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The effect of flavonoid derivatives on doxorubicin transport and metabolism

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Abstract—This study investigated the effect of naturally occurring flavonoids and synthetic aurone derivatives on the formation of cardiotoxic doxorubicinol and transport of doxorubicin in breast cancer cells. Quercetin significantly inhibited the formation of doxorubicinol. Quercetin and aurones did not significantly affect transport of [^{14}C]doxorubicin in human resistant breast cancer cells. In conclusion, quercetin should be further tested for its potency to decrease doxorubicin-mediated toxicity.

1. Introduction

Anthracyclines are among the most effective and widely used cytotoxic agents in the treatment of solid and hemopoietic tumors.[1] The leading anthracycline doxorubicin (DOX) is used predominantly in the chemotherapy of breast cancer and leukemia.[2,3] The main mechanisms of the anticancer effects of DOX include DNA intercalation and strand breakage and inhibition of topoisomerase II.[4,5] The dose-limiting side effect of DOX is the development of acute and chronic cardiotoxicity leading to arrhythmia and congestive heart failure.[6,7] Another impediment to successful chemotherapy with DOX is the development of multidrug resistance.[8] DOX shows reduced effectiveness against tumor cell populations that exhibit the typical P-glycoprotein (EC 3.6.3.44, P-gp) multidrug resistance phenotype.[9]

The chronic phase of DOX toxicity is probably mediated by preferred metabolic conversion of DOX to doxorubicinol.[7] The DOX metabolism to doxorubicinol occurs 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 doxorubicinol toxicity is its interaction with iron and subsequent formation of ROS affecting biomacromolecules.[7]

Flavonoids are the most abundant polyphenolic components of 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 believed to be beneficial for human health.[10] Several flavonoids have been shown to be able to increase accumulation of anticancer drugs in resistant human cancer cells. Quercetin and its methoxylated derivative inhibited the efflux of rhodamine-123 and restored sensitivity to DOX in MCF-7 breast cancer cells.[11] Quercetin was shown to bind to purified P-gp and efficiently inhibit its activity.[12] Morin, biochanin A, phloretin, and silymarin increased the accumulation of [^{3}H]-daunomycin in P-gp overexpressing MCF-7/ADR cells.[13] The antioxidant properties of flavonoids and their ability to chelate free iron could also be effective in reducing

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the cardiotoxicity of DOX. To potentiate the beneficial properties of natural flavonoids, new synthetic analogues based on their structure are being tested as antioxidants or resistance reversal agents. Structures of these derivatives belong to different subclasses of natural flavonoids and include: aurones, chalcones, flavones, flavonols, chromones, and isoflavones. Those flavonoid analogues, which successfully interact with the NBD2 domain of P-gp, are potential resistance modulators and reversing agents of tumor resistance to cytotoxic drugs.[14–16] Moreover, some of these flavonoids effectively decreased uptake of another typical P-gp substrate, paclitaxel.[17]

In general, the aim of this study was to find potentially effective inhibitors of mechanisms leading to DOX toxicity and test new promising P-gp modulators, which could increase the therapeutic effect of DOX in resistant tumors. The specific objectives of this investigation were: (1) to determine the effect of several naturally occurring flavonoids and their synthetic derivatives (4-hydroxy-6-methoxyauron and 4-iodochalcon) on the formation of the DOX metabolite doxorubicinol and cytoplasmic NADPH dependent aldo-keto reductase activity in human cytosolic liver fractions and (2) to determine the effect of P-gp inhibitor verapamil and MRP1/2 inhibitor MK-571 as well as the effects of a natural flavonoid quercetin and synthetic flavonoid derivatives, namely aurones, on P-gp mediated DOX accumulation and efflux in sensitive (MDA-MB-435) and resistant (NCI-ADR-RES) human breast cancer cell lines. Previously, we found that NCI-ADR-RES cells express high levels of P-gp protein, which the MDA-MB-435 cells are lacking.[17] Therefore, we expected to find significant differences in DOX transport between the two cell lines.

2. Results

2.1. In vitro metabolism of doxorubicin

Metabolism of DOX was investigated in human liver cytosolic and microsomal fractions. Approximately 4.5% (4.63% ± 0.53%) of the amount of DOX incubated with liver microsomes was converted to DOX aglycon. Doxorubicinol or its aglycone was not detected in microsomal incubations. The cytosolic incubations of liver preparations catalyzed the reductive conversion of DOX to doxorubicinol in accordance with data reported in the literature.[18] About 15% (15.10% ± 3.09%) of DOX was converted to its main human metabolite doxorubicinol. No other significant metabolites (DOX aglycone or doxorubicinol aglycone) were detected in cytosolic incubations.

