Flavonoid-Related Modulators of Multidrug Resistance: Synthesis, Pharmacological Activity, and Structure−Activity Relationships

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A series of 28 flavonoid derivatives containing a *N*-benzylpiperazine chain have been synthesized and tested for their ability to modulate multidrug resistance (MDR) in vitro. At 5 μM, most compounds potentiated doxorubicin cytotoxicity on resistant K562/DOX cells. They were also able to increase the intracellular accumulation of JC-1, a fluorescent molecule recently described as a probe of P-glycoprotein-mediated MDR. This suggests that these compounds act, at least in part, by inhibiting P-glycoprotein activity. As in other studies, lipophilicity was shown to influence MDR-modulating activity but was not the only determinant. Diverse di- and trimethoxy substitutions on *N*-benzyl were examined and found to affect the activity differently. The most active compounds had a 2,3,4-trimethoxybenzylpiperazine chain attached to either a flavone or a flavanone moiety (**13**, **19**, **33**, and **37**) and were found to be more potent than verapamil.

Introduction

The development of multidrug resistance (MDR) represents a major problem in the treatment of cancer.[1] The best documented mechanism by which tumor cells acquire this MDR phenotype is the overexpression of an ATP-dependent membrane glycoprotein termed P-glycoprotein (Pgp).[2,3] Although the precise molecular mechanism involved is not yet known,[4] Pgp activity results in a decreased intracellular accumulation of a broad range of structurally and functionally unrelated drugs, such as antitumor agents (anthracyclines, epipodophyllotoxins, Vinca alkaloids, taxol), lipophilic cations (TPP^+, rhodamine-123, JC-1), and steroids (aldosterone).[5−8] During the past decade, numerous compounds which are able to overcome MDR by restoring the intracellular accumulation of antitumor agents[9] in resistant cells have been identified. Some of these compounds, termed MDR modulators, are currently being evaluated for clinical circumvention of MDR malignancies.[10] Structure−activity relationship (SAR) studies have shown that a cyclic tertiary amine and the presence of several aromatic ring systems are structural features frequently observed in MDR modulators.[11]

Recently, controversial results have been reported concerning the MDR modulating activity of some natural flavonoid polyphenols, such as kaempferol, quercetin, and genistein (chemical structures are shown in Figure 1). On one hand, the laboratory of Yeh showed that flavonols such as kaempferol and quercetin *stimulated* the Pgp-mediated efflux of 7,12-dimethylbenz[*a*]-anthracene and doxorubicin (DOX).[12,13] This stimulation was associated with an increase in DOX resistance in two different MDR cell lines. On the other hand, Scambia et al. reported that quercetin *inhibited* rhodamine-123 efflux and decreased DOX resistance.[14] Moreover, Shapiro and Ling, using a reconstituted Pgp system, showed that quercetin inhibited Pgp-mediated transport of the fluorescent probe Hoechst 33342, at least in part by inhibiting Pgp ATPase activity.[15] Finally, the isoflavone genistein was initially suggested to be inactive on Pgp-mediated MDR,[16] but this conclusion has been recently challenged.[17]

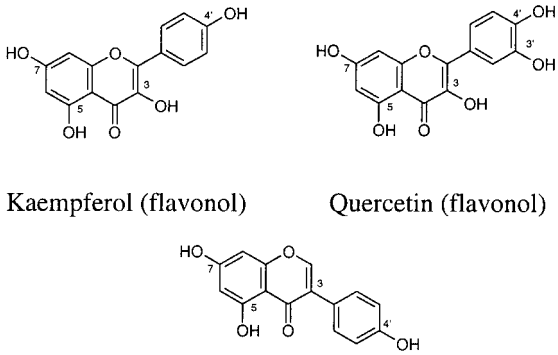

Figure 1. Chemical structures of flavonoids used in previous MDR-modulating studies.

In the present study, we have synthesized and evaluated the MDR-modulating activity of flavonoid derivatives containing a *N*-benzylpiperazine side chain. These new derivatives were designed with the aim to both introduce a basic nitrogen and to increase their lipophilic properties, two parameters shared by many other MDR modulators. The MDR-modulating activity of the resulting molecules was tested in vitro in two ways:

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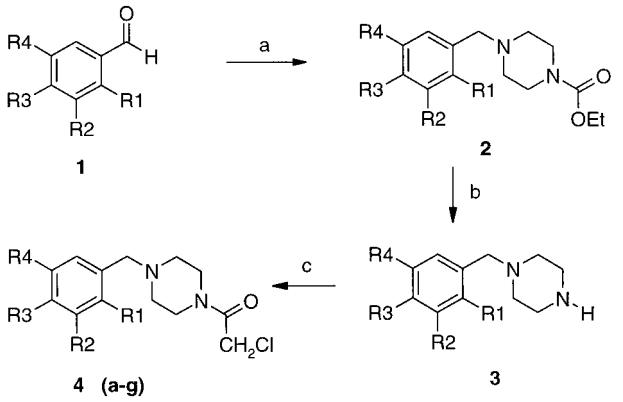
Scheme 1[a]



	R_1	R_2	R_3	R_4
4a	H	H	H	H
4b	Cl	H	H	H
4c	H	H	Cl	H
4d	OMe	OMe	H	H
4e	H	OMe	OMe	H
4f	OMe	OMe	OMe	H
4g	H	OMe	OMe	OMe

[a] Reagents: (a) ethyl 1-piperazinecarboxylate, $NaBH_3CN$ in AcOH; (b) KOH in EtOH−H_2O; (c) $ClCOCH_2Cl$ in CH_2Cl_2.

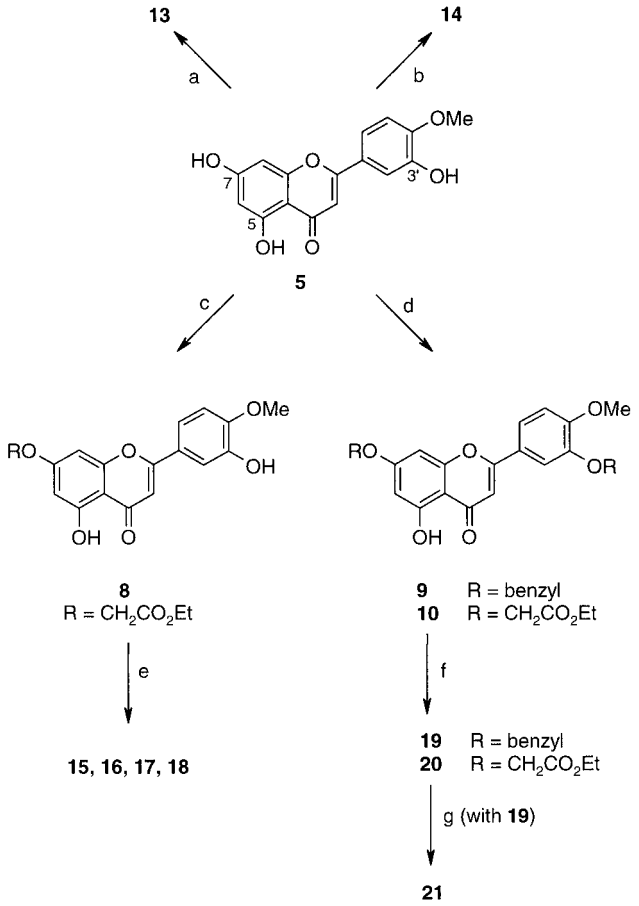
their ability to restore the intracellular accumulation of JC-1 (a fluorescent dye recently shown to be a probe of Pgp transport activity[8]) and their ability to potentiate DOX cytotoxicity on MDR cells. The position of the linked phenol, the substitution on the *N*-benzyl group, and the structure of the starting polyphenol were varied in order to study structure−activity relationships. Most of the 28 *N*-benzylpiperazine flavonoids (BPF) synthesized displayed MDR-modulating activity, some of them being more potent than the calcium-channel blocker verapamil, a standard MDR-reversing agent. It appeared that certain modifications led to marked changes in the potency of the molecules.

Chemistry

The synthetic strategy for the preparation of compounds **13**−**40** involved the preparation of substituted *N*-(chloroacetyl)benzylpiperazines **4** and their coupling through the phenol alkylation of an appropriate flavonoid. *N*-(Chloroacetyl)benzylpiperazines **4** were synthesized from the appropriate benzaldehyde **1** according to Scheme 1. Reductive amination of **1** by ethyl 1-piperazinecarboxylate in the presence of $NaBH_3CN$ yielded crude **2** which was saponified and decarboxylated to give **3**. Condensation of **3** with chloroacetyl chloride provided the chloroacetamides **4**.

