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Modulation of paclitaxel transport by flavonoid derivatives in human breast cancer cells. Is there a correlation between binding affinity to NBD of P-gp and modulation of transport?

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Abstract—We have investigated the effect of 13 flavonoid derivatives on [^{14}C]paclitaxel transport in two human breast cancer cell lines, the adriamycin-resistant NCI/ADR-RES and sensitive MDA-MB-435. For this study, we selected representatives of aurones, chalcones, flavones, flavonols, chromones, and isoflavones with known binding affinity toward nucleotide-binding domain (NBD2) of P-glycoprotein and for which no reported work is available regarding paclitaxel transport. Aurones **CB-284**, **CB-285**, **CB-287**, and **ML-50** most effectively inhibited P-gp related transport in the resistant line in comparison with chalcones, flavones, flavonols, chromones, and isoflavone derivatives and accordingly increased the accumulation of [^{14}C]paclitaxel and decreased its efflux. Those agents efficiently modulated paclitaxel transport in P-gp highly expressing resistant human breast cancer cells and they could increase the efficiency of chemotherapy in paclitaxel-resistant tumors. In contrast, the sensitive cell line responded reversely in that **CB-284**, **CB-285**, **CB-287**, and **ML-50** significantly inhibited accumulation of [^{14}C]paclitaxel and especially **CB-287**, which significantly stimulated its efflux. Some, but not all, of the data correlated with the binding of flavonoid derivatives to P-gp, and indicated that even in the P-gp highly expressing NCI/ADR-RES cells, the binding was not the only factor influencing the transport of [^{14}C]paclitaxel. Opposite effects of flavonoid derivatives on the P-gp highly expressing and MDA-MB-435 non-expressing cell lines indicate that paclitaxel is not only transported by P-gp and let us assume that Mrp2 or ABCC5 seem to be good transport-candidates in these cells. The inhibition of paclitaxel accumulation and stimulation of its efflux are potentially unfavorable for drug therapy and since they could be due to modulation of drug transporters other than P-gp, their expression in tumors is of great significance for efficient chemotherapy.

1. Introduction

Tumor cells frequently possess or develop resistance, which constitutes a major problem for cancer chemotherapy.[1,2] A significant part of intrinsic or acquired resistance of tumors to chemotherapy, called multidrug resistance (MDR), is caused by high expression of ABC transporters responsible for the efflux of drugs from tumors. The MDR phenotype often correlates with high expression of membrane ABC transporter P-glycoprotein (P-gp). P-gp is a transmembrane ATP-dependent efflux pump which significantly reduces the intracellular levels of many anticancer drugs in tumors.[3]

Important anticancer drugs belonging to taxanes (paclitaxel and docetaxel) and anthracyclines (doxorubicin) are excellent substrates for the drug transporter P-gp.[4,5] The resistance to paclitaxel and doxorubicin in patients with breast cancer is correlated with increased P-gp expression.[6]

P-gp reversers offer a possible therapeutic approach for increasing drug concentrations in tumors; more than 40 have been described[7] and their number is steadily increasing. Well-known specific inhibitors of P-gp

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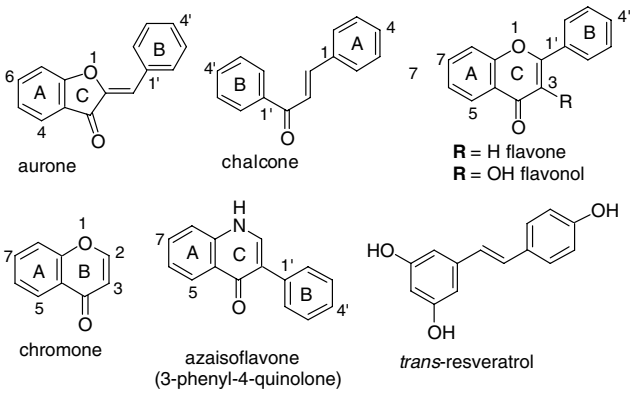
transport such as cyclosporine A and verapamil suppress MDR and they appear to be competitive inhibitors and substrates of P-gp.[8] High levels of those modulators are required, which produce undesirable side effects.[9] That is why new modulators, which are not themselves effluxed by P-gp, are being looked for.

