expression, the cells were cultured in serum-free DMEM for 6 h and then incubated in the growth medium only for a further 2 to 16 h. Phosphorylated ERK was found to have slightly increased in each of the four cell lines by the addition of FBS to the serum-starved culture, and this occurred simultaneously with increased P-gp expression levels (Fig. 4B). These data indicate that FBS itself activates the MEK-ERK-RSK pathway and increases P-gp expression. Moreover, the strong enhancement of P-gp expression by the stimulation of EGF and FBS persisted for a longer duration compared with the stimulation with FBS alone (Fig. 4A and B). However, although the P-gp levels were enhanced by EGF stimulation, the corresponding *MDR1* mRNA levels were unchanged, as indicated by our RT-PCR analysis (Fig. 4C; Supplementary Fig. S1B).[3] We next did bFGF treatments at 10 μg/L for 8 or 12 h using the same procedures described for Fig. 4A and a bFGF-dependent enhancement of P-gp expression was also observed in each cell line (Fig. 4D).

We next determined the effects upon P-gp expression levels by Western blotting when HCT-15 cells were transiently transfected with cDNAs corresponding to genes of either the MEK-ERK-RSK or PI3K-Akt signaling pathways. The activation of the MEK-ERK-RSK pathway by transfection with WT cDNAs for *H-Ras*, *Raf-1*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *RSK1*, or *RSK2* was found to enhance

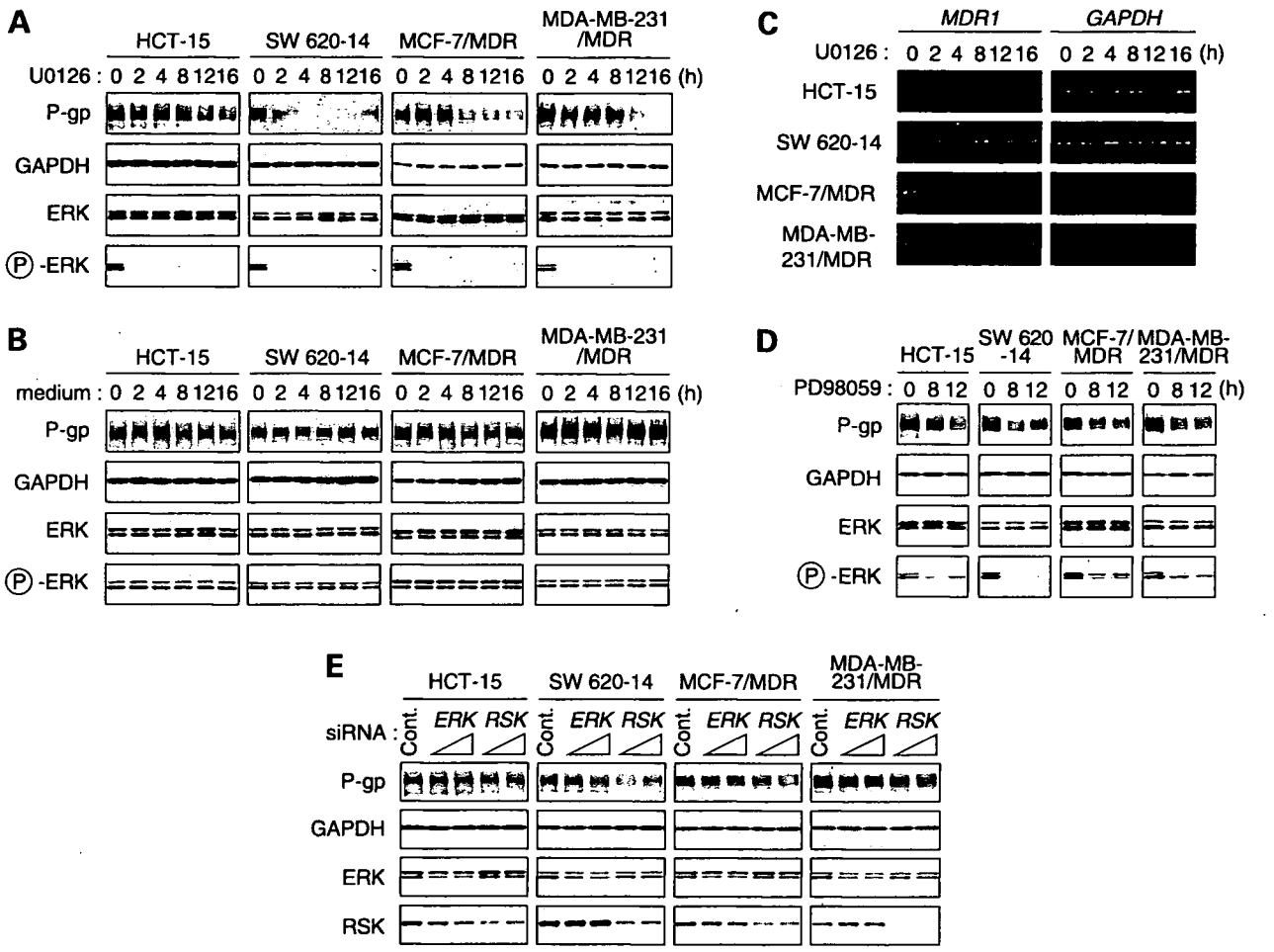

Figure 2. Down-regulation of P-gp by treatment with MEK inhibitors or by RNA interference of *MEK* and *ERK*. **A,** Western blot analysis of P-gp expression levels after a time course of U0126 treatment. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were treated with medium alone or with 10 μmol/L U0126 for 2 to 16 h. Cellular membrane and cytoplasmic fractions were prepared using lysis buffer containing 0.2% NP40 and subjected to Western blotting with the indicated antibodies. **B,** Western blot analysis of P-gp expression levels after a time course of treatment with medium alone. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were treated with medium alone for 2 to 16 h and processed as described in **A. C,** RT-PCR analysis of *MDR1* mRNA levels over a time course of U0126 treatment. After treatment with medium alone or with 10 μmol/L U0126 for 2 to 16 h in HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells, total RNAs were extracted from the cells. The mRNA levels of the *MDR1* and *GAPDH* genes were analyzed by RT-PCR as described in Materials and Methods. **D,** P-gp expression levels in PD98059-treated HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells. The cells were treated with medium alone or with 50 μmol/L PD98059 for 8 or 12 h and processed as described in **A. E,** the effects of *ERK* and *RSK* siRNAs on P-gp expression levels. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were transfected with siRNAs in the following combinations: 50 μmol/L negative control siRNA (*Cont.*); 25 or 50 μmol/L (each 12.5 or 25 μmol/L) of an *ERK1+ERK2* siRNA combination; or 25 or 50 μmol/L (each 8.3 or 16.7 μmol/L) of an *RSK1/RSK2/RSK3* siRNA mixture. After transfection for 48 h, the cells were harvested and lysed with lysis buffer containing 0.2% NP40. Cell lysates were then subjected to Western blotting with the indicated antibodies.

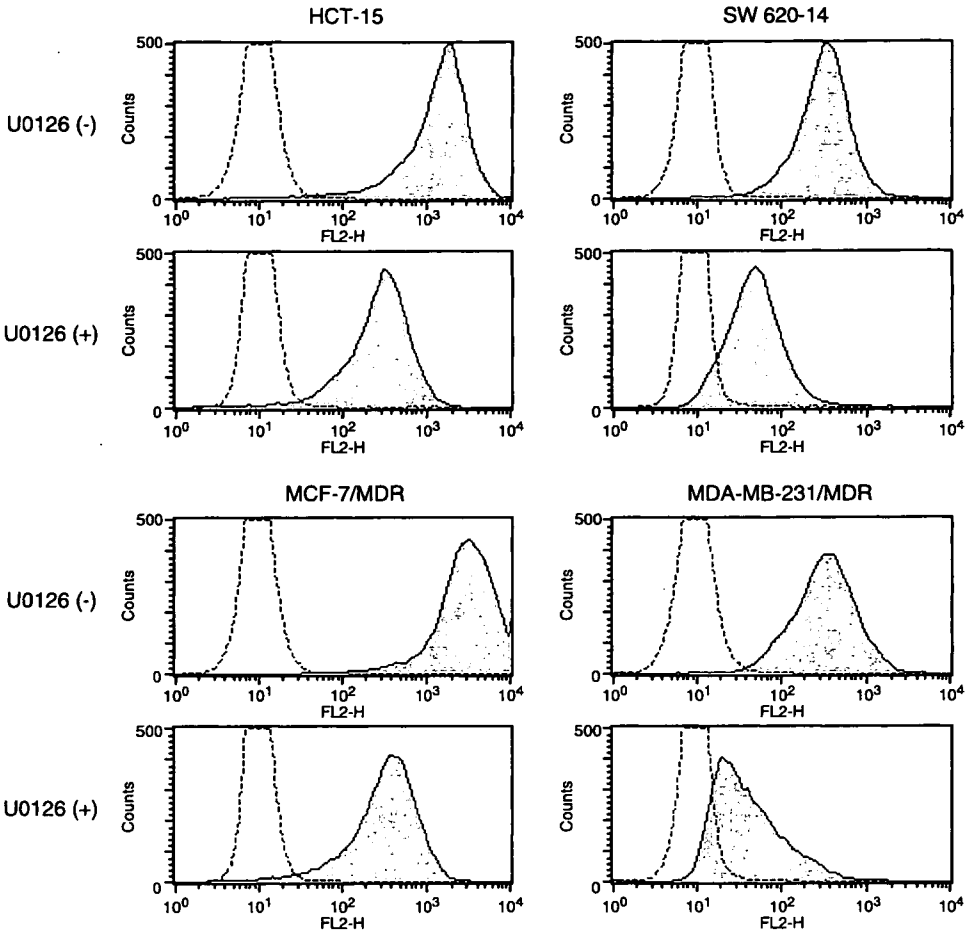

Figure 3. The down-regulation of cell surface P-gp expression by U0126. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were treated with medium alone or with 10 μmol/L U0126 for 72 h, with medium replacement every 24 h. The cells were then harvested with trypsin, washed with PBS, and incubated with (*closed areas*) or without (*open areas*) a biotinylated $F(ab')_2$ fragment of the MRK16 antibody. The cells were then washed with PBS and incubated with R-phycoerythrin–conjugated streptavidin. Fluorescence staining was then evaluated using FACSCalibur.

the P-gp expression levels, whereas the activation of PI3K-Akt signaling pathway by transfection with *p85α* (WT) regulatory subunit of *PI3K*, *akt1* (WT), *PTEN* (C124S), or *PTEN* (WT) DNA did not affect P-gp expression levels (Supplementary Fig. S2).[3] These results further indicate that P-gp expression is positively regulated by the MEK-ERK-RSK pathway.

