Enhanced Oral Bioavailability of Morin Administered in Mixed Micelle Formulation with PluronicF127 and Tween80 in Rats

Yeon Ah Choi, You Hyun Yoon, Kwangik Choi, Mihwa Kwon, Soo Hyeon Goo, Jin-Sun Cha, Min-Koo Choi, Hye Suk Lee, and Im-Sook Song

Regular Article

To overcome the low oral bioavailability of morin, a mixed micelle formulation with pharmaceutical excipients that facilitate solubilization and modulate P-glycoprotein (P-gp) was developed and evaluated in vitro and in vivo rats. Morin-loaded mixed micelle formulation with a morin–PluronicF127–Tween80 ratio of 1 : 10 : 0.02 (w/w/w) was prepared by a thin-film hydration method. The solubility, size distribution, drug encapsulation efficiency, and percent drug loading of the formulation were characterized. Subsequently, in vivo pharmacokinetic parameters of morin loaded in a PluronicF127 and Tween80 mixed-micelle formulation were investigated in rats. Absolute bioavailability of morin was dramatically increased by the oral administration of morin-loaded PluronicF127 and Tween80 mixed micelle formulation compared with that of morin alone. P-gp inhibition by cyclosporine A (CsA) increased absorptive permeability of morin 2.4-fold but decreased the efflux of morin by 52%, which was consistent with increased plasma concentration of morin in the pretreatment of CsA in rats. The morin formulation inhibition P-gp transport activity by 83.1% at 100 µM as morin concentration. Moreover, morin formulation increased paracellular permeability of Lucifer yellow by 1.6–1.8 fold. In conclusion, enhanced oral bioavailability of morin from morin-loaded PluronicF127 and Tween80 mixed micelle formulation can be attributed to increased intestinal permeation of morin, which was mediated at least by P-gp inhibition and enhanced paracellular route.

Key words morin; mixed micelle; P-glycoprotein (P-gp); oral bioavailability

Flavonoids are present in fruits, vegetables, plant-derived beverages, and many herbal products. The average daily intake of total flavonoids in the U.S. diet is estimated to be 0.2–1 g.[1][2] Flavonoids have recently been the focus of much attention owing to their antiproliferative effect on various cancer cells, as well as their antioxidative potential.[3]

Morin (3,5,7,2′,4′-pentahydroxyflavone) is one of the flavonoids found in fruits, vegetables, tea, and a number of Asian medicinal herbs.[4] It has been reported to possess anti-inflammatory, anti-cancer, anti-oxidant, anti-hypertensive, and anti-estrogenic activities.[5–9] Recently, Jin et al. reported that morin inhibited growth of highly metastatic breast cancer cell line MDA-MB-231.[10] The effective concentration of morin required for anti-inflammatory, anti-cancer, and anti-oxidant effect has been reported as about 10–100 µM in in vitro cell system,[10,11] suggesting that higher plasma and tissue concentrations of morin are necessary to show in vivo efficacy. However, the absolute bioavailability of morin after a single oral dose is very low (less than 1%),[12] likely reflecting low aqueous solubility and low intestinal permeability caused by efflux transporters such as P-glycoprotein (P-gp).[13] Therefore, for the better therapeutic effect of morin on oxidative stress, inflammatory disease, and cancerous pathophysiology, an effort for the enhancement of oral bioavailability and plasma concentration of morin to reach its effective concentration should be required.

P-gp is expressed on the plasma membranes of tumor cells and actively extrudes a wide variety of anticancer agents from the cells, resulting in multidrug resistance that is a significant obstacle to successful chemotherapy of many cancers.[14] Furthermore, P-gp is localized in the apical membrane of hepatocytes, kidney, and enterocytes, and plays an important role in the efflux of xenobiotics from the tissues, functioning as barriers for tissue concentration of drugs and blocking the intestinal absorption of substrate drugs.[15] Therefore, the co-administration of a P-gp inhibitor with compounds with low intestinal permeability can be a strategy for increasing the oral bioavailability of these drugs.

