Abstract. A preliminary exploration of coumarin derivatives as novel multidrug resistance (MDR) modulators was carried out to determine the basic features of the structure responsible for the MDR reversal activity. Among 44 coumarins, 14 compounds moderately induced reversal of MDR (fluorescence activity ratio (FAR) values >1). The most active compound, 6-hydroxy-3-(2-hydroxyethyl)-4-methyl-7-methoxycoumarin [C34], was equally potent as a MDR modulator verapamil. These data show a relationship between the chemical structure and MDR-reversal effect on tumor cells. All coumarins tested were more cytotoxic against tumor cells than normal cells. Several compounds displayed potent cytotoxic activities (CC50 15-29 µg/mL in HSC cells), comparable with that of gallic acid (CC50=24 µg/mL). Both 6-hydroxy-7-methoxy-4-methyl-3-isopropylcoumarin [C43] and 3-ethyl-6-hydroxy-7-methoxy-4-methylcoumarin [C44] showed the highest tumor-specific cytotoxicity (SI value=4.1 and 3.6, respectively). We conclude that coumarins are potentially potent new MDR modulators with low toxicity against normal cells. A deeper understanding of the relationship between their structures and their potency will contribute to the design of optimal agents.

Although recent developments in molecular cancer chemotherapeutics has been successful and encouraging, effectiveness has often been limited by cytotoxic effects on normal tissues and by drug resistance of tumors. Our studies have focused on molecular cancer chemotherapeutics, mainly in the areas of drug resistance and tumor-specific cytotoxic drugs.

Multidrug resistance (MDR) of human tumors is one of the major reasons for the failure of chemotherapy in refractory cancer patients (1). Reversal of MDR has been accomplished by a number of agents such as verapamil and cyclosporin A (2, 3). Unfortunately, the concentration of many of these agents necessary to reverse drug resistance is difficult to achieve in vivo (4). Thus, there is considerable interest in the search for new P-glycoprotein (P-gp) inhibitors that do not show significant toxicity at doses required for P-gp inhibition.

Coumarins constitute a major class of widely distributed O-heterocyclic natural products isolated from citrus fruits and vegetables (5). Naturally occurring coumarins possess a variety of biological activities, including antitumor activity (6). In contrast, little information is available on the MDR-modulating potency of simple coumarins, which are considered to exert low mammalian toxicity. Due to the presence of coumarins in the human diet and medicinal plants, a study on the MDR modulatory effects of coumarins is of significance (7). Three coumarin derivatives, 6-methylcoumarin, 7-methylcoumarin and ethyl 3-coumarin carboxylate, were not effective on the MDR efflux pump P-gp of mouse lymphoma cells in vitro (8). 7-Hydroxycoumarin and 8-nitro-7-hydroxycoumarin were shown to be potent cytotoxic agents against the human renal cell carcinoma cell line. However, these compounds were not a substrate for P-gp-mediated MDR (9, 10). Pyranocoumarin derivatives, racemic cis-3’-angeloyl-4’-acetoxy-khellactone,
were suggested to possess the ability to circumvent the MDR phenotype conferred by the overexpression of P-gp (11). In drug combination studies, the pyranoocoumarins increased intracellular doxorubicin accumulation in the P-gp-overexpressing human oral epidermoid carcinoma cell line KB-V1, but not in the drug-sensitive human oral epidermoid carcinoma cell line KB-3-1.

In our previous paper, coumarin derivatives [C1-23] were screened for their cytotoxic activity against human tumor cells and some compounds were found to exhibit potent cytotoxic activity (12). These studies led to the identification of a 6,7-dihydroxycoumarin derivative as a lead molecule with tumor cell-specific cytotoxicity. It is also suggested that proper substitution at the 3 and/or 4 positions of the molecule makes it possible to design more cytotoxic agents. In a continuing search for potent and selective cytotoxic agents, we prepared another 22 coumarin derivatives [C24-45] and evaluated their cytotoxic effects against human oral tumor cells. In the present work, we also investigated the MDR reversal activities of 45 coumarins [C1-45] against mouse lymphoma cells transfected with the human MDR1 gene.

Materials and Methods

General. Melting points were determined by an Electrothermal or a Büchi B-545 instrument. 1H and 13C-NMR spectra were determined using a Bruker AC 200 or Avance 200 spectrometer (200 MHz for 1H and 50.3 MHz for 13C both) in DMSO-d6. The chemical shifts refer to TMS (1H-NMR) or to DMSO-d6 (13C-NMR, ‰=39.5 ppm), respectively. Combustion analyses were carried out on "Mikroanalytisches Labor Hein", Moembris, Germany. TLC (Thin layer chromatography) was performed using Merck Kieselgel 60 F254 (Merck 5549, USA).

