

항산화제인 아피제닌이 에토포시드의 생체이용률 및 약동학에 미치는 영향

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Effects of Apigenin, an Antioxidant, on the Bioavailability and Pharmacokinetics of Etoposide

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에토포시드와 아피제닌의 약동학적 상호작용 연구를 위하여 아피제닌 (0.4, 2.0 또는 8 mg/kg)과 에토포시드의 경구 (6 mg/kg) 및 정맥 (2 mg/kg) 투여 하여 본 연구를 실시하였다. 아피제닌이 cytochrome P450 (CYP) 3A4 활성과 P-glycoprotein (P-gp)의 활성화에 미치는 영향도 평가하였다. 아피제닌의 CYP3A4의 50% 효소활성억제는 1.8 μ M 이었다. 아피제닌은 MCF-7/ADR 세포의 로다마인-123 세포 축적을 증가 시키므로 P-gp를 억제시켰다. 아피제닌은 에토포시드의 혈장곡선하면적과 최고혈장농도 (AUC and C_{max})를 유의성 있게 증가시켰으나, 에토포시드의 최고혈장농도 도달시간 (T_{max})과 생물학적 반감기 ($t_{1/2}$)에는 영향을 미치지 않았다. 따라서, 아피제닌 존재하에 에토포시드의 절대적생체이용률 (AB)은 대조군과 비교하여 유의성있게 증가되었다. 경구투여시와는 대조적으로, 아피제닌은 정맥 내로 투여된 에토포시드에서는 약동학적 파라미터에 어떤 영향도 미치지 않았다. 따라서 아피제닌이 에토포시드의 생체이용률을 증가시킨 것은 아피제닌이 소장과 간장에서 CYP3A4를 억제 및 소장에서 P-gp를 억제 시켰기 때문으로 사료된다.

□ Key words - etoposide, apigenin, bioavailability, pharmacokinetics, CYP3A, P-gp, rats

Etoposide (VP-16-213) [4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene)- β -D-glucopyranoside], is a semi-synthetic derivative of podophyllotoxin extracted from the roots and rhizomes of *Podophyllum peltatum* or *Podophyllum emodi*.^{1,2)} Etoposide exhibits anticancer activity against the wide range of malignancies including small cell lung cancer, acute leukemia, lymphoma and testicular cancer²⁾ via the inhibition of topoisomerase II enzyme and/or the induction of direct DNA breaks.³⁾ Etoposide undergoes the cytochrome P450 (CYP)-catalyzed O-demethylation in human and rat liver microsomes.^{4,5)} Among CYP enzymes, CYP3A4 has been identified as a

major enzyme responsible for the metabolism of etoposide and to a minor degree, CYP1A2 and 2E1.⁶⁾ Etoposide was also reported to be a substrate for P-glycoprotein (P-gp).⁷⁾ Consequently, CYP3A4 and P-gp inhibitors could affect the pharmacokinetics of etoposide.⁷⁻⁹⁾ For examples, cyclosporine and its analogue PSC833 have been reported to increase the plasma concentration of orally administered etoposide in rats^{8,9)} and quinidine enhanced the uptake of etoposide from rat *in situ* perfused intestinal loops.⁷⁾

Flavonoids represent a group of phytochemicals that are produced by various plants in high quantities.¹⁰⁾ Among flavonoids, apigenin (4,5,7-trihydroxyflavone) is present in citrus fruits and guava and exhibits various biological activities including antioxidation, anti-mutagenesis, anti-inflammatory, anti-tumor, anti-inflammatory and anti-cancer effects.¹¹⁻¹⁶⁾ Furthermore, apigenin is reported to interact with CYP3A4 as well as the P-gp efflux pump.¹⁷⁻