2.2. The effect of flavonoids and their synthetic derivatives on doxorubicin metabolism

The effect of naturally occurring flavonoids quercetin, fisetin, and eriodictyol and their synthetic derivatives, 4-hydroxy-6-methoxyauron and 4-iodochalcon, on the formation of doxorubicinol in human liver cytosolic fractions is shown in Figure 2. The data indicate marked differences in the effects of the tested flavonoids. Quercetin proved to be the most efficient inhibitor of DOX metabolic transformation to doxorubicinol (IC_{50} = 16.25 μM). Synthetic flavonoid derivatives, 4-hydroxy-6-methoxyauron and 4-iodochalcon, exerted significant inhibition only at higher concentrations (30 μM), while fisetin and eriodictyol did not have any significant effect on DOX metabolism.

2.3. The transport of doxorubicin in breast cancer cell lines

The uptake and efflux of DOX and its modulation were investigated in sensitive (MDA-MB-435) and resistant (NCI-ADR-RES) human breast cancer cell lines. During 4 h incubation, MDA-MB-435 cells accumulated a 3 times higher amount of [^{14}C]DOX than NCI-ADR-RES cells (Fig. 3A). The subsequent efflux of 100 nM [^{14}C]DOX within 120 min reached 66% in NCI-ADR-RES cells compared to 23% efflux in MDA-MB-435 cells. The efflux of [^{14}C]DOX was 2.3 times lower from the MDA-MB-435 cells than from the NCI-ADR-RES cells after 120 min (Fig. 3B).

2.4. The effect of verapamil and MK-571 on doxorubicin transport

The presence of 10 μM verapamil did not influence the accumulation of [^{14}C]DOX in sensitive and resistant cells. In contrast, the presence of 100 μM verapamil increased [^{14}C]DOX intracellular concentration 7.6 times in NCI-ADR-RES cells and 3.7 times in the MDA-MB-435 cells (Fig. 4A). The efflux of [^{14}C]DOX after 120 min loading with 100 nM [^{14}C]DOX was significantly inhibited by 100 μM verapamil in NCI-ADR-RES cells. In contrast, the efflux of [^{14}C]DOX from MDA-MB-435 cells was not significantly influenced by verapamil (Fig. 4B). The presence of 100 μM MK-571 did not influence the accumulation of [^{14}C]DOX in MDA-MB-435 cells and only slightly (1.27 times) enhanced the accumulation of the drug in the NCI-ADR-RES cells. The efflux of [^{14}C]DOX after 120 min loading with 100 nM [^{14}C]DOX was significantly influenced by 100 μM MK-571 neither in MDA-MB-435 nor in NCI-ADR-RES cells (data not shown).

2.5. Cytotoxicity of tested flavonoid derivatives in breast cancer cell line

The tested flavonoids (10–30 μM) did not exert significant cytotoxicity in resistant breast cancer NCI-ADR-RES cells but they were (except 4′-chloro-4-hydroxy-6-methoxyauron) significantly ($P < 0.05$) cytotoxic in sensitive MDA-MB-435 cells. Therefore, MDA-MB-435 cells were not used in further experiments to assess the effects of flavonoids on DOX transport.

2.6. The effect of quercetin and synthetic flavonoid derivatives on doxorubicin transport in resistant breast cancer cell line

We initially investigated the effect of quercetin (5–30 μM) on [^{14}C]DOX accumulation in NCI-ADR-

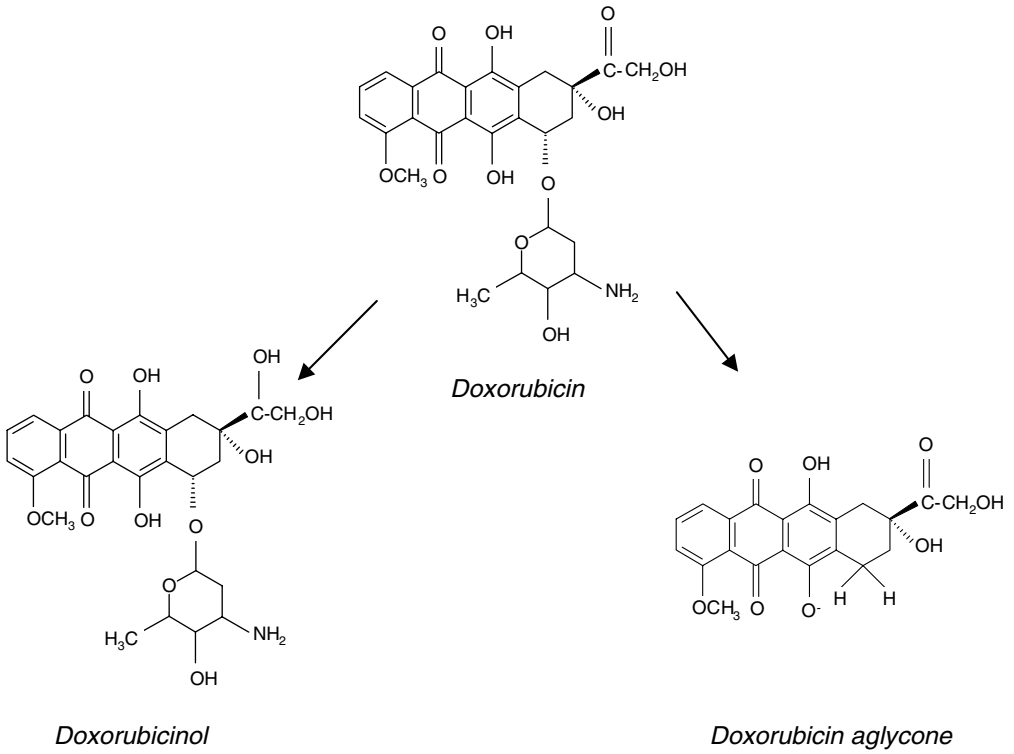


Figure 1. The chemical structure of doxorubicin and its main metabolites.