U0126 Promotes P-gp Degradation but Does Not Inhibit Its Biosynthesis

Based on our observation that U0126 suppresses P-gp expression levels without affecting its gene transcription (Fig. 2A and C), we further examined the biosynthesis and degradation of P-gp using pulse-chase experiments in which MDA-MB-231/3HisMDR cells were treated with (+) or without (−) 10 μmol/L U0126. During the pulse labeling procedure for 0.5 or 1 h, the labeled P-gp levels were observed to gradually increase in both the untreated and U0126-treated cells (Fig. 5A and B), and were found to be almost equivalent in both cases. To subsequently examine the effects of U0126 on P-gp degradation, ^{35}S metabolic labeling was done for 1 h using MDA-MB-231/3HisMDR cells in the absence (−) or presence (+) of 10 μmol/L U0126. The cells were then chased for 2 to 12 h in the growth medium without (−) or with (+) 10 μmol/L U0126. The labeled P-gp expression levels were found to be largely unchanged in the untreated cells, but a significant reduction in P-gp was observed in the U0126-treated cells at the 8 and 12 h time points of the chase period (Fig. 5C and D). Moreover, the quantities of labeled P-gp at 12 h were ~50% of the 0 h levels (no chase; Fig. 5C and D). These results suggest that U0126 promotes P-gp degradation but does not affect its biosynthesis.

The U0126-Mediated Down-regulation of P-gp Enhances the Sensitivity of Cells to Paclitaxel and Rhodamine123 Uptake

To examine whether the U0126-mediated down-regulation of P-gp has any effect on anticancer agent–mediated apoptosis, we treated HCT-15, SW620-14, MCF-7/MDR, and MDA-MB-231/MDR cells with (+) or without (−) 10 μmol/L U0126 for 72 h to down-regulate P-gp expression on cell surface. We then treated the cells with (+) or without (−) 10 μmol/L U0126 combined with different doses of paclitaxel for an additional 24 h. In this experiment, the IC_{50} values (the dosage at which a 50% inhibition of cell growth occurs) for paclitaxel had been previously determined for each cell line, and the cells were treated with both this dose and a 3-fold higher concentration of the drug. As shown in Fig. 6A, paclitaxel increased the levels of cleaved PARP, which is a substrate of caspase-3, as reported previously (17). When the cells were

pretreated with U0126, the extent of this PARP cleavage by paclitaxel was enhanced in each cell lines, particularly at the 3-fold IC_{50} concentrations (Fig. 6A). These results indicate that paclitaxel-mediated apoptosis signaling via the activation of caspase-3 is enhanced by the U0126-mediated suppression of P-gp.

Finally, we examined the effects of U0126 on rhodamine123 uptake in P-gp–expressing cells. As shown in Fig. 6B, cells treated with U0126 accumulated higher levels of rhodamine123 compared with untreated cells. Hence, the U0126-mediated down-regulation of P-gp leads to an increase in the intracellular concentration of P-gp substrates.

Discussion

Many previous studies have evaluated P-gp inhibitors to effectively reverse P-gp–mediated drug resistance. In the 1980s, verapamil was initially identified as a P-gp inhibitor (18, 19), as it increases the intracellular concentration of various anticancer agents in multidrug-resistant cells by binding P-gp and inhibiting drug efflux. Subsequently, many P-gp inhibitors such as valspoder (PSC-833), dofequidar fumarate (MS-209), tariquidar (XR9576), and thiosemicarbazone derivative (NSC73306), have been developed (20–23). Clinical trials using such P-gp inhibitors have shown *in vivo* increases in the intracellular concentrations of coadministered anticancer agents in P-gp–positive tumor cells (24). However, phase III trials of these agents have not been successful, and no significant survival benefit as a result of P-gp inhibition has yet been achieved (25, 26). Further clinical studies using new P-gp inhibitors and new combination-treatment regimens have been devised, and some are currently ongoing.

The regulatory mechanisms underlying the expression of ABC transporters, including P-gp and BCRP, have not yet been well clarified. We have previously shown that physiologic levels of estrogens suppress P-gp and BCRP in estrogen receptor α–expressing breast cancer cells via posttranscriptional processes, and that this occurs without any affects upon transcription (14, 15). Other groups have also shown that the stability of P-gp is regulated by the ubiquitin-proteasome system (27, 28). Furthermore, Akt signaling has been shown to modulate a side population cell phenotype by regulating the expression of Bcrp1 in mouse (29). The PI3K inhibitor, LY294002, and a dominant-negative form of Akt have also been reported to down-regulate BCRP expression levels (16). Thus, the association between the expression of ABC transporter proteins and either cell growth signaling or the ubiquitin-proteasome system has recently generated some interest. In our present

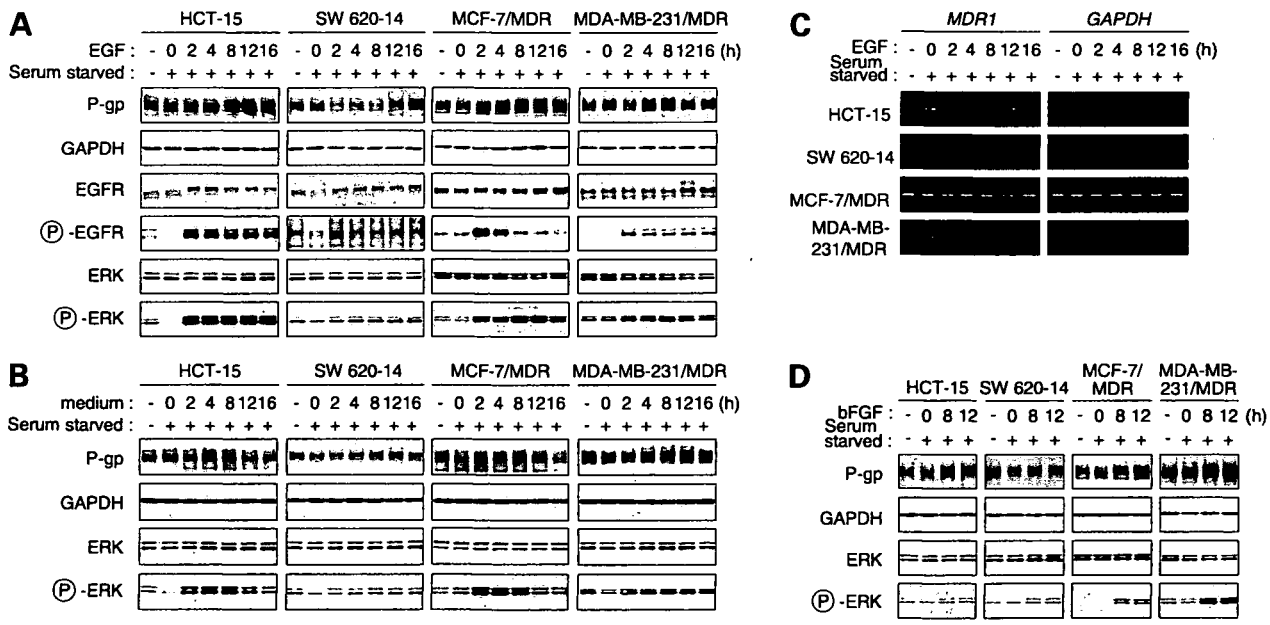

Figure 4. The up-regulation of P-gp by EGF or bFGF in a time-dependent manner. **A,** Western blot analysis of P-gp expression levels after the EGF-dependent activation of the MEK-ERK-RSK pathway. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were cultured in medium without serum for 6 h (*Serum starved*; +). The medium was then replaced with the growth medium supplemented with 100 μg/L EGF, and the cells were incubated for a further 2 to 16 h. Negative control cells were not serum starved and were untreated (*Serum starved*; – and *EGF*; –). Cellular membrane and cytoplasmic fractions were lysed with lysis buffer containing 0.2% NP40 and subjected to Western blotting with the indicated antibodies. **B,** Western blot analysis of P-gp expression levels after FBS-dependent activation of the MEK-ERK-RSK pathway. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were cultured in medium without serum for 6 h (*Serum starved*; +). The medium was then replaced with the flesh growth medium, and the cells were incubated for a further 2 to 16 h. Negative control cells and cell processing were as described in **A. C,** RT-PCR analysis of *MDR1* mRNA levels after EGF treatment. After treatment with EGF as in **A**, total RNAs were extracted from the cells and the mRNA levels of the *MDR1* and *GAPDH* genes were analyzed by RT-PCR. **D,** Western blot analysis of P-gp expression levels after bFGF treatment. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were cultured in medium without serum (*Serum starved*; +) and after incubation for 6 h, the medium was replaced with the growth medium supplemented with 10 μg/L bFGF, and the cells were cultured for a further 8 or 12 h. Negative control cells and cell processing were as described in **A.**

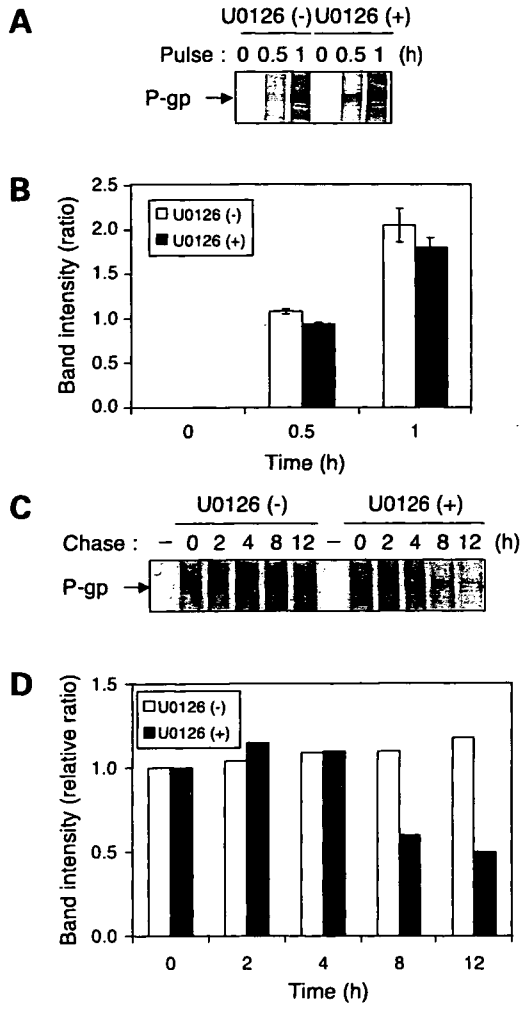

Figure 5. U0126 promotes the degradation of P-gp but does not suppress its biosynthesis. **A** and **B**, biosynthesis of P-gp. MDA-MB-231/3HisMDR cells were cultured in methionine- and cysteine-free medium for 1.5 h and then metabolically labeled with 300 μCi/mL of ^{35}S for 0.5 or 1 h. For the U0126 treatment, the cells were treated with 10 μmol/L U0126 from 4 h before beginning the experiments to the end of the ^{35}S-labeling period. ^{35}S-labeled P-gp was immunoprecipitated from 500 μg of cell lysates, subjected to SDS-PAGE, and autoradiographed. Band intensities were measured using an NIH Image densitometer (**B**). *Columns*, means; *bars*, SD; calculated from three independent experiments. **C** and **D**, degradation of P-gp. MDA-MB-231/3HisMDR cells were cultured in methionine- and cysteine-free medium for 1.5 h, metabolically labeled with 300 μCi/mL of ^{35}S for 1 h, and chased for a further 2 to 12 h. For U0126 treatment, the cells were treated with 10 μmol/L U0126 from the time of ^{35}S labeling to the end of the chase period. ^{35}S-labeled P-gp was immunoprecipitated from 500 μg of cell lysates, subjected to SDS-PAGE, and autoradiographed. The cells without ^{35}S labeling were also prepared, and unlabeled P-gp was analyzed in the same manner as the ^{35}S-labeled P-gp (*Chase*; −). Band intensities were again measured using an NIH Image densitometer (**D**).

study, we have attempted to further clarify the regulatory mechanisms underlying ABC transporter protein expression, focusing on P-gp, to identify inhibitors that specifically target these expression mechanism(s).

