicin used to generate hydroxyl radicals in this study present a model system that differs from the in-vivo situation in both the concentration of doxorubicin (peak plasma concentration during treatment of cancer patients reaches up to 5 μmol/l; [34]) and the target organ of toxicity (heart vs. liver). Taking into consideration the limitations described above and these data the 100 μmol/l effective concentrations of flavonoids observed in this study may not be reached *in vivo*. However, the 30 μmol/l doxorubicin concentration was recently successfully used for testing of effect of 7-monohydroxyethylrutoside against DOX-induced toxicity in in-vitro and ex-vivo organ bath studies [35]. Moreover, a dose of 1500 mg/m^2 of 7-monohydroxyethylrutoside (maximum peak plasma concentration reached 360 ± 69.3 μmol/l in six healthy volunteers) was suggested as a feasible and safe dose to be evaluated in a phase II study to investigate its protective properties against doxorubicin-induced cardiotoxicity in cancer patients [36].

According to the experimental data in the literature, the mechanism by which flavonoids decreased doxorubicin-mediated production of hydroxyl radicals seems to be a mixture of chelation of free iron [37,38], direct scavenging

of radicals (both semiquinone and ROS) [39], and maintaining doxorubicin in the reduced state. Which of these events prevails or whether there are additional mechanisms remains to be discovered [40]. From our initial screening results (measured peroxidation of lipids in microsomal system) it was obvious, that flavonols have been the most interesting candidates for investigation in ESR measurement. This clearly points to the importance of the benzopyran-4-one system and the *ortho*-dihydroxyphenyl moiety. This is an expected result because of the role of both moieties in the antioxidant activity of flavonoids. Although the number of investigated flavonoids is limited, the structure–activity relationship drawn from the subsequent ESR study points to the positive role of the 3-hydroxybenzopyran-4-one (eriodictyol and 4-hydroxy-6-methoxyaurone vs. the remaining molecules shown in Table 2). The 5-hydroxy group seems not to be required for the activity (fisetin vs. myricetin and kaempferol). This may indicate that metal chelation properties of flavonol are ensured by the 3-hydroxy-4-carbonyl system. The substitution pattern of the B-ring seems to tolerate the presence of one, two, or three hydroxyl groups. The *ortho*-dihydroxylation substitution pattern is crucial for the antioxidant activity (fisetin vs. quercetin). From this preliminary structure–activity relationship analysis, it can be concluded that the metal chelation is principal for the antioxidant activity, as most of the active flavonoids contained a 3-hydroxybenzopyran-4-one and an *ortho*-dihydroxyphenyl moiety, both of which are known for their metal chelation properties.

In conclusion, this study has found the inhibition of doxorubicin-induced formation of hydroxyl radicals in minipig liver microsomes by several natural flavonoids. Considering the wide agreement on their antioxidant effects these flavonoids should be further tested as potential agents to reduce doxorubicin-mediated toxicity. The model system and the experimental approach presented here seems to be quite convenient for prescreening of further candidate antioxidants at cell-free level.

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