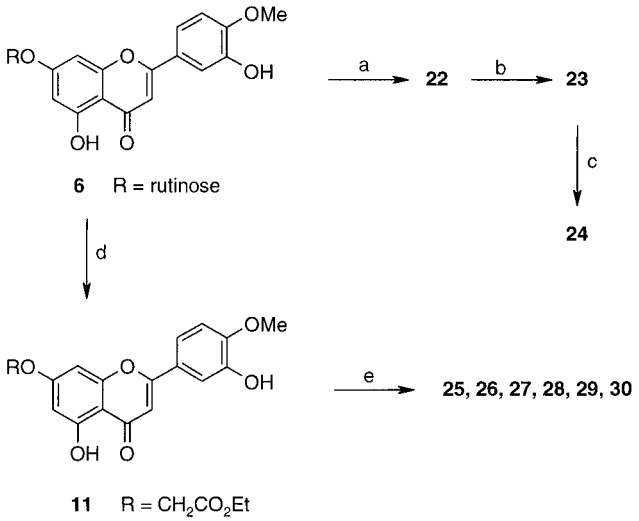
The readily available flavones diosmetin (**5**) and diosmin (**6**) and the flavanone hesperetin (**7**) were the starting flavonoids. In the flavone series, we succeeded in coupling both moieties through any phenol group at C-7, C-3′, or C-5: both 7- and 3′-phenol groups could be alkylated with the DMF−$KHCO_3$ system, while the chelated 5-phenol group, almost unreactive under these conditions, required K_2CO_3. Benzylation and ethoxycarbonylmethylation of 7- and 3′-phenol groups were carried out to prevent these groups from alkylation by the *N*-(chloroacetyl)benzylpiperazine **4** and to enhance the lipophilicity (Scheme 2).

Scheme 2[a]



[a] Reagents: (a) $KHCO_3$ (1 equiv), **4f** in DMF; (b) $KHCO_3$ (2 equiv), **4f** in DMF; (c) $KHCO_3$ (1 equiv), $ClCH_2CO_2Et$ in DMF; (d) $KHCO_3$ (2 equiv), $ClCH_2CO_2Et$ or $C_6H_5CH_2Cl$ in DMF; (e) $KHCO_3$ (1.5 equiv), **4** in DMF; (f) K_2CO_3 (5 equiv), **4f** in DMF; (g) H_2, Pd−C in DMF.

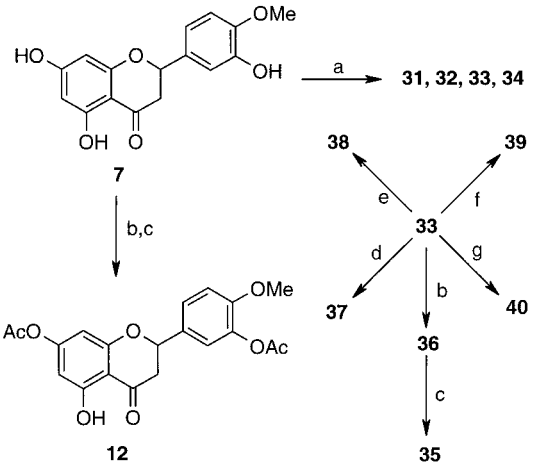
Scheme 3[a]



[a] Reagents: (a) $KHCO_3$ (1.3 equiv), **4f** in DMF; (b) naringinase in aqueous solution at pH 4; (c) β-glucosidase in aqueous solution at pH 3.95; (d) $KHCO_3$ (1 equiv), $ClCH_2CO_2Et$ in DMF, HCl (11 N), TsOH in abs. EtOH; (e) $KHCO_3$ (1.5 equiv), **4** in DMF.

With diosmin (Scheme 3), the rutinose chain acted as a natural protecting group of the 7-phenol, allowing

Scheme 4[a]



[a] Reagents: (a) $KHCO_3$ (1 equiv), **4** in DMF; (b) Ac_2O in pyridine; (c) CF_3CO_2H; (d) pivaloyl chloride in pyridine; (e) MeI in CH_2Cl_2; (f) $KHCO_3$ in DMF; (g) H_2, Pd−C in aqueous NaOH.

for the regioselective alkylation of the 3′-phenol group by a chloroacetamide **4** or by ethyl chloroacetate. The removal of the sugars was performed either by a one-step acidic hydrolysis or, as in the case of **22**, by a two-step enzymatic procedure with successive isolation of the 7-*O*-*β*-glucoside (**23**) and then isolation of **24**.

In the flavanone series (Scheme 4), only the most acidic 7-phenol group could be alkylated easily with the DMF−$KHCO_3$ system, since excess base resulted in heterocycle cleavage products. Starting from the coupled compound **33**, lipophilicity was either enhanced by acylation of the remaining phenol groups (**35**−**37**) or strongly decreased by quaternization of the amine function (**38**). Furthermore, an alkaline treatment of **33** provided the chalcone **39** and, under hydrogenation conditions, the dihydrochalcone **40**. Finally, 28 flavonoids with a *N*-benzylpiperazine chain were synthesized including 18 flavones (**13**−**30**) (Tables 1−3), 8 flavanones (**31**−**38**), the chalcone **39**, and the dihydrochalcone **40** (Table 4). These compounds differed from each other by the position (7, 3′, or 5) and the structure of the *N*-benzylpiperazine chain and also by the nature of the remaining phenol groups (free or substituted).

Results and Discussion

Assessment of MDR-Modulating Activity: 1. Cell Cytotoxicity Assay. The compounds were tested for their ability to potentiate DOX cytotoxicity on K562/DOX MDR cells, which were shown to overexpress P-glycoprotein.[18] Cells were incubated with increasing DOX concentrations, in the presence of 1 or 5 μM of the tested compound. Cell survival was assayed by MTT conversion, and IC_{50} values for DOX were determined. The MDR-modulating activity was expressed as a modulation factor: MF = IC_{50}(DOX alone)/IC_{50}(DOX + tested compound). Verapamil, one of the first identified MDR modulators, and S9788, a more potent triazinoaminopiperidine modulator,[19] were also tested for comparison.

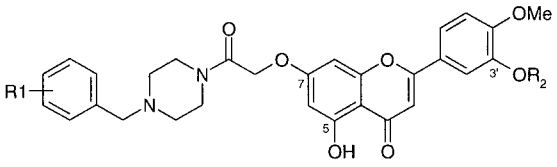
2. JC-1 Accumulation Assay. DOX cytotoxicity experiments cannot, by themselves, ascertain the effect of the tested compounds on Pgp transport activity. Therefore, we completed our screening procedure by an assay of Pgp transport function, using JC-1 as a substrate. Similarly to rhodamine-123, JC-1 is a membrane potential-sensitive fluorescent probe initially used for the measurement of mitochondrial membrane potential.[20] It has recently been shown that it could also be used for functional assay of Pgp-mediated cell resistance.[8] The unique feature of this carbocyanine dye is its propensity to form, above a critical concentration, J-aggregates with spectral properties distinct from those of the monomer form.[21] When sensitive cells are exposed to JC-1 in its monomer form, its mitochondrial accumulation, driven by the transmembrane electrical potential, leads to its aggregation which can be detected through its red (600 nm) emission fluorescence. In MDR cells, the intracellular concentration of JC-1 is reduced, due to Pgp transport activity, and aggregates do not form. Known Pgp modulators such as verapamil, S9788, or cyclosporin A were shown to restore JC-1 accumulation in resistant cells, thus leading to the recovery of its red fluorescence.[8] JC-1 thus provides a sensitive and versatile means to evaluate the Pgp-modulating activity of new compounds. For the screening of the BPF series, cells were incubated with JC-1 and the tested compound concomitantly until a plateau value was reached, which usually occurred within 2 h. To allow for comparison of independent experiments, S9788 was used as a positive control since, at 5 μM, it restored JC-1 accumulation to a level similar to that of the sensitive parental K562 cell line (data not shown).

Pharmacological Results. The pharmacological results are summarized in Tables 5 and 6 for flavone and flavanone derivatives, respectively. It appeared that these closely related molecules displayed a wide range of activities. The use of two different types of assays, namely, a short-term evaluation of intracellular accumulation and a long-term viability assay, allowed us to gain insight into different aspects of the pharmacological profile of these molecules. The JC-1 assay would be expected to reflect inhibition of Pgp transport activity,[8] while the MTT assay allows assessment of other cellular effects of the compound tested. For example, at 5 μM, compound **14** exhibited good modulating activity on JC-1 accumulation but proved to be toxic over a 72-h time scale.

At 1 μM, 8 compounds had MF ≥ 8, superior to that of verapamil (MF = 7). At 5 μM, 18 out of 28 compounds restored DOX cytotoxicity on K562/DOX cells by a factor > 20, which is in the same range as verapamil activity (MF = 31). The five most active compounds (**13**, **33**, **37**, **39**, and **19**) all contain a 2,3,4-trimethoxybenzylpiperazine chain. In the case of **13**, **33**, **37**, and **39**, the side chain is attached at position 7 of the flavonoid moiety, while in **19**, it is attached at position 5. Compound **19** is distinct from all other flavonoid derivatives, since it contains two additional benzyl groups (Table 2).