We initially found that inhibitors of the MEK-ERK-RSK pathway suppressed P-gp expression (Fig. 1). In particular, the MEK inhibitor U0126 was found to potently down-regulate endogenous P-gp expression (Fig. 1), and both the U0126- and PD98059-mediated down-regulation of P-gp could be observed in both endogenous and exogenous P-gp–expressing cells (Fig. 2A and D). Moreover, these phenomena were found not to be the result of transcriptional regulation (Fig. 2C; Supplementary Fig. S1A).[3] The suppression of P-gp by MEK inhibitors was also far more rapid compared with estrogens (Fig. 2A and D, compared with ref. 15). ERK and RSK knockdown by siRNAs also down-regulated P-gp expression (Fig. 2E). Conversely, the activation of the MEK-ERK-RSK pathway by the overexpression of WT proteins for H-Ras, Raf-1, MEK1, MEK2, ERK1, ERK2, RSK1, or RSK2 enhanced P-gp expression in HCT-15 cells, whereas the activation of the PI3K-Akt signaling pathway by overexpressing the phosphatase inactive form of PTEN (C124S), p85α (WT) regulatory subunit of PI3K, or Akt (WT) did not affect P-gp in these cells (Supplementary Fig. S2).[3] These results suggest that the stability of P-gp is regulated by the MEK-ERK-RSK pathway, and that the kinase activities of RSK are necessary for this stabilization. Although BCRP has been shown to be regulated by the PI3K-Akt signaling pathway (16), it is likely that P-gp is regulated by different mechanisms. We additionally examined P-gp expression in cells treated with EGF and bFGF, which are activators of the MEK-ERK-RSK pathway via EGFR and FGF receptor, respectively. As expected, the stimulation of either EGF or bFGF enhanced P-gp expression levels without affecting its transcription (Fig. 4A–D; Supplementary Fig. S1B).[3] Moreover, the presence of FBS in the growth medium slightly enhanced the P-gp expression levels with the activation of the MEK-ERK-RSK pathway (Fig. 4B). These data thus support our earlier findings that P-gp expression is positively regulated by the MEK-ERK-RSK pathway.

In the present study, we have shown that U0126 treatment down-regulated P-gp expression for 12 h, but that SP600125 (a JNK inhibitor) and SB203580 (a p38MAPK inhibitor) did not alter these expression levels in HCT-15 and SW620-14 cells (Fig. 1). The components of MAPK comprise three subfamilies, ERK, JNK, and p38MAPK. In previous studies, the JNK or p38MAPK pathways have been reported to alter P-gp expression levels. In addition, adenoviral transduction of *JNK* has been shown to down-regulate P-gp expression, whereas SP600125 treatment for 24 h did not affect its expression in human gastric and pancreatic cancer cells (30). In another report, SP600125 treatment for 24 h was shown to enhance P-gp expression in human prostate cancer DU145 spheroids (31). SB203580 has been shown to decrease P-gp expression levels in DU145 spheroids and vincristine-resistant murine leukemia L1210/VCR cells (31, 32). U0126 treatment was also reported to up-regulate P-gp expression after 24 h in DU145 spheroids (31). The discrepancies between the data from these previous reports and our present experiments may be due to differences in the cell lines and treatment protocols used. We observed that the down-regulation of phosphorylated ERK by U0126 was slightly recovered at 24 h in each of the cell lines tested in this study (data not shown),

suggesting that U0126 may be degraded. Therefore, we replenished the U0126-containing medium every 24 h in the experiments shown in Figs. 3 and 6.

Although U0126 suppressed both endogenous and exogenous P-gp, the *MDR1* mRNA levels were unaffected by treatment with this agent (Fig. 2C; Supplementary Fig. S1A).[3] These data strongly indicate the existence of U0126-mediated posttranscriptional P-gp regulation mechanism(s), most likely to be translation and degradation processes. To further elucidate this, we established MDA-MB-231/3HisMDR cells and found that the rate of P-gp biosynthesis in U0126-treated cells was virtually equivalent to the untreated cells (Fig. 5A and B). In contrast, however, the degradation rate of P-gp in U0126-treated cells was higher than in untreated cells (Fig. 5C and D). We subsequently did a pulse-chase experiment to confirm these observations and found that the P-gp expression levels in untreated cells were constant up to the 12 h time point,

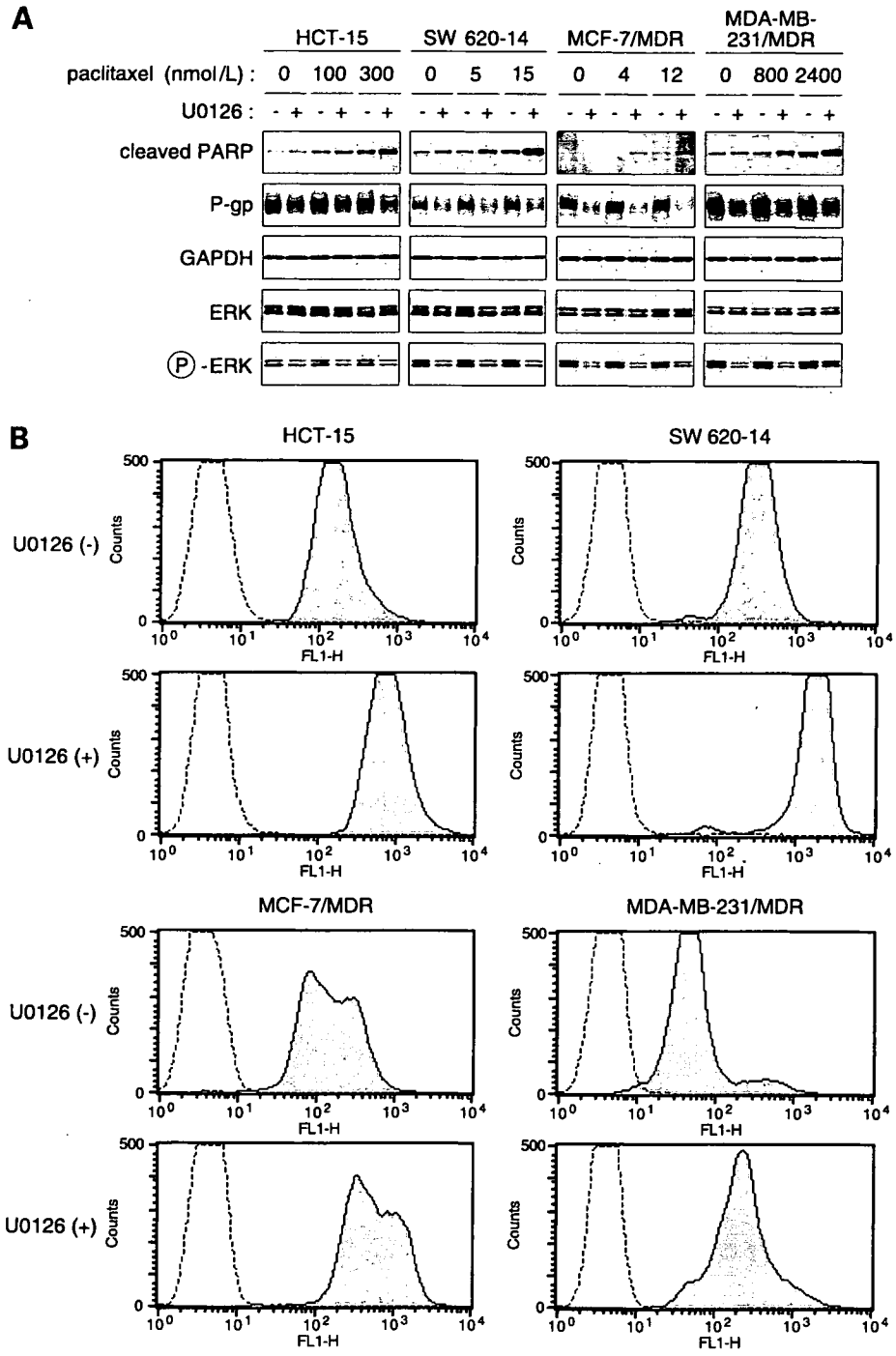

Figure 6. The physiologic effects of U0126 upon the function of P-gp as an efflux pump. **A,** Western blot analysis of cleaved PARP after paclitaxel treatment combined with U0126. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were treated with medium alone (*U0126*; −) or with 10 μmol/L U0126 (*U0126*; +) for 72 h, with medium replacement every 24 h. The cells were then further treated with or without 10 μmol/L U0126 and/or paclitaxel as the indicated combinations for 24 h. Paclitaxel was used at both 1× and 3× its predetermined IC_{50} concentration for each cell line. The cells were harvested with lysis buffer containing 0.2% NP40 and the cell lysates were subjected to Western blotting with the indicated antibodies. **B,** fluorescence-activated cell sorting analysis of rhodamine123 uptake in U0126-treated cells. HCT-15, SW620-14, MCF-7/MDR, or MDA-MB-231/MDR cells were treated with medium alone [*U0126 (−)*] or with 10 μmol/L U0126 [*U0126 (+)*] for 72 h, with medium replacement every 24 h. The cells were then trypsinized and adjusted to a concentration of 5 × 10^5/mL with medium. The cells were incubated with 300 nmol/L rhodamine123 for 20 min at 37°C and washed twice with ice-cold PBS. The intracellular accumulation of rhodamine123 was detected using FACSCalibur.

but that the levels in the U0126-treated cells had decreased by ~50% during the same 8 to 12 h chase period (Fig. 5C and D). These results indicate that U0126 suppresses P-gp expression by promoting its degradation.

To analyze the physiologic responses to the U0126-mediated down-regulation of P-gp, we examined the possible effects of this response upon the enhancement of apoptosis by paclitaxel, and also upon the accumulation of rhodamine123, both of which are substrates of P-gp (33). Because PARP is cleaved by active caspase-3, an apoptosis inducer (17, 34), we used this as the index of the paclitaxel-mediated enhancement of apoptosis signaling. P-gp expression on the cell surface decreased when the cells were treated with U0126 for 72 h (Fig. 3). The cells in this experiment were therefore pretreated with U0126 for 72 h and then cotreated with U0126 and paclitaxel for a further 24 h. The combination of U0126 and paclitaxel was found to enhance the levels of cleaved PARP, compared with paclitaxel exposure alone (Fig. 6A). In addition, the intracellular rhodamine123 levels were also found to accumulate after U0126 treatment for 72 h (Fig. 6B). These results indicate that the U0126-mediated down-regulation of P-gp can reverse the P-gp–mediated resistance to anticancer agents.