Preparation of coumarins [C24, 29-31, 33-44]. The following coumarins were prepared by the Pechmann reaction (14), unless otherwise noted.

One equivalent of the (2-substituted) β-ketoester was added dropwise to a solution of the appropriate phenol in sulfuric acid (75% in water) at 0 to 25°C. The mixture was kept at room temperature for at least 30 min and was then placed on ice or diluted with ice/water. The product was filtered off and recrystallized.

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7,8-Dihydroxy-3-(2-hydroxyethyl)-4-methyl-2H-1-benzopyran-2-one [C35]: starting material 2-acetyl-Á-butyrolactone; yield 66%; mp. 221-222°C (ethanol/water)(18).

7-Hydroxy-3-(2-hydroxyethyl)-6-methoxy-4-methyl-2H-1-benzopyran-2-one [C36]: starting material 2-acetyl-Á-butyrolactone; yield 70%; mp. 225-227°C (dec.)(2-propanol): 1H NMR (DMSO-d6, 200 MHz) δ: 2.40 (s, 3H), 2.72 (t, 2H, J=6.9 Hz), 3.50 (td, 2H, J=6.7, 5.9 Hz), 3.86 (s, 3H), 4.06 (t, 1H, J=5.5 Hz), 6.74 (s, 1H), 7.13 (s, 1H), 10.12 (s, 1H). 13C NMR (DMSO-d6, 50.3 MHz) δ: 15.2 (CH2), 30.9 (CH3), 55.2 (CH), 59.4 (CH2), 102.5 (CH), 107.1 (CH), 111.9 (C), 118.6 (C), 141.5 (C), 147.2 (C), 150.0 (C), 161.3 (C). Anal. Calculated for C13H12O6: C, 59.09; H, 4.58. Found: C, 58.52; H, 4.72.

5,7-Dihydroxy-3-(2-hydroxyethyl)-4-methyl-2H-1-benzopyran-2-one [C37]: starting material 2-acetyl-Á-butyrolactone; yield 89%; mp. 243-245°C (ethanol/water)(17).

6-(Hydroxy-7-methoxy-4-propyl-2H-1-benzopyran-2-one [C40]: yield 68%; mp. 165-164°C (2-propanol/ethanol): 1H NMR (DMSO-d6, 200 MHz) δ: 0.98 (t, 3H, J=7.3 Hz), 1.64 (sext, 2H, J=7.5 Hz), 2.66 (t, 2H, J=7.5 Hz), 3.88 (s, 3H), 6.12 (s, 1H), 7.01 (s, 1H), 7.09 (s, 1H), 9.16 (s, 1H). 13C NMR (DMSO-d6, 50.3 MHz) δ: 13.7 (CH3), 21.1 (CH3), 33.1 (CH3), 56.1 (CH), 100.2 (CH), 108.8 (CH), 110.3 (CH), 111.5 (C), 143.4 (C), 147.9 (C), 151.8 (C), 160.3 (C), 166.9 (C). Anal. Calculated for C14H16O4: C, 67.73; H, 6.50. Found: C, 67.57; H, 6.55.

6-Hydroxy-7-methoxy-4-propyl-2H-1-benzopyran-2-one [C41]: purification by chromatography over silica gel before recrystallisation; yield 38%; mp. 126-127°C (TBME/2-propanol): 1H NMR (DMSO-d6, 200 MHz) δ: 1.24 (d, 6H, J=6.8 Hz), 3.20 (sept, 1H, J=6.8 Hz), 3.88 (s, 3H), 6.12 (s, 1H), 7.03 (s, 1H). 13C NMR (DMSO-d6, 50.3 MHz) δ: 13.0 (CH3), 14.4 (CH3), 20.2 (CH), 56.0 (CH), 99.7 (CH). 102.9 (CH), 112.8 (C), 123.7 (C), 143.3 (C), 145.9 (C), 146.1 (C), 150.5 (C), 161.0 (C). Anal. Calculated for C14H14O5: C, 62.39; H, 5.64. Found: C, 62.32; H, 5.71.

