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THE EFFECT OF KAEMPFEROL AND MORIN ON THE BIOAVAILABILITY OF TAMOXIFEN IN RATS

조선대학교 대학원

식품의약학과

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이 논문을 약학박사학위신청 논문으로 제출함.

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ABSTRACT

THE EFFECT OF KAEMPFEROL AND MORIN ON THE BIOAVAILABILITY OF TAMOXIFEN IN RATS

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Multidrug resistance (MDR) proteins as well as drug metabolism catalyzed by phase I and phase II enzymes play a major role in the first-pass extraction that contribute to the low oral bioavailability of many anticancer agents. Tamoxifen is a substrate of MDR transporters, specifically P-gp, and the phase I and II metabolizing enzymes, specifically CYP3A. Some natural flavonoids were suggested to inhibit CYP3A and P-gp in vitro. If they do not elicit toxicity or side effects, flavonoids might have some advantages in improving the oral bioavailability of many anticancer agents via the dual inhibition of CYP3A and P-gp. Therefore, the present study investigated the effect of two flavonoids (flavonols), kaempferol and morin, on the pharmacokinetics of tamoxifen and one of its metabolite, 4-hydroxytamoxifen, in rats. A single dose of tamoxifen was administered intravenously (2 mg/kg), and orally (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) or morin (3 and 10 mg/kg). Plasma concentration of tamoxifen was determined by HPLC equipped with fluorescence detector.

Following an oral administration of tamoxifen, the presence of flavonoids (i.e., kaempferol and morin) significantly altered the pharmacokinietics of tamoxifen.

Compared to the oral control group (given tamoxifen alone), the presence of kaempferol and morin significantly ($p < 0.05$) reduced the total body clearance (CL/F) of tamoxifen, followed with the significantly ($p < 0.05$) increased the area under the plasma concentration-time curve ($AUC_{0-\infty}$) and peak plasma concentration (C_{max}) of tamoxifen. Consequently, the absolute bioavailability (AB) of tamoxifen in the presence of flavonoids was elevated significantly ($p < 0.05$) compared to that from the control group. Relative bioavailability (RB) of tamoxifen was increased approximately 1.5-fold in the presence of kaempferol or morin. The enhanced bioavailability of tamoxifen is likely to be mainly due to the decreased first-pass extraction in the intestine and liver. The presence of kaempferol and morin did not show significance in the time to reach C_{max} (T_{max}) and the terminal half-life ($t_{1/2}$) of tamoxifen.

The presence of the two flavonoids reduced the CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, but not significantly except for morin at a dose of 10 mg/kg ($p < 0.05$). The metabolite-parent ratio (MR) of 4-hydroxytamoxifen was not changed significantly by the flavonoids, suggesting that the flavonoids do not inhibit the production of the active metabolite, 4-hydroxytamoxifen considerably.

In summary, the presence of kaempferol and morin enhanced the oral bioavailability of tamoxifen and did not affect the $AUC_{0-\infty}$ of 4-hydroxytamoxifen considerably in the present study. Kaempferol and morin as the nature flavonoids with many health-benificial activities and non-consistent side-effect might be effective MDR modulators to improve the bioavailability of tamoxifen in humans. These results also suggest that the concomitant use of these flvonoids or dietary supplements containing these flavonoids with tamoxifen should require close monitoring for potential diet-drug interactions.

Key words: tamoxifen, flavonoids, kaempferol, morin, P-glycoprotein (P-gp), CYP3A, pharmacokinetics, bioavailability, rats.

국 문 초 록

켐페롤과 모린이 타목시펜의 생체이용율에 미치는 영향

설 효 찬

지 도 교 수: 최 준 식

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많은 항암약물들은 경구투여 하였을 때 생체내이용률이 낮아서 경구투여가 불가능하다. 소장과 간에 존재하는 1 상 및 2 상반응의 대사효소(특히 CYP3A) 및 ATP-binding cassette (ABC) 수송체(특히 P-당단백질)등의 상가작용으로 인한 초회통과효과가 낮은 생체내이용률을 초래하는 주요원인이다. 타목시펜은 P-당단백질와 CYP3A 의 기질물질이다. 시험관실험에서 프라보노이드류인 켐페롤과 모린은 P-당단백질과 CYP3A 에 대한 억제작용이 있는 것으로 보고되었다. 따라서, 본 실험에서는 흰쥐에게 타목시펜을 켐페롤 혹은 모린과 함께투여시켰을 때 타목시펜과 그의 활성 대사체인 4-hydroxytamoxifen 의 약물동태에 미치는 영향을 연구하였다. 실험동물(웅성 Sprague-Dawley 흰쥐)을 각각 6 개의 실험군으로 분류하여 1 군과 2 군에는 타목시펜을 단독 정맥(2 mg/kg) 또는 경구(10 mg/kg) 투여하였으며 나머지 설험군에는 켐페롤(3 및 10 mg/kg) 혹은 모린(3 및 10 mg/kg)을 각각 경구투여한 후, 30 분후에 타목시펜을 경구투여 하였다. 혈장중 타목시펜의 농도를 형광검출기를 사용한 HPLC 에서 측정하였다.