Although we show that inhibition of the MEK-ERK-RSK pathway suppresses P-gp expression, and that this involves the kinase activities of RSKs (Fig. 2E; Supplementary Fig. S2A),[3] it remains unclear how MEK inhibitors promote P-gp degradation or whether the RSKs directly regulate P-gp expression. If these molecules indirectly regulate P-gp degradation, the question of which factors are associated with this mechanism remains to be elucidated and will require further molecular analyses.

Many P-gp inhibitors that have been developed are competitors of anticancer agents that are also P-gp substrates. Because RSKs have been shown to positively regulate P-gp expression in our present study, we speculate that MEK, ERK, and RSK inhibitors, and also RNA interferences may have potential as novel therapeutic agents for the reversal of P-gp–mediated anticancer drug resistance. During cellular hyperplasia, the MAPK pathway is often activated and provides a variety of growth signals, promotes cell cycle progression, and suppresses apoptosis (9). Inhibitors of the MEK-ERK-RSK pathway would thus be expected to have significant benefits as chemotherapeutics against P-gp–mediated drug-resistant cancer cells.

In conclusion, we show that a blockade of the MEK-ERK-RSK pathway suppresses cell surface P-gp expression by promoting its degradation. Our data therefore provide new insights into the regulation of P-gp expression and suggest potential new strategies for the reversal of P-gp–mediated anticancer drug resistance.

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ORIGINAL ARTICLE

Flavonoids inhibit breast cancer resistance protein-mediated drug resistance: transporter specificity and structure–activity relationship

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Abstract
Purpose ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-related protein 1 (MRP1), confer resistance to various anticancer agents. We previously reported that some flavonoids have BCRP-inhibitory activity. Here we show the reversal effects of an extensive panel of flavonoids upon BCRP-, P-gp-, and MRP1-mediated drug resistance.
Methods Reversal effects of flavonoids upon BCRP-, P-gp-, or MRP1-mediated drug resistance were examined in the *BCRP*- or *MDR1*-transduced human leukemia K562 cells or in the *MRP1*-transfected human epidermoid carcinoma KB-3-1 cells using cell growth inhibition assays. The IC_{50} values were determined from the growth inhibition curves. The RI_{50} values were then determined as the concentration of inhibitor that causes a twofold reduction of the IC_{50} in each transfectant. The reversal of BCRP activity was tested by measuring the fluorescence of intracellular topotecan.
Results The BCRP-inhibitory activity of 32 compounds was screened, and 20 were found to be active. Among these active compounds, 3′,4′,7-trimethoxyflavone showed the strongest anti-BCRP activity with RI_{50} values of 0.012 μM for SN-38 and 0.044 μM for mitoxantrone. We next examined the effects of a panel of 11 compounds on P-gp- and MRP1-mediated drug resistance. Two of the flavones, 3′,4′,7-trimethoxyflavone and acacetin, showed only low anti-P-gp activity, with the remainder displaying no suppressive effects against P-gp. None of the flavonoids that we tested inhibited MRP1.
Conclusion Our present results thus indicate that many flavonoids selectively inhibit BCRP only. Moreover, we examined the structure–BCRP inhibitory activity relationship from our current study.

Keywords BCRP/ABCG2 · P-glycoprotein/ABCB1 · MRP1/ABCC1 · Flavonoid · Growth inhibition assay

Abbreviations

ABC	ATP-binding cassette
BCRP	Breast cancer resistance protein
MDR	Multidrug resistance
P-gp	P-glycoprotein
MRP1	Multidrug resistance-related protein 1
SN-38	7-Ethyl-10-hydroxycamptothecin (the active metabolite of irinotecan)
VP-16	Etoposide

Introduction

Tumor cells often acquire multidrug resistance, characterized by cross-resistance to other structurally unrelated

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agents [1]. Multidrug-resistant cells express ABC transporters, such as the *MDR1* gene product P-glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, and multidrug resistance-related protein 1 (MRP1)/ABCC1, that pump out various structurally unrelated anticancer agents in an ATP-dependent manner. In the 1980s, verapamil was firstly found to increase the intracellular concentration of anticancer agents in multidrug-resistant cells by binding P-gp and inhibiting the P-gp-mediated drug efflux [2, 3]. Subsequently, many P-gp inhibitors such as valspoder (PSC-833), dofequidar fumarate (MS-209), tariquidar (XR9576), and thiose-micarbazone derivative (NSC73306) have been developed that also interact with P-gp and reverse P-gp-mediated drug resistance [4–7]. Clinical trials using such P-gp inhibitors have shown an in vivo increase in the intracellular concentration of coadministered anticancer agents in P-gp-positive tumor cells [8]. However, phase III trials of these agents have not been successful and no significant survival benefit of P-gp inhibition has yet been achieved [9, 10]. Further clinical studies using new P-gp inhibitors and new combination treatment regimens have been devised however, and some are now ongoing.

BCRP is a half-molecule ABC transporter with an NH_2-terminal ATP-binding site and COOH-terminal transmembrane domain [11–15]. Recently, we reported that BCRP forms homodimers via a disulfide bridge between Cys603, a residue on the third outer-membrane domain of the BCRP monomer [16, 17]. The homodimeric BCRP complex acts as an efflux pump for various anticancer agents including SN-38, mitoxantorone, and topotecan, and thus prevents the build up of high intracellular concentrations of such anticancer agents and decreases their cytotoxic effects. BCRP is reportedly also expressed in various normal human tissues and cells, such as the placenta, liver, brain, spinal code, adrenal gland, testes, prostate, uterus, kidney, heart, bone marrow, and small intestine [18]. Furthermore, BCRP is expressed in hematopoietic stem cells and is thought to be a stem cell marker [19]. We previously reported that estrone and 17β-estradiol inhibit BCRP-mediated drug transport and resistance. In addition, we have found from our studies that BCRP transports sulfated estrogens as physiologic substrates but not as free estrogens [20, 21]. We further demonstrated that some flavonoids, such as genistein and naringenin, diminished the function of BCRP as an efflux pump and reversed BCRP-mediated resistance to anticancer agents [22]. Flavopiridol, a flavonoid-derived antitumor agent, is a substrate of BCRP [23], and flavonoids and estrogenic compounds thus possess BCRP inhibitory properties.

In our current study, we screened a further panel of flavonoids possessing inhibitory activity for BCRP, including 29 flavonoids and 3 flavonoid-related compounds (total 32), by cell growth inhibition assay. We find that 20 of these compounds harbor inhibitory activity against BCRP. However, although two of the flavonoids that we tested induced a weak reversal of P-gp-mediated multidrug resistance, none of the other compounds displayed any inhibitory properties toward P-gp. Additionally, none of the flavonoids screened in this study were found to inhibit MRP1. We thus conclude that they selectively target BCRP only.

Materials and methods

Reagents

Flavonoid compounds were purchased from Funakoshi (Tokyo, Japan). Fumitremorgin C (FTC) was purchased from Alexis (San Diego, CA, USA). Anti-BCRP polyclonal antibody (3488) was raised by immunizing rabbits with a KLH-conjugated 20-mer peptide corresponding to the amino acid region 340–359 of the human BCRP protein [16]. The anti-P-gp monoclonal antibody (C219) was purchased from Zymed (South San Francisco, CA, USA), and the anti-MRP1 monoclonal antibody (MRPm6) was obtained from Nichirei (Tokyo, Japan).

Cells and cell culture

K562/BCRP and K562/MDR cells were established from human leukemia K562 cells in our laboratory [22], and grown in RPMI 1640 medium supplemented with 7% fetal bovine serum at 37°C in 5% CO_2. Human epidermoid carcinoma KB-3-1 cells were cultured in DMEM supplemented with 7% fetal bovine serum at 37°C in 5% CO_2. KB/MRP1 cells were established using the following procedures: KB-3-1 cells were transfected with the pCAL-MRP1 construct bearing the human *MRP1* cDNA insert by the use of the Mammalian Transfection Kit (Stratagene, La Jolla, CA, USA). This was followed by selection with increasing concentrations of etoposide (VP-16). The cells were subcloned, and the MRP1 expression levels of each clone was confirmed by western blotting with an anti-MRP1 monoclonal antibody. The western blotting procedure used has been described previously [16]. Subclone 14 showed the highest expression of MRP1 and these cells were thus further selected with increasing concentrations of doxorubicin for 4 weeks and designated as KB/MRP1 cells.

Growth inhibition assay

The effects of flavonoids on the sensitivity of cells to various cytotoxic agents were evaluated by measuring cell growth inhibition after incubation at 37°C for 5 days in the absence or presence of various concentrations of anticancer drugs in combination with the test compounds. Cell numbers were determined with a Coulter counter. The IC_{50} values (drug dose causing 50% inhibition of cell growth) were determined from the growth inhibition curves. The RI_{50} values were then determined as the concentration of inhibitor that causes a twofold reduction of the IC_{50} in each transfectant. RI_{50}^{-1}, the reciprocal value of RI_{50}, was also used as a reverse activity measurement of drug resistance.

Topotecan uptake

The intracellular accumulations of topotecan were determined by measuring the fluorescence spectrophotometrically. K562 or K562/BCRP cells (2×10^6 cells) were suspended in 1 ml of RPMI 1640 medium containing 0.5 μM topotecan and appropriate concentrations of the compounds. The cells were incubated at 37°C for 30 min, and washed with ice-cold PBS. The intracellular topotecan was extracted from the cells with 1 ml of ethanol. The intensity of topotecan fluorescence was measured using a fluorescent spectrophotometer.

