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Effects of baicalein, an antioxidant, on the bioavailability of doxorubicin in rats: possible role of P-glycoprotein inhibition by baicalein

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The purpose of this thesis is to investigate the effects of baicalein, an antioxidant, on the bioavailability and pharmacokinetics of Doxorubicin (DOX) in rats. Thus, DOX was administered intravenously (i.v.; 10 mg · kg^{-1}) or orally (p.o.; 50 mg · kg^{-1}) with or without oral baicalein (0.3, 1.5 and 6 mg · kg^{-1}). In the presence of 1.5 and 6 mg · kg^{-1} of baicalein, the total area under the plasma concentration-time curve from time zero to time infinity (AUC) and the peak concentration (C_{max}) of DOX were significantly greater and higher, respectively, than those of the control. Consequently, the absolute bioavailability (AB) of DOX in the presence of baicalein was 3.5–4.4%, which was significantly enhanced compared with that of the control group (2.2%). The relative bioavailability (RB) of DOX was 1.20 to 1.96 times higher than that of the control group. Compared to the intravenous control, the presence of oral baicalein increased the i.v. AUC of DOX and other pharmacokinetic parameters were not significantly affected. The enhanced bioavailability of oral DOX by oral baicalein may be due to the inhibition of both P-glycoprotein (P-gp) and the cytochrome P450 (CYP) 3A subfamily by baicalein in the intestine and/or liver. This result suggests that the development of oral DOX is feasible by combination with baicalein that would be more convenient than the i.v. dosage forms. Furthermore, since the present study raises the awareness about potential drug interactions by concomitant use of DOX with baicalein, the dosage regimen of DOX should be taken into consideration, if this result is confirmed in clinical studies.

1. Introduction

Many researchers have attempted to circumvent inhibition of P-glycoprotein (P-gp) during cytotoxic drug administration. For example, P-gp inhibitors, such as verapamil, cyclosporine, valspodar, GF120918 or LY357739 have been used to enhance intracellular accumulation of drugs in multidrug-resistant (MDR) cells (Avendano et al. 2002; Gottesman et al. 2002). P-gp, an important member of the ATP binding cassette (ABC) family that effluxes substrates out of cells, is highly expressed in solid tumors of epithelial origin, such as colon (Cordon-Cardo et al. 1990), kidney (Fojo et al. 1987) and breast (Merkel et al. 1989). When P-gp is over-expressed by tumors, P-gp-mediated efflux of drugs from resultant MDR tumor cells is a major obstacle to successful clinical cancer chemotherapy.

The cytochrome P450 (CYP) 3A subfamily, major phase I drug metabolizing enzymes, are co-localized with P-gp in the liver and intestine (Wang et al. 2001; Fakhoury et al. 2005). Thus, the combined actions of P-gp and the CYP3A subfamily could decrease the oral bioavailability of drugs that are substrates of P-gp and the CYP3A subfamily.

Doxorubicin (DOX), an anthracycline glycosidic anticancer drug, impairs DNA synthesis during tumor cell division. The main mechanisms of the anticancer effects of DOX include DNA intercalation and strand breakage and inhibition of topoisomerase II (Cutts et al. 2005; Ramiji et al. 2003). It is most commonly used for the treatment of lymphoma, osteosarcoma and other sarcomas, carcinomas, and melanoma (Schwarzbach et al. 2002; Langer et al. 2006; Lind et al. 2007; Lundberg et al. 2007; Smylie et al. 2007). DOX is a substrate of P-gp (Gustafson et al. 2005), and one or more enzymes of the CYP3A subfamily plays a role in DOX metabolism (Kivistö et al. 1995).

Flavonoids are the most abundant polyphenolic compounds present in human diet and are found in fruits, vegetables, tea, and red wine. Flavonoids have a variety of beneficial pharmacological properties including antitumor, anti-oxidative, antiviral, and anti-inflammatory activities (Middleton et al. 2000). The antioxidant properties of flavonoids and their ability to chelate free iron could also effectively reduce the cardiotoxicity of DOX. On the other hand, flavonoids are reported to modulate the CYP3A subfamily and/or P-gp (Lee et al. 1994; Chieli et al. 1995; Di Pietro et al. 2002).