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¹⁹⁾ Nguyen et al.²⁰⁾ reported that apigenin significantly increased the cellular accumulation of vinblastine, a P-gp substrate in Panc-1 cells. Ho et al.¹⁸⁾ reported very weak inhibition effect of apigenin on CYP3A4 mediated metabolism but recently, others reported potent inhibition effect of apigenin against CYP3A4 activity.²¹⁾ Therefore, the effect of apigenin on the inhibition of CYP enzyme activity is inconclusive. Hence we attempted to re-evaluate the inhibition potency of apigenin on CYP3A4 and P-gp activity using CYP inhibition assay and rhodamine-123 retention assay in MCF-7/ADR cells overexpressing P-gp. In addition, apigenin and etoposide could be used together for the treatment of cancer as a combination therapy, but the possible effects of apigenin on the bioavailability of etoposide have not been reported *in vivo*. Therefore, the present study also investigated the effect of apigenin on the bioavailability and pharmacokinetics of etoposide after oral and intravenous administration in rats.

MATERIALS AND METHODS

Materials

Etoposide, apigenin and podophyllotoxin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Etoposide injectable solution was purchased from Boryung Chemical Co. (Seoul, Republic of Korea). Methanol and tert-butylmethylether were acquired from Merck Co. (Darmstadt, Germany). Other chemicals were of reagent grade or HPLC grade.

Animal Studies

Male Sprague-Dawley rats weighing 270-300 g were purchased from the Dae Han Laboratory Animal Research Co. (Eumsung, Republic of Korea) and were given access to tap water and a normal standard chow diet (No. 322-7-1, Superfeed Co., Wonju, Republic of Korea) *ad libitum*. Animals were kept in these facilities for at least one week before the experiments. The experiments were carried out in accordance with the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA). Rats

were randomly divided into eight groups containing six animals in each group: (1) an oral control group, administered 6 mg/kg etoposide orally; (2-4) the oral combination groups, administered apigenin (0.4, 2 or 8 mg/kg) orally 30 min prior to oral etoposide; (5) an IV control group, intravenous administration of 2 mg/kg of etoposide; (6-8) the IV combination groups, administered apigenin (0.4, 2 or 8 mg/kg) orally 30 min prior to intravenous administration of etoposide. The rats were fasted for at least 24 h prior to the experiments. The etoposide solution for oral administration was prepared by diluting 6 mg/kg of etoposide injectable solution in distilled water, and the solution for intravenous administration was diluted with 0.9% NaCl-injectable. Blood samples (0.4 mL) were collected into heparinized tubes via the femoral artery at 0, 0.017, 0.1, 0.25, 0.5, 1, 2, 4, 6 and 10 h after an IV administration and at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 10 h after an oral administration of etoposide. The blood samples were centrifuged at 13,000 rpm for 5 min, and the obtained plasma was stored at -40°C until HPLC analysis.

HPLC Analysis

The plasma concentrations of etoposide were determined by a HPLC assay reported by Li et al.²²⁾ with a slight modification. Briefly, 0.5 mL of 50 ng/mL podophyllotoxin and 1.2 mL of tert-butylmethylether were mixed with 0.2 mL of the plasma sample. The mixture was stirred vigorously for 1 min and centrifuged at 13,000 rpm for 10 min. One mL of the upper layer was transferred to another clean microtube and evaporated under nitrogen at 38°C. The residue was dissolved in 0.2 mL of 50% methanol and 50 µL of the solution was injected into the HPLC system.

The fluorescence detector was operated at an excitation wavelength of 230 nm with an emission cut-off filter of 330 nm. A Symmetry[®] C₁₈ column (4.6×150 mm, 5 µm, Waters Co., Milford, MA, USA) was used at 30°C. The mobile phase consisted of methanol:water:acetic acid (50:50:0.5, v/v/v). The flow rate was maintained at 1.0 mL/min. The retention times of etoposide and the internal standard were approximately 5.4 and 11.1 min, respectively. The detection limit of etoposide in plasma was 5 ng/

mL. The intra- and inter-day variation coefficients of etoposide in plasma samples were below 15%.