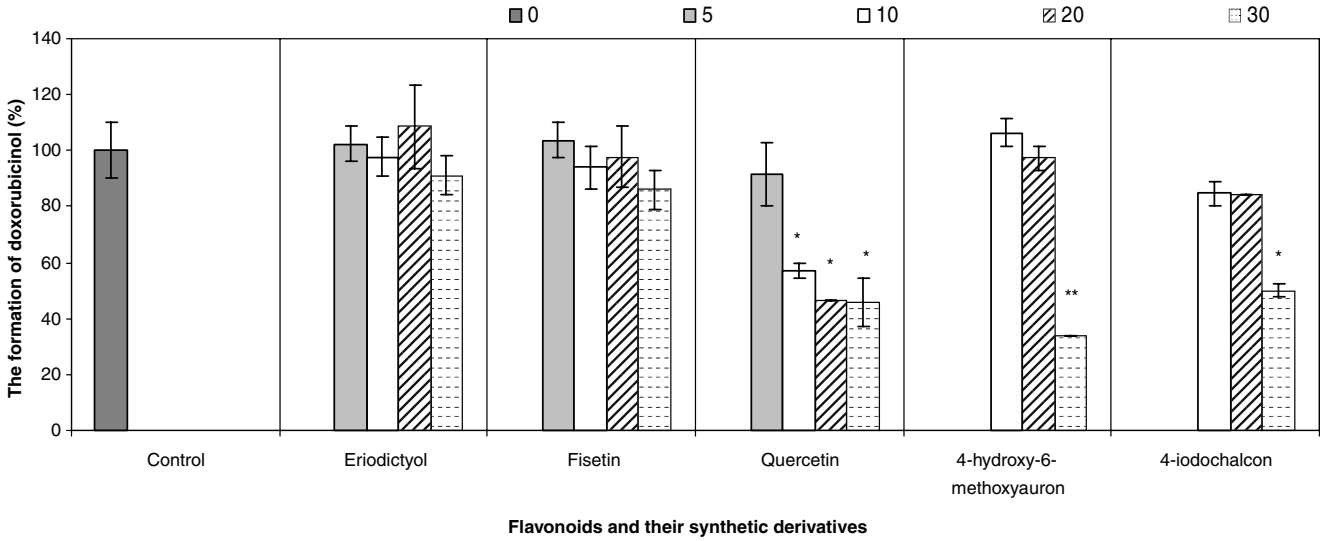


Figure 2. The effect of quercetin, fisetin, eriodictyol, 4-hydroxy-6-methoxyauron, and 4-iodochalcon on the formation of doxorubicinol in human liver cytosolic fractions. Concentrations of DOX (10 μM) and flavonoids were as follows: 5, 10, and 30 μM quercetin, fisetin, and eriodictyol, and 10 and 30 μM 4-hydroxy-6-methoxyauron and 4-iodochalcon, respectively. Inhibition experiments were performed as described in Section 5. Data are expressed as the percentage of remaining activity relative to the control sample. Means ± SD of individual experiments ($n \geqslant 2$) with pooled human liver samples are displayed. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's t test.

RES cells. The highest concentration of this flavonoid weakly decreased [^{14}C]DOX accumulation (max 51.3% reduction at 120 min) in these cells (Fig. 5) and it did not influence the efflux of [^{14}C]DOX from NCI-ADR-RES cells (data not shown). We subsequently incubated NCI-ADR-RES cells with 100 nM [^{14}C]DOX without or with 5–30 μM synthetic flavonoid derivatives of natural flavonoid auron, namely: 4,6,3′,4′,6′-pentamethoxyauron, 4′-chloro-4-hydroxy-6-methoxyauron, 4′-ethyl-4-hydroxy-6-methoxyauron, and 4-hydroxy-6-methoxyauron. The accumulation of [^{14}C]DOX was measured after 30, 60, and 120 min. None of the tested flavonoid derivatives exerted any significant effect on the accumulation of [^{14}C]DOX in NCI-ADR-RES cells (results not shown).