Baicalein are the major flavonoids of *Scutellariae radix* and are mainly present as their glucuronide forms. Baicalein glucuronides can constitute up to 20% of the dry weight of *Scutellariae radix*, respectively (Sagara et al. 1985; Takino et al. 1987). After digestion, the glucuronides are readily hydrolyzed by intestinal bacteria (Manach et al. 1996). The evidence suggests that baicalein and related flavonoids are the major components responsible

for the pharmacological effects of *Scutellariae radix* (Lin and Shieh 1996; Matsuzaki et al. 1996). Baicalein inhibits testosterone 6bβ-hydroxylation (CYP3A4) activity with an IC_{50} of 17.4 μM. Baicalein is the inhibitor of P-gp in the KB/MDR cell system (Lee et al. 2004), but the effect of baicalein on P-gp inhibition **is** partially ambiguous. Thus, we reevaluated P-gp activity using rhodamine-123 retention assay in P-gp-overexpressing MCF-7/ADR cells. The effect of baicalein was similar to that of quercetin (Kitagawa et al. 2005). Baicalein and doxorubicin could be prescribed for the treatment or prevention of cancer as a combination therapy. However, little information is available on the *in vivo* effects of these flavonoids on the pharmacokinetics of drug interaction.

Therefore, the aim of this study was to examine the bioavailability and pharmacokinetics of DOX after oral or intravenous administration of DOX with baicalein in rats.

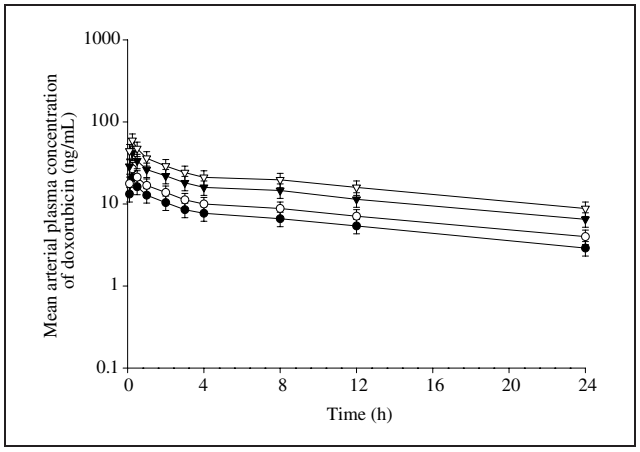

Fig. 1: Mean arterial plasma concentration-time profiles of DOX after oral administration of DOX (50 mg · kg^{-1}) in the presence of 0.3 (○; n = 6), 1.5 (▼; n = 6) and 6 mg · kg^{-1} (▽; n = 6) baicalein or its absence (●; n = 6) to rats. Bars represent standard deviation

2. Investigations and results

The mean plasma concentration-time profiles of DOX following oral administration to rats in the presence or absence of oral baicalein are shown in Fig. 1. The mean pharmacokinetic parameters of DOX are listed in Table 1. As shown in Table 1, baicalein significantly altered the pharmacokinetic parameters of DOX. Compared with the control group (given oral DOX alone), the presence of baicalein significantly ($p < 0.05$ at 1.5 mg · kg^{-1}; $p < 0.01$ at 6 mg · kg^{-1}) increased the area under the plasma concentration-time curve from zero to time infinity (AUC) and the peak concentration (C_{max}) of DOX by 55.1–96.4% and 45.0–80.0%, respectively, and significantly reduced the total body clearance (CL/F) of DOX ($p < 0.05$, 1.5 and 6 mg · kg^{-1}) by 35.5–49.0%. The absolute bioavailability (AB) of DOX was significantly elevated ($p < 0.05$ at 1.5 mg · kg^{-1}; $p < 0.01$ at 6 mg · kg^{-1}) by 3.5–4.4%, compared with the control group (2.2%). The relative bioavailability (RB) of DOX in the presence of baicalein (0.3, 1.5 and 6 mg · kg^{-1}) was 1.20 to 1.96 times higher. There was no significant difference in the time to reach peak concentration (T_{max}) and the terminal half-life ($t_{1/2}$) of DOX in the presence of baicalein.

The mean plasma concentration-time profiles of DOX following intravenous administration to rats in the presence or absence of oral baicalein are illustrated in Fig. 2. The mean pharmacokinetic parameters of DOX are listed in Table 2. The corresponding pharmacokinetic parameters of DOX are listed in Table 2. Compared with the control group, the presence of baicalein increased bioavailability of i.v. DOX, but the pharmacokinetic parameters did not change significantly.

In this study, a cell-based P-gp activity test using rhodamine-123 also showed that a low concentrations range of baicalein (3 and 10 μM) significantly inhibited P-gp activity (Fig. 3).