CYP Inhibition Assay

The inhibition assays of human CYP3A4 enzyme activity were performed in a multiwell plate using the CYP inhibition assay kit (BD Bioscience, San Jose, CA). Briefly, human CYP enzymes were obtained from baculovirus-infected insect cells. CYP3A4 substrate (7-Benzylloxy-4-(trifluoromethyl)couamrin (BFC)) was incubated with or without test compounds in a reaction mixture containing 1 pmol of CYP3A4 enzyme and the NADPH-generating system (1.3 mM NADP, 3.54 mM glucose 6-phosphate, 0.4 U/mL glucose 6-phosphate dehydrogenase and 3.3 mM MgCl₂) in potassium phosphate buffer (pH 7.4). Reactions were terminated by adding stop solution after 45 min. Metabolite concentrations were measured with a spectrofluorometer (Molecular Device, Sunnyvale, CA) set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive control (1 μM ketoconazole) was run on the same plate and produced 99% inhibition. All experiments were performed in duplicate, and the results are expressed as the percent of inhibition.

Rhodamine-123 Retention Assay

MCF-7/ADR cells were seeded into 24-well plates at a seeding density of 10⁵ cells. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed to Hanks' balanced salt solution and the cells were incubated at 37°C for 30 min. After incubation of the cells with 20 μM rhodamine-123 for 90 min, the medium was completely removed. The cells were then washed three times with ice-cold phosphate buffer (pH 7.0). After cell lysis, the rhodamine-123 in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and are presented as the ratio to control values.

Pharmacokinetic Analysis

The plasma concentration data were analyzed by the

noncompartmental method using WinNonlin software version 4.1 (Pharsight Co., Mountain View, CA). The elimination rate constant (K_{el}) was calculated by log-linear regression of etoposide concentration data during the elimination phase, and the terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The peak concentration (C_{max}) and the time to reach peak concentration (T_{max}) of etoposide in plasma were obtained by visual inspection of the data from the concentration–time curve. The area under the plasma concentration–time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero to infinity ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el} . Total body clearance was calculated by $Dose/AUC$. The absolute bioavailability (AB) of etoposide was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral} \times 100$ and the relative bioavailability (RB) of etoposide was estimated by $AUC_{etoposide+apigenin}/AUC_{etoposide} \times 100$.

Statistical Analysis

All mean values were presented with their standard deviation (Mean ± SD). The pharmacokinetic parameters were compared using one-way ANOVA, followed by *a posteriori* testing with the Dunnett correction. Differences were considered significant at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Inhibition Effect of Apigenin on CYP3A4

The inhibitory effect of apigenin on CYP3A4 activity is shown in Fig. 1. Apigenin inhibited CYP3A4 enzyme activity in a concentration-dependent manner and the 50% inhibition concentration (IC_{50}) values of apigenin on CYP3A4 activity was determined as 1.8 μM.

Rhodamine-123 Retention Assay

As shown in Fig. 2, accumulation of rhodamine-123, a P-gp substrate, was raised in MCF-7/ADR cells overexpressing P-gp compared to that in MCF-7 cells lacking P-gp. The concurrent use of apigenin enhanced the