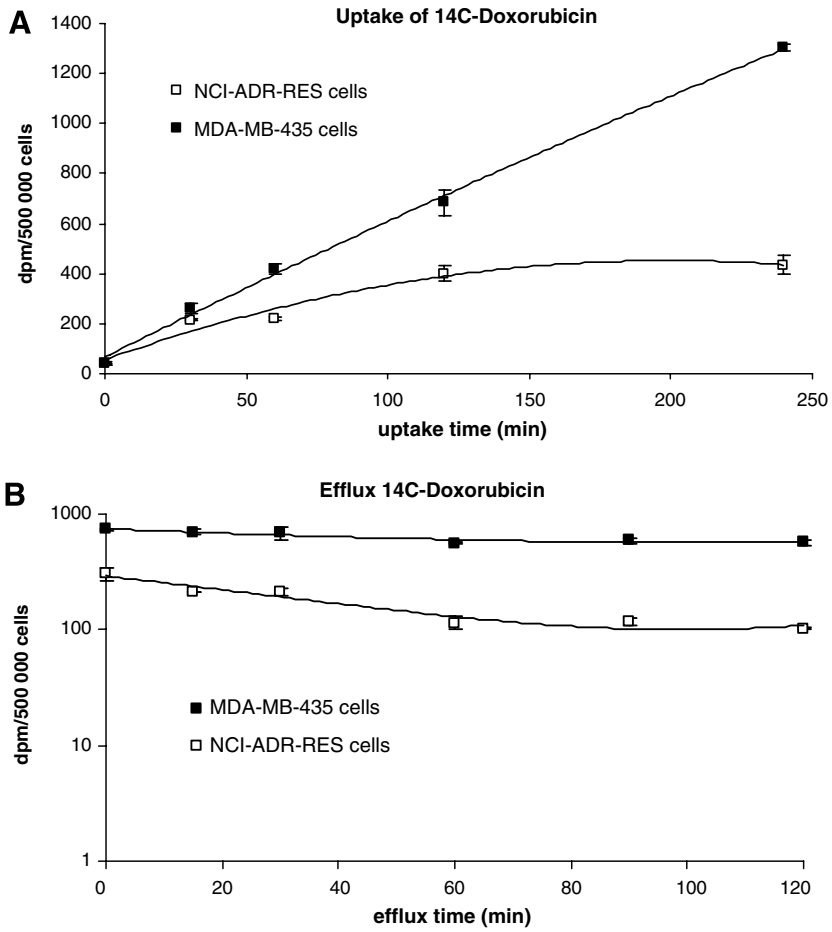


Figure 3. Uptake and efflux of doxorubicin by NCI-ADR-RES and MDA-MB-435 cells. (A) Uptake of 100 nM [^{14}C]DOX after 30, 60, 120, and 240 min incubation. (B) Efflux of [^{14}C]DOX after 120 min loading at 100 nM concentration. Efflux times were 15, 30, 60, 90, and 120 min. The levels of DOX were measured by liquid scintillation. Data are shown as means of duplicate determinations ± SD and expressed as dpm units/500000 cells/well.

3. Discussion

We investigated the possible modulating effect of flavonoids and their synthetic derivatives on DOX metabolism and transport as important mechanisms of DOX toxicity in various model systems. The major goal of this study was to find potentially effective inhibitors of mechanisms leading to DOX toxicity and find some new promising P-gp modulators, which could increase the therapeutic effect of DOX in resistant tumors.

The main DOX metabolite detected in liver cytosolic fractions was doxorubicinol. This metabolite is considered to be responsible for chronic cardiotoxicity of DOX, because it was found to be accumulated in strips of atrial or ventricular cardiac tissues after a multiple dosing regimen in rabbits.[19] In addition, an increased cardiotoxicity with accompanying iron overload and decreased cardiotoxicity after use of iron chelators supported the view that iron-DOX redox cycling is involved. The fact that doxorubicinol has markedly higher toxic effects than the parent drug suggests that inhibition of DOX transformation to doxorubicinol could significantly decrease the toxicity of DOX. The natural flavonoid quercetin, common in human diet,[20] exerts antioxidant effects and it is well known to influence the activity of different biotransformation enzymes, for instance cytochromes P450.[21] In our study, quercetin strongly inhibited the formation of doxorubicinol in human liver cytosolic fractions. The ability of quercetin to inhibit the formation of doxorubicinol suggested that this flavonoid might influence the catalytic activity of human reductases. This finding supports the beneficial role of antioxidant flavonoid quercetin in the prevention of DOX toxicity due to the metabolism of this drug. Synthetic flavonoid derivatives (4-iodochalcon and 4-hydroxy-6-methoxyauron) inhibited doxorubicinol formation as well, but their effects were notable at the highest concentration (30 μM), so those synthetic flavonoid derivatives are not good candidates for the study of DOX metabolism modulation in vivo. Although the plasma concentrations of these derivatives have not yet been characterized, it is highly probable that 30 μM levels can hardly be reached by nutritional intervention. For instance, the mean human plasma concentration of quercetin from high-vegetable diet was found to be 0.052 μM.[22] Olthof et al.[23] 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 μM, respectively.