Table 1: Mean (± S.D.) pharmacokinetic parameters of DOX after oral administration of DOX (50 mg · kg^{-1}) in the presence or absence (control) of baicalein (0.3, 1.5 and 6 mg · kg^{-1}) to rats (n = 6, each)

Parameters	DOX (Control)	DOX + Baicalein		
		0.3 mg · kg^{-1}	1.5 mg · kg^{-1}	6 mg · kg^{-1}
AUC (ng · h · mL^{-1})	167 ± 41.1	201 ± 56.2*	250 ± 84.6**	328 ± 107**
C_{max} (ng · mL^{-1})	20.0 ± 4.30	24.1 ± 5.60	29.2 ± 8.82*	36.4 ± 12.3**
T_{max} (h)	0.25	0.25	0.25	0.25
CL/F (mL · min^{-1} · kg^{-1})	4.98 ± 0.98	4.15 ± 0.85	3.21 ± 0.76*	2.54 ± 0.54*
$t_{1/2}$ (h)	12.1 ± 2.90	12.6 ± 3.11	13.7 ± 3.12	14.4 ± 3.17
AB (%)	2.2 ± 0.55	2.7 ± 0.74*	3.5 ± 0.87**	4.4 ± 0.13**
RB (%)	100	120	155	196

* $p < 0.05$, ** $p < 0.01$ significant difference compared with the control
AUC: area under the plasma concentration-time curve from zero to time infinity; C_{max}: peak concentration; T_{max}: time to reach peak concentration; CL/F: total body clearance; $t_{1/2}$: the terminal half-life; AB (%): absolute bioavailability; RB (%): relative bioavailability

Table 2: Mean (± S.D.) pharmacokinetic parameters of DOX after intravenous administration of DOX (10 mg · kg^{-1}) in the presence or absence (control) of baicalein (0.3, 1.5 and 6 mg · kg^{-1}) to rats (n = 6, each)

Parameters	DOX (Control)	DOX + Baicalein		
		0.3 mg · kg^{-1}	1.5 mg · kg^{-1}	6 mg · kg^{-1}
AUC (ng · h · mL^{-1})	1492 ± 312	1719 ± 377	1830 ± 397	1897 ± 416*
CL_t (mL · min^{-1} · kg^{-1})	112 ± 25.2	97.0 ± 19.6	91.0 ± 14.4	88.0 ± 13.7
$t_{1/2}$ (h)	7.9 ± 1.7	8.1 ± 1.8	8.3 ± 1.9	8.5 ± 2.0

* $p < 0.05$, significant difference compared with the control
AUC: area under the plasma concentration-time curve from zero to infinity; CL_t: total body clearance; $t_{1/2}$: terminal half-life

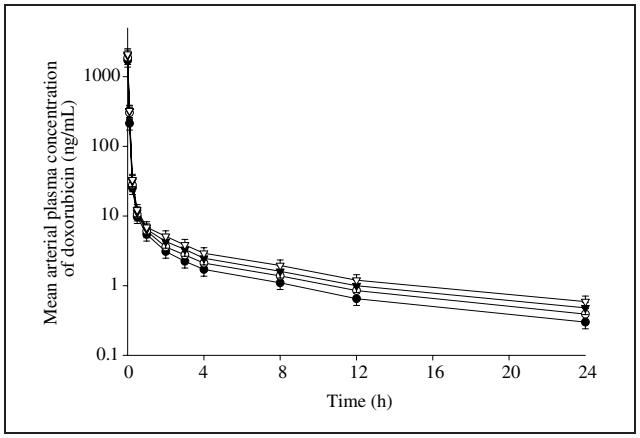

Fig. 2: Mean arterial plasma concentration-time profiles of DOX after intravenous administration of DOX ($10\ mg \cdot kg^{-1}$) in the presence of 0.3 (○; n = 6), 1.5 (▼; n = 6) and $6\ mg \cdot kg^{-1}$ (▽; n = 6) baicalein or its absence (●; n = 6) to rats. Bars represent standard deviation