In recent years, various pharmaceutical excipients have emerged as not only solubilizing agents but also as potential alternatives to currently used P-gp inhibitors.[16] For instance, Pluronic block copolymers, amphiphilic synthetic polymers containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks arranged in a triblock structure (PEO–PPO–PEO) have been reported to reverse P-gp-mediated efflux in multidrug resistant cancer cell lines.[17,18] Moreover, doxorubicin containing mixed micelle formulation composed of Pluronic L61 and F127 (i.e., SP1049C, Supratek Pharma Inc., Montreal, Canada) showed increased cellular doxorubicin concentration and has reached phase 3 stage in clinical testing for its superior antitumor activity to doxorubicin standard formulation.[19]

Therefore, the purpose of this study was to develop a morin-loaded mixed micelle formulation with PluronicF127 and Tween80 and to investigate the enhancement of morin bioavailability through the use of this formulation in rats. The
underlying mechanisms of intestinal absorption of morin from the proposed formulation were also investigated using Caco-2 cells and P-gp overexpressed cells (i.e., LLC-PK1-P-gp).

MATERIALS AND METHODS

MaterialsMorin, PluronicF127, Tween80, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), Hanks’ balanced saline solution (HBSS), cyclosporine A (CsA), lucifer yellow, and 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) and Dulbecco’s modified medium (MTT) were purchased from Biochrom Bio, Inc. (Daegu, Korea). Animals were acclimatized to a relative humidity of 55±5% and a 12 h illumination cycle (07:00–19:00). Food and water were supplied ad libitum. All animal procedures were approved by the Animal Care and Use Committee of Kyungpook National University.

The femoral artery and vein were cannulated with polyethylene tubes (PE-50; Jungdo, Seoul, Korea) under light anesthesia with isoflurane. The rats were not restrained at any time during the study. Heparinized isotonic saline (10 µL/mL) was used to flush the catheters to prevent blood clotting. The rats were fasted for at least 12 h before the oral administration of drugs.

Morin was dissolved in dimethyl sulfoxide (DMSO)–polyethylene glycol–DDW (2:6:2, v/v/v), and a 10 mg/kg dose was injected via the femoral vein (vehicle dosing volume, 1 mL/kg). Blood samples (approximately 250 µL each) were collected from the femoral artery at 0, 0.017, 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, and 10 h following intravenous administration of morin.

In the group administered morin in a mixed micelle formulation, the composition of the morin formulation was morin–PluronicF127–Tween80=1:10:0.02 (w/w/w). The morin formulation was dissolved in DDW and administered to rats as a single oral dose corresponding to 20 mg/kg morin (vehicle dosing volume, 3 mL/kg). Morin samples were collected from the femoral artery at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h following oral administration of morin. After centrifugation of blood samples at 13000 rpm for 5 min, plasma samples (100 µL) were collected and stored at −80°C until analysis.

To investigate the effect of P-gp inhibition on the pharmacokinetics of morin, 0.5% methyl cellulose suspension of morin was administered to rats as a single oral 100 mg/kg dose (vehicle dosing volume, 3 mL/kg) in the presence and absence of CsA (50 mg/kg). CsA was dissolved in DMSO–polyethylene glycol–DDW (2:6:2, v/v/v), and a 10 mg/kg dose (vehicle dosing volume, 3 mL/kg). Blood samples were collected from the femoral artery at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h following oral administration of morin. After centrifugation of blood samples at 13000 rpm for 5 min, plasma samples (100 µL) were collected and stored at −80°C until analysis.

Aliquots of 50 µL plasma were added to 200 µL acetonitrile containing 0.5 mg/mL of propranolol (an internal standard). After vortex-mixing for 10 min and centrifugation for 10 min at 13000 rpm, the supernatant was transferred to a clean tube and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted in 150 µL of mobile phase, and a 10 µL aliquot was injected directly into LC-MS/MS system.