Nowadays, there is an intensive search for new potent MDR-reversal agents with minimal adverse effects. Naturally occurring flavonoids and resveratrol may be suitable candidates for: (a) being natural and low-toxic antioxidant constituents of food, (b) bind to P-gp,[10] (c) inhibiting CYP-catalyzed inactivation of paclitaxel,[11] and (d) exerting anticancer activity of their own.[12] It was reported that flavonoids are a new class of bifunctional modulators, which partly overlap the ATP-binding site and the vicinal hydrophobic region interacting with steroids within the cytosolic domain of P-gp.[13] Flavonoids such as kaempferol, galangin, quercetin, their hydrophobic derivatives, and genistein, which modulate drug efflux in MDR cancer cells, bind with similar affinities and relative efficiencies to purified H_6-NBD2 (nucleotide-binding domain) cytosolic domain of P-gp.[13] Perez-Victoria et al. suggested that only flavonoids, which bind with high affinity to the cytosolic domain of P-gp, are able to increase daunomycin accumulation in *Leishmania tropica* line overexpressing this transporter and inhibit the parasite's growth in the presence of the drug.[9] However, contradictory effects were reported; quercetin and its methoxylated derivative inhibited the efflux of rhodamine-123 and restored sensitivity to adriamycin in MCF-7 breast cancer cells[14] and quercetin bound to purified P-gp and efficiently inhibited its activity.[15] In contrast, quercetin, kaempferol, and galangin increased adriamycin efflux in HCT-15 colon cells supposedly via a P-gp-mediated mechanism.[16] Among other types of plant polyphenols, green tea polyphenols, such as (−)-epigallocatechin gallate, inhibited the binding and efflux of two P-gp substrates, rhodamine-123 and [^{3}H]-vinblastine, in resistant Chinese hamster ovary cells (CH^RC5) and human colon adenocarcinoma cells (Caco-2).[17]

In view of these contradictory results, we focused our study on a new generation of synthetic flavonoid derivatives as more promising modulators of P-gp-mediated transport. Those flavonoid analogues, which successfully interact with the NBD2 domain of P-gp are potential MDR modulators and reversing agents of cancer resistance to cytotoxic drugs.[18–21] The purpose of the present study was to investigate flavonoid derivatives with known binding affinities to NBD2 of P-gp for their effect on [^{14}C]paclitaxel transport in both resistant and sensitive human breast cancer cells. In addition to the evaluation of the effect on paclitaxel accumulation and efflux, we decided to shed light on the existence or not of a correlation between binding affinity to NBD2 of P-gp and modulation activity. Structures of these derivatives belong to different subclasses of natural flavonoids and include: aurones, chalcones, flavones, flavonols, chromones, and isoflavones. Their structures and binding affinities to NBD2 of P-gp are shown in Table 1. For the sake of comparison, we selected quercetin for its proved binding to P-gp. Moreover, quercetin was the most effective inhibitor of CYP metabolism of paclitaxel out of eight flavonoids and resveratrol was selected as an even more effective inhibitor of paclitaxel metabolism.[11]

Table 1. Structures of tested flavonoid derivatives and their binding affinity to P-gp