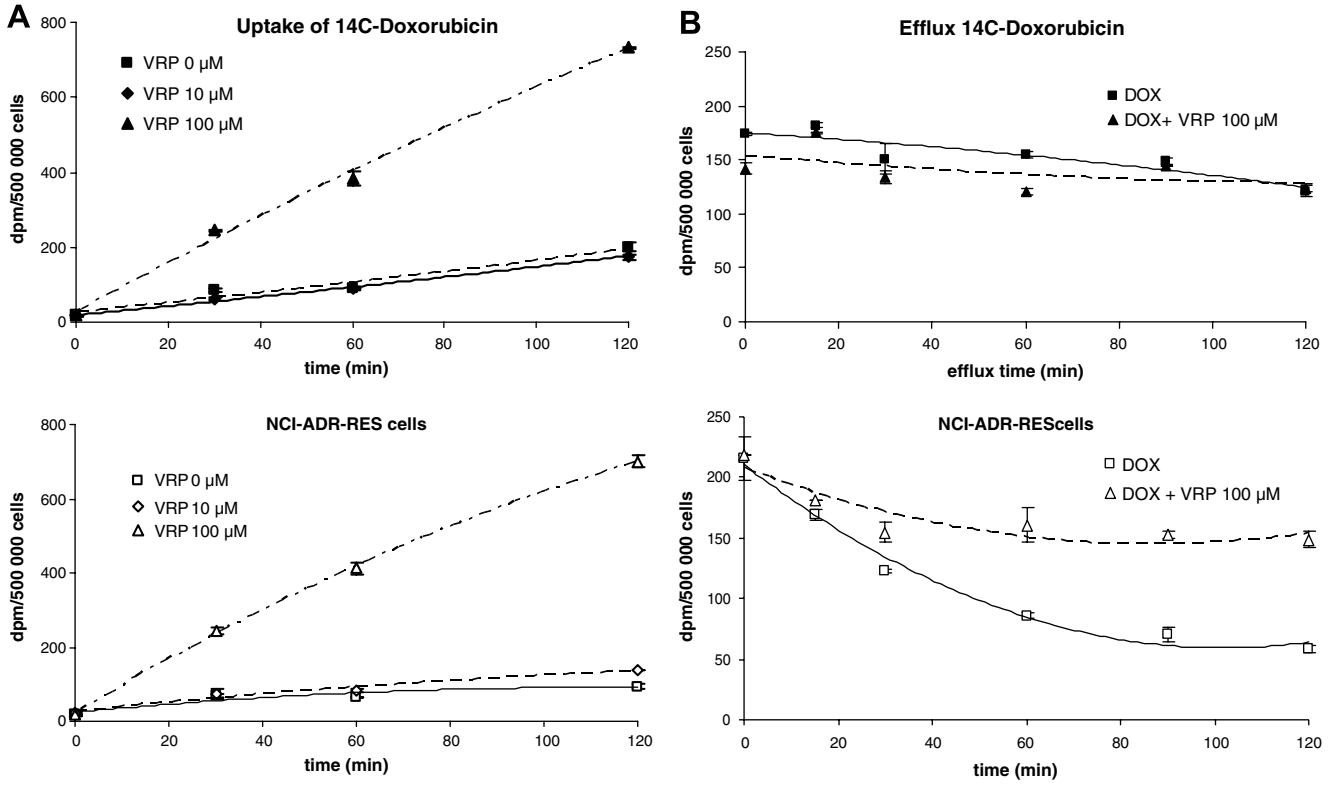


Figure 4. Effect of verapamil on doxorubicin uptake and efflux in NCI-ADR-RES and MDA-MB-435 cells. (A) The effect of 10 and 100 μM verapamil on uptake of 100 nM [^{14}C]DOX after 0, 30, 60, and 120 min. incubation. (B) The effect of 100 μM verapamil on efflux of 100 nM [^{14}C]DOX. Eflux times were 0, 15, 30, 60, 90, and 120 min. The levels of DOX were measured by liquid scintillation. Data are shown as means of duplicate determinations ± SD and expressed as dpm units/500 000 cells/well.

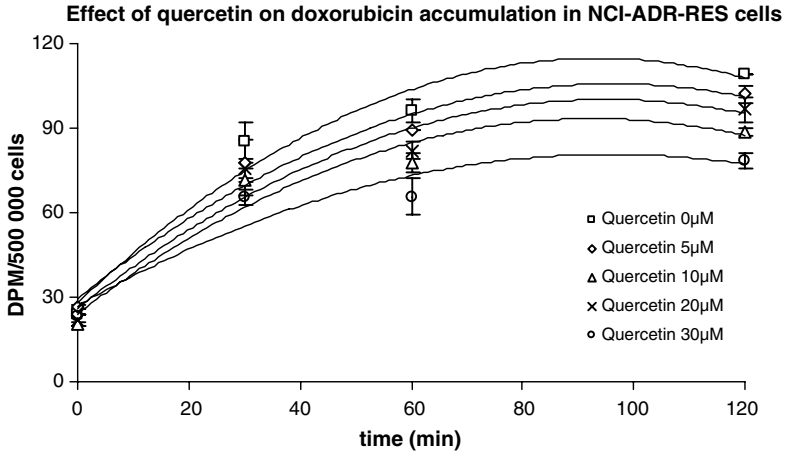


Figure 5. The effect of quercetin on doxorubicin accumulation in NCI-ADR-RES cells. Incubation of 100 nM [^{14}C]DOX in the presence of 0, 5, 10, 20, and 30 μM quercetin lasted 30, 60, 90, and 120 min. The levels of DOX were measured by liquid scintillation. Data are shown as means of duplicate determinations ± SD and expressed as dpm units/500 000 cells/well.