From the data in Tables 5 and 6, it appeared that cytotoxicity results at 1 μM could be more satisfactorily compared to JC-1 accumulation results at 5 μM. This may reflect the difference in incubation times between the two assays. Figure 2 represents the scatter graph of JC-1 and cytotoxicity results at 5 and 1 μM, respectively. Two clusters can be defined: the first one comprises compounds which substantially restored both DOX cytotoxicity (MF ≥ 8) and JC-1 accumulation (≥50%). The second one comprises compounds with

Table 1. Chemical Structures and Physical Data of 7-(*N*-Benzylpiperazinyl)flavones



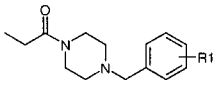
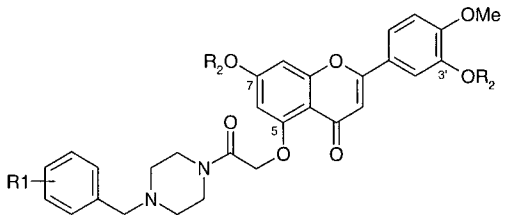
compd	R_1	R_2	formula	mp(°C)	recryst solvent
13	2,3,4-$(MeO)_3$	H	$C_{32}H_{34}N_2O_{10}$	amorph	-
14	2,3,4-$(MeO)_3$		$C_{48}H_{56}N_4O_{14}$	amorph	-
25	H	CH_2CO_2Et	$C_{33}H_{34}N_2O_9$	150-151	MeOH
26	4-Cl	CH_2CO_2Et	$C_{33}H_{33}N_2O_9Cl$	109-111	MeOH
27	2,3-$(MeO)_2$	CH_2CO_2Et	$C_{35}H_{38}N_2O_{11}$	138-140	MeOH
28	3,4-$(MeO)_2$	CH_2CO_2Et	$C_{35}H_{38}N_2O_{11}$	107-109	MeOH
29	2,3,4-$(MeO)_3$	CH_2CO_2Et	$C_{36}H_{40}N_2O_{12}$	103-104	MeOH
30	3,4,5-$(MeO)_3$	CH_2CO_2Et	$C_{36}H_{40}N_2O_{12}$	134-136	MeOH

Table 2. Chemical Structures and Physical Data of 5-(*N*-Benzylpiperazinyl)flavones

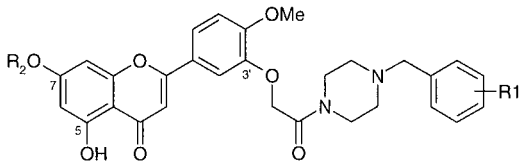


compd	R_1	R_2	formula	mp(°C)	recryst solvent
19	2,3,4-$(MeO)_3$	$C_6H_5CH_2$	$C_{46}H_{46}N_2O_{10}$	93-95	MeOH
20	2,3,4-$(MeO)_3$	CH_2CO_2Et	$C_{40}H_{46}N_2O_{14}$	86-88	MeOH
21	2,3,4-$(MeO)_3$	H	$C_{32}H_{34}N_2O_{10}$	148-150	MeOH

lower activity in both tests (MF ≤ 5 and JC-1 accumulation ≤35%). Hence, for most compounds, general agreement was observed between the two assays. This suggests that MDR-modulating activity of BPF compounds could be mediated, at least in part, by inhibition of Pgp transport activity.

Four compounds showed different results in the two tests (**14**, **17**, **18**, and **39**; Figure 2). One of these (**14**) differed markedly in structure from the other BPF compounds, since it had two *N*-benzylpiperazine side chains (Table 1). On the other hand, the chalcone **39** is interconvertible with the isomeric flavanone **33**, through

Table 3. Chemical Structures and Physical Data of 3′-(*N*-Benzylpiperazinyl)flavones



compd	R_1	R_2	formula	mp(°C)	recryst solvent
15	2-Cl	CH_2CO_2Et	$C_{33}H_{33}N_2O_9Cl$	138-140	EtOH
16	3,4-$(MeO)_2$	CH_2CO_2Et	$C_{35}H_{38}N_2O_{11}$	95-97	EtOH
17	2,3,4-$(MeO)_3$	CH_2CO_2Et	$C_{36}H_{40}N_2O_{12}$	105-106	EtOH
18	3,4,5-$(MeO)_3$	CH_2CO_2Et	$C_{36}H_{40}N_2O_{12}$	97-98	EtOH
22	2,3,4-$(MeO)_3$	rutinose	$C_{44}H_{54}N_2O_{19}$	184-186	nBuOH -H_2O
23	2,3,4-$(MeO)_3$	β-glucose	$C_{38}H_{44}N_2O_{15}$	132-134	MeOH
24	2,3,4-$(MeO)_3$	H	$C_{32}H_{34}N_2O_{10}$	200-202	MeOH

a pH-dependent equilibrium.[22] Both compounds had similar activities on DOX cytotoxicity, while the chalcone **39** was much less potent in restoring JC-1 accumulation. This suggests that flavanone is the active form and that chalcone could be, at least in part, converted into flavanone during the 72-h incubation period. In contrast, this transformation may not occur during the 2-h incubation used for JC-1 accumulation experiments. Finally, compounds **17** and **18** form, with compounds **15** and **16**, the series of 3′-substituted derivatives. They appeared to be more active in short-term JC-1 experiments than in long-term DOX cytotoxicity tests. One possible explanation could be the metabolic inactivation of these compounds.

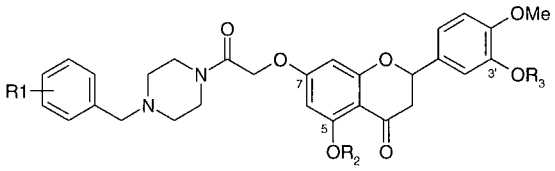
Structure−Activity Relationships. The parent compound diosmetin (**5**) had no effect on JC-1 accumulation up to 50 μM and did not enhance DOX cytotoxicity at 5 μM (data not shown). This was not unexpected, since diosmetin does not have the structural features commonly displayed by MDR modulators, namely, two or more lipophilic ring systems separated by a linker containing a basic nitrogen.[11] The addition of the *N*-benzylpiperazine side chain conferred these structural features, and most of the resulting derivatives showed MDR-modulating activity. However, it appeared that other parameters such as the type of flavonoid moiety and the nature of the substituents on the flavonoid phenols and on the benzyl ring were critical in determining the potency of the molecule.

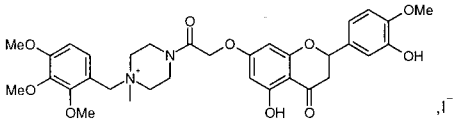
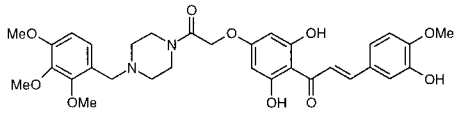
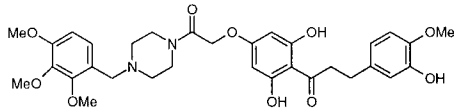
1. Influence of the Flavonoid Moiety. Replacement of the flavone ring by flavanone did not markedly alter MDR-modulating efficiency but reduced the inherent toxicity (**13** vs **33**). The chalcone derivative **39** was less efficient in restoring JC-1 accumulation than its flavanone isomer **33**, while the dihydrochalcone derivative **40** was inefficient. Nevertheless, **33** and **39** were equally potent in restoring DOX cytotoxicity. As already mentioned, this discrepancy could be accounted for by the existence of a pH-dependent equilibrium between these isomers, the chalcone being more stable at alkaline pH than flavanone. On the other hand, the loss of activity of dihydrochalcone **40** as compared with the flavanone could be due its inability to undergo isomerization, as well as to an increase in polarity upon reduction of the double bond.

Overall, it appeared that the presence of the heterocyclic ring in the flavonoid structure was important for MDR-modulating activity.

2. Influence of Substitution on Flavone Phenol Groups. It has previously been noted in a QSAR study that phenol groups were generally detrimental to MDR-modulating activity.[23] In the case of flavone derivatives, the influence of OH substitution on MDR-modulating activity appeared to be highly dependent on the position of substitution. At position 7, OH substitution, either by acylation or by a *N*-benzylpiperazine chain, appeared to be necessary, since derivatives with a free OH at this position were devoid of MDR-modulating activity (Table 5; **13** vs **21** and **24**). This could be explained by the acidic property of 7-OH. Indeed, it has been reported that the pK_a of this phenol in 7-OH-flavone and 7-OH-5,3′,4′-OMe-flavone was about 7.3−7.4.[24] Thus, this phenol would be expected to be at least partly ionized at physiological pH. The presence of this highly polar and

Table 4. Chemical Structures and Physical Data of 7-(*N*-Benzylpiperazinyl)flavanones, -chalcone, and -dihydrochalcone



compd	R_1	R_2	R_3	formula	mp(°C)
31	2,3-$(MeO)_2$	H	H	$C_{31}H_{34}N_2O_9$	amorph
32	3,4-$(MeO)_2$	H	H	$C_{31}H_{34}N_2O_9$	amorph
33	2,3,4-$(MeO)_3$	H	H	$C_{32}H_{36}N_2O_{10}$	amorph
34	3,4,5-$(MeO)_3$	H	H	$C_{32}H_{36}N_2O_{10}$	amorph
35	2,3,4-$(MeO)_3$	H	$-COCH_3$	$C_{34}H_{38}N_2O_{11}$	amorph
36	2,3,4-$(MeO)_3$	$-COCH_3$	$-COCH_3$	$C_{36}H_{40}N_2O_{12}$	amorph
37	2,3,4-$(MeO)_3$	$-COC(CH_3)_3$	$-COC(CH_3)_3$	$C_{42}H_{52}N_2O_{12}$	amorph
38				$C_{33}H_{39}N_2O_{10}I$	185-186[a]
39				$C_{32}H_{36}N_2O_{10}$	173-175[b]
40				$C_{32}H_{38}N_2O_{10}$	207-208[b]

[a] Crystals from CH_2Cl_2. [b] Crystals from MeOH.

negatively charged group could be detrimental to MDR-modulating activity, as noted in the aforementioned QSAR study.[23] In contrast, at position 5, the presence of an unsubstituted OH appeared to be preferable (Table 5; **20** vs **17** and **29**). This different behavior could be related to the presence of an intramolecular hydrogen bond between this phenol and the adjacent ketone group (see, for example, Scheme 2). This creates a pseudoring structure, which brings an additional lipophilic contribution to the flavonoid nucleus. The disruption of this hydrogen bond leads to a decreased lipophilicity of the flavone structure, as evidenced by a marked decrease in retention factors measured by TLC (data not shown). This may account for the lower activity of **20**. Indeed, increasing the lipophilicity of **20** by benzylation of the remaining phenol groups resulted in a more active compound (**19**). Finally, substitution at position 3′ appeared to be less critical, with respect to MDR-modulating activity, since active compounds could be found among both substituted and unsubstituted derivatives (**13** and **29**). Nevertheless, at 5 μM, compound **13** appeared more toxic per se, as compared with **29**. Further investigation is needed to determine the possible involvement of OH at position 3′ in the intrinsic toxicity of BPF compounds.