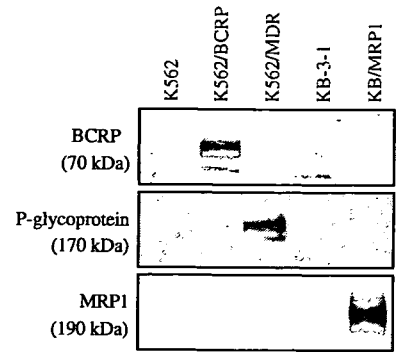


Fig. 1 Analysis of the expression levels of BCRP, P-gp, and MRP1 in stably transfected cells by western blot. Cell lysates (20 μg/lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The expression levels of BCRP, P-gp, or MRP1 were then detected by incubation of the membrane with anti-BCRP polyclonal antibody (3488), anti-P-gp monoclonal antibody (C219), or anti-MRP1 monoclonal antibody (MRPm6), respectively

Table 1 Drug resistance of each resistant cells

Resistant protein	Resistant cells	Relative resistance factor			
		SN-38	VCR	Dox	VP-16
BCRP	K562/BCRP	21	ND	ND	ND
P-gp	K562/MDR	ND	160	ND	ND
MRP1	KB/MRP	2.6	25	8.8	12

Parental or resistant cells were cultured for 5 days with increasing concentrations of the indicated drugs. Cell numbers were counted with a Coulter counter, and IC_{50} was determined. Relative resistance factor is the ratio of IC_{50} for the resistant cells divided by that for the parental cells

VCR Vincristine, *Dox* doxorubicin, ND not determined

Results

To screen the various flavonoids under analysis, we used *MDR1*, *BCRP*, and *MRP1* cDNA transfectants. The expression of the ABC transporters in each transfectant was first confirmed by western blot. The parental cells, K562 or KB-3-1, did not express any of the three ABC transporters, whereas the K562/BCRP, K562/MDR, and KB/MRP1 cells expressed exogenous BCRP, P-gp, and MRP1, respectively (Fig. 1). These transfectants did not express any of other transporters (Fig. 1). We next examined the degree of resistance to various anticancer agents in the K562/BCRP, K562/MDR, and KB/MRP1 cells, compared with the corresponding parental cells. K562/BCRP cells showed a 21-fold higher resistance to SN-38 than K562 cells (Table 1). K562/MDR cells showed 160-fold higher resistance to vincristine than K562 cells (Table 1). In addition, the KB/MRP1 cells that we established in this study showed resistance to VP-16, doxorubicin, vincristine, and SN-38 (Table 1), which is similar to the cross-resistant patterns in other MRP-expressing cells [23–25].

The structures, symbols, and compound names of the flavonoids and their related agents used in this study are shown in Fig. 2. Each compound itself showed no or only marginal growth inhibitory effect on the cells in the concentrations used in this study (data not shown). We examined the effects of these compounds on SN-38 and mitoxantrone (MXR) resistances in K562/BCRP cells (Figs. 3, 4). The results shown in Fig. 3a indicate that 3′,4′,7-trimethoxyflavone (Fig. 2; 1-a) strongly suppressed BCRP-mediated multidrug resistance. This is evident from the growth inhibition curves of the K562/BCRP cells treated with this flavonoid at low concentrations (0.03 and 0.1 μM; closed triangle and lozenge, respectively) as they are well shifted to the left compared with the untreated cells (closed circle). The growth inhibition curve of K562/BCRP cells treated with 1 μM apigenin (Fig. 2;

A

1. Flavone	5	7	3'	4'	
1-a		OCH_3	OCH_3	OCH_3	3',4',7-trimethoxyflavone
1-b	OH	OH		OCH_3	Acacetin
1-c	OH	OH	OH	OCH_3	Diosmetin
1-d	OH	OH		OH	Apigenin
1-e	OH	OH			Chrysin
1-f	OH	OH	OH	OH	Luteolin
1-g	OH	OH	OH	G_3	Luteolin-4'-O-glucoside
1-h	OH	G_1			Rhoifolin
1-i	OH	G_2	OH	OCH_3	Diosmin

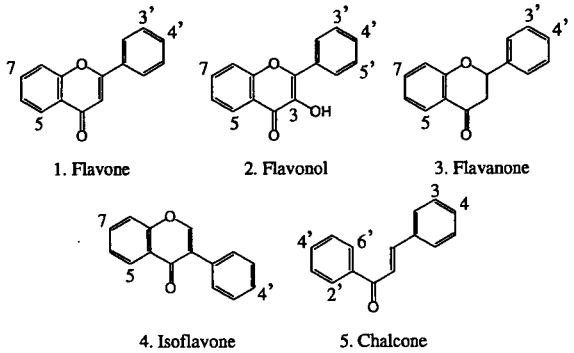
2. Flavonol	3	5	7	3'	4'	5'	
2-a		OH	OH		OCH_3		Kaempferide
2-b		OH	OH		OH		Kaempferol
2-c		OH	OH				Galangin
2-d		OH	OH	OH	OH	OH	Myricetin
2-e		OH	OH	OH	OH		Quercetin
2-f	G_3	OH	OH		OH		Kaempferol-3-O-glucoside
2-g		OH	G_1		OH		Kaempferol-7-O-nephesperidose
2-h			OH	OH	OH		Fisetin
2-i	G_4	OH	OH	OH	OH		Peltatoside
2-j		OH	OH	OCH_3	OH		Rutin

3. Flavanone	5	7	3'	4'	
3-a	OH	OH	OH	OCH_3	Hesperetin
3-b	OH	G_1		OH	Naringenin-7-O-glucoside
3-c	OH	OH		OH	Naringenin
3-d	OH	OH	OH	OH	Eriodictyol

4. Isoflavone	5	7	4'	
4-a	OH	OH	OH	Genistein
4-b		OH	OH	Daidzein

5. Chalcone	3	4	2'	4'	6'	
5-a	OCH_3	OCH_3				3,4-dimethoxychalcone
5-b		OH	OH	OH	OH	Phloretin

B



C

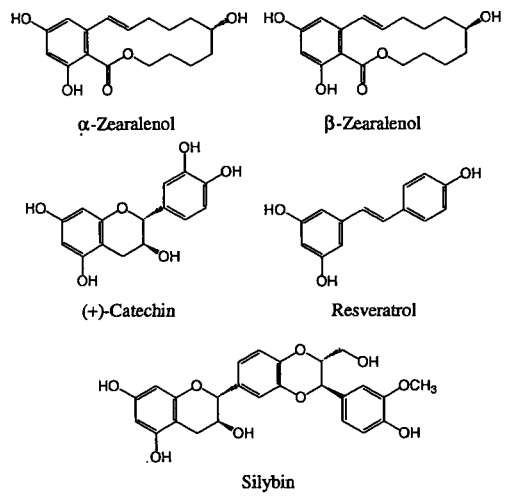


Fig. 2 Chemical structures of the flavonoids tested in the present study. **a** The structures of the flavones (9 compounds), flavonols (10 compounds), flavanones (4 compounds), isoflavones (2 compounds), and chalcones (2 compounds) screened in the present study. G_1, *O*-neohesperidoside; G_2, *O*-rutinoside; G_3, *O*-glucoside; G_4, *O*-arabinoglucoside. **b** Core structures of the five groups of compounds screened in the present study. **c** The flavonoid-related agents tested in the present study

1-d) (closed upward triangle) was also found to have shifted to the left compared with cells treated with 0.3 μM apigenin (closed downward triangle) or untreated (closed circle). This indicated that apigenin inhibits BCRP-mediated resistance to SN-38 and MXR (Fig. 3b). In contrast, diosmin (Fig. 2; 1-i) did not suppress BCRP-mediated drug resistance at any concentration (Fig. 3c). The reversal indices (RI_{50}) for SN-38 of 3',4',7-trimethoxyflavone, apigenin, and diosmin were measured as 0.012, 0.39, and >3 μM, respectively, and those for MXR were measured as 0.044, 0.62, and >3 μM, respectively. Other compounds were also examined using identical analyses, and the RI_{50} values for SN-38 were obtained from each growth inhibition curve. The RI_{50}^{-1} of the total panel of 32 compounds that we screened in this study are presented in Fig. 4, which shows that 20 of these 32 compounds can reverse BCRP-mediated SN-38 resistance. The lack of any reversal properties of the remaining 12 compounds was confirmed by treatments at the highest concentrations used in these experiments (data not shown). We also examined the reversal effects of a well-known BCRP inhibitor, FTC, as a positive control on BCRP-mediated drug resistances (Fig. 3d). As expected, FTC suppressed these resistances at the concentrations of 0.3 and 1 μM. The RI_{50} of FTC for SN-38 and that for MXR were measured as 0.24 and 0.23 μM, respectively.

We then examined the effects of the compounds on the BCRP-mediated efflux of topotecan. The intracellular topotecan in K562/BCRP cells was threefold lower than that in K562 cells (Fig. 5). 3',4',7-Trimethoxyflavone

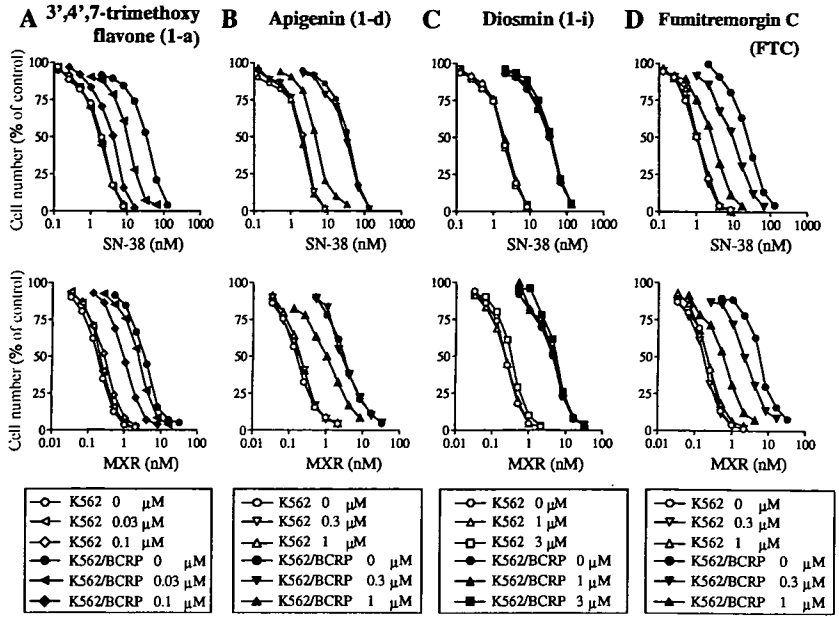


Fig. 3 Reversal effects of flavonoids on BCRP-mediated anticancer drug resistance. K562 (*open symbols*) and K562/BCRP (*closed symbols*) cells were cultured for 5 days in the absence (*circle*) or presence of 0.03 μM (*leftward triangle*), 0.1 μM (*lozenge*), 0.3 μM (*downward triangle*), 1 μM (*upward triangle*), or 3 μM (*square*) of the indicated compounds under increasing concentrations of SN-38. Cell numbers were determined with using a Coulter counter. **a** Effects of 3',4',7-trimethoxyflavone (*1-a*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **b** Effects of Apigenin (*1-d*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **c** Effects of Diosmin (*1-i*) on the sensitivity to SN-38 in K562 and K562/BCRP cells. **d** Effects of Fumitremorgin C on the sensitivity to SN-38 in K562 and K562/BCRP cells. Data points are the measurements of the mean ± SD from triplicate determinations

(1-a) at 1 μM increased the intracellular topotecan in K562/BCRP cells to a similar level as that in K562 cells (Fig. 5). Apigenin (1-d) at 10 μM also increased the intracellular accumulation of topotecan in K562/BCRP cells (Fig. 5). Diosmin (1-i) treatment did not alter the intracellular levels of topotecan (Fig. 5). These results clearly indicate that 3',4',7-trimethoxyflavone (1-a) and apigenin (1-d) increase the intracellular concentrations of BCRP substrate anticancer agents, but diosmin (1-i) does not.