Class/name	Substituents	K_D[a] (μM)
Stilbenes		
trans-resveratrol	3,5,4′-Trihydroxystilbene	ND
Aurones		
CB-284	4,6-OMe; 4′-Br	0.82 ± 0.08[b]
CB-285	4,6-OMe; 4′-Cl	0.99 ± 0.2[b]
CB-287	4,6,3′,4′,6′-OMe	92 ± 43[b]
ML-50	4-OH; 6-OMe	1.32 ± 0.33[b]
ND-285	4-OH; 6-OMe; 4′-Cl	0.46 ± 0.08[b]
ML-30	4-OH; 6-OMe; 4′-CH_2CH_3	10 ± 0.15
A-55B	4-OH; 6-OMe; 7-I; 4′-CN	ND
Chalcone		
FBB-14	4-I; 2′,4′,6′-OH	0.25 ± 0.06[c]
Flavone		
CB-436	3-OMe; 5,7-OH; 4′-Br	>100
Flavonols		
Kaempferid	5,7-OH; 4′-OMe	5.0 ± 0.2[d]
AB-2DE	5,7-OH; 4′-I	1.06 ± 0.08[d]
Quercetin	5,7,3′,4′	7.0 ± 0.5[e]
Chromone		
MH-11[f]	2-CO–N⟨⟩N·CH_3; 5-OH	>100
Azaisoflavone		
A-12[f]	3-Phenyl; 5,7-OH	>100

ND, K_D was not measured.
[a] Dissociation constant of binding to P-gp.
[b] Ref. 20.
[c] Ref. 18.
[d] Ref. 19.
[e] Ref. 10.
[f] **MH-11** and **A-12** enhanced accumulation of rhodamine-123 in K562R resistant cells 5-fold and 2-fold higher than cyclosporine A at 1 μM concentration.[25]

2. Results

2.1. Flavonoid enhancement of [^{14}C]paclitaxel accumulation in resistant human breast cancer cells

On the basis of known similarity between CYP3A and P-gp substrates, we initially investigated the effect of two natural polyphenols; flavonoid quercetin and stilbene *trans*-resveratrol on [^{14}C]paclitaxel (a P-gp sub-

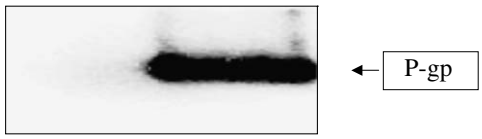


Figure 1. The difference in expression of P-gp in sensitive (MDA-MB-435) and adriamycin-resistant (NCI/ADR-RES) human breast cancer cells.

strate) accumulation. These two substances are among the most efficient inhibitors of paclitaxel metabolism by human and rat CYP3As.[11] However, at 100 μM (25 μM was ineffective), quercetin weakly increased [^{14}C]paclitaxel accumulation (30% increase) in both NCI/ADR-RES cells (selected for resistance to the P-gp substrate adriamycin) and sensitive MDA-MB-435 cells (Fig. 2A). This weak and similar effect in both cell lines indicates that P-gp was not responsible for the enhancement. In contrast, resveratrol (100 μM) inhibited uptake (30%) of [^{14}C]paclitaxel in MDA-MB-435 (but not in NCI/ADR-RES) cells during 30 min, suggesting an inhibition of inward active transport. The effect was apparently too weak to be significant in vivo, decreased until 60 min of incubation and 25 μM resveratrol was ineffective (Fig. 2A).

We subsequently investigated the effects of flavonoid derivatives on transport of [^{14}C]paclitaxel in NCI/ADR-RES. Figure 1 illustrates that resistant NCI/ADR-RES cells expressed high levels of P-gp protein, which the sensitive MDA-MB-435 cells were lacking. Thus, we expected meaningful differences in paclitaxel transport in the two cell lines.

In our transport studies, the cells were incubated with [^{14}C]paclitaxel without or with one of 13 different synthetic flavonoids, namely: aurones (**CB-284**, **CB-285**, **CB-287**, **CB-436**, **ND-285**, **ML-302**, **ML-50**, and **A-55B**), chalcone (**FBB-14**), flavones (**CB-436**), flavonols (**AB-2DE** and kaempferid), chromone (**MH-11**), and azaisoflavones (**A-12**).

The accumulation of [^{14}C]paclitaxel was measured after 30 min and 60 min (Fig. 2B). Six out of the 13 flavonoids strongly enhanced the accumulation of [^{14}C]paclitaxel in the resistant cells after 30 min in the following order: **CB-287** ⩾ **ML-50** > **CB-284** ⩾ **CB-285** > **CB-436** > **A-12** (Fig. 2B). After 60 min, the order was somewhat different, but the first four most effective flavonoids remained most effective as well.