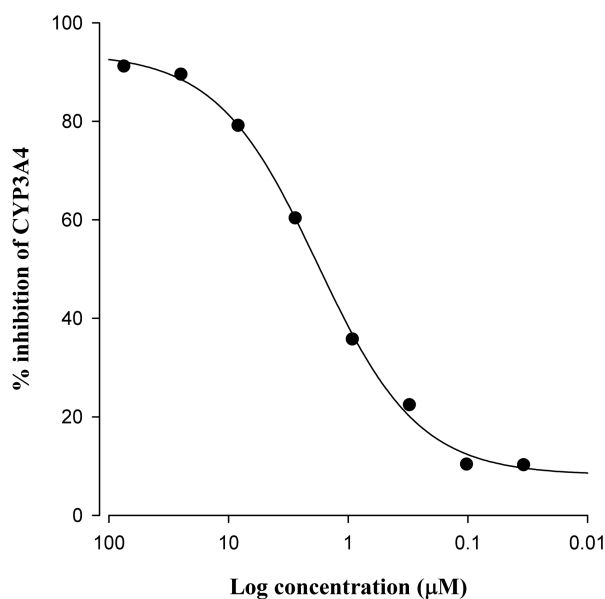


Fig. 1. Inhibitory effect of apigenin on CYP3A4 activity. All experiments were performed in duplicate and the results are expressed as the percent of inhibition.

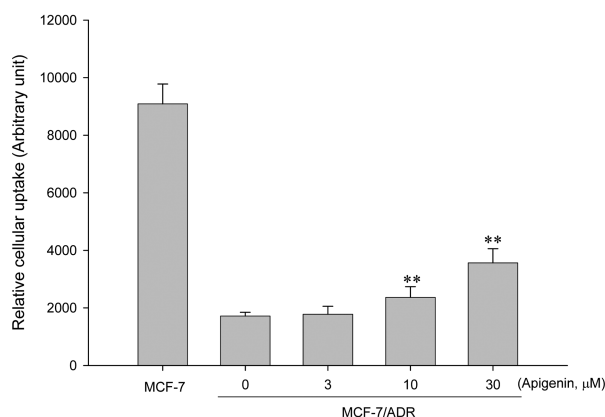


Fig. 2. Effect of apigenin on the cellular accumulation of rhodamine-123 in MCF-7 and MCF-7/ADR cells. Data represents mean±SD of 6 separate samples (**p<0.01).

cellular uptake of rhodamine-123 in a concentration dependent manner and showed statistically significant ($p<0.01$) increase at the concentration range of 10-30 μM . This result suggests that apigenin significantly inhibits P-gp activity.

Effect of Apigenin on the Pharmacokinetics of Etoposide

The plasma concentration-time profiles of etoposide

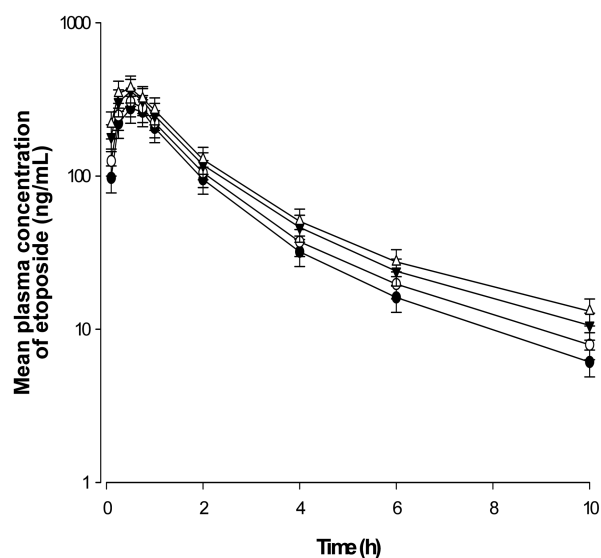


Fig. 3. Mean plasma concentration-time profiles of etoposide after an oral administration of etoposide (6 mg/kg) to rats in the presence or absence of apigenin (Mean±SD, n=6). (●) Control (etoposide 6 mg/kg, oral), (○) with 0.4 mg/kg apigenin, (▼) with 2 mg/kg apigenin, (△) with 8 mg/kg apigenin.