3. Effect of Quaternization. As shown in Table 6, quaternization of the basic nitrogen also proved to be detrimental to activity (**38**). This suggests a critical requirement for MDR-modulating agents to display a basic nitrogen instead of a quaternary salt, as already observed in other chemical series.[23,25] The inefficiency of the permanently charged compound (**38**) could reflect its inability to cross the lipid membrane, rather than an inability to interact with the putative target. Indeed, Kirk et al. showed that an impermeant quaternized derivative of tamoxifen did not modulate cellular drug

Table 5. MDR-Modulating Activities of (*N*-Benzylpiperazinyl)flavones on K562/DOX Cells

compd	IC_{50}[a] (μM)	relative JC-1 accumulation[b] 1 μM	5 μM	10 μM	modulation factor[c] 1 μM	5 μM
13	6	4	50	30	11	78[d]
14	2	22	60	53	1	nd[d]
15	>20	7	13	24	2	7
16	9	4	26	31	2	25
17	>20	7	56	59	3	17
18	11	5	56	55	5	25
19	8	28	50	56	14	45
20	>20	1	10	59	nd	1
21	>20	0	0	0	nd	1
22	>20	0	0	0	nd	1
23	>20	0	0	0	nd	1
24	>20	0	0	0	nd	1
25	>20	10	35	38	4	21
26	14	6	14	23	4	22
27	>20	2	22	28	5	24
28	>20	3	28	27	4	23
29	>20	14	69	73	9	22
30	>20	2	26	24	3	31
S9788	12	nd	100	nd	17	58
verapamil	37	nd	nd	nd	7	31

[a] IC_{50} of the tested compound alone. [b] Ratio [FI_{590}(JC-1 + tested compound) − FI_{590}(JC-1 alone)]/[FI_{590}(JC-1 + 5 μM S9788) − FI_{590}(JC-1 alone)] (100, where FI_{590} represents fluorescence intensity at 590 nm. Results are the mean of at least two independent experiments. Interexperimental variations were less than 20%. [c] Ratio of IC_{50}(DOX alone)/IC_{50}(DOX + modulator). Results are the mean of at least two independent experiments. Interexperimental variations were less than 15%. [d] Cell survival in the presence of the tested compound alone was 60% and 25% for **13** and **14**, respectively; nd, not determined.

Table 6. MDR-Modulating Activities of 7-(*N*-Benzylpiperazinyl)flavanones, -chalcone, and -dihydrochalcone on K562/DOX Cells

compd	IC_{50}[a] (μM)	relative JC-1 accumulation[b] 1 μM	5 μM	10 μM	modulation factor[c] 1 μM	5 μM
31	>20	0	2	6	4	28
32	>20	0	9	31	4	31
33	16	4	50	69	11	42
34	>20	0	3	8	2	21
35	>20	33	82	91	8	31
36	>20	2	55	75	10	31
37	>20	72	82	75	18	45
38	>20	0	0	0	nd	1
39	>20	3	17	21	9	45
40	>20	2		5	nd	3
S9788	12	nd	100	nd	17	58
verapamil	37	nd	nd	nd	7	31

[a] IC_{50} of the tested compound alone. [b] Ratio [FI_{590}(JC-1 + tested compound) − FI_{590}(JC-1 alone)]/[FI_{590}(JC-1 + 5 μM S9788) − FI_{590}(JC-1 alone)] (100, where FI_{590} represents fluorescence intensity at 590 nm. Results are the mean of at least two independent experiments. Interexperimental variations were less than 20%. [c] Ratio of IC_{50}(DOX alone)/IC_{50}(DOX + modulator). Results are the mean of at least two independent experiments. Interexperimental variations were less than 15%; nd, not determined.

resistance, although it was as effective as tamoxifen in inhibiting vinblastine accumulation in Pgp-containing inverted vesicles.[26] Likewise, quaternized derivatives of quinidine and ajmaline were shown to be even more efficiently transported into Pgp-containing inverted vesicles than their parent compounds.[27] This suggests that BPF modulators exert their activity on the cytoplasmic side of the membrane, possibly through a direct interaction with Pgp, as suggested for other modulators.[28,29] This hypothesis is supported by the observation

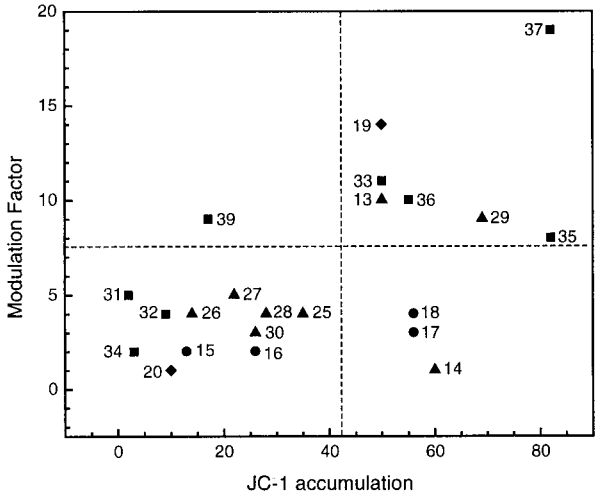


Figure 2. Scatter graph of MDR modulation factor values (MF) obtained in cytotoxicity assays versus results obtained in JC-1 accumulation assays in the presence of 1 and 5 μM of the tested compound, respectively: ▲, 7-substituted flavones; ◆, 5-substituted flavones; ●, 3′-substituted flavones; ■, 7-substituted flavanones.

that some flavone and chalcone derivatives were able to interact with a site located near the ATP-binding domains of P-glycoprotein, which are located on the cytoplasmic side of the membrane.[30]

4. Substitution of *N*-Benzyl. We systematically examined the effect of *o*-dimethoxy and trimethoxy substitution patterns on MDR-modulating activity. Some chloro derivatives were also synthesized. The results indicated a complex relationship between the benzyl substitution pattern and Pgp-modulating potency, as evidenced by the JC-1 accumulation assay.

In the 7-substituted flavone series, only the 2,3,4-trimethoxy (**29**) showed better potency in restoring JC-1 accumulation than the unsubstituted benzyl (**25**), while 4-chloro (**26**) or other di- and trimethoxy substitution patterns (**27**, **28**, and **30**) showed slightly decreased activities. In the 3′-substituted flavone series, 2,3,4- and 3,4,5-trimethoxy (**17** and **18**) appeared equivalent, while 3,4-dimethoxy (**16**) and 2-chloro (**15**) were less active.

In the flavanone series, we observed a pattern similar to that in the 7-substituted flavone series, the order of potency being 2,3,4-trimethoxy (**33**) ≫ 2,3-dimethoxy (**32**) > 3,4-dimethoxy (**31**) ∼ 3,4,5-trimethoxy (**34**). The comparison between the trimethoxylated isomers **33** and **34** is most interesting. At 5 μM, the first compound efficiently restored JC-1 accumulation while the second had only a marginal effect. This difference was also reflected in the potentiation of DOX cytotoxicity at 1 μM modulator.

On the whole, 2,3,4-trimethoxy substitution clearly appeared to be the most efficient one for inhibition of Pgp activity. This may be related to the electron-donating effect of methoxy substituents on the benzyl moiety. Indeed, as previously noted for a series of *N*-benzylpiperazine derivatives, the electron-donating abilities described by the Hammet parameter σ are in the order 2,3,4-trimethoxy ($\sigma = -0.42$) > 3,4-dimethoxy ($\sigma = -0.15$) > 3,4,5-trimethoxy ($\sigma = -0.03$).[31] Different hypotheses could explain the effect of benzyl substitution on MDR-modulating activity. First, the aromatic moieties of Pgp substrates and modulators have been

suggested to participate in $\pi-\pi$ interactions with Pgp aromatic residues:[32] different substitution patterns on the aromatic ring could alter these interactions. Second, electron-donating groups could form H-bonds with suitable amino acids on Pgp, as suggested by Seelig.[33] Alternatively, the electron-donating effect on the benzyl moiety increases the basicity of the piperazine nitrogen atom.[31] This suggests that, in the case of BPF compounds, MDR-modulating activity essentially results from the protonated cationic species. However, the permanently charged derivative **38** was devoid of activity (see above). Taken together, these results suggest that the neutral form of the drug would be necessary for transmembrane diffusion, whereas the protonated form would be involved in Pgp inhibition.