Pharmacokinetic Study Male Sprague–Dawley (SD) rats (8–9 weeks, 250–300 g) were purchased from the Hyechang Bio, Inc. (Daegu, Korea). Animals were acclimatized for 1 week in a temperature controlled room (23±2°C), with a relative humidity of 55±10%, an illumination intensity of 150–300 lx, a frequency of air ventilation of 15–20 times/h, and a 12 h illumination cycle (07:00–19:00). Food and water were supplied ad libitum. All animal procedures were approved by the Animal Care and Use Committee of Kyungpook National University.

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Transpeptithelial Transport Study Caco-2 cells (ATCC, Rockville, MD, U.S.A.) were grown in tissue culture flasks in DMEM supplemented with 20% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin-streptomycin. LLC-PK1-P-gp cells (BD-Corning, Corning, NY, U.S.A.) were grown in tissue culture flasks in Medium 199 supplemented with 10% fetal bovine serum, 50 µg/mL of gentamycin, and 50 µg/mL of hygromycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO2–95% air. The cells were grown and seeded on filter inserts of 12 transwell plates at a
density of $5 \times 10^5$ cells/insert. The integrity of the cell monolayers was evaluated prior to the transport experiments by measuring transepithelial electrical resistance, with the values in the range of 300–650 Ω cm$^2$ used in the transport experiment.20

To investigate the effect of morin formulation on the P-gp transport activity, we measured the basal to apical (BA) transport of paclitaxel in LLC-PK1-P-gp cell monolayers. Aliquots (1.5 mL) of HBSS medium containing 5 μM paclitaxel and morin (1, 10, 100 μM) or morin formulation (1, 10, 100 μM as morin) was added to the basal side, and 0.5 mL of fresh HBSS medium was added to the apical side. The transport medium in the apical side was replaced with 0.35 mL of fresh incubation medium every 15 min for 1 h. Aliquots (100 μL) of samples were added to a 100 μL acetonitrile containing 1 ng/mL of propranolol. After vortex-mixing for 10 min and centrifugation for 10 min at 13000 rpm, the supernatant was transferred to a clean tube and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted in 150 μL aliquot of mobile phase, and a 10 μL aliquot was injected directly into the LC-MS/MS system.

Intestinal permeability of morin and morin formulation was assessed in Caco-2 cells. For measurement of the apical to basal (AB) transport of morin, 0.5 mL of HBSS supplemented with 10 mM HEPES (pH 7.4) medium containing morin (100 μM) or morin formulation (100 μM as morin) in the presence and absence of cyclosporine A (CsA, 25 μM) was added to the apical side and 1.5 mL of fresh HBSS medium was added to the basal side of the insert. The insert was transferred to a well containing fresh HBSS medium every 15 min for 1 h. For measurement of the BA transport, 1.5 mL of HBSS medium containing morin (100 μM) or morin formulation (100 μM as morin) in the presence and absence of CsA (25 μM) was added to the basal side, and 0.5 mL of fresh HBSS medium was added to the apical side. The transport medium on the apical side was replaced with 0.35 mL of fresh incubation medium every 15 min for 1 h. A 100 μL aliquot of each sample was added to a 100 μL aliquot of acetonitrile containing 1 ng/mL of propranolol. After vortex-mixing for 10 min and centrifugation for 10 min at 13000 rpm, the supernatant was transferred to a clean tube and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted in 150 μL aliquot of mobile phase, and a 10 μL aliquot was injected directly into the LC-MS/MS system.

To investigate the effect of morin formulation on the paracellular transport activity, we measured the AB transport of Lucifer yellow in the presence of morin (1, 10, 100 μM) or morin formulation (1, 10, 100 μM as morin) in Caco-2 cell monolayers. Aliquots (0.5 mL) of HBSS containing 10 μM Lucifer yellow and morin or morin formulation were added to the apical side and 1.5 mL of fresh HBSS was added to the basal side of the insert. The insert was transferred to a well containing fresh HBSS medium every 15 min for 1 h. A 100 μL aliquot of samples was transferred to 96 well plates and the fluorescence of Lucifer yellow was read directly in a fluorescence plate reader using a 485 nm excitation and an emission filter of 535 nm.