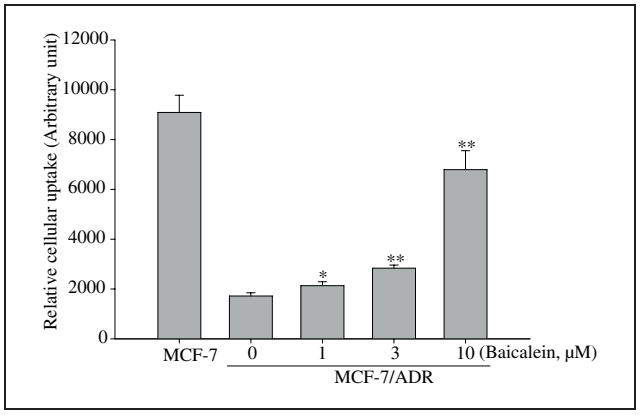

Fig. 3: Rhodamine-123 (R-123) retention. MCF-7/ADR cells were preincubated with baicalein for 24 h. After incubation of MCF-7/ADR cells with 20 μM R-123 for 90 min, the R-123 fluorescence values in cell lysates were measured using excitation and emission wavelengths of 480 and 540 nm, respectively. The values were divided by total protein content in each sample. Data represents mean ± SD of 6 separate samples (significant versus control MCF-7 cells, * $p < 0.05$, ** $p < 0.01$)

3. Discussion

With the great interest in herbal products as alternative medicines, much effort is currently being expended to identify natural compounds from plant origins that modulate P-gp metabolic enzymes. However, there is far less information on the pharmacokinetic interactions between herbal products and anticancer agents. Therefore, more preclinical and clinical investigations on herbal constituent-drug interactions should be performed to prevent potential adverse reactions or to use such interactions for therapeutic benefits. We investigated the possible modulation effect of flavonoids and their synthetic derivatives on DOX metabolism and transport as important mechanisms of DOX toxicity in various model systems. Therefore, we evaluated the effects of baicalein, a naturally occurring flavonoid, on the bioavailability of DOX in rats, in order to examine possible drug interactions between baicalein and DOX via the dual inhibition of the CYP3A subfamily and P-gp by baicalein.

Based on the broad overlap in their substrate specificities as well as their co-localization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A and P-gp have been recognized as a concerted barrier to drug absorption (Cummins et al. 2002; Benet et al. 2003). Therefore, dual inhibitors against both CYP3A and P-gp could greatly impact the bioavailability of many drugs.

CYP3A subfamily and P-gp inhibitors might interact with DOX and substantially alter its pharmacokinetic parameters. Since cyclosporin and verapamil, both substrates for CYP3A, increase DOX plasma concentrations, it is possible that one or more enzymes of the CYP3A subfamily play a role in DOX metabolism (Kivistö et al. 1995).

As listed in Table 1, the presence of baicalein significantly increased the AUC and C_{max} of oral DOX. These results were similar to those of Buening et al. (1981) and Zhang and Morris (2003), who suggested that the presence of morin might inhibit the CYP3A and the P-gp pathway because orally administered DOX is a substrate for P-gp-mediated efflux and metabolized by the CYP3A subfamily in the intestine and/or liver. Baicalein might improve the oral bioavailability of DOX by altering its absorption pattern or reducing the gut wall metabolism of this drug.

As listed in Table 2, the presence of baicalein had no effect on pharmacokinetic parameters of i.v. DOX, although the AUC of i.v. DOX increased with increasing oral baicalein doses. This suggests that the inhibition of DOX metabolism via the CYP3A subfamily is not considerable.

Collectively, the bioavailability of oral DOX was significantly increased by the concomitant use of baicalein via the inhibition of P-gp mediated efflux and first-pass metabolism of DOX in the intestine and/or liver. This result suggests that the development of oral DOX preparations combined with baicalein is feasible, that would be more convenient than the i.v. dosage forms.

Furthermore, since the present study raises the awareness of the potential drug interactions by concomitant use of DOX with baicalein, the dosage regimen of DOX should be taken into consideration, if this result is confirmed in clinical studies.

Baicalein enhanced the oral bioavailability of DOX, which might be attributed to the promotion of intestinal absorption and a reduction of the first-pass metabolism of DOX. This result may suggest that the development of oral DOX preparations in combination with baicalein is feasible that would be more convenient than the i.v. dosage forms.

4. Experimental

4.1. Chemicals

DOX was obtained from Boryung Pharmaceutical Co. (Seoul, Republic of Korea). Baicalein and daunorubicin [internal standard for the high-performance liquid chromatographic (HPLC) analysis of DOX] were purchased from Sigma–Aldrich Co. (St. Louis, MO). Other chemicals were of reagent or HPLC grade.