5. Role of Lipophilicity. It has previously been shown from different structure−activity relationship studies that lipophilicity was an important parameter affecting MDR-modulating efficiency.[11,34] In the present series, minimal hydrophobicity seemed to be required, since the most polar compounds, such as the glycosylated BPFs **22** and **23** or the 7-OH-containing **24**, were devoid of activity.

More lipophilic derivatives were obtained from the 7-substituted flavanone **33** by mono- or diacetylation and dipivaloylation of the phenol groups at positions 5 and 3′ (compounds **35**−**37**, respectively). As expected, both TLC and reverse-phase HPLC showed that their lipophilicity increased in the order **33** < **36** < **35** < **37** (data not shown). For these compounds, MDR-modulating activity seemed to be correlated with their lipophilic character since the same order of potency was found when considering their effect on JC-1 accumulation at 1 and 5 μM (Table 6). In contrast, compounds **33** and **31**, which displayed similar lipophilicity (same retention times in RP-HPLC experiments), showed markedly different effects on JC-1 accumulation. This latter result suggested that lipophilicity was not the only parameter governing MDR-modulating activity.

Similarly, in a previous study on propafenone-derived MDR modulators,[35] it was concluded that lipophilicity, by itself, does not determine MDR-modulating activity but may be regarded as a favorable parameter within a homologous series.

Conclusion

We have synthesized and evaluated a series of new modulators of multidrug resistance derived from flavonoids. The most active compounds had a 2,3,4-trimethoxybenzylpiperazine chain attached on either a flavone or a flavanone moiety (**13**, **19**, **33**, and **37**) and were found to be more potent than verapamil. From both DOX sensitization assays and JC-1 accumulation experiments, these compounds can be suggested to act, at least in part, by inhibiting Pgp transport activity.

The present study supports the observation that lipophilic compounds containing several ring systems and a tertiary amine are good candidates for MDR modulation. A study of structure−activity relationships pointed to a specific role for the methoxy substitution pattern on the benzyl group. *o*-Dimethoxy- or trimethoxyphenyl moieties have been previously identified as good pharmacophores for MDR-modulating activity.[22,33] The results obtained with BPF compounds further suggest that, for a given number of methoxy substituents, their relative position may be critical in determining the MDR-modulating activity, possibly because of the electron-donating capacity of each pattern.

Experimental Section

NMR spectra were carried out at 200 or 500 MHz on Bruker AC-200 or Bruker AMX-500 spectrometers in $CDCl_3$ or in DMSO-d_6 using TMS as internal reference. CIMS were recorded on a Nermag R10-10C and FABMS on a Finnigan MAT 95Q (BEQ geometry) mass spectrometer. IR spectra were recorded on a Perkin-Elmer 457 spectrophotometer. Microanalyses were performed on a Carlo Erba EA 1108 apparatus, and melting points were determined with a micro-Koffler and are uncorrected. Satisfactory C, H, N analyses (±0.4%) were obtained for all compounds.

General Procedure for the Preparation of *N*-(Chloroacetyl)benzylpiperazines 4. The synthesis of **4e** is given to illustrate the general procedure.

4-(Chloroacetyl)-1-[(2,3-dimethoxyphenyl)methyl]piperazine (4e). To a solution of 3,4-dimethoxybenzaldehyde (1.66 g, 10 mmol) in AcOH (25 mL) at 0 °C were added successively ethyl 1-piperazinecarboxylate (10 mL, 6.8 mmol) and then $NaBH_3CN$ (0.5 g, 8 mmol), and the solution was left at room temperature for 16 h. The solution was diluted with iced water, heated with aqueous 2 N NaOH until pH 6, and extracted with CH_2Cl_2. The organic layer was washed with water, dried over Na_2SO_4, filtered, and evaporated. To the dried residue (3 g) in solution in EtOH (30 mL) was added aqueous KOH (36 g in 25 mL), and the mixture was stirred for 2.5 h under nitrogen at 130 °C. Extraction with CH_2Cl_2 and then standard treatment provided crude **3e** (1.85 g) as a colorless oil. A solution of this residue in 150 mL of the mixture MeOH−AcOEt−12 N HCl (48/100/2) was concentrated under vacuum until crystallization started and then kept overnight at room temperature. Filtration of the white crystals yielded pure **3e**, 2HCl (2.13 g, 69%): mp 220−223 °C; ^{1}H NMR (DMSO-d_6) δ 3.2−3.5 (m, 8H), 3.73 (s, 3H), 3.75 (s, 3H), 4.25 (s, 2H), 6.98 (d, J = 8.5 Hz, 1H), 7.10 (dd, J = 8.5 and 2 Hz, 1H), 7.39 (d, J = 2 Hz, 1H), 9.7 (br s, 2H, NH_2^+), 12.2 (br s, 1H, NH^+).

A solution of chloroacetyl chloride (0.37 mL, 4.6 mmol) in CH_2Cl_2 (3 mL) was added dropwise to a stirred solution of **3e** (1.09 g, 4.6 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 15 min at room temperature, the mixture was poured into iced water, adjusted to pH 8 with 5% $NaHCO_3$ aqueous solution, and extracted with CH_2Cl_2. Standard workup of the organic layer yielded pure **4e** (1.34 g, 93%) as a colorless oil: IR (CH_2Cl_2) 1650 cm^{-1} (amide).

General Procedure for the Alkylation of the 7-Phenol Group by 4. (a) From Diosmetin (5). 5,3′-Dihydroxy-4′-methoxy-7-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (13). A mixture of diosmetin (0.3 g, 1 mmol) and $KHCO_3$ (0.1 g, 1 mmol) in DMF (5 mL) was stirred for 3 min at 115 °C under nitrogen; then **4f** (0.36 g, 1.05 mmol) in solution in DMF (3 mL) was added. After 2.5 h under the same conditions, the reaction mixture was cooled, diluted with CH_2Cl_2 (15 mL), filtered, and evaporated to dryness. The dried residue was purified by flash chromatography (SiO_2, CH_2Cl_2−acetone, 3/1) to provide pure **13** (0.145 g, 24%) as a yellowish amorphous compound: ^{1}H NMR ($CDCl_3$) (benzylpiperazinylcarbonylmethyl chain) δ 2.4 (m, 4H), 3.35 (s, 2H), 3.4−3.6 (m, 4H), 3.75−3.80 (3s, 9H), 4.70 (s, 2H), 6.58 (d, J = 8.5 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H); (flavone moiety) δ 3.90 (s, 3H), 6.28 (d, J = 2 Hz, 1H), 6.48 (s + d, J = 2 Hz, 2H), 6.88 (d, J = 8.5 Hz, 1H), 7.34 (m, 2H), 12.7 (s, 1H, chelated OH); CIMS (NH_3) m/z 607 $(M + 1)^+$; IR (Nujol) 3500−2500 (OH), 1660 (amide, cetone), 1610 (C=C) cm^{-1}.

(b) From Diosmin (6). 5,7-Dihydroxy-3′-(ethoxycarbonylmethoxy)-4′-methoxyflavone (11). A mixture of diosmin (15.2 g, 25 mmol) and $KHCO_3$ (2.5 g, 25 mmol) in DMF (125 mL) was stirred for 3 min at 115 °C under nitrogen; then

$ClCH_2CO_2Et$ (13 mL, 120 mmol) was added. After 3 h under the same conditions, the reaction mixture was cooled, filtered, and evaporated to dryness. A solution of the dried residue in aqueous 11 N HCl (150 mL) was stirred between 50 and 55 °C until crystallization occurred (0.75−1 h). After 2 h at room temperature, the crystals were filtered, washed several times with water, and then dried with P_2O_5 under vacuum to yield a crude residue of 3′-*O*-(carboxymethyl)diosmetin (7 g). In the next step, this dried residue was stirred under reflux for 5 h in anhydrous ethanol (100 mL) in the presence of TsOH (0.35 g). Concentration of the mixture gave 6.8 g of the ester which yielded pure **11** (4.4 g, 46% from diosmin) as pale-yellow crystals after recrystallization from THF−MeOH: mp 226−227 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (t, J = 7.5 Hz, 3H), 3.85 (s, 3H), 4.15 (q, J = 7.5 Hz, 2H), 4.90 (s, 2H), 6.21 (d, J = 2 Hz, 1H), 6.48 (d, J = 2 Hz, 1H), 6.95 (s, 1H), 7.14 (d, J = 8.5 Hz, 1H), 7.52 (d, J = 2 Hz, 1H), 7.68 (dd, J = 8.5 and 2 Hz, 1H), 10.8 (br s, 1H, OH), 12.9 (s, 1H, chelated OH).