after an oral administration of etoposide (6 mg/kg) in the absence or presence of apigenin (0.4, 2 or 8 mg/kg) are illustrated in Fig. 3, and the pharmacokinetic parameters for etoposide are summarized in Table 1. As shown in Table 1, the presence of apigenin (2 or 8 mg/kg) significantly altered the pharmacokinetic parameters of oral etoposide. Compared to the control group (given 6 mg/kg of etoposide alone), the presence of apigenin significantly (2 mg/kg, $p<0.05$; 8 mg/kg, $p<0.01$) increased (28.8-47.6%; 22.8-33.3%) the area under the plasma concentration-time curve (AUC) and the peak plasma concentration (C_{max}) of the oral etoposide. Consequently, the absolute bioavailability (AB) of etoposide was significantly (2 mg/kg, $p<0.05$; 8 mg/kg, $p<0.01$) increased (40.5-60.8%) by the concurrent use of apigenin. The presence of apigenin tends to prolong the terminal plasma half-life ($t_{1/2}$) of oral etoposide but this effect was not statistically significant. The presence of apigenin did not alter the time to reach peak concentration (T_{max}) of oral etoposide.

For the intravenous administration of etoposide (2 mg/kg) in the presence or absence of oral apigenin

Table 1. Mean pharmacokinetic parameters of etoposide after an oral administration of etoposide (6 mg/kg) to rats in the presence or absence of apigenin (0.4, 2 or 8 mg/kg) (mean±SD, n=6)

Parameters	Control	Etoposide+Apigenin		
		0.4 mg/kg	2.0 mg/kg	8 mg/kg
AUC (ngh/mL)	605±96.8	689±117	779±140*	893±170**
C _{max} (ng/mL)	276±49.2	304±57.9	339±69.0*	368±78.2**
T _{max} (h)	0.46±0.10	0.46±0.10	0.42±0.13	0.42±0.13
t _{1/2} (h)	2.10±0.34	2.20±0.37	2.4±0.43	2.5±0.48
AB (%)	7.4±1.2	9.2±1.6	10.4±1.9*	11.9±2.3**
RB (%)	100	114	129	148

* p<0.05, ** p<0.01 significant difference compared to the control group.

AUC: area under the plasma concentrationtime curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach peak plasma concentration; t_{1/2}: terminal half-life; AB(%): absolute bioavailability; RB(%): relative bioavailability.

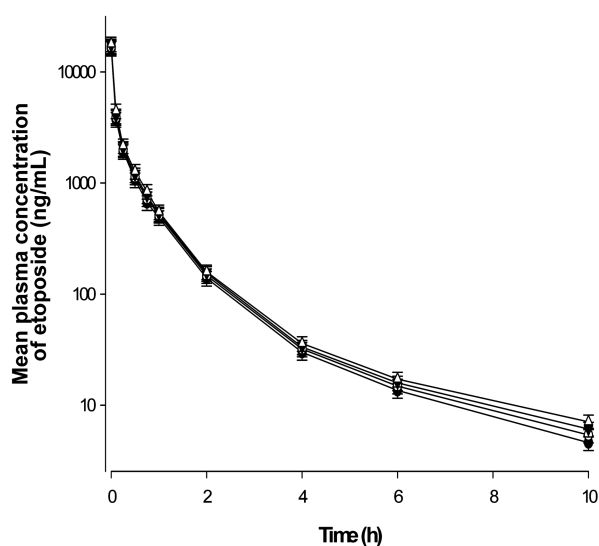


Fig. 4. Mean plasma concentrationtime profiles of etoposide after an intravenous administration of etoposide (2 mg/kg) to rats in the presence or absence of apigenin (Mean±SD, n=6). (●) Control (etoposide 2 mg/kg, IV), (○) with 0.4 mg/kg apigenin, (▼) with 2 mg/kg apigenin, (△) with 8 mg/kg apigenin.