4.2. Animals

Male Sprague-Dawley rats, 7–8 weeks old and weighing 270–300 g, were purchased from the Dae Han Laboratory Animal Research Company (EumSung, Republic of Korea) and given free access to a commercial rat chow diet (No. 322-7-1; Superfeed Company, Wonju, Republic of Korea) and tap water. They were maintained in a clean room (College of Pharmacy, Chosun University) at a temperature of 22 ± 2 °C with 12 h light and dark cycles and a relative humidity of 50–60%. The rats were acclimated under these conditions for at least 1 week. All protocol in this animal study were approved by the Animal Care Committee of Chosun University (Gwangju, Republic of Korea). Each rat was fasted for at least 24 h prior to the start of the experiment. The left femoral artery (for blood sampling) and the left femoral vein (for drug administration in the intravenous study only) were cannulated using polyethylene tube (SP45; i.d., 0.58 mm, o.d., 0.96 mm; Natsume Seisakusho Co. Ltd, Tokyo, Japan) while each rat was under light ether anesthesia.

4.3. Intravenous and oral administration of DOX

Rats were randomly divided into two groups (n = 6, each): the oral group [50 mg (5 mL) · kg^{-1} of DOX dissolved in distilled water] without (control) or with 0.5, 3 and 10 mg · kg^{-1} of oral baicalein (mixed in distilled water; total oral volume of 3.0 mL · kg^{-1}) and the intravenous group (10 mg · kg^{-1} of DOX dissolved in 0.9% NaCl solution; total injection volume of 1.5 mL · kg^{-1}). A feeding tube was used for oral administration of DOX and baicalein. Baicalein was administered 30 min prior to oral administration of DOX. A blood sample (0.45 mL) was collected into a heparinized tube via the femoral artery at 0 (control), 0.017 (end of infusion), 0.1, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h for intravenous study, and at 0, 0.1, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 12 and 24 h for oral study. Whole blood (approximately 1 mL) collected from untreated rats was infused via the femoral artery at 0.25, 1, 3, 8 and 12 h, respectively, to replace blood-loss due to blood sampling. The blood samples were centrifuged (13,000 rpm, 5 min), and a 200 µl aliquots of plasma samples were stored at −40 °C until the HPLC analysis.

4.4. HPLC analysis of DOX

The HPLC assay of Andersen et al. (1993) was used to analyze DOX levels, with minor modifications. Briefly, a 50 µl aliquot of daunorubicin (1 µg · mL^{-1}; internal standard), and 1 mL of acetonitrile was added to each 200 µl sample to precipitate proteins and extract DOX. The mixture was then stirred for 2 min and centrifuged (13,000 rpm, 10 min). A 0.8 mL aliquot of the upper layer was transferred to another clean microtube, and then evaporated under a gentle stream of nitrogen gas at 38 °C. The residue was reconstituted in 200 µl of mobile phase prior to injection (50 µl) onto a C_{18} reversed phase column (ODS ThermoHypersil; 4.6 mm, i.d. × 150 mm; 5.0 µm; Thermo Electron Co., MA, USA). The mobile phase consisted of 20 mM phosphate buffer (pH 3.8) : acetonitrile : methanol (45 : 20 : 35; v/v/v). The flow-rate of the mobile phase was 1.0 mL · min^{-1} and the column eluent was monitored using a fluoresence detector at an excitation wavelength of 460 nm with an emission cut-off filter of 580 nm. The retention times of DOX and daunorubicin (an internal standard) were approximately 3.5 and 5.8 min, respectively. The detection limit of DOX in rat plasma was 2 ng · mL^{-1}. The intra- and inter-day coefficients of variation of DOX were below 11.3%.

4.5. Pharmacokinetic analysis

The following pharmacokinetic data were analyzed using the non-compartmental method (WinNonlin software version 4.1; Pharsight Corporation, Mountain View, CA, USA). The 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 DOX were directly read from the experimental data. 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}. The total body clearance for intravenous (CL_t) and oral administration (CL/F) was calculated from the quotient of the dose (D) and $AUC_{0-\infty}$. The absolute bioavailability (AB) was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral} \times 100$, and the relative bioavailability (RB) was estimated by $AUC_{with\ baicalein}/AUC_{control} \times 100$.

4.6. Rhodamine-123 retention assay

MCF-7/ADR cells were seeded in 24-well plates. 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 in the presence or absence of baicalein (1, 3 and 10 µM) for 90 min, the medium was completely removed. The cells were then washed three times with ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. The rhodamine-123 fluorescence 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 presented as the ratio to control.

4.7. Statistical analysis

A p-value < 0.05 was deemed to be statistically significant using the Duncan's multiple range test of Statistical Package of Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) program among the three means for the unpaired data. All data are expressed as mean ± standard deviation.

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