3′-(Ethoxycarbonylmethoxy)-5-hydroxy-4′-methoxy-7-[4-(phenylmethyl)piperazin-1-ylcarbonylmethoxy]flavone (25). A mixture of **11** (0.39 g, 1 mmol) and $KHCO_3$ (0.15 g, 1.5 mmol) was stirred for 3 min in DMF (5 mL) at 115 °C under nitrogen; **4a** (0.3 g, 1.2 mmol) in DMF (3 mL) was added. After 2.5 h under the same conditions, the reaction mixture was cooled, diluted with CH_2Cl_2 (15 mL), filtered, and evaporated to dryness. The dried residue yielded pure **25** (0.46 g, 76%) as light-yellow crystals after flash chromatography (SiO_2, CH_2Cl_2−acetone, 4/1) and crystallization from MeOH: ^{1}H NMR ($CDCl_3$) (benzylpiperazinylcarbonylmethyl and ethoxycarbonylmethyl chains) δ 1.30 (t, J = 7.5 Hz, 3H), 2.4 (m, 4H), 3.48 (s, 2H), 3.5−3.6 (m, 4H), 4.28 (q, J = 7.5 Hz, 2H), 4.80 (s, 4H), 7.3 (m, 5H); (flavone moiety) δ 3.90 (s, 3H), 6.35 (d, J = 2 Hz, 1H), 6.50 (s + d, J = 2 Hz, 2H), 6.96 (d, J = 8.5 Hz, 1H), 7.3 (m, 1H), 7.52 (dd, J = 8.5 and 2 Hz, 1H), 12.8 (s, 1H, chelated OH); ^{13}C NMR ($CDCl_3$) δ 14.1 (CH_3), 42.1 (CH_2), 45.2 (CH_2), 52.5 (CH_2), 52.3 (CH_2), 56.0 (OCH_3), 61.4 (CH_2), 62.7 (CH_2), 66.6 (CH_2), 67.3 (CH_2), 93.1 (CH), 98.6 (CH), 104.7 (CH), 106.0 (C), 111.9 (CH), 112.5 (CH), 121.3 (CH), 123.5 (C), 127.3 (CH), 128.3 (2CH), 134.0 (2CH), 137.3 (C), 147.5 (C), 152.9 (C), 157.5 (C), 162.1 (C), 163.5 (C), 163.7 (C), 165.1 (C), 168.4 (C), 182.2 (C); FABMS (thioglycerol−glycerol matrix) in positive mode m/z 603 $(M + H)^+$; IR (Nujol) 3400−2600 (OH), 1750 (ester), 1660 (amide, cetone), 1610 (C=C) cm^{-1}.

(c) From Hesperetin (7). 5,3′-Dihydroxy-4′-methoxy-7-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavanone (33). Alkylation with **4f** was carried out from hesperetin (3.6 g, 12 mmol) under the same conditions as the **5** → **13** procedure. The dried residue was purified by flash chromatography (SiO_2, CH_2Cl_2−MeOH, 97.5/2.5) and provided pure **33** (3.6 g, 49%) as a light-yellow amorphous compound: ^{1}H NMR ($CDCl_3$) (benzylpiperazinylcarbonylmethyl chain) δ 2.47 (m, 4H), 3.52 (s, 2H), 3.5−3.6 (m, 4H), 3.90 (3s, 9H), 4.67 (s, 2H), 6.88 (d, J = 8.5 Hz, 1H), 6.98 (d, J = 8.5 Hz, 1H); (flavanone moiety) δ 2.81 (dd, J = 12.7 and 2.5 Hz, 1H), 3.08 (dd, J = 16.5 and 12.7 Hz, 1H), 3.90 (s, 3H), 5.32 (dd, J = 12.7 and 2.5 Hz, 1H), 6.08 (s, 2H), 6.65 (d, J = 8.5 Hz, 1H), 6.92 (dd, J = 8.5 and 2 Hz, 1H), 7.04 (d, J = 2 Hz, 1H), 12.0 (s, 1H, chelated OH); ^{13}C NMR ($CDCl_3$) δ 42.1 (CH_2), 43.1 (CH_2), 45.1 (CH_2), 52.3 (CH_3), 52.7 (CH_2), 55.9 (2OCH_3), 56.3 (CH_2), 60.7 (OCH_3), 61.1 (OCH_3), 66.9 (CH_2), 78.9 (CH), 94.3 (CH), 95.7 (CH), 103.5 (C), 106.9 (CH), 110.6 (CH), 112.7 (CH), 118.1 (CH), 123.1 (C), 125.0 (CH), 131.3 (C), 142.3 (C), 145.9 (C), 147.0 (C), 152.6 (C), 153.1 (C), 162.8 (C), 163.9. (C), 164.9 (C), 165.9 (C), 196.1 (C); CIMS (NH_3) m/z 609 $(M + 1)^+$; IR (CH_2Cl_2) 3500−2500 (OH), 1660 (amide, cetone).

General Procedure for the Alkylation of the 3′-Phenol Group by 4. (a) From Diosmetin (5). 5,3′-Dihydroxy-7-(ethoxycarbonylmethoxy)-4′-methoxyflavone (8). Alkylation of diosmetin (6 g, 20 mmol) with $ClCH_2CO_2Et$ was carried out under the same conditions as described for diosmin. Two successive crystallizations of the dried residue from THF−MeOH and then CH_2Cl_2−acetone systems provided pure 7-*O*-(carboxymethyl)diosmetin (**8**) (4 g, 52%) as bright-yellow crystals: mp 194−195 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (t, J = 7.5 Hz, 3H), 3.90 (s, 3H), 4.20 (q, J = 7.5 Hz, 2H), 4.93 (s, 2H), 6.40 (d, J = 2 Hz, 1H), 6.75 (d, J = 2 Hz, 1H), 6.82 (s, 1H), 7.10 (d, J = 8.5 Hz, 1H), 7.45 (d, J = 2 Hz, 1H), 7.55 (dd, J = 8.5 and 2 Hz, 1H), 9.5 (s, 1H, OH), 12.95 (s, 1H, chelated OH).

3′-[4-(2-Chlorophenyl)methyl]piperazinyl-1-ylcarbonylmethoxy]-7-(ethoxycarbonylmethoxy)-5-hydroxy-4′-methoxyflavone (15). Alkylation of **8** (0.39 g, 1 mmol) with **4b** was carried out according to the **11** → **25** procedure. Crystallization of the dried residue from EtOH and then purification of the crude crystals by flash chromatography (SiO_2, CH_2Cl_2−MeOH, 96/4) yielded pure **15** (0.44 g, 69%) as white-yellowish crystals: ^{1}H NMR ($CDCl_3$) similar to that of **25** except for one fewer proton signal at δ 7.15−7.3; ^{13}C NMR ($CDCl_3$) δ 14.1 (CH_3), 42.1 (CH_2), 45.2 (CH_2), 52.6 (CH_2), 53.0 (CH_2), 56.0 (OCH_3), 59.0 (CH_2), 61.6 (CH_2), 65.2 (CH_2), 68.5 (CH_2), 93.4 (CH), 98.3 (CH), 104.7 (CH), 106.0 (C), 111.8 (CH), 112.2 (CH), 121.3 (CH), 123.6 (C), 126.6 (CH), 128.4 (CH), 129.5 (CH), 130.7 (CH), 134.3 (C), 135.1 (C), 147.6 (C), 152.8 (C), 157.4 (C), 162.2 (C), 163.3 (C), 163.7 (C), 165.7 (C), 167.8 (C), 182.2 (C); FABMS (thioglycerol−glycerol matrix) in positive mode m/z 637−635 $(M + H)^+$; IR see **25**.

(b) From Diosmin (6). 7-[[6-*O*-6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-4′-methoxy-3′-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (22). A mixture of diosmin (11.9 g, 19.5 mmol) and $KHCO_3$ (2.5 g, 25 mmol) in DMF (80 mL) was stirred for 3 min at 115 °C under nitrogen, and 4f (8.67 g, 25 mmol) in solution in DMF (20 mL) was added. After 3 h under the same conditions, the mixture was cooled, filtered, diluted with water (200 mL), and thoroughly extracted first with AcOEt and then with *n*-butanol. The collected *n*-butanol layers were concentrated under vacuum until crystallization started and then left at room temperature for 12 h. Crude crystals were recrystallized in the same manner to provide pure **22** (11.6 g, 65%) as pale-brown crystals: ^{13}C NMR (DMSO-d_6) (sugar carbons) δ 17.7, 66.2, 68.3, 69.8, 70.4, 70.9, 72.2, 73.2, 75.9, 76.5, 100.2, 100.6; (benzylpiperazinylcarbonylmethyl chain carbons) δ 41.6, 44.6, 52.3, 52.7, 55.8, 55.9, 60.2, 60.9, 67.1, 107.8, 123.3, 124.8, 142.0, 152.0, 152.6, 165.6; (flavone moiety carbons) δ 55.9, 95.1, 99.7, 104.1, 105.6, 111.7, 112.6, 120.9, 122.7, 147.7, 152.6, 156.9, 161.3, 163.0, 163.8, 182.0; FABMS (glycerol matrix) in positive mode m/z 915 $(M + H)^+$.