We next examined whether any of the compounds showing reversal effects against BCRP-mediated drug resistance showed any cross-reactivity against other ABC transporter-mediated drug resistance pathways. As shown in Fig. 6a and b (left panels), 3',4',7-trimethoxyflavone (1-a) weakly suppresses P-gp- but not MRP1-mediated drug resistance. In addition, apigenin (1-d) and diosmin (1-i) do not reverse either P-gp- or MRP1-mediated drug resistance (Fig. 6a, b, middle and right panels). Among the representative 11 compounds that we chose to analyze in this experiment from the 32 compound panel, only two (1-a and -b) in fact inhibited P-gp-mediated drug resistance and none suppressed MRP1-mediated drug resistance (Table 2). These compounds also did not show growth inhibitory effects against K562 and KB-3-1 cells at the highest concentrations used in these experiments (data not shown). These data indicate that many flavonoids are select inhibitors of BCRP only.

Discussion

Estrogens such as estrone and 17β-estradiol have been found to contain inhibitory activity against the BCRP-mediated multidrug resistance pathways [20]. In addition, sulfated estrogens are found to be physiological substrates of BCRP, suggesting that they compete with anticancer agents on efflux from cells [21]. Synthesized estrogen antagonists and agonists have also been demonstrated to reverse drug resistance and have structural similarities that can directly inhibit BCRP function and/or reduce its expression levels [26]. It is noteworthy also that some flavonoids have structures that somewhat resemble the estrogens and display weak estrogenic activity [27]. Significantly, we and others have now shown that flavonoids possess anti-BCRP activity [22, 28–31].

Some flavonoids have been reported to interact with and competitively inhibit ABC transporters, including P-gp, MRP1, MRP2, and cystic fibrosis transmembrane conductance regulator [32–37]. In this regard,

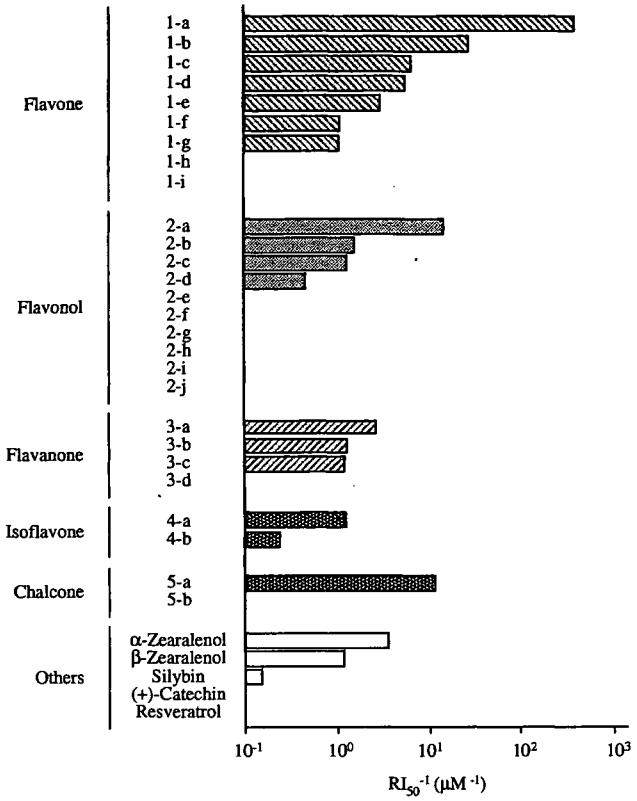


Fig. 4 Inhibitory effects of flavonoids on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of the appropriate concentrations of flavonoids with increasing concentrations of SN-38. Cell numbers were determined with a Coulter counter. RI_{50} values were obtained graphically from the IC_{50} values of the K562 and K562/BCRP cells as described in Materials and methods. RI_{50}^{-1}, the reciprocal value of RI_{50}, was used to show the extent of drug resistance reversal of the compounds. These data are averaged from triplicate experiments

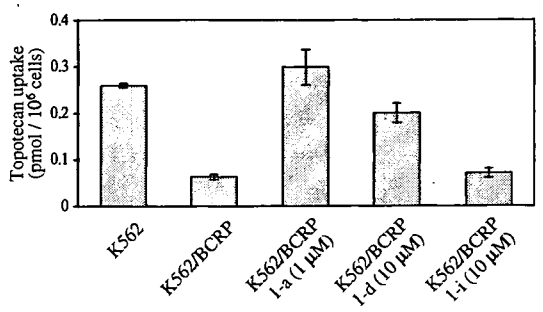


Fig. 5 Effect of flavonoids on topotecan uptake in K562/BCRP cells. K562 and K562/BCRP cells were incubated with 0.5 μM topotecan in the absence or presence of flavonoids at indicated concentrations at 37°C for 30 min. The intracellular topotecan was extracted from the cells with 1 ml of ethanol. The fluorescence intensity of topotecan was measured using a fluorescence spectrophotometer. Each *vertical bar* represents the mean ± SD of the measurements from triplicate determinations

genistein, naringenin, acacetin, kaempferol, quercetin, and flavopiridol have shown reversal effects against BCRP-mediated drug resistance in the previous studies from our laboratory and from other groups [22, 28, 38]. We therefore set out to screen additional flavonoids that may also reverse the effects of BCRP-mediated multidrug resistance in our present study. Of the 32 compounds that we tested, including the above flavonoids except flavopiridol, 20 compounds showed reversal effect for the resistance (Fig. 4). Of interest is that 3′,4′,7-trimethoxyflavone (1-a) has stronger inhibitory activity against BCRP than acacetin (1-b) that has been the strongest BCRP inhibitor in the flavonoids demonstrated in the previous study (Fig. 4). Almost all of the flavones, including 3′,4′,7-trimethoxyflavone, acacetin, diosmin, apigenin, chrysin, luteolin, luteolin-4′-*O*-glucoside, show BCRP-reversing activity (Figs. 3, 4). Moreover, many of the flavones, isoflavones, and chalcones tested in this study also reverse BCRP-mediated drug resistance, but 50% of the flavonols examined had no impact upon BCRP (Fig. 4).

From our present results, we propose a structure–activity relationship for BCRP inhibition by flavonoids (Fig. 7). We postulate that: (a) The double bond between position 2 and 3 of the C-ring is associated with high inhibitory activity against BCRP (Fig. 7a). As an example of this, apigenin (1-b) shows stronger suppressive activity toward BCRP than naringenin (3-c), and luteolin (1-f) is also more potent in this regard than eriodictyol (3-d) (Figs. 2, 4). (b) The 4′-*O*-methoxylation of the B-ring or the 4′-hydroxylation of the B-ring is also associated with more potent BCRP inhibition (Fig. 7b, c). In the former instance, hesperetin (3-a) has stronger BCRP inhibitory activity than eriodictyol (3-d), diosmetin (1-c) is more strongly inhibitory than luteolin (1-f), acacetin (1-b) is more potent than apigenin (1-d), and kaempferide (2-a) is a better inhibitor of BCRP than kaempferol (2-b) (Figs. 2, 4). In the case of 4′-hydroxylation of the B-ring, apigenin (1-d) showed slightly stronger BCRP suppression than chrysin (1-e), and kaempferol (2-b) is marginally more potent than galangin (2-c) (Figs. 2, 4). (c) The 3-hydroxylation of the C-ring or 3′-hydroxylation of the B-ring reduce BCRP inhibitory activity (Fig. 7d, e). In the former case, we mentioned our present results with galangin (2-c) compared with chrysin (1-e), kaempferol (2-b) compared with apigenin (1-d), kaempferide (2-a) compared with acacetin (1-b), and quercetin (2-e) compared with luteolin (1-f) (Figs. 2, 4). In the latter instance, our available examples include, luteolin (1-f) versus apigenin (1-d), eriodictyol (3-d) versus naringenin (3-c), and quercetin (2-e) versus kaempferol (2-b) (Figs. 2, 4). It is noteworthy also that in our present study, some glycosilated flavonoids showed anti-BCRP

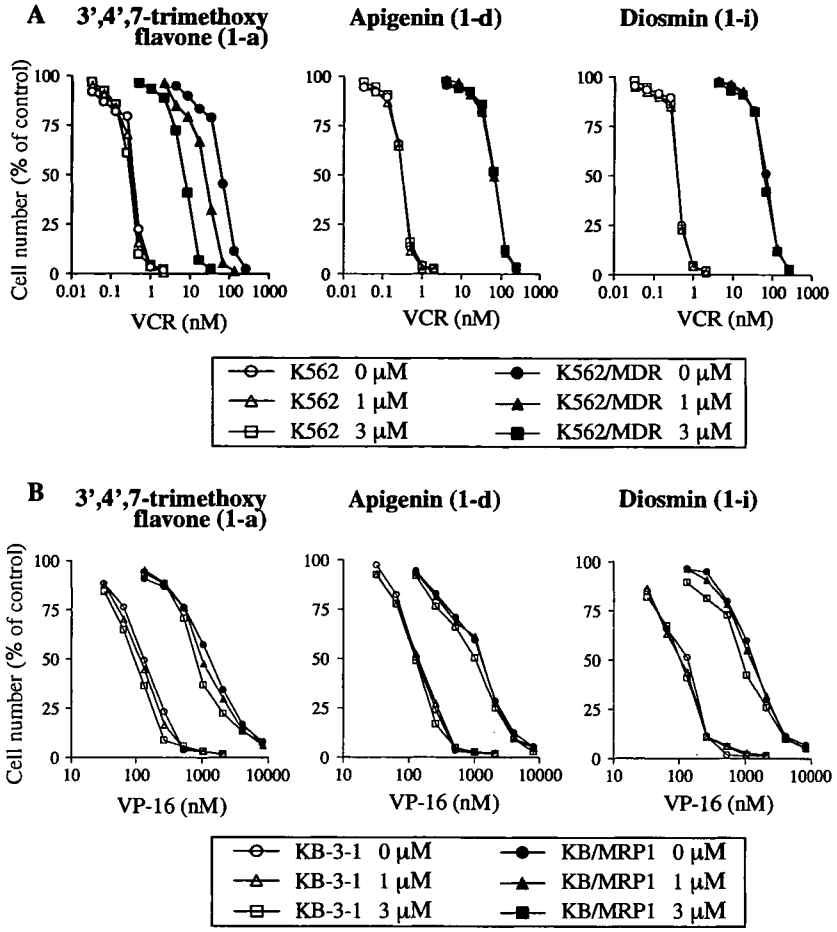


Fig. 6 Reversal effects of flavonoids on P-gp- or MRP1-mediated anticancer drug resistance. Parental cells (*open symbols*) and transfected cells (*closed symbols*) were cultured for 5 days in the absence (*circle*) or presence of 1 μM (*triangle*), or 3 μM (*square*) of the specific compounds indicated under increasing concentrations of anticancer agents. Cell numbers were determined using a Coulter counter. **a** Effects of flavonoid treatment upon the sensitivity to vincristine (VCR) in K562 and K562/MDR cells. **b** Effects of flavonoid treatment on the sensitivity to VP-16 in KB-3-1 and KB/MRP1 cells. Data points are measurements of the mean ± SD from triplicate determinations

Table 2 Reversal of BCRP-, P-gp-, and/or MRP1-mediated anticancer drug resistance by flavonoids

	RI_{50} (μM)		
	BCRP	P-gp	MRP1
1. Flavone			
1-a	0.012	0.68	>3
1-b	0.11	1.5	>3
1-d	0.39	>3	>3
1-e	0.67	>3	>3
1-f	1.5	>3	>3
1-h	>3	>3	>3
1-i	>3	>3	>3
2. Flavonol			
2-a	0.17	>3	>3
2-b	1.1	>3	>3
2-g	>3	>3	>3
2-h	>3	>3	>3

Parental or resistant cells were cultured for 5 days with increasing concentrations of anticancer drugs together with or without flavonoids. Cell numbers were counted with a Coulter counter, and RI_{50} was determined

activity. Glycosilated flavonoids may be useful for clinical practice because they are soluble in water and we would predict that both they and/or water-soluble derivatives of flavonoids will be developed as BCRP inhibitors in the future.