Maximum plasma concentration of kaempferol 6 h after endive consumption was 0.1 ± 0.01 μM.[24]

The development of multidrug resistance in cancer cells decreases the overall uptake of DOX as a result of increased efflux, increased intracellular sequestration, and decreased membrane permeability.[25] Resistance is mostly associated with the expression of P-gp encoded by MDR1 gene but another ATP-binding transport protein MRP has also been found elevated in multidrug resistant cell lines.[25] The objective of this study was to confirm the different pattern of DOX accumulation and efflux in cell lines sensitive (MDA-MB-435) and resistant (NCI-ADR-RES) to DOX and to test potentially efficient modulators of DOX transport in resistant tumor cell line. The NCI-ADR-RES cell line differs from sensitive cell line in high expression of the ABC transporter genes, especially MDR1.[17] Our results

suggest that human breast cancer cell line NCI-ADR-RES was threefold more resistant to DOX than the P-gp non-expressing MDA-MB-435 cell line. This result supports the very important role of P-gp transporter expression in cell membranes of tumor cells and its role in sensitivity of tumors to DOX. A P-gp modulator, verapamil, significantly increased [^{14}C]DOX uptake and decreased its efflux in NCI-ADR-RES cells. The fact that verapamil increased the accumulation of [^{14}C]DOX in MDA-MB-435 (Pgp non-expressing) cells 3.7-fold suggests that the resistance to DOX is caused not only by P-gp overexpression. On the other hand, verapamil did not have any significant effect on the efflux of [^{14}C]DOX in MDA-MB-435 cells. Thus, verapamil may potentiate the antitumor effect of DOX on cancer cells highly expressing P-gp but also slightly influence the sensitive MDA-MB-435 cells. It is possible that DOX forms an intracellular chemical complex with verapamil, which is not a suitable substrate for the P-gp transport pump. MRP proteins are other membrane transporters involved in the development of multidrug resistance potentially responsible for the efflux of [^{14}C]DOX. However, we have not found any significant effect of an MRP1/2 inhibitor, MK-571, on [^{14}C]DOX transport in sensitive and resistant human breast cancer cell lines. Thus, MRP1/2 transporters are probably not involved in the development of resistance to DOX.

In a previous study,[26] we found that synthetic flavonoids, especially aurones, increased the accumulation and decreased the efflux of [^{14}C]paclitaxel, another P-gp substrate, in NCI-ADR-RES cells. Moreover, synthetic flavonoid derivatives exerted effective binding affinity toward the nucleotide binding-domain (NBD2) of P-gp.[14–16] Therefore, we investigated the potential modulatory effect of the natural flavonoid, quercetin, and synthetic flavonoid derivatives: 4,6,3′,4′,6′-pentamethoxyauron, 4′-chloro-4-hydroxy-6-methoxyauron, and 4′-ethyl-4-hydroxy-6-methoxyauron, 4-hyroxy-6-methoxyauron on DOX transport. In this study however, none of the tested auron derivatives as well as quercetin exerted any significant effect on the accumulation of [^{14}C]DOX in NCI-ADR-RES cells. Thus, the tested flavonoids cannot be considered appropriate modulators of DOX transport in human resistant breast cancer cells, although they effectively decreased uptake of another typical P-gp substrate paclitaxel.[26] The modulating effect of synthetic flavonoid derivatives on P-gp-mediated transport therefore strongly depends on the type and structure of P-gp substrate.

4. Conclusions

Our study revealed the inhibition of DOX metabolism to toxic doxorubicinol by naturally occurring flavonoid quercetin and determined its IC_{50} value in human liver cytosolic fractions. Quercetin did not significantly inhibit the accumulation of DOX in resistant human breast cancer cell line. Synthetic flavonoid derivatives from auron class did not significantly modify DOX accumulation and efflux in a resistant human breast cancer cell line, and their use in combating the resistance to DOX is thus limited. Finally, MRP1/2 transporters did not significantly participate in DOX transport as shown by the lack of effect of a specific inhibitor, MK-571.