Chalcone **FBB-14**, flavonol **AB-2DE**, aurones **ND-285** and **ML-30** exerted a significant, but mild effect, whereas

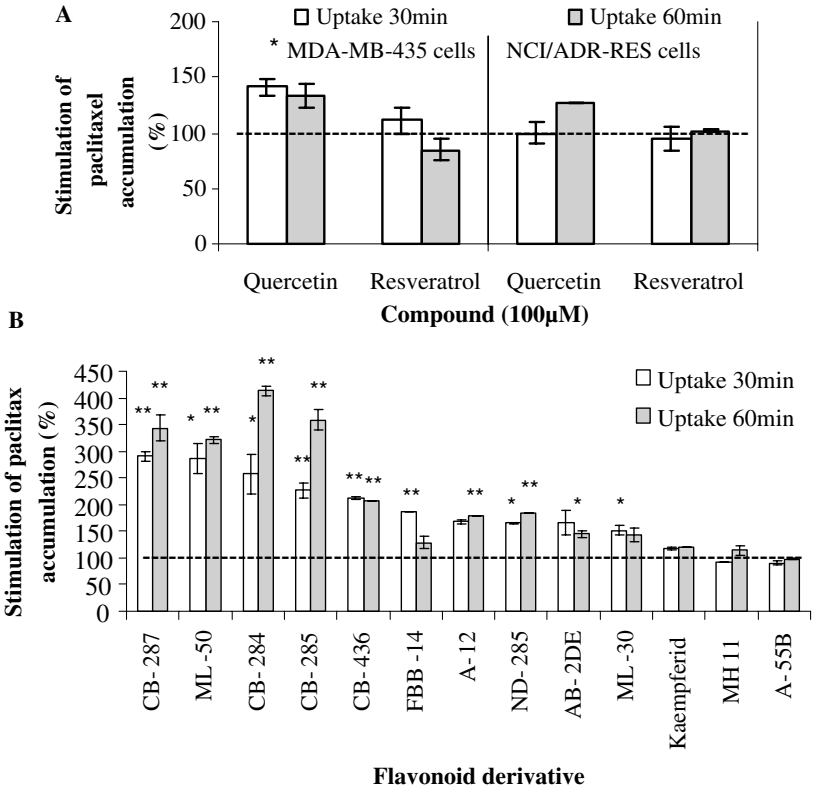


Figure 2. (A) Effect of quercetin and resveratrol on [^{14}C]paclitaxel accumulation in NCI/ADR-RES and MDA-MB-435 human breast cancer cell lines. NCI/ADR-RES and MDA-MB-435 cells were loaded with [^{14}C]paclitaxel for 30 and 60 min in the presence of flavonoid derivatives (100 μM) or DMSO as control. (B) Effect of flavonoid derivatives on [^{14}C]paclitaxel accumulation in NCI/ADR-RES human breast cancer cell lines. NCI/ADR-RES cells were loaded with [^{14}C]paclitaxel for 30 and 60 min in the presence of flavonoid derivatives (10 μM) or DMSO (0.1%) as control. Values (means ± SD, $n \geqslant 2$) are expressed as a percentage of stimulated accumulation. Asterisks denote a difference from the 100% accumulation at $*p < 0.05$ and $**p < 0.01$, using a two-tailed t-test.

three flavonoids, chromone **MH-11**, and aurone **A-55B** did not influence paclitaxel accumulation.

2.2. Flavonoid inhibition of [^{14}C]paclitaxel efflux in resistant human breast cancer cells

The efflux of [^{14}C]paclitaxel alone in the resistant NCI/ADRE-RES cells was 4-fold higher than in the sensitive MDA-MB-435 cells with low P-gp expression (data not shown).