Analysis of Morin and Paclitaxel The concentrations of morin were analyzed using a modified LC-MS/MS method reported by He et al.21 Briefly, Agilent 6430 Triple Quad LC/MS-MS system (Agilent, Wilmington, DE, U.S.A.) coupled with an Agilent 1260 series HPLC system was used. The separation was performed on an Eclipse Plus C18 column (4.6 mm i.d.×150 mm, 5 μm, Agilent) using a mobile phase that consisted of acetonitrile and DDW (50:50, v/v) with 0.1% formic acid at a flow rate of 0.25 mL/min. Mass spectra were recorded by electrospray ionization with a positive mode. Quantification was carried out using selected reaction monitoring (SRM) at m/z 301.1→151 for morin and m/z 260.0→116.0 for propranolol. In this study, the lower limit of quantitation (LLOQ) was determined to be 1 ng/mL and intra- and inter-day precision and accuracy were found to be within the acceptance criteria for assay validation guidelines.

The concentrations of paclitaxel were analyzed using a modified LC-MS/MS method reported by Li et al.22 Briefly, Agilent 6430 Triple Quad LC/MS-MS system coupled with an Agilent 1260 series HPLC system was also used. The separation was performed on a Polar RP column (4.6 mm i.d.×150 mm, 5 μm, Phenomenex) using a mobile phase that consisted of methanol and DDW (90:10, v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min. Mass spectra were recorded by electrospray ionization with a positive mode. Quantification was carried out using SRM mode at m/z 876.4→308.1 for morin and m/z 260.0→116.0 for propranolol. The peak areas for all components were automatically integrated using MassHunter B 01.03. In this study, the LLOQ was determined to be 5 ng/mL and the linearity was shown in the standard range from 5 ng/mL to 2000 ng/mL. The intra- and inter-day precision and accuracy were found to be within the acceptance criteria for assay validation guidelines.

Cell Viability Test MDA-MB-231 cells (ATCC), LLC-PK1-mock and LLC-PK1-P-gp cells (BD-Corning) grown in 96 well plates (10$^4$ cells/well) were incubated in the absence and the presence of morin (100 μM), PluronicF127 (0.3 mg/mL), Tween80 (0.6 μg/mL), blank formulation, and morin formulation for 48 h. A mixed micelle formulation consisting of PluronicF127 (0.3 mg/mL) and Tween80 (0.6 μg/mL) in the absence and presence of 100 μM morin was added. Then, 200 μL of MTT (0.5 mg/mL) was added to each well, and the plate was incubated for 4 h. The medium was removed and the purple formazan products were solubilized with 120 μL DMSO. The cell contents were measured by the absorbance at 570 nm. Cell viability was indicated as a percentage of live cells compared with control group after the background correction.

Data Analysis Standard methods were used to calculate the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin ver 2.0; Pharsight, Mountain View, CA, U.S.A.). The area under the plasma concentration–time curve from zero to 12 h (AUC$_{0-12}$) was calculated using the linear trapezoid method and AUC from 12 h to infinity (AUC$_{12-\infty}$) was estimated by dividing the last measured concentration in plasma by the terminal rate constant. The terminal elimination half-life ($t_{1/2}$) was calculated by dividing 0.693 by the slope of terminal phase. Systemic clearance (CL) was determined by dividing dose by AUC. Dose-normalized bioavailability (BA) was calculated by dividing AUC$_{oral}$/oral dose by AUC$_{iv}$/iv dose. The peak plasma concentration (C$_{max}$) and time to reach C$_{max}$ (T$_{max}$) were directly read from the experimental data.