(0.4, 2 or 8 mg/kg), the plasma concentrationtime profiles of etoposide were illustrated in Fig. 4 and the pharmacokinetic parameters were summarized in Table

2. After an intravenous administration of etoposide with apigenin, the total body clearance (CL) of etoposide tends to be decreased and consequently AUC was increased, although there was no statistical significance. The terminal plasma half-life (t_{1/2}) of etoposide was not altered by the combined use of apigenin. The metabolism of etoposide is mainly mediated by CYP3A4⁶⁾ and also etoposide is a substrate of P-gp during the intestinal absorption.⁷⁾ P-gp is colocalized with CYP3A4 in the apical membrane of the intestine,^{23,24)} and they act synergistically in regulating the first-pass metabolism and bioavailability of many orally ingested anticancer agents. Therefore, it may be possible to improve the oral bioavailability of etoposide via the combined use with CYP3A4 and/or P-gp inhibitors. The inhibition effect of apigenin against CYP3A4-mediated metabolism was confirmed by the employment of recombinant CYP3A4 enzyme. As shown in Fig. 1, apigenin exhibited inhibition effect against CYP3A4-mediated metabolism with IC₅₀ of 1.8 μM. This result is comparable to the recent studies done by Kimura et al.²¹⁾ In their studies, apigenin showed competitive-non-competitive

Table 2. Mean pharmacokinetic parameters of etoposide after an intravenous administration of etoposide (2 mg/kg) to rats in the presence or absence of apigenin (0.4, 2 or 8 mg/kg) (mean±SD, n=6)

Parameters	Control	Etoposide+Apigenin		
		0.4 mg/kg	2.0 mg/kg	8 mg/kg
AUC (ngh/mL)	2500±524	2620±572	2720±577	2900±604
CL (mL/min/kg)	13.3±3.04	12.7±2.87	12.3±2.74	11.5±2.48
t _{1/2} (h)	2.2±0.48	2.3±0.49	2.4±0.53	2.6±0.58
RB (%)	100	105	109	116

AUC: area under the plasma concentrationtime curve from 0 h to infinity; CL: total body clearance; t_{1/2}: terminal half-life; RB(%): relative bioavailability.

mixed type of inhibition patterns for CYP3A4 with K_i values of 0.67 μM .²¹⁾ Furthermore, the cell-based assay using rhodamine-123 indicated that apigenin (10-30 μM) significantly ($p < 0.01$) inhibited P-gp-mediated drug efflux (Fig. 2). Those results suggest that apigenin might be effective to improve the bioavailability of etoposide, a dual substrate of CYP3A4 and P-gp. Therefore, the pharmacokinetic characteristics of etoposide were evaluated in the absence and the presence of apigenin in rats. As CYP3A9 expressed in rat is corresponding to the ortholog of CYP3A4 in human,²⁵⁾ rats were selected as an animal model in this study to evaluate the potential pharmacokinetic interactions mediated by CYP3A4, although there should be some extent of difference in enzyme activity between rat and human.²⁶⁾

As summarized in Table 1, the presence of apigenin significantly increased the oral exposure of etoposide. This result is coincident with previous studies using other flavonoids.^{22,27-29)} For examples, morin and quercetin significantly increased the AUC and C_{max} of etoposide by inhibiting P-gp and CYP3A4 in rat intestine and/or liver.^{22,27)} Kaempferol and genistein also significantly increased oral exposure of tamoxifen and paclitaxel, respectively.^{28,29)} In contrast to the oral pharmacokinetics, the intravenous pharmacokinetics of etoposide was not significantly affected by the concurrent use of apigenin. Although there was slight decrease in the total body clearance in the presence of apigenin, there was no statistical significance, implying that the inhibition effect of apigenin against hepatic metabolism might be minimal. Therefore, the enhanced oral bioavailability of etoposide in the presence of apigenin could be mainly due to enhanced absorption in the gastrointestinal tract rather than the reduced hepatic elimination of etoposide. The increase in oral bioavailability of etoposide in the presence of apigenin should be taken into consideration of potential drug interactions between etoposide and apigenin.

CONCLUSION

The presence of apigenin enhanced the bioavailability of oral etoposide in rats, suggesting that the combined use of apigenin may be helpful to improve the bioavailability of etoposide in the chemotherapeutic applications. The increase in oral bioavailability of etoposide with apigenin should be taken into consideration of potential drug interactions between etoposide and apigenin.

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