Based on the results obtained from the accumulation assay, we went forward to investigate whether flavonoids which significantly increased [^{14}C]paclitaxel accumulation would inhibit efflux of this drug in NCI/ADR-RES cells as a proof of P-gp-mediated transport. It was found that, in the presence of CB-287, the remaining intracellular amount of [^{14}C]paclitaxel after 15 and 30 min of efflux was higher than control. For example, in the control cells, the efflux eliminated 33% of the onset level of [^{14}C]paclitaxel versus 3% in those exposed to **CB-287** (10 μM), meaning that the latter decreased the efflux 10 times. Under the same conditions, the other flavonoid derivatives **CB-284**, **CB-285**, **ML-50**, **A-12** and **CB-436** did not significantly influence elimination of [^{14}C]paclitaxel (Fig. 3). These data suggest that at 10 μM and higher levels, the efflux of [^{14}C]paclitaxel in human resistant cancer cells could be decreased by **CB-287**, the flavonoid derivative of the aurone subclass.

2.3. Different effects of flavonoids on [^{14}C]paclitaxel transport in sensitive human breast cancer cells

Four compounds, which most effectively stimulated [^{14}C]paclitaxel accumulation in NCI/ADR-RES cell lines, were also tested in the sensitive MDA-MB-435 cells. Contrary to their effects in resistant NCI/ADR-RES cells, all of them significantly decreased the uptake of [^{14}C]paclitaxel in sensitive human breast cancer cells (Fig. 4A). For example, after 60 min, the uptake of [^{14}C]paclitaxel was 6-fold inhibited by **ML-50** as compared with the control. These results suggest that

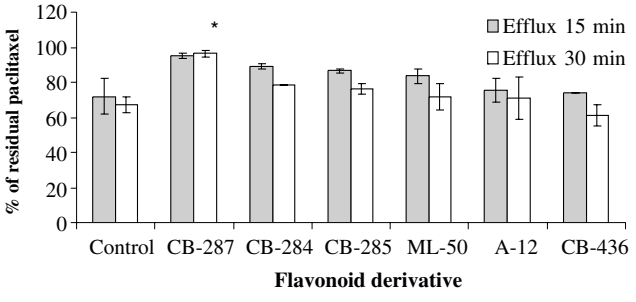


Figure 3. The effect of flavonoid derivatives which influenced accumulation of [^{14}C]paclitaxel on efflux in NCI/ADR-RES cells. Cells were loaded with [^{14}C]paclitaxel for 1 h and then allowed to efflux for 15 or 30 min in the presence of flavonoid derivative (10 μM) or DMSO (0.1%) as control. The values (means ± SD, $n \geqslant 2$) represent residual cellular radioactivity after the efflux expressed as percentage of values found at the onset of efflux. Asterisks denote a difference from the 100% accumulation at $*p < 0.05$ and $**p < 0.01$, using a two-tailed *t*-test.