In each transport experiment, the apparent permeability ($P_{app}$) of the drug was calculated dividing the initial transport rate ($V_i$, pmol/cm$^2$/min) of the drug by initial drug concentra-
tions in the donor compartment of the insert (C) and the surface area of the insert (A):

\[ P_{\text{app}} = \frac{V}{C \times A} \]

All data are expressed as the mean±S.D. of three independent experiments. The statistical significance of differences between treatments was evaluated using the unpaired \( t \)-test, and a value of \( p<0.01 \) was taken to be statistically significant.

RESULTS

Characterization of Morin-Loaded Mixed Micelles

Morin solubility was dramatically increased with the increasing ratio of PluronicF127 (Fig. 1A). Enhancement of the amount of Tween80 in the range of 0.01–0.05 also gradually increased morin solubility when the ratio of morin–PluronicF127 was fixed at 1:10 (Fig. 1B). The size of morin formulation dispersed in water may be very different from the actual size in the systemic circulation. For example, the size of morin–PluronicF127 micelle was 91.97±25.80 nm in water and 1241±65.31 nm in plasma. However, the presence of Tween80 stabilized the size increase of morin formulation and the size distribution was relatively constant with the Tween80 content in the range of 0.01–0.05 when the ratio of morin and PluronicF127 was fixed at 1:10 (Fig. 2). Based on the solubility enhancement and size distribution in water and plasma, we selected the ratio of morin–PluronicF127–Tween80 as 1:10:0.02. After the preparation of mixed micelles, the physical characteristics of morin-loaded mixed micelle formulation were evaluated. EE and DL of morin in the mixed micelle were determined to be 84.19±3.30% and 9.15±0.61%, respectively.

Pharmacokinetic Properties of Morin-Loaded Mixed Micelles

Bioavailability of morin administered using the optimized mixed-micelle formulation was assessed and compared with that of morin vehicle following both intravenous and oral administration (Fig. 3A, Table 1). Plasma concentration profile of morin administered intravenously into the blood circulation showed a multi-exponential elimination process.
was dramatically increased (Fig. 3A, Table 1; intravenous administration as morin-loaded mixed micelle formulation). As expected, the plasma concentration of morin following its administration as morin formulation was similar to the profile observed following intravenous administration of morin.

To investigate the effect of P-gp inhibition on in vivo plasma concentrations of morin, CsA (50 mg/kg) was administered to the rats by oral gavages at 30 min prior to the oral administration of morin at a dose of 100 mg/kg. As shown in Fig. 3B and Table 1, dose normalized C\text{max} and AUC\text{∞} values of morin (C\text{max}/D and AUC\text{∞}/D) in the CsA pretreatment group were increased without a significant change in t\text{1/2} and CL. Consequently, the oral bioavailability (BA) of morin was found to be increased 33.5-fold, as compared to the administration of morin alone. Moreover, the plasma concentration profile of morin after 2 h administered as morin formulation was similar to the profile observed following intravenous administration of morin.

Cell Permeability of Morin-Loaded Mixed Micelles
To investigate the underlying mechanism of increased oral bioavailability of morin administered in the mixed micelle formulation, a cell permeability study was performed using Caco-2 cells and P-gp overexpressed cells (i.e., LLC-PK1-P-gp).

At first, inhibitory effect of morin and morin formulation on the P-gp-mediated paclitaxel transport was investigated. Morin inhibited secretory permeability (P\text{app,BA}) of paclitaxel in a concentration dependent manner in LLC-PK1-P-gp cells (Fig. 4A). Morin-loaded mixed micelle also inhibited P-gp-mediated paclitaxel transport in a concentration dependent manner (Fig. 4B). However, the inhibitory potency of morin formulation is much greater than morin itself, suggesting the role in the modulation of P-gp function.