Cell culture. Normal cells, human gingival fibroblast (HGF), human pulp cell (HPC) and human periodontal ligament fibroblast (HPLF), were obtained from human periodontal tissue after informed consent, according to the guidelines of Meikai University Ethics Committee, Japan (No. 0206). Since normal cells have a limited lifespan (19), cells at 3-7 population doubling level (PDL) were used for the present study. The human oral squamous cell carcinoma cell lines (HSC-2, HSC-3) were supplied by Prof. Nagumo, Showa University and Dr. Fukuda, Meikai University, Japan, respectively. All cells used were cultured as a monolayer culture at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO2 atmosphere, and subcultured by trypsinization.

Cytotoxic activity. The relative viable cell number of adherent cells was determined by MTT methods. In brief, the cells were treated for 24 h without (control) or with various concentrations of the test samples. The cells were washed once with phosphate-buffered saline without Mg2+ or Ca2+ (PBS), and further incubated for 4 h with 0.2 mg/mL MTT in DMEM + 10% FBS. After removal of the medium, the cells were lysed with 0.1 mL of dimethyl sulfoxide (DMSO). The absorbance at 540 nm of the solubilized formazan pellet (which reflects the relative viable cell number) was then determined by a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose-response curve, the 50% cytotoxic concentration (CC50) was determined (20). Tumor-specific cytotoxicity (SI value) was determined by the following equation: SI = CC50(GHF + HPC + HPLF)/CC50(HSC-2 + HSC-3) x 2.3.

Cell and fluorescence uptake. The MDRI/A-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDRI phenotype (21). The L5178 MDR cell line and the L5178 Y parent cell line were subcultured by trypsinization. The L5178 MDR cell line and the L5178 Y parent cell line were obtained from human periodontal tissue after limited lifespan (19), cells at 3-7 population doubling level (PDL) were used for the present study. Normal cells, human gingival fibroblast (HGF), human pulp cell (HPC) and human periodontal ligament fibroblast (HPLF), were obtained from human periodontal tissue after informed consent, according to the guidelines of Meikai University Ethics Committee, Japan (No. 0206). Since normal cells have a limited lifespan (19), cells at 3-7 population doubling level (PDL) were used for the present study. The human oral squamous cell carcinoma cell lines (HSC-2, HSC-3) were supplied by Prof. Nagumo, Showa University and Dr. Fukuda, Meikai University, Japan, respectively. All cells used were cultured as a monolayer culture at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO2 atmosphere, and subcultured by trypsinization.

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Figure 1. Chemical structures and calculated log p-values of coumarin derivatives [C1-44].
for a further 20 min at 37°C, washed twice and resuspended in 0.5 mL PBS (pH 7.4) for analysis. The fluorescence of the cell population was measured by flow cytometry using the Beckton Dickinson FACScan instrument. (±)-Verapamil was used as the positive control in R123 accumulation experiments (22). The R123 accumulation was calculated from the fluorescence of one height values. Then, the percentage of mean fluorescence intensity was calculated in treated MDR1 and parental cell lines, compared to untreated cells. The fluorescence activity ratio (FAR) was calculated by the following equation (21, 22):

\[ \text{MDR1 reversal activity} = \frac{\text{MDR1-treated/MDR1 control}}{\text{parental-treated/parental control}} \]

Results

Cytotoxic activity. The in vitro cytotoxicities of 21 coumarin derivatives [C24-44] (see structural formulae in Figure 1) were evaluated in two human tumor cells (HSC-2, HSC-3) and three normal cells (HGF, HPC, HPLF) by the MTT assay and the results are summarized in Table I. In general, the coumarin derivatives were more cytotoxic against HSC-2 than HSC-3 cells. Five compounds, C29, C39, C40, C43 and C44, displayed potent cytotoxic activities, ranging from CC50 15 to 24 µg/mL in HSC-2 cells. Their potencies were comparable with that of gallic acid (CC 50=24 µg/mL). Both C43 and C44 showed the highest tumor-specific cytotoxicity (SI value=CC 50(HGF + HPC + HPLF)/CC 50(HSC-2 + HSC-3) x 2/3=4.1 and 3.6, respectively).