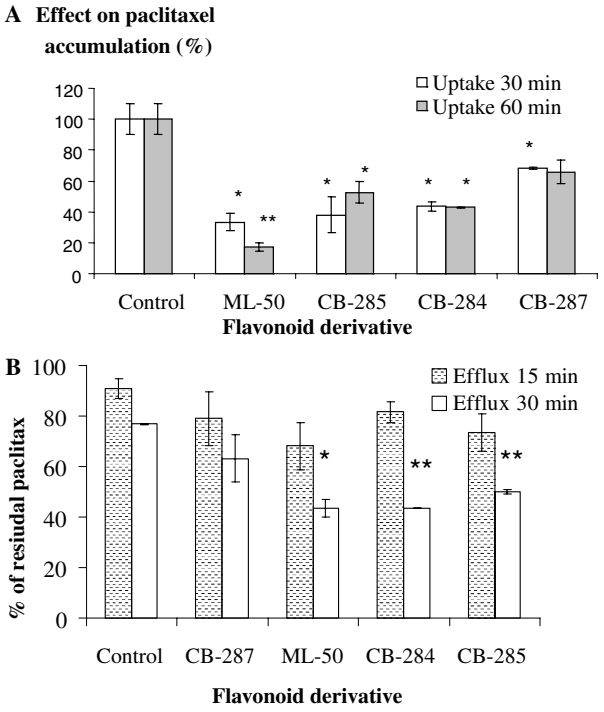


Figure 4. (A) The effect of flavonoid derivatives on [^{14}C]paclitaxel uptake in MDA-MB-435 sensitive human breast cancer cells. MDA-MB-435 cells were loaded with [^{14}C]paclitaxel for 30 min and 60 min in the presence of flavonoids (10 μM) or DMSO (0.1%) as control. Values (means ± SD, $n \geqslant 2$). (B) The effect of flavonoid derivatives on [^{14}C]paclitaxel efflux in MDA-MB-435 cells. Cells were loaded with [^{14}C]paclitaxel for 1 h and then allowed to efflux for 15 or 30 min in the presence of flavonoid derivative (10 μM) or DMSO (0.1%) as control. The values (means ± SD, $n \geqslant 2$) represent residual cellular radioactivity after the efflux expressed as percentage of values found at the onset of efflux. Asterisks denote a difference from the 100% accumulation at $*p < 0.05$ and $**p < 0.01$, using a two-tailed *t*-test.

Table 2. Cytotoxicity of tested flavonoid derivatives in MDA-MB-435 and NCI/ADR-RES cells

Flavonoid derivative	Cell line	
	MDA-MB-435	NCI/ADR-RES
Control[a]	0.931 ± 0.076*	0.554 ± 0.052
Kaempferid	0.690 ± 0.107*	0.488 ± 0.025
MH-11	0.721 ± 0.095*	0.471 ± 0.046
A-12	0.696 ± 0.112*	0.488 ± 0.065
FBB-14	0.100 ± 0.044*	0.608 ± 0.069
A-55B	0.534 ± 0.033*	0.290 ± 0.042*
CB-287	0.705 ± 0.103*	0.525 ± 0.098
CB-285	0.657 ± 0.087*	0.483 ± 0.050
ML-50	0.745 ± 0.110*	0.525 ± 0.038
CB-284	0.551 ± 0.087*	0.538 ± 0.030
CB-436	0.641 ± 0.180*	0.550 ± 0.132
ML-30	0.628 ± 0.069*	0.468 ± 0.042
AB-2DE	0.746 ± 0.080*	0.550 ± 0.035
ND-285	0.852 ± 0.066	0.579 ± 0.064

Effect of flavonoid derivatives on the growth and survival of MDA-MB-435 and NCI/ADR-RES cells. Cells were seeded at 10×10^3 cells/100 μL of medium in the well. The number of living cells was determined after 96 h of incubation. Numbers in the table indicate the mean of absorbance values (A^{570}) of eight separate cultures ± SEM. The data were evaluated by one-way ANOVA with Newman–Keuls post hoc comparison (post hoc test).

[a] Control, control cells without flavonoid derivatives.

* $P < 0.05$ compared with control values.

different transporters may be involved in the two cell lines. Moreover, in contrast to the resistant NCI/ADR-RES cells, the efflux of [^{14}C]paclitaxel in MDA-MB-435 cells during 30 min was significantly stimulated in the presence of **ML-50**, **CB-284**, and **CB-285** (Fig. 4B).

2.4. Cytotoxicity of tested flavonoid derivatives in NCI/ADR-RES and MDA-MB-435 human breast cancer cell lines

The tested flavonoids (10 μM) did not have any significant effect on cell cytotoxicity of NCI/ADR-RES cells except for **A-55B**. On the other hand, all the tested flavonoid derivatives (except ND-285) were significantly ($P < 0.05$) cytotoxic for sensitive cells MDA-MB-435 (Table 2). These results support different behavior of flavonoid derivatives in sensitive MDA-MB-435 and resistant NCI/ADR-RES human breast cancer cell lines.