Apparent permeability of secretion (P\text{app,BA}) of morin was 5.3-fold greater than the absorption permeability (P\text{app,AB}) in Caco-2 cell monolayer, with the P\text{app,AB} of 9.6×10^{-7} cm/s. Moreover, by the presence of CsA (a inhibitor of P-gp), the P\text{app,BA} of morin was decreased whereas the P\text{app,AB} of this compound was 2.4-fold increased (Fig. 5A). These results suggested that the intestinal absorption of morin was restricted by the action of an efflux system such as P-gp, which is consistent with previously published reports,\textsuperscript{12,13} and the inhibition of P-gp could increase absorption of morin. When absorptive permeability measured using the morin-loaded mixed micelle formulation, P\text{app,AB} of morin was found to be 3.6-fold higher compared to the permeability of morin itself. Moreover, P\text{app,BA} of the morin formulation was decreased and, as results, the efflux ratio (P\text{app,BA}/P\text{app,AB}) of morin formulation was found to be 0.62 (Fig. 5B). The presence of CsA did not alter the permeability of morin formulation, contrary to the case of morin. The results suggested that the use of the mixed micelle formulation could increase morin permeability and diminish the efflux of morin through the P-gp inhibition.

We also investigated the effect of morin formulation on the paracellular route of Caco-2 cells. As shown in Figs. 6A, P\text{app,AB} of Lucifer yellow was very low, consistent with the previous report,\textsuperscript{23} and it was not affected by the presence
PK1-P-gp cells. Each data point represents the mean ± S.D.; per oral; CsA, cyclosporine A; C_{max}, maximum plasma concentration; dose normalized

Fig. 4. Inhibitory Effect of Morin-Loaded Mixed Micelles on P-gp Transport Activity

Table 1. Pharmacokinetic Parameters of Morin Following Intravenous (i.v.) and per oral (p.o.) Administration of Morin and Morin-Loaded Mixed Micelle Formulation

<table>
<thead>
<tr>
<th>Parameters&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Administration of morin</th>
<th>Administration of morin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) i.v., Vehicle</td>
<td>(B) p.o., Vehicle</td>
</tr>
<tr>
<td></td>
<td>(10mg/kg)</td>
<td>(100mg/kg)</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>—</td>
<td>49.59±27.39</td>
</tr>
<tr>
<td>C_{max}/D (ng/mL/mg/kg)</td>
<td>—</td>
<td>0.4959±0.2739</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>—</td>
<td>3.17±2.75</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;12h&lt;/sub&gt; (ng/mL-h)</td>
<td>14594±957.8</td>
<td>364.0±290.0</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;12h)/D (ng/mL-h/mg/kg)</td>
<td>1459±95.78</td>
<td>3.64±0.900</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;c&lt;/sub&gt; (ng/mL-h)</td>
<td>14656±999.9</td>
<td>517.1±495.1</td>
</tr>
<tr>
<td>AUC/D (ng/mL-h/mg/kg)</td>
<td>1466±99.99</td>
<td>5.17±4.951</td>
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<tr>
<td>t_{1/2} (h)</td>
<td>1.53±0.15</td>
<td>4.31±2.04</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>11.41±0.81</td>
<td>18.84±12.12</td>
</tr>
<tr>
<td>BA (%)</td>
<td>—</td>
<td>0.35</td>
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</table>

<sup>a</sup> Each data represents Mean±S.D.; n=3. *p<0.05, **p<0.01 statistically significant compared with a group of orally administered morin (p.o., vehicle; B group). †p<0.05, ††p<0.01 statistically significant compared with a group of orally administered morin alone (morin, 100mg/kg; D group). Abbreviations: i.v., intravenous injection; p.o., per oral; CsA, cyclosporine A; C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; AUC, area under the plasma concentration-time curve; C_{max}/D and AUC/D, dose normalized C_{max} and AUC, respectively; t_{1/2}, terminal elimination half-life; CL, systemic clearance; BA, dose-normalized bioavailability.