5. Experimental

5.1. Chemicals

DOX hydrochloride and daunorubicin hydrochloride, solvents, quercetin, fisetin, β-NADP, and glucose-6-phosphate were purchased from Sigma–Aldrich (Prague, CR). Radiolabeled DOX ([^{14}C]Doxorubicin hydrochloride=[^{14}C]Adriamycin, 925 kBq, 25 μCi) was obtained from Amersham Biosciences (New Jersey, USA). Verapamil was purchased from Sigma–Aldrich (Prague, CR) and MK-571 sodium salt was product of Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was purchased from J. Kysilka (Brno, CR), L-glutamine, Hepes, penicillin, streptomycin, and trypsin were obtained from PAN Biotech GmbH (Aidenbach, Germany). Glucose-6-phosphate dehydrogenase was from Boehringer (Mannheim, Germany). Eriodictyol was a generous gift of Yune-Fang Ueng (National Research Institute of Chinese Medicine, Taipei, Taiwan, Republic of China). Synthetic flavonoid derivatives, 4-hydroxy-6-methoxyauron, pentamethoxyauron, 3-phenyl-5-7-dihydroxyazaisoflavon, and 4-iodochalcon were prepared according to Bois et al.[14] and Boumendjel et al.[16] The structures of DOX and its metabolites are shown in Figure 1. The chemical and common names of examined naturally occurring flavonoids and their synthetic derivatives are given in Table 1.

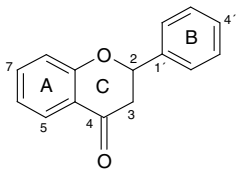
5.2. Preparation and characterization of human microsomal preparations

Human liver samples were obtained from transplantation donors in the period 1992–1997. The use of human liver samples was in accordance with the law. Livers were collected from male patients ($N = 14$) with unknown drug history, who invariably died from head injury. During the heart or kidney transplantation procedure the corpses were infused with cold physiological saline and the liver samples were frozen in liquid nitrogen immediately after excision and maintained there until use. The time period between the beginning of blood exchange and freezing the liver in liquid nitrogen was 9–46 min and this period as well as the age of the donor did not correlate with various cytochrome P450 (EC 1.14.14.1) activities and immunochemical levels.[27] Microsomal and cytosolic fractions of the liver were prepared by differential centrifugation as described in detail previously.[28] The concentrations of microsomal and cytosolic protein were determined according to Lowry et al.[29] with bovine serum albumin as standard.

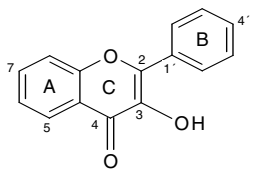
5.3. Assay of doxorubicin metabolism

The incubation mixture contained human microsomal or cytosolic protein, 1 mg/ml, in 0.1 M phosphate buffer

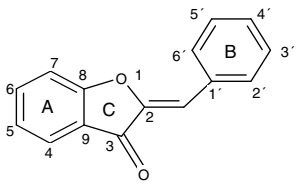
Table 1. Structures of tested natural flavonoids and synthetic flavonoid derivatives



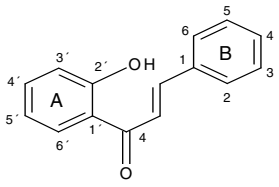
Flavanone



Flavonol



Aurone



Chalcone

Class	Name	Substituents
	Natural flavonoids	
Flavanones	Eriodictyol (5,7,3′,4′-tetrahydroxyflavon)	5,7,3′,4′-OH
Flavonols	Fisetin (3,7,3′,4′- tetrahydroxyflavon)	3,7,3′,4′-OH
	Quercetin (3,5,7,3′,4′-pentahydroxyflavon)	3,5,7,3′,4′-OH
	Synthetic flavonoid derivatives	
Aurones	4,6,3′,4′,6′- pentamethoxyauron	4,6,3′,4′,6′-OMe
	4-Hydroxy-6-methoxyauron	4-OH; 6-OMe
	4′-Chloro-4-hydroxy-6-methoxyauron	4-OH; 6-OMe; 4′-Cl
	4′-Ethyl-4-hydroxy-6-methoxyauron	4-OH; 6-OMe; 4′-CH_2CH_3
Chalcones	4-Iodochalcon	4-I; 2′,4′,6′-OH

(pH 7.4) and DOX (5–25 μM) added as 1 mM aqueous solution in a total volume of 1 ml. The reaction was initiated by adding an NADPH-generating system (NADPH-GS, final concentration 1 mM NADP, 10 mM glucose-6-phosphate, 10 mM $MgCl_2$, and 0.5 U/ml glucose-6-phosphate dehydrogenase). The samples were incubated at 37 °C for 60 min in a shaking water bath. The reaction was stopped by addition of 500 μl of ice-cold ethanol. Samples were then centrifuged for 15 min at 12000 rpm and their supernatants were analyzed by HPLC with daunorubicin as internal standard. HPLC analysis was performed with fluorescence detection using the Agilent HP1100 system (Agilent Technologies, Santa Clara, CA, USA) equipped with fluorescence detector type HP 1046A (Agilent Technologies, Santa Clara, CA, USA). The 4×125 mm column with Nucleosil 100-5 C18 (Macherey-Nagel, Düren, Germany) and 0.05 M ammonium formate buffer (pH = 7.4):acetonitrile (70:30, v/v) as the mobile phase was used for the separation of metabolites. Flow rate was 1.0 ml/min and the injection volume was 40 μl. For the determination of DOX, doxorubicinol, aglycon of DOX, and the internal standard (daunorubicin), fluorescence with excitation at 233 nm and emission at 550 nm was used. Chromatograms were analyzed by CSW software version 1.7 (Chromatography Station for Windows, DataApex, Prague, CR) and the peak height was used for quantification. The metabolites were identified by comparing their known retention time ratios to daunorubicin and were quantified assuming similar molar extinction coefficients of DOX and daunorubicin.