Flavonoids are safe nutrients, being the most abundant polyphenolic compounds present in the human diet in fruits, vegetables, and plant-derived beverages. It has been reported that a human adult normally assimilates 200–300 mg of flavonoids per day in their diet [39]. For example, 100 g of soybean contains 100–200 mg of isoflavones comprising genistein, daidzein, glycitein, and their corresponding glycosides. In the case of an intake of 50 mg of genistein, the peak plasma concentration of this compound was reported to reach a level of approximately 1 μM in healthy premenopausal women [40]. Therefore, flavonoids contained in foods can be considered to have a positive effect on the pharmacokinetics of anticancer agents. Hence, dietary controls will be necessary for patients undergoing cancer chemotherapy and it will also be important that the peak plasma concentrations of anticancer agents are continually monitored in these individuals.

In conclusion, we have shown that flavonoids selectively reverse BCRP-mediated drug resistance, that these compounds may be useful as BCRP inhibitors,

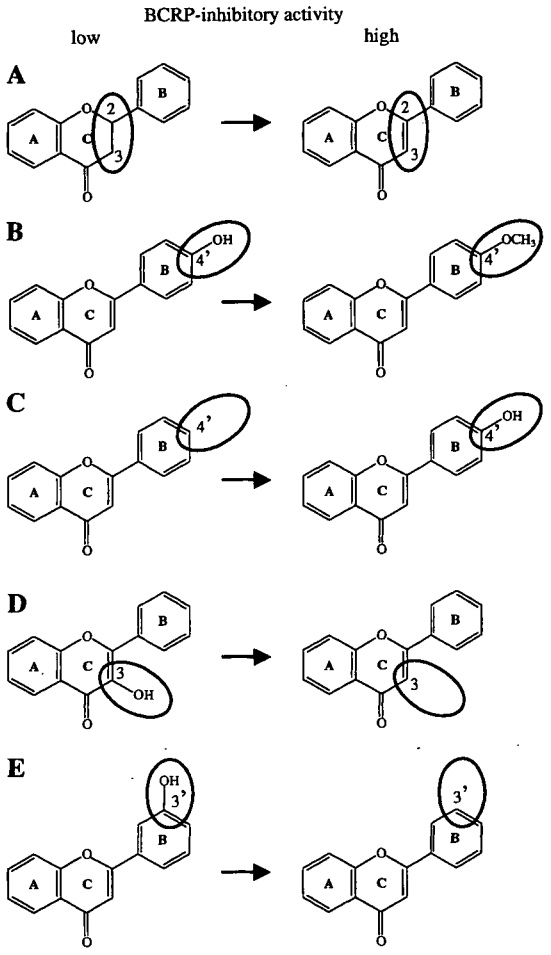


Fig. 7 Structure–activity relationship of BCRP inhibition by flavonoids

and that they are likely to bring clinical benefits via more effective and safer cancer chemotherapy treatments.

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Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan

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Background: To compare the efficacy and toxicity of three platinum-based combination regimens against cisplatin plus irinotecan (IP) in patients with untreated advanced non-small-cell lung cancer (NSCLC) by a non-inferiority design.
Patients and methods: A total of 602 patients were randomly assigned to one of four regimens: cisplatin 80 mg/m^2 on day 1 plus irinotecan 60 mg/m^2 on days 1, 8, 15 every 4 weeks (IP) carboplatin AUC 6.0 min × mg/mL (area under the concentration–time curve) on day 1 plus paclitaxel 200 mg/m^2 on day 1 every 3 weeks (TC); cisplatin 80 mg/m^2 on day 1 plus gemcitabine 1000 mg/m^2 on days 1, 8 every 3 weeks (GP); and cisplatin 80 mg/m^2 on day 1 plus vinorelbine 25 mg/m^2 on days 1, 8 every 3 weeks (NP).
Results: The response rate, median survival time, and 1-year survival rate were 31.0%, 13.9 months, 59.2%, respectively, in IP; 32.4%, 12.3 months, 51.0% in TC; 30.1%, 14.0 months, 59.6% in GP; and 33.1%, 11.4 months, 48.3% in NP. No statistically significant differences were found in response rate or overall survival, but the non-inferiority of none of the experimental regimens could be confirmed. All the four regimens were well tolerated.
Conclusion: The four regimens have similar efficacy and different toxicity profiles, and they can be used to treat advanced NSCLC patients.
Key words: carboplatin, cisplatin, gemcitabine, irinotecan, non-small-cell lung cancer, paclitaxel, randomized phase III study, vinorelbine

introduction

Nearly 60 000 patients in Japan died of lung cancer in 2004, and the mortality rate is still increasing [1]. Even old-generation cisplatin-based chemotherapy provides a survival benefit and symptom relief in patients with inoperable non-small-cell lung cancer (NSCLC) [2]. Several anticancer agents including irinotecan, paclitaxel, docetaxel, gemcitabine, and vinorelbine, were developed in the 1990s and most of them have mechanisms of action that differ from those of the old-generation agents [3–7]. The combinations of platinum and these new agents developed in the 1990s are more useful against advanced NSCLC than old-generation combination chemotherapy, and doublets of platinum and new-generation anticancer agents are considered standard chemotherapy regimens for advanced NSCLC, although no consistent standard regimens have yet been established [8–17].

Two phase III studies comparing cisplatin plus irinotecan (IP) with cisplatin plus vindesine for advanced NSCLC have been conducted in Japan [18, 19]. Fukuoka et al. [20] reported the results of a combined analysis of the 358 eligible stage IV patients in these studies. They carried out a multivariate analysis using the Cox regression model with adjustment for well-known prognostic factors, and the Cox regression analysis demonstrated that treatment with IP was one of significant independent favorable factor. Based on their data, we selected IP for the reference arm in our study.

The Ministry of Health, Labour and Welfare of Japan approved the prescription of paclitaxel, gemcitabine, and

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vinorelbine for NSCLC in 1999 and requested a phase III study to confirm the efficacy and safety of these agents. The Japanese investigators and the pharmaceutical companies decided to conduct a four-arm randomized phase III study for NSCLC, the so-called FACS, Four-Arm Cooperative Study. The purpose of the study was to compare the efficacy and toxicity of three platinum-based combination regimens, carboplatin plus paclitaxel (TC), cisplatin plus gemcitabine (GP), cisplatin plus vinorelbine (NP), with IP as the reference arm.

patients and methods

patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for participation in the study. Each patient had to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as malignant pleural effusion, pleural dissemination, malignant pericardiac effusion, or metastatic lesion in the same lobe), at least one target lesion >2 cm, no prior chemotherapy, no prior surgery and/or radiotherapy for the primary site, age 20–74 years, Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, adequate hematological, hepatic and renal functions, partial pressure of arterial oxygen (paO_2) ≥60 torr, expected survival >3 months, able to undergo first course treatment in an inpatient setting, and written informed consent. The study was approved by the Institutional Review Board at each hospital. Written informed consent was obtained from every patient.

treatment schedule

All patients were randomly assigned to one of the four treatment groups by the central registration office by means of the minimization method. Stage, PS, gender, lactate dehydrogenase (LDH) and albumin values, and institution were used as adjustment variables. The first group received the reference treatment, 80 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan on days 1, 8, and 15, and the cycle was repeated every 4 weeks. The second group received 200 mg/m^2 of paclitaxel (Bristol-Myers K.K., Tokyo, Japan) over a 3-h period followed by carboplatin at a dose calculated to produce an area under the concentration–time curve of 6.0 min × mg/mL on day 1 and the cycle was repeated every 3 weeks. The third group received 80 mg/m^2 of cisplatin on day 1 and 1000 mg/m^2 of gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. The fourth group received 80 mg/m^2 of cisplatin on day 1 and 25 mg/m^2 of vinorelbine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. Each treatment was repeated for three or more cycles unless the patient met the criteria for progressive disease or experienced unacceptable toxicity.

response and toxicity evaluation

Response was evaluated according to the Response Evaluation Criteria in Solid Tumors, and tumor markers were excluded from the criteria [21]. Objective tumor response in all responding patients was evaluated by an external review committee with no information on the treatment group. Toxicity grading criteria in National Cancer Institute Common Toxicity Criteria Ver 2.0 were used to evaluate toxicity.

quality of life assessment

Quality of life (QoL) was evaluated by means of the Functional Assessment of Cancer Therapy—Lung (FACT-L) Japanese version and the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QoL-ACD), before treatment, immediately before the second cycles of chemotherapy, and 3 and 6 months after the start of treatment [22–24].

statistical analysis and monitoring

The primary end point of this study was overall survival (OS), and the secondary end points were response rate, response duration, time to progressive disease (TTP), time to treatment failure (TTTF), adverse event, and QoL. The 1-year survival rate of the control group in this study was estimated to be 43% based on the data in published papers, and the 1-year survival rate in the other treatment group was expected to be 50%. The lower equivalence limit for 1-year survival rate was set as '−10%'. The criterion for the non-inferiority of each treatment was a lower limit of the two-sided 95% confidence interval (CI) of the 1-year survival rate of treatment minus that of control larger than the lower equivalence limit. Because the non-inferiority of each treatment versus the control was to be evaluated independently, a separate null hypothesis was stated for each treatment, and for that reason no multiple comparison adjustment was included in the study. Based on the above conditions and binomial distribution, 135 patients were needed per arm for a one-sided Type I error of 2.5% and 80.0% power. In view of the possibility of variance inflation due to censoring, the sample size was set at 600 (150 per arm).