7-(β-D-Glucopyranosyloxy)-5-hydroxy-4′-methoxy-3′-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (23). A solution of **22**, hemitartrate (4 g, 3.9 mmol) in water (100 mL) was set at pH 4 by addition of 0.5 N aqueous HCl and heated at 40 °C. Naringinase (1.2 g; Sigma N 1385) was added, and the reaction was stirred at 40 °C for 2 h. The cloudy mixture was diluted with DMF (20 mL), neutralized with 10% aqueous $NaHCO_3$, and extracted with *n*-butanol. After evaporation of the organic layer, the dried residue was crystallized twice from MeOH to yield pure **23** (2.4 g, 80%) as pale-brown crystals: ^{1}H NMR (DMSO-d_6) (β-glucose) δ 3.2−3.5 (m, 6H), 4.6−5.4 (4H, OH), 5.04 (d, J = 7 Hz, 1H); (benzylpiperazinylcarbonylmethyl chain) δ 2.40 (m, 4H), 3.30 (s, 2H), 3.2−3.5 (m, 2H), 3.7 (m, 2H), 3.73−3.77 (3s, 9H), 4.93 (s, 2H), 6.76 (d, J = 8.5 Hz, 1H), 7.16 (d, J = 8.5 Hz, 1H); (flavone moiety) δ 3.87 (s, 3H), 6.46 (d, J = 2 Hz, 1H), 6.84 (d, J = 2 Hz, 1H), 6.99 (s + d, J = 8.5 Hz, 2H), 7.54 (d, J = 2 Hz, 1H), 7.72 (dd, J = 8.5 and 2 Hz, 1H), 12.9 (s, 1H, chelated OH); FABMS (glycerol matrix) in positive mode m/z 769 $(M + 1)^+$.

7,5-Dihydroxy-4′-methoxy-3′-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (24). To a solution of **23** (1.54 g, 2 mmol) in water (40 mL) adjusted to pH 3.95 with 1 N aqueous H_3PO_4 and heated at 37 °C was added β-glucosidase (0.055 g; Sigma type II G8625). Then the reaction was stirred for 48 h at the same temperature. A similar workup to that in the **22** → **23** procedure yielded a dried residue (1.17 g) which was purified by crystallization from MeOH followed by flash chromatography (SiO_2, CH_2Cl_2−acetone, 1/1) to provide pure **24** (0.94 g, 77%) as pale-yellow

crystals: ^{1}H NMR (DMSO-d_6) (benzylpiperazinylcarbonylmethyl chain) see **23**; (flavone moiety) δ 3.87 (s, 3H), 6.18 (d, J = 2 Hz, 1H), 6.45 (d, J = 2 Hz, 1H), 6.88 (s, 1H), 7.12 (d, J = 8.5 Hz, 1H), 7.45 (d, J = 2 Hz, 1H), 7.68 (dd, J = 8.5 and 2 Hz, 1H), 12.9 (s, 1H, chelated OH); CIMS (NH_3) m/z 607 $(M + 1)^+$.

General Procedure for the Alkylation of the 5-Phenol Group by 4. 7,3′-Bis(benzyloxy)-5-hydroxy-4′-methoxyflavone (9). Dibenzylation of diosmetin (3 g, 10 mmol) with benzyl chloride (7 mL, 60 mmol) in the presence of $KHCO_3$ (2 g, 20 mmol) was carried out according to the general workup described for 7- and 3′-alkylations. The dried residue was purified by flash chromatography (SiO_2, CH_2Cl_2) and then crystallization from MeOH to yield pure 7,3′-*O*-dibenzyldiosmetin (**9**) (3.65 g, 76%) as yellow crystals: mp 176−177 °C; ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 5.07 (s, 2H), 5.17 (s, 2H), 6.38 (d, J = 2 Hz, 1H), 6.43 (s, 1H), 6.48 (d, J = 2 Hz, 1H), 6.92 (d, J = 8.5 Hz, 1H), 7.2−7.5 (m, 12H), 12.95 (s, 1H, chelated OH).

7,3′-Bis(benzyloxy)-4′-methoxy-5-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (19). A mixture of **9** (1.15 g, 2.4 mmol) and K_2CO_3 (1.65 g, 12 mmol) in DMF (10 mL) was stirred for 3 min at 115 °C under nitrogen; then **4f** (0.9 g, 2.64 mmol) in solution in DMF (5 mL) was added. After 2.5 h under the same conditions, the reaction mixture was cooled, diluted with CH_2Cl_2 (15 mL), filtered, and then evaporated to dryness. The dried residue was purified by flash chromatography (SiO_2, CH_2Cl_2−MeOH, 97/3) and then crystallized from MeOH to yield pure **19** (1.28 g, 68%) as white crystals: ^{1}H NMR ($CDCl_3$) (benzyl groups) see **9**; (benzylpiperazinylcarbonylmethyl chain) see **13**; (flavone moiety) δ 3.89 (s, 3H), 6.45 (s, 1H), 6.54 (s, 2H), 6.85 (d, J = 8.5 Hz, 1H), 7.2−7.5 (m, 2H); CIMS (NH_3) m/z 787 $(M + 1)^+$; IR (Nujol) 1640 (amide, cetone), 1605 (C=C) cm^{-1}.

7,3′-Dihydroxy-4′-methoxy-5-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavone (21). A solution of **19** (0.39 g, 0.5 mmol) in DMF (10 mL) was hydrogenated under 1 atm pressure hydrogen with 10% Pd−C (0.1 g) at room temperature for 3 h. The catalyst was removed and the filtrate concentrated to dryness. Crystallization of the dried residue from CH_2Cl_2−MeOH yielded pure **21** (0.19 g, 63%) as white crystals: ^{1}H NMR (DMSO-d_6) (benzylpiperazinylcarbonylmethyl chain) see **23**; (flavone moiety) δ 3.90 (s, 3H), 6.33 (d, J = 2 Hz, 1H), 6.55 (s, 1H), 6.58 (d, J = 2 Hz, 1H), 7.00 (d, J = 8.5 Hz, 1H), 7.42 (d, J = 2 Hz, 1H), 7.52 (dd, J = 8.5 and 2 Hz, 1H), 9.45 and 10.8 (2s, 2H, OH); ^{13}C NMR (DMSO-d_6) δ 41.5 (CH_2), 44.5 (CH_2), 52.2 (CH_3), 52.6 (CH_2), 55.6 (CH_2), 55.6 (OCH_3), 55.7 (OCH_3), 60.2 (OCH_3), 60.8 (OCH_3), 67.3 (CH_2), 95.7 (CH), 97.9 (CH), 106.5 (CH), 107.4 (C), 107.5 (CH), 112.1 (CH), 112.5 (CH), 117.9 (CH), 123.2 (C), 123.3 (C), 124.7 (CH), 141.8 (C), 146.7 (C), 150.4 (C), 151.9 (C), 152.5 (C), 158.9 (C), 159.0. (C), 159.7 (C), 162.1 (C), 165.3 (C), 175.4 (C); CIMS (NH_3) m/z 607 $(M + 1)^+$.

General Procedure for the Dialkylation of the 7- and 3′-Phenol Groups by 4. 7,3′-Bis[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]-5-hydroxy-4′-methoxyflavone (14). Synthesis of **14** from diosmetin (0.6 g, 2 mmol) with **4f** (1.44 g, 4.2 mmol) in the presence of $KHCO_3$ (0.4 g, 4 mmol) was similar to the preparation of **13**. The dried residue was purified by flash chromatography (SiO_2, acetone−CH_2Cl_2, 2/1) and provided pure **14** (1 g, 55%) as a yellowish amorphous compound: ^{1}H NMR ($CDCl_3$) (benzylpiperazinylcarbonylmethyl chain) see **13**; (flavone moiety) similar to **25**; ^{13}C NMR ($CDCl_3$) δ 42.2 ($2CH_2$), 45.3 ($2CH_2$), 52.3 (CH_3), 52.4 (CH_2), 52.8 ($2CH_2$), 55.9 (OCH_3), 56.0 ($2OCH_3$), 56.3 ($2CH_2$), 60.7 ($2OCH_3$), 61.1 ($2OCH_3$), 67.3 (CH_2), 68.5 (CH_2), 93.2 (CH), 98.7 (CH), 104.7 (CH), 106.0 (C), 106.9 (2CH), 111.7 (CH), 112.3 (CH), 121.3 (CH), 123.2 (C), 123.3 (C), 123.6 (C), 125.0 (2CH), 142.3 (2C), 147.6 (C), 152.5 (C), 152.8 (2C), 153.1 (2C), 157.5 (C), 162.1 (C), 163.5 (C), 163.7 (C), 165.1. (C), 165.6 (C), 182.2 (C); FABMS (glycerol matrix) in positive mode m/z 913 $(M + 1)^+$.

Acylations of the Flavanone 33 into Monoacetate 35, Diacetate 36, and Dipivalate 37. 3′-Acetoxy-5-hydroxy-4′-methoxy-7-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavanone (35) and 5,3′-Diacetoxy-4′-methoxy-7-[4-[(2,3,4-trimethoxyphenyl)methyl]-piperazin-1-ylcarbonylmethoxy]flavanone (36). A solution of **33** (0.61 g, 1 mmol) in the mixture Ac_2O−pyridine, 1/1 (12 mL), was left for 18 h at room temperature. A standard extraction of the reaction mixture provided a dry residue of pure diacetate **36** (0.63 g, 91%) as a white amorphous compound; 0.35 g of **36** (0.5 mmol) in solution in TFA (5 mL) was left at room temperature for 24 h. A standard workup of the solution yielded pure monoacetate **35** as a yellowish amorphous compound (0.29 g, 89%): ^{1}H NMR ($CDCl_3$) spectrum is very similar to that of **33** but displays the additional signal of the acetate group at δ 2.38 (s, 3H) and shifted signals for the B-ring protons of the flavanone moiety at δ 6.70 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 2 Hz, 1H), and 7.30 (dd, J = 8.5 and 2 Hz, 1H); IR (CH_2Cl_2) 3300−2500, 1760 (ester), 1660−1640 (cetone, amide) cm^{-1}. Diacetate **36**: ^{1}H NMR ($CDCl_3$) spectrum is very similar to that of **35** except for a deshielding of A-ring proton signals of the flavanone at δ 6.28 and 6.35 (2d, J = 2 Hz, 2H), the additional signal of an acetate group at δ 2.35 (s, 3H), and loss of the chelated phenol group at δ 12.0.