MDR reversal on tumor cells. The MDR-reversing effect of 44 coumarins [C1-44] was compared to that of (±)-verapamil, using a mouse lymphoma cell line (L-5178 cells) (Table II). The effects were measured by determination of the fluorescence activity ratio (FAR) between treated and untreated group of cells. Among 44 coumarins, fourteen compounds [C5, 19, 21, 22, 24, 28, 33, 34, 36, 39, 40, 42, 43 and 44] were able to moderately increase the amount of rhodamine 123 accumulated by resistant lymphoma cells at 40 µg/mL concentration (FAR values >1) (data not shown). However, most compounds, except C34 and C43, had a 10

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<tr>
<th>Table I. Cytotoxic activity of coumarin derivatives [C24-44] against cultured human tumor and normal cells.</th>
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<tr>
<td><strong>Compound</strong></td>
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<td><strong>Human tumor cell lines</strong></td>
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<td>C43</td>
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<td>C44</td>
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<td>Gallic acid</td>
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Near confluent cells were incubated for 24 hours without or with various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm of the lysate of MTT-stained cells) was determined by the MTT method. The CC50 was determined from the dose-response curve. Each value represents the mean from duplicate determinations. aAbsorbance at 540 nm of the lysate of MTT-stained control cells.
times lower FAR value than that of the MDR modulator verapamil (used as a positive control at concentration of 10 μg/mL). Another thirty compounds were not active at 40 μg/mL concentration (FAR values <1). The most tumor-specific compound, C43 (FAR=5.66 at 40 μg/mL), was three times less active than (±)-verapamil, while the compound C34 (FAR=17.98 at 40 μg/mL) was equipotent to (±)-verapamil (FAR=18.34 at 10 μg/mL) (Table II).

To check if there is any correlation between a compound’s hydrophobicity and its MDR-reversing potency, we calculated the octanol/water partition coefficient (log P) of the coumarins (Figure 1) (23). Among 44 coumarins, the log P values ranged from 0.70 to 4.79. The log P values of the potent C34 and C43 were 0.82 and 3.21, respectively.

**Discussion**

No or only minimal cytotoxicity of most coumarins could be detected in normal cells in contrast to the positive control gallic acid. On the other hand, several of them showed concentration-dependent cytotoxicity against the tumor cell lines HSC-2 and HSC-3. Among them, 6-hydroxy-7-methoxycoumarin derivatives [C29, C30, C39, C40, C41, C42 and C43] were cytotoxic to the tumor cell line HSC-2. However, C34 and C38 were relatively non-toxic to all cell lines used. It is suggested that the presence of polar substituents, such as an ester and alcohol group at the C3 and/or C4 position, was unfavorable for cytotoxicity. This observation confirms the recent findings that 3,4-dimethyl-[C17] and 3,4-cyclopentano-6-hydroxy-7-methoxycoumarin [C22] showed marked cytotoxic effects (12). However, it should be noted that 4-methyl-6-hydroxy-7-methoxycoumarin [C10] and 7-hydroxy-6-methoxycoumarin (scopoletin)[C4] were inactive. Furthermore, C43 showed the highest tumor-specific cytotoxicity (SI value=4.1), which was more potent than that of gallic acid (SI=2.4). In the series of 3-(2-hydroxyethyl)-4-methylcoumarins [C34-37], enhancement of cytotoxicity was observed in the following order: 7-hydroxy-6-methoxy [C36] < 6-hydroxy-7-methoxy [C34] < 5,7-dihydroxy [C37] < 7,8-dihydroxy [C35].

We also studied the influence of coumarin derivatives on Pgp transport activity by the use of flow cytometric functional test. When the fluorescence activity ratio (FAR) values are greater than 1, reversal of MDR has taken place. Among 44 coumarins, fourteen compounds [C5, 19, 21, 22, 24, 28, 33, 34, 36, 39, 40, 42, 43 and 44] were able to moderately increase the amount of rhodamine 123 accumulated by resistant lymphoma cells at 40 μg/mL concentration (FAR values >1) (data not shown). However, the majority of the compounds, except C34 and C43, had a 10 times lower FAR value than that of the MDR modulator verapamil (used as a positive control at a concentration of 10 μg/mL). The most active compound C34 was equally potent to verapamil. It is suggested that the presence of the 2-hydroxyethyl group is favorable for the activity. Then, C34 might be an anti-MDR inducing agent of great interest (3, 24).

We have often been suggested that hydrophobicity is an important feature of MDR modulators (25, 26), which is why the log P values of the coumarins studied was calculated. The most active C34 showed low hydrophobicity concerning the log P value. Therefore, hydrophobicity alone was not an essential parameter for the direct MDR-modulating activity of the present series of compounds.
Although a number of agents have been developed to modify MDR, none of them are currently used in the clinic due to their lack of potency or of side-effects at that concentrations necessary for efficacy. At the same time, there is an urgent need to improve cancer chemotherapy for resistant tumors. Therefore, we have to find and select effective compounds, at least in vitro. To fulfil this need, the coumarins were tested in this study and, after further modifications, the coumarins might be MDR reversal compounds.

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23. Pomona College Medicinal Chemistry Project, Claremont, CA, USA.


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