Central registration with randomization, monitoring, data collection, and the statistical analyses were independently carried out by a contract research organization (EPS Co., Ltd, Tokyo, Japan).

results

patient characteristics

From October 2000 to June 2002, a total of 602 patients were registered by 44 hospitals in Japan. All patients had been followed up for >2 years, and 447 patients had died as of June 2004. Of the 602 patients registered, 151 were allocated to the reference treatment, IP, and 150, 151, and 150 patients were allocated to TC, GP, and NP, respectively. Since 10 patients did not receive chemotherapy and 11 patients were subsequently found to be ineligible, 592 patients were assessable for toxicity and 581 patients were assessable for efficacy. Four patients did not receive chemotherapy due to electrolytic disorder, fever, symptomatic brain metastases, and rapid tumor progression in IP, two patients due to refusal and pneumonia in TC, four patients due to lower WBC counts (two patients), rapid tumor progression, and nephritic syndrome in NP. Two patents were ineligible due to wrong stage in IP, two patients were wrong stage and one patient had double cancer in TC, two patients were wrong diagnosis, one patient had massive pleural effusion, one patient received prior chemotherapy in GP, one patient had no target lesions in NP. Age, gender, PS, stage, and LDH and albumin values were well balanced in each arm (Table 1). Fewer patients with adenocarcinoma and more patients with squamous cell carcinona were, however, entered in three experimental arms than in IP.

objective tumor response and response duration

Objective tumor response is shown in Table 2. Forty-five partial responses occurred in the 145 assessable patients in the reference arm, IP, for an objective response rate of 31.0% with a median response duration of 4.8 months. The response rate and median response duration were 32.4% and 4.0 months in TC, 30.1% and 3.5 months in GP, and 33.1% and 3.4 months in NP. The response rates in TC, GP, and NP were not statistically different from the rate in IP according to the results of the χ^2 test.

Table 1. Patient characteristics and treatment delivery

	Cisplatin + irinotecan	Carboplatin + paclitaxel	Cisplatin + gemcitabine	Cisplatin + vinorelbine
Assessable patients	145	145	146	145
Gender (male/female)	97/48	99/46	101/45	101/44
Age, median (range)	62 (30–74)	63 (33–74)	61 (34–74)	61 (28–74)
PS (0/1)	44/101	44/101	45/101	45/100
Histology				
Adenocarcinoma	121	104	108	109
Squamous cell carcinoma	16	31	29	29
Others	8	10	9	7
Stage (IIIB/IV)	31/114	28/117	30/116	26/119
No. of cycles				
Mean ± SD	3.0 ± 1.3	3.5 ± 1.5	3.2 ± 1.2	3.1 ± 1.3
Median	3	3	3	3
Range	1–7	1–10	1–7	1–8

PS, performance status; SD, standard deviation.

Table 2. Survival, TTP, TTTF, response rate, and response duration

	N	Median survival, months	1-year survival (%)	Difference in 1-year survival from IP	2-year survival (%)	TTP (median), months	TTTF (median), months	Response rate (%)	Response duration (median), months
Cisplatin + irinotecan	145	13.9	59.2	–	26.5	4.7	3.3	31.0	4.8 (n = 45)
Carboplatin + paclitaxel	145	12.3	51.0	−8.2% (95% CI −19.6% to 3.3%)	25.5	4.5 (P = 0.355)[a]	3.2 (P = 0.282)[a]	32.4 (P = 0.801)[b]	4.0 (n = 47)
Cisplatin + gemcitabine	146	14.0	59.6	0.4% (95% CI −10.9% to11.7%)	31.5	4.0 (P = 0.170)[a]	3.2 (P = 0.567)[a]	30.1 (P = 0.868)[b]	3.5 (n = 44)
Cisplatin + vinorelbine	145	11.4	48.3	−10.9% (95% CI −22.3% to 0.5%)	21.4	4.1 (P = 0.133)[a]	3.0 (P = 0.091)[a]	33.1 (P = 0.706)[b]	3.4 (n = 48)

[a]Compared with IP by the generalized Wilcoxon test.

[b]Compared with IP by the χ^2 test.

CI, confidence interval; IP, cisplatin plus irinotecan; TTP, time to progressive disease; TTTF, time to treatment failure.

OS, TTP disease, and TTTF

OS and TTP are shown in Figure 1. Median survival time (MST), the 1-year, and 2-year survival rate in IP were 13.9 months, 59.2%, and 26.5%, respectively. The MSTs, 1-year, and 2-year survival rates were, respectively, 12.3 months, 51.0%, and 25.5% in TC; 14.0 months, 59.6%, and 31.5% in GP; and 11.4 months, 48.3%, and 21.4% in NP. The lower limits of the 95% CI of the difference in 1-year survival rate between IP and TC (−19.6%), GP (−10.9%), and NP (−22.3%) were below −10%, which was considered the lower equivalence limit (Table 2). Thus, the results did not show non-inferiority in three experimental regimens compared with reference treatment. Median TTP and median TTTF were 4.7 and 3.3 months, respectively in IP. Median TTP and TTTF were, respectively, 4.5 and 3.2 months in TC, 4.0 and 3.2 months in GP, and 4.1 and 3.0 months in NP. There were no statistical differences in either TTP or TTTF in TC, GP, or NP, compared with IP according to the results of the generalized Wilcoxon test (Table 2).

hematologic and non-hematologic toxicity

In IP, 47.6% and 83.7% of patients developed grade 3 or worse leukopenia and neutropenia, respectively (Table 3). The incidences of grade 3 or worse leukopenia (33.1%, P = 0.010) and neutropenia (62.9%, P < 0.001) were significantly lower in GP than in IP. The incidence of grade 3 or worse leukopenia (67.1%, P < 0.001) was significantly higher in NP than in IP. Grade 3 or worse thrombocytopenia developed in 5.4% of the patients in IP, and the incidence was significantly higher in GP (35.1%, P < 0.001). The incidence of febril neutropenia in IP was 14.3%, and was significantly lower in GP (2.0%, P < 0.001).

Grade 2 or worse nausea, vomiting, anorexia, and fatigue occurred in 60.5%, 51.0%, 65.3%, and 38.8%, respectively, of the patients in IP. The incidences of grade 2 or worse nausea (TC: 25.0%, P < 0.001, NP: 47.3%, P = 0.022), vomiting (TC: 22.3%, P < 0.001, NP: 36.3%, P = 0.011), and anorexia (TC: 32.4%, P < 0.001, NP: 49.3%, P = 0.005) were significantly lower in TC and NP than in IP. Grade 2 or worse diarrhea was

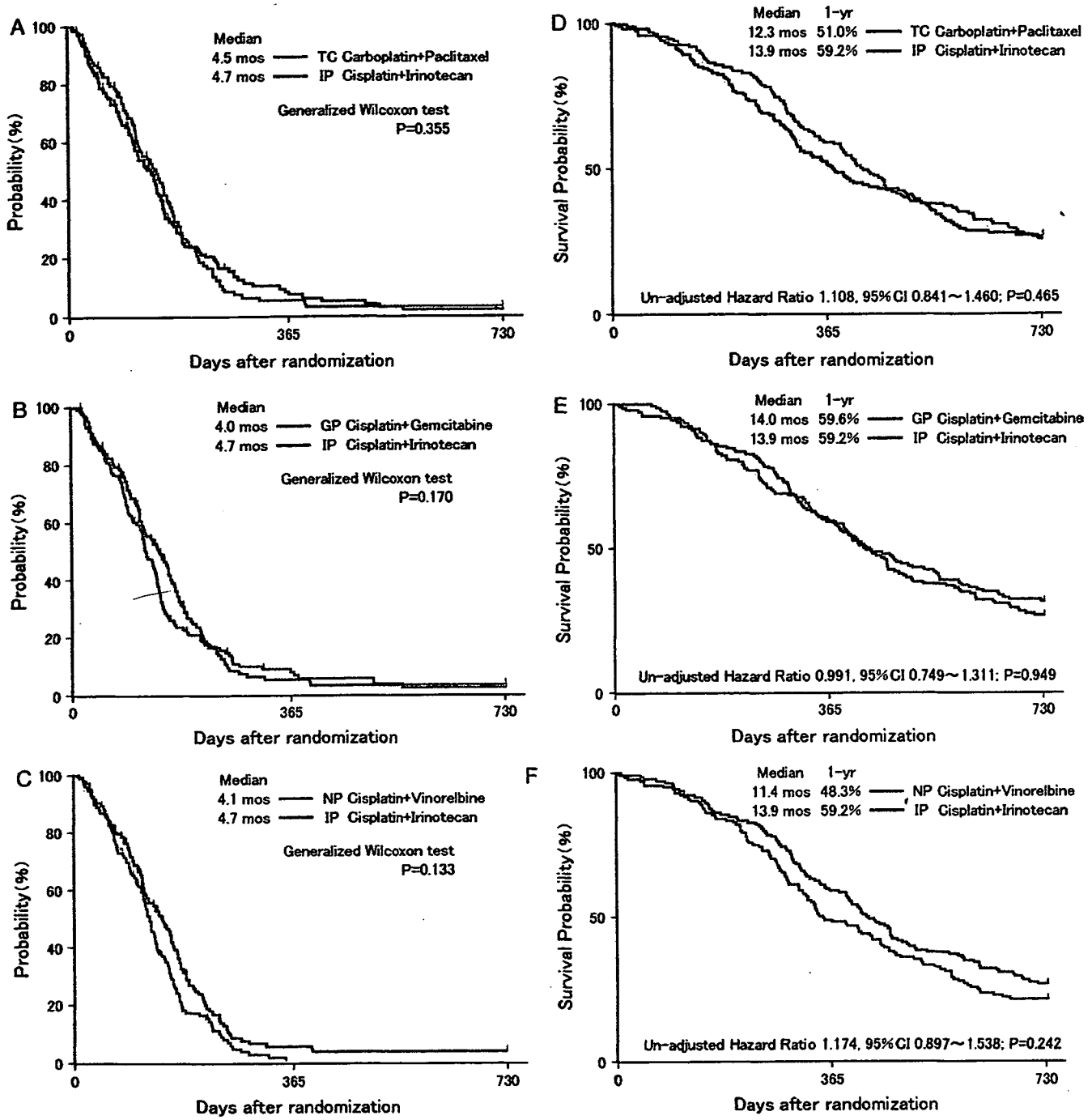

Figure 1. Overall survival (OS) and time to progressive (TTP) disease. TTP and OS in the carboplatin plus paclitaxel (TC) (A, D), cisplatin plus gemcitabine (GP) (B, E), and cisplatin plus vinorelbine (NP) (C, F) were not statistically significantly different from the values in the cisplatin plus irinotecan.

significantly less frequent in TC (6.8%), GP (8.6%), and NP (11.6%) than in IP (48.3%, $P < 0.001$). The incidences of grade 2 or worse sensory neuropathy (16.9%, $P < 0.001$), arthralgia (21.6%, $P < 0.001$), and myalgia (17.6%, $P < 0.001$) were significantly higher in TC than in IP. Grade 2 alopecia occurred in 30.6% of the patients in IP, and its incidence was significantly higher in TC (44.6%, $P = 0.013$) and significantly lower in GP (15.2%, $P = 0.001$) and NP (8.9%, $P < 0.001$). Grade 2 injection site reactions were more frequent in NP (26.7%) than in IP (4.8%, $P < 0.001$).

A total of five patients died of treatment-related toxicity: three in IP (cerebral hemorrhage, interstitial pneumonia, acute circulatory failure/disseminated intravascular coagulation: 2.0%), one in TC (acute renal failure: 0.7%), and one in NP (pulmonary embolism: 0.7%).

second-line treatment

Data on second-line treatment, but not third-line or later treatment, was available in this study, and they showed that