3. Structure–activity relationship and discussion

Quercetin, a naturally occurring flavonoid, known to bind to and influence P-gp related transport, only slightly inhibited transport of the P-gp substrate [^{14}C]paclitaxel in P-gp highly expressing NCI/ADR-RES cells. In this study, we selected representatives of different flavonoid-subclasses (aurones, chalcones, flavones, flavonols, chromones, and isoflavones) with known binding affinity toward the nucleotide-binding domain (NBD2) of P-gp (Table 1) and investigated them as modulators of paclitaxel transport. We found that synthetic flavonoids, especially aurones, increased the accumulation and decreased the efflux of [^{14}C]paclitaxel in NCI/ADR-RES cells. As paclitaxel is a P-gp substrate, the effect occurring in the P-gp expressing cells and not in P-gp-lacking MDA-MB-435 cells indicates that aurones are acting very likely via P-gp inhibition.

The decreased accumulation and increased efflux of [^{14}C]paclitaxel in MDA-MB-435 cells are completely contradictory to their effects in NCI/ADR-RES cells. It is known that paclitaxel is significantly transported by another ABC transporter.[22] The fact that MDA-MB-435 cells do not express P-gp, but strongly express Mrp2 and ABCC5 (lacking in the NCI/ADR-RES cells),[23] suggests their possible involvement in paclitaxel transport in MDA-MB-435 cells. The role of other ABC transporters in effects of flavonoids on transport of doxorubicin (P-gp substrate) in different cell lines was already suggested before.[14–16] It is noteworthy to highlight that an ABC protein is able to transport P-gp substrates, but it is not inhibited by P-gp inhibitors.[24]

Some of these flavonoids increased the accumulation of [^{14}C]paclitaxel, with the most active compounds being members of the aurone class. Structural features enhancing the activity of aurones on paclitaxel transport were methoxy groups on both A- and B-rings (**CB-287**) and to a lesser extent, the simultaneous presence of methoxy groups on the A-ring and a halogen at 4′-position with Br being more effective than Cl (**CB-285** and **CB-284**). Replacing the 4-methoxy group with a hydroxyl was disadvantageous for the activity (**CB-285** vs **ND-285**). This is quite surprising, because in an earlier study it was reported that the presence of a hydroxy group at the 4-position of aurones is essential for the binding affinity to NBD2 of P-gp.[20] The highest activity of 4,6-dimethoxyaurones can be explained by their high hydrophobic character in comparison to 4-hydroxyaurones, as it is known that most P-gp inhibitors are hydrophobic compounds.

The apparent lack of correlation between the power of binding affinity and high modulation activity addresses the issue related to whether or not one can transpose in vitro effective compounds (affinity to NBD2) to the cellular test which measures MDR-reversing activity. Dissociation constant (K_D) values which are measured on the cytosolic NBD2 of P-gp may indicate potential interaction with P-gp but do not ensure a significant reversing activity.

The latter assumption is confirmed by the investigation of the only compound belonging to the chalcone subclass tested in this study, 4-iodo-2′,4′,6′-trihydroxychalcone (**FBB-14**). **FBB-14** possesses the highest binding affinity toward NBD2 ($K_D = 0.25$ μM),[18] but shows a moderate activity on [^{14}C]paclitaxel transport, because it increased the accumulation only to 155% of control values. Flavonol (**AB-2DE**) behaves in the same manner as chalcone **FBB-14**. In this study, we included a chromone representative (**MH-11**) and an isoflavone analog (**A-12**) which have very low binding affinity toward NBD2, but potentiated daunorubicin cytotoxicity in an adriamycin-resistant human myeloid leukemia cell line (K562) and increased the intracellular accumulation of rhodamine-123, a probe of P-gp-mediated transport.[25] The observation that **MH-11** at 20 μM did not significantly influence accumulation of the P-gp substrate [^{14}C]paclitaxel in NCI/ADR-RES cell line questions the specificity of these two substrates for P-gp. This difference in effect on accumulation may be due to the fact that these two studies were performed on different P-gp expressing cell types.

It is obvious that establishing a relation between binding affinity and modulation activity on a single cell type can be correctly done. Unfortunately, when cells of different types and origins are used, it renders the correlation more complicated. Moreover, potential human use of these inhibitors will require to prove their efficiency of MDR-reversal in vivo, for example, by positron emission tracer imaging as described, for example, in Ref. 26.