5.4. Chemical inhibition of doxorubicin metabolism

Inhibition studies were performed in the presence of 0, 10, 20, and 30 μM fisetin, quercetin, eriodictyol, 4-hydroxy-6-methoxyauron, and 4-iodochalcon in the incubation mixtures. Required volumes of stock solutions (1 mM in methanol) were evaporated under nitrogen stream and redissolved in the incubation mixtures by sonication at 80 °C for 10 min. The concentration of DOX was 10 μM. The incubation and HPLC conditions were the same as mentioned above.

5.5. Cells and culture conditions

The human breast carcinoma cell lines MDA-MB-435 and NCI-ADR-RES were obtained from the National Cancer Institute (Frederick, MD, USA). Cells were maintained in the basic medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. RPMI 1640 medium containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), Hepes (15 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used as a basic medium. The cells were trypsinized before use by 0.2% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS).

5.6. Cell growth and survival—MTT assay

Cells maintained in 10% FBS medium were harvested by low speed-centrifugation, washed with FBS medium, and seeded at 10×10^3 cells/100 μl of medium into wells of a 96-well plastic plate. Cell growth and survival were evaluated under control conditions (FBS medium) or after exposure to flavonoid derivatives (10 μM) for 96 h. Then 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 g l^{-1}) was added to the cells in each well, and incubated for 2 h at 37 °C in a humidified incubator (5% CO_2). After the incubation, 80 μl of medium was aspirated, 150 μl of 0.04 N HCl in isopropanol was added, and the mixture was resuspended. Absorbance was measured at 570 nm using a Spectra Sunrise microplate reader (Tecan, Salzburg, Austria).

5.7. Transport of [^{14}C]doxorubicin

For the uptake assay, cells were exposed to fresh medium or medium containing 10–100 nM [^{14}C]DOX, dissolved in PBS. After incubation for 30–240 min, medium was quickly aspirated and the adherent cells were washed three times with ice-cold PBS. The cells were released by 2×400 μl of trypsin and EDTA (humid atmosphere, 37 °C, 15 min) and then ejected into scintillation vials using Bray solution. Sodium dodecyl sulfate (200 μl) was added up to 2% final concentration for lysis of cells and release of the drug. The efflux measurements were performed after a 2 h loading with 100 nM [^{14}C]DOX. Cells were then washed twice with PBS, placed in fresh medium, and analyzed after 15, 30, 60, 90, and 120 min. The releasing of cells was the same as described above.

5.8. Effect of verapamil, MK-571 and flavonoids on [^{14}C]doxorubicin transport

For the study of [^{14}C]DOX uptake modulation, cells were preincubated for 30 min with fresh medium or medium containing 10 and 100 μM verapamil, 100 μM MK-571 or 10–30 μM flavonoid (quercetin, 4,6,3′,4′, 6′-pentamethoxyauron, 4′-chloro-4-hydroxy-6-methoxyauron, 4′-ethyl-4-hydroxy-6-methoxyauron, 4-hyroxy-6-methoxyauron) dissolved in DMSO (maximum concentration in medium 0.3%, v/v). The medium was rapidly replaced by fresh medium with 100 nM [^{14}C]DOX or 100 nM [^{14}C] DOX with the above-specified modulators. Cells were incubated for 30, 60, 90, 120, and 240 min. Cells were then rapidly washed three times with ice-cold PBS, released and dissolved as described above. In the efflux assays, cells were preincubated for 120 min with 100 nM [^{14}C] DOX. The medium was then replaced with lukewarm fresh medium with or without 100 μM verapamil or medium containing MK-571 and cells were incubated at 37 °C for 15, 30, 60, 90, and 120 min. Cells were then rapidly washed three times with ice-cold PBS, released, and dissolved as described above.

5.9. Effect of quercetin on [^{14}C]doxorubicin efflux

In the efflux assays, MDA-MB-435 and NCI-ADR-RES cells were preincubated with 100 nM [^{14}C] DOX for 2 h. The medium was then replaced with lukewarm fresh medium or medium with 30 μM quercetin and the cells were incubated at 37 °C for time periods specified in the figures. The cells were then rapidly washed 3 times with ice-cold PBS, released, and dissolved as described above for measurement of radioactivity.

5.10. Statistical analysis

All values are expressed as means ± standard deviations (SD) of the estimates. Differences in the effects of flavonoid derivatives on DOX metabolism were estimated with unpaired two-tailed Student's *t*-test ($^{*}p < 0.05$ and $^{**}p < 0.01$ considered significant).

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