Fig. 4. Inhibitory Effect of Morin-Loaded Mixed Micelles on P-gp Transport Activity

Secretory permeability of paclitaxel (P_{app,AB}) was measured in the presence of (A) morin (1, 10, 100 μM) or (B) morin formulation (1, 10, 100 μM as morin) in LLC-PK1-P-gp cells. Each data point represents the mean±S.D. of three independent experiments. *p<0.01, statistically significant compared with a control group. †p<0.01, statistically significant compared with a morin group.

of morin at a concentration range of 1–100 μM. However, the presence of morin formulation increased the P_{app,AB} value 1.6 to 1.8-fold (Fig. 6B). The increase in the paracellular transport route could contribute, in part, to the increased permeability of morin in Caco-2 cells.

Cell Viability of Morin-Loaded Mixed Micelles To confirm the safety of the excipients, we measured cell viability after incubation with the excipients alone and blank formulation, which are the same amounts used in mixed micelle formulation. Cell viability in MDA-MB-231 and LLC-PK1-mock and P-gp cells was not significantly changed by the treatment of PluronicFl127 and Tween80 alone or blank formulation (Fig. 7). However, the viability in MDA-MB-231 cells was decreased to 66.9% after 48h incubation with 100μM morin and it was synergistically decreased to 11.7% by the treatment of morin formulation (Fig. 7A), suggesting the enhanced cytotoxic effect of morin by the treatment of mixed micelle formulation.

To investigate whether the enhanced cytotoxic effect of morin formulation was associated with P-gp modulation, cell viability test was performed in mock cells and P-gp overexpressed cells. As shown in Figs. 7B and C, the cytotoxic effect of morin and morin formulation was greater in LLC-PK1-mock cells than in LLC-PK1-P-gp cells. The results suggest that the expression level of P-gp was important for the morin sensitivity and, therefore, the efficacy of morin could be sensitized by modulating P-gp function.

DISCUSSION

Low aqueous solubility and limited intestinal absorption are recognized barriers for oral absorption and the cause of
low plasma concentration of therapeutics, reflected in the inadequate therapeutic response. At first, we evaluated the enhancement of water-solubility of morin in the presence of hydrophilic polymers such as β-cyclodextrin, Kollidon, polyethylene glycol, PluronicF127, Tween20, Tween80, Span20, and Span80. Among these polymers, PluronicF127 and Tween80 were found to be most profound increase of morin solubility (data not shown). Therefore, we selected PluronicF127 and Tween80 as excipients for morin formulation and further optimized the formulation containing these components.

Moreover, pharmaceutical excipients such as PluronicF127 and Tween80 that we used in this study are known to have the ability to modulate the P-gp-mediated efflux of low permeable drugs.24–26)

Morin-loaded mixed micelle formulation consisting of morin–PluronicF127–Tween80 = 1 : 10 : 0.02 (w/w/w) was developed and the bioavailability of morin using this formulation was dramatically increased from 0.35% (in case of morin itself) to 11.62% (Fig. 3). The observed enhancement of bioavailability is likely caused by increased intestinal permeability since the administration of morin formulation resulted in the increased C_max/D and AUC/D with decreasing T_max. Moreover, the pharmacokinetic parameters related to drug disposition such as CL and t_1/2 were not changed by the administration of morin formulation. The results, taken together, suggested that enhancing the limited oral absorption of morin by the use of an appropriate formulation may overcome the low plasma concentration of this compound following oral administration.

The underlying mechanisms for the increased intestinal
absorption were investigated in an in vitro model. Since P-gp is involved in the transport of morin, the inhibitory effect of morin formulation on the P-gp function was investigated in LLC-PK1-P-gp cells. Paclitaxel was used as a substrate for P-gp. As shown in Fig. 4B, P-gp-mediated efflux of paclitaxel was inhibited by the morin formulation in a concentration dependent manner and the morin-loaded mixed micelle formulation (100 µM as morin) inhibited 83.1% of P-gp function. However, the P-gp inhibition was caused not only by morin but also by Pluronic F127 and Tween80.