5,3′-Dipivaloxy-4′-methoxy-7-[4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]flavanone (37). A solution of **33** (0.1 g, 0.16 mmol) in 4 mL of the mixture pivaloyl chloride−pyridine, 1/1 (12 mL), was left for 48 h at room temperature. A standard extraction of the reaction mixture and then purification of the dried residue by flash chromatography (alumina, CH_2Cl_2−MeOH, 99/1) provided pure dipivalate **37** (0.052 g, 42%) as a white amorphous compound: ^{1}H NMR ($CDCl_3$) spectrum is similar to that of **36** except for the replacement of acetate by pivalate signals (2s, 18H) at δ 1.30 and 1.35.

Isomerization of the Flavanone 33 into the Chalcone 39. 1-[2,6-Dihydroxy-4-[(2,3,4-trimethoxyphenyl)methyl]-piperazin-1-ylcarbonylmethoxy]-3-(3-hydroxy-4-methoxy)-2-propen-1-one (39). A mixture of **33** (0.06 g, 0.1 mmol) and $KHCO_3$ (0.02 g, 0.2 mmol) in DMF (3 mL) was heated at 125 °C for 1 h under nitrogen. The reaction mixture was diluted with water (20 mL) and extracted by CH_2Cl_2. Standard workup of the organic layer afforded a quantitative dried residue. Purification of this residue by TLC (SiO_2, CH_2Cl_2−MeOH, 97/3) and then crystallization from MeOH provided pure **39** (0.02 g, 30%) as bright-yellow crystals: ^{1}H NMR (DMSO-d_6) (benzylpiperazinylcarbonylmethyl chain) see **23**; (chalcone moiety) δ 3.80 (s, 3H), 5.92 (s, 2H), 6.95 (d, J = 8.5 Hz, 1H), 6.9−7.15 (m, 2H), 7.58 and 7.94 (2d, J = 16 Hz, 2H), 9.3 (s, 1H, OH), 12.6 (s, 1H, chelated OH); CIMS (NH_3) m/z 609 $(M + 1)^+$.

Hydrogenation of the Flavanone 33 into the Dihydrochalcone 40. 1-[2,6-Dihydroxy-4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-1-ylcarbonylmethoxy]-3-(3-hydroxy-4-methoxy)-1-propanone (40). A solution of **33** (0.12 g, 0.2 mmol) in aqueous 0.1 N NaOH (20 mL) was hydrogenated under 1 atm pressure hydrogen with 10% Pd−C (0.1 g) at room temperature for 4 h. The catalyst was separated; the filtrate was adjusted to pH 8 with aqueous 0.2 N HCl and extracted thoroughly with CH_2Cl_2. The organic layer was evaporated to dryness, and the residue (0.06 g) was crystallized from MeOH to provide pure white crystals of dihydrochalcone **40** (0.031 g, 25%): ^{1}H NMR (DMSO-d_6) (benzylpiperazinylcarbonylmethyl chain) see **23**; (dihydrochalcone moiety) δ 2.73 and 3.22 (2t, J = 7.5 Hz, 4H), 5.90 (s, 2H), 6.58 (d, J = 8.5 and 2 Hz, 1H), 6.60 (d, J = 2 Hz, 1H), 6.77 (d, J = 7.5 Hz, 1H), 8.9 (s, 1H, OH), 12.4 (s, 1H, chelated OH); CIMS (NH_3) m/z 611 $(M + 1)^+$.

Quaternization of the Flavanone 33 into the Methiodide 38. 5,3′-Dihydroxy-4′-methoxy-7-[4-methyl-4-[(2,3,4-trimethoxyphenyl)methyl]piperazin-4-ium-1-ylcarbonylmethoxy]flavanone, Iodide (38). A solution of **33** (0.06 g, 0.1 mmol) in CH_2Cl_2 (5 mL) was added to MeI (0.2 mL, 3.2 mmol) and then left at room temperature for 96 h until crystallization was complete. Filtration of these white crystals provided pure methiodide **38** (0.032 g, 43%): ^{1}H NMR (DMSO-d_6) (benzylpiperazinylcarbonylmethyl chain) δ 3.00 (s, 3H),

3.2−4.2 (m, 8H), 3.80−3.85 (2s, 9H), 4.50 (s, 2H), 4.92 and 5.02 (2d, J = 14.7 Hz, 2H), 6.85 (m, 1H), 7.15 (d, J = 8.5 Hz, 1H); (flavanone moiety) δ 2.71 (dd, J = 12.7 and 2.5 Hz, 1H), 3.22 (dd, J = 16.5 and 12.7 Hz, 1H), 3.90 (s, 3H), 5.45 (dd, J = 12.7 and 2.5 Hz, 1H), 6.08 (s, 2H), 6.8−7.0 (m, 3H), 9.1 (s, 1H, OH), 12.1 (s, 1H, chelated OH).

Cell Lines and Culture Conditions. K562/DOX cell line was provided by Dr. Tapiero (Faculté de Pharmacie, Châtenay-Malabry, France) and was initially established in Dr. Tsuruo's laboratory (Cancer Chemotherapy Center, Tokyo, Japan). This cell line was derived from the parental erythroleukemia human cell line K562 by stepwise selection with doxorubicin and has been shown to overexpress P-glycoprotein.[18] Using the MTT-based assay (see below), we found K562/DOX cells to be 300-fold resistant to doxorubicin, as compared with the parental cell line (data not shown). Although selection with an anticancer agent probably induces several mechanisms of resistance, Pgp appears to be the major mechanism of MDR in this cell line since (i) altered accumulation of MDR-type agents such as anthracyclines, Rh-123, and JC-1 was observed and (ii) Pgp modulators such as verapamil or S9788 could completely restore doxorubicin cytotoxicity (data not shown). Cells were kept in RPMI-1640 medium supplemented with 10% fetal calf serum under standard culture conditions.

MTT-Based Assay. The assay is based on the intracellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Saint-Quentin-Fallavier, France) in mitochondria of viable cells to water-insoluble formazan. The assays were performed in 96-well plates, essentially as described by Mosmann.[36] Cells were exposed for 72 h to both doxorubicin (seven graded concentrations, six wells per concentration) and the tested compound (1 or 5 μM). Stock solutions of BPF were prepared in DMSO and diluted directly in the culture medium. The final DMSO content did not exceed 0.1%, which had no effect on cell growth. Water-insoluble formazan crystals were dissolved in DMSO, and absorbance was read at 570 nm using an Elx800 BioTek microplate reader (OSI Fischer Scientific). Cell survival was calculated by dividing absorbance at a given DOX concentration by the absorbance of control wells (no DOX, no tested compound). The IC_{50} value was determined by linear interpolation from the survival curve as a function of DOX concentration. The MDR-modulating activity was expressed as the modulation factor: MF = IC_{50}(DOX alone)/IC_{50}(DOX + tested compound). Most compounds were not toxic per se at 5 μM, except compounds **13** and **14** (63% and 25% cell viability at 5 μM, respectively).

JC-1 Accumulation Assay. JC-1 was purchased from Molecular Probes, Eugene, OR. The incubation solution was prepared by diluting JC-1 (1 mg/mL in DMSO) to a final concentration of 5 μg/mL, in PBS containing 1 g/L glucose. The solution was subsequently filtered on a 0.45-μm cellulose acetate filter in order to eliminate JC-1 aggregates. The final JC-1 monomer concentration was 0.2 μM. Cells were washed once with PBS, resuspended at a concentration of 5×10^5 cell/mL in the incubation solution, and distributed in 24-well plates. Tested compounds were then added from stock solutions. The final DMSO content was identical for every concentration and did not exceed 0.5%. The plates were incubated at 37 °C, and at regular time points, fluorescence was measured using FL600 BioTek fluorescence microplate reader (OSI Fischer Scientific), until the accumulation reached a plateau. Emission and excitation filters were set at 485 ± 10 and 590 ± 17 nm, respectively. On each plate, a positive control was included, using 5 μM S9788, a known modulator of the MDR phenotype. Results are expressed relative to S9788, to allow comparison of independent experiments. The percentage of JC-1 accumulation was calculated using the following formula:

$$\%\ \text{accumulation} = [\text{FI(JC-1 + tested compound)} - \text{FI(JC-1)}]/[\text{FI(JC-1 + S9788)} - \text{FI(JC-1)}]$$

where FI is the fluorescence intensity at 590 nm.

HPLC Measurements. The high-performance liquid chromatography apparatus (Varian 2000LC) was equipped with a Lichrosorb RP-18 column (5 μm, 30 cm) and a UV detector (Varian) set at 270 nm for flavone-derived compounds and at 290 nm for flavanone-derived compounds. The eluent was water/acetonitrile/mesylic acid (60:40:0.1). The flow was kept constant at 1.2 mL/min. Compounds were dissolved directly in the eluent and injected through a 20-μL loop. At least three injections were performed for each compound.

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