4. Conclusion

In conclusion, some derivatives of flavonoids, especially 4,6-dimethoxyauronies **CB-284**, **CB-285**, and **CB-287** and 4-hydroxy-4-methoxyaurone (**ML-50**), proved to be efficient modulators of paclitaxel transport in P-gp highly expressing resistant human breast cancer cells. Moreover, they are significantly more efficient than natural flavonoids such as quercetin, reported to possess that

activity. These agents could increase the efficiency of chemotherapy with paclitaxel in P-gp highly expressing breast tumors. However, care should be taken of the fact that drug transport in the P-gp non-expressing tumors may be influenced quite differently due to other transporters. Further studies are needed to investigate whether these derivatives are sufficiently potent to increase the effects of P-gp transported anticancer drugs in vivo.

5. Experimental

5.1. Chemicals

[^{14}C]paclitaxel (TAXOL®) was purchased from Moravek Biochemicals (Brea, CA). Fetal bovine serum (FBS) was purchased from J. Kysilka (Brno, CZ), L-glutamine, HEPES, penicillin, streptomycin, and trypsin were obtained from PAN Biotech GmbH (Aidenbach, Germany). Monoclonal antibody MDR H-241 (source: rabbit) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated goat anti-rabbit IgG was from Sigma (St. Louis, MO). Blocker Blotto in TBS and other reagents for Western blotting and ECL detection of P-gp were from Pierce (Rockford, IL). Hyperfilm ECL for the high-speed detection of chemiluminescent signal from protein blots was purchased from Amersham Biosciences (Buckinghamshire, UK). All other chemicals used were purchased from Sigma (St. Louis, MO).

5.2. 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 the basic medium. The cells were trypsinized before use (0.2% trypsin and 0.02% EDTA in PBS).

5.3. Cell growth and survival—MTT assay

Cells maintained in 10% FBS medium were harvested by low-speed centrifugation, washed with FBS medium, and seeded 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. Ten microliters of 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).

5.4. Effect of flavonoids on [^{14}C]paclitaxel uptake

The cells were preincubated with 100 nM [^{14}C]paclitaxel for 30 min either with fresh medium or 5–20 μM flavonoid derivative (specified in figures) or 100 μM quercetin and resveratrol dissolved in DMSO (maximum concentration in medium 0.1%, v/v). The medium was then rapidly replaced with fresh medium containing 100 nM [^{14}C]paclitaxel with or without flavonoids. The cells were incubated at 37 °C for various periods as specified in the figures and then the cells were rapidly 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). Sodium dodecyl sulfate in water (200 μL) was added up to 2% final concentration for lysis of the cells and Bray solution (10 mL) was used for liquid scintillation.

5.5. Effect of flavonoid derivatives on [^{14}C]paclitaxel efflux

In the efflux assays, cells were preincubated with 100 nM [^{14}C]paclitaxel for 2 h. The medium was then replaced with lukewarm fresh medium or medium with flavonoids and cells were incubated at 37 °C for time periods specified in the figures. The cells were then rapidly washed three times with ice-cold PBS, and released and dissolved as described above for measurement of radioactivity.

5.6. P-gp detection

Samples of cell lysates dissolved in sample lysis buffer (SLB) (15 μg of protein/15 μL) were resolved on 7.5% SDS–PAGE gels and transferred to a nitrocellulose membrane in an ice-cooled Transblot system for 1 h at 300 mA at 4 °C. Blots were incubated with the MDR H-241 anti-P-gp antibody (diluted 1000-fold) in Bloto blocking buffer with 0.05% Tween-20 overnight at 4 °C. The blots were washed five times (10 min each) with Tris-buffered saline containing 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature according to Huang et al.[22] The blots were then washed three times (10 min each) with Tris-buffered saline and antibody detection was performed using the ECL method (Pierce).

5.7. Statistical analysis

All experimental values are expressed as means ± standard deviations (SD) of the estimates. Significant differences ($*p < 0.05$ and $**p < 0.01$) in the effects of flavonoid derivatives were estimated with two-tailed Student's *t*-test.

Acknowledgments

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References and notes

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