The inhibition of P-gp by pharmaceutical excipients such as Pluronic block copolymers and Tweens was reported to relate to the concentration of the excipients. For example, monomethoxy polyethylene glycol–polyepsilon–caprolactone diblock copolymer increased the uptake of rhodamine 123 in Caco-2 cells at concentrations 10-fold higher than that of critical micelle concentration (CMC), while the intracellular accumulation of rhodamine 123 reached maximal levels at or near the CMC of Pluronic P85. When 0.0025–0.01% of Tween20 was combined with doxorubicin, it significantly increased the sensitivity of MDR cells by modulating P-gp function and increasing the intracellular concentration of doxorubicin. In this study, 100 µM of morin was used to evaluate the permeability. Considering the composition of the formulation (morin–Pluronic F127–Tween80 = 1: 10: 0.02) and CMCs of Pluronic F127 and Tween80 (35.3 and 0.16 µg/mL, respectively), the concentrations of Pluronic F127 and Tween80 used in Caco-2 cell permeability study were 8.5- and 3.7-fold higher than their CMC. Thus, we can conclude that the enhanced permeability of morin elicited by the reversing of P-gp function is likely a result of the mixed micelle formulation consisting of Pluronic F127 and Tween80.

The presence of a representative P-gp inhibitor, CsA, also increased morin permeability but not affected the permeability of morin formulation (Fig. 5). These results can be attributed to the P-gp inhibition by the pre-existing pharmaceutical excipients from the morin formulation. Taken together, the reversal of P-gp mediated morin efflux could be a major factor contributing to the increased permeability of morin across the intestinal epithelium. To further investigate the P-gp inhibition could contribute the increased in vivo BA of morin, we compared plasma concentration of morin in the group of CsA pretreatment via oral administration (50 mg/kg) with that in the control group. As shown in Fig. 3B, the plasma concentrations of morin were increased by the P-gp inhibition, but the BA enhancement of morin was lower than that in oral administration of morin formulation, suggesting the involvement of additional mechanism in addition to P-gp blockade in the BA enhancement of morin.

Similarly the increase in the morin permeability from morin-loaded mixed micelle formulation (3.6-fold) was greater than the increased morin permeability in the presence of CsA (2.4-fold), suggesting that other mechanisms may be involved in the increased intestinal permeability of morin. We investigated whether the increased permeability of morin is mediated by a paracellular route and caused by the opening of tight junctions since the excipients used in this study are known to exhibit surfactant properties. In this investigation, Lucifer yellow was used as a marker of paracellular transport. As results, the presence of morin formulation increased the permeability of Lucifer yellow, suggesting the enhancement of paracellular route could contribute, in part, to the increased intestinal absorption of morin as well. In addition to this, we should note that in vivo BA of morin was assessed as a morin suspension with 0.5% methyl cellulose, therefore incomplete solubility of morin might also affect the lower BA of morin compared with that administered as mixed micelle formulation.

The therapeutic response was assessed by cell viability test in MDA-MB-231 cells, a breast cancer cell line, in which
anti-cancer effect of morin was previously shown and P-gp was also expressed.\textsuperscript{10,31} Cytotoxic effect of morin treated as a mixed micelle formulation was significantly increased compared with that of morin alone (Fig. 7A), suggesting the synergetic role of PluronicF127 and Tween80 as P-gp modulators. To differentiate the underlying mechanism, we compared the cytotoxicity of morin and morin formulation in LLC-PK1-mock and LLC-PK1-P-gp cells (Figs. 7B, C). LLC-PK1-mock cells, which showed limited P-gp expression, were more sensitive to morin formulation compared with LLC-PK1-P-gp cells, which showed P-gp overexpression. These results suggest that cytotoxic efficacy of morin can be enhanced by the mixed micelle formulation, in which P-gp modulating excipients can serve as additional chemosensitizing agents.

In conclusion, higher bioavailability and better therapeutic response of morin could be achieved through the use of a PluronicF127 and Tween80 mixed micelle formulation that enhances the solubility, inhibits P-gp-mediated efflux of morin, and increases paracellular pathway as well.

**Conflict of Interest** The authors declare no conflict of interest.

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