프라보노이드류를 전투여하였을 때 타목시펜의 약물동태학적 파라미터는 유의성 있게 변화되었다. 대조군에 비해 전투여군에서 타목시펜의 전신클리어런스(CL/F)는 유의성($p<0.05$) 있게 감소되었고, 혈장농도곡선하 면적($AUC_{0-\infty}$)과 최고혈중농도(C_{max})도 유의성($p<0.05$) 있게 증가되었다. 절대적 생체이용률(AB)도 대조군에 비해 전투여군에서 유의성($p<0.05$) 있게 증가되었으며, 상대적생체이용률(RB)은 약 1.5 배로 증가되었다. 소실반감기($t_{1/2}$)와 최고혈중농도도달시간(T_{max})은 유의성 있게 변화되지 않았다.

프라보노이드류인 켐페롤과 모린을 전투여하였을 때 타목시펜의 대사물질인 4-hydroxytamoxifen 의 약물동태학적 파라미터는 다음과 같았다. 10 mg/kg 모린 전투여군외 기타 전투여군에서는 CL/F 는 감소되었고 $AUC_{0-\infty}$는 증가되었으나 유의성은 없었다. 대사체와 모체의 $AUC_{0-\infty}$비율(MR)도 감소되었으나 유의성 있는 변화는 없었다. 따라서, 켐페롤과 모린은 타목시펜의 4-hydroxytamoxifen 으로의 대사과정에 현저한 영향을 미치지는 않는 것으로 보인다.

본 연구에서 프라보노이드류인 켐페롤과 모린를 함께투여 시켰을 때 경구투여시킨 타목시펜의 생체이용률은 현저히 높아졌으나, 약물활성이 타목시펜에 비해 수십배로 강한 4-hydroxytamoxifen 의 생성에 대하여는 현저한 영향을 주지 않았다. 본 연구결과를 토대로, 천연음식물에 흔히 존재하는 프라보노이드류들은 타목시펜과 함께 섭취할 때 식품과 약물간에 상호작용을 일으킬 가능성이 있는 것으로 사료된다.

Part I. The effect of kaempferol on the bioavailability of tamoxifen in rats

Abstract

Kaempferol is supposed to inhibit CYP3A and P-gp in vitro. This study investigated the effect of kaempferol on the pharmacokinetics of tamoxifen and its metabolite, 4-hydroxytamoxifen in rats. A single dose of tamoxifen was administered intravenously (2 mg/kg) to the male Sprague-Dawley rats, and orally (10 mg/kg) in the presence or absence of kaempferol (3 or 10 mg/kg). Plasma concentration of tamoxifen was determined by HPLC equipped with fluorescence detector.

The presence of kaempferol (3 and 10 mg/kg) significantly altered the pharmacokinetics of orally administered tamoxifen. Compared to the oral control group (given tamoxifen alone), the CL/F of tamoxifen reduced significantly ($p < 0.05$, 28.6-32.4%), and the $AUC_{0-\infty}$ of tamoxifen increased significantly ($p < 0.05$, 39.9-48.1%). AB of tamoxifen in the presence of kaempferol was 33.5-35.5%, which was enhanced significantly ($p < 0.05$) compared to the oral control group (23.9%), and RB of tamoxifen elevated 1.39- to 1.48-fold. The enhanced bioavailability of tamoxifen is mainly due to the decreased first-pass effect in the intestine and liver.

In regarding of the active metabolite, 4-hydroxytamoxifen, even if the presence of kaempferol decreased the CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, it was not significant. The MR of 4-hydroxytamoxifen was not altered significantly, implying orally administered kaempferol cannot affect the production of 4-hydroxytamoxifen considerably.

The enhanced oral bioavailability of tamoxifen was observed in this study in

combination of kaempferol. Kaempferol might be an effective modulator in improving the oral bioavailability of tamoxifen in humans. The pharmacokinetic interaction between tamoxifen and kaempferol and the dietary complements containiny kaempferol should be taken into consideration in the clinical setting to avoid the toxic reactions of tamoxifen.

Key words: tamoxifen, kaempferol, P-glycoprotein (P-gp), CYP3A, pharmacokinetics, bioavailability, rats.

1. Introduction

Oral administration of drugs has many advantages over intravenous injection because it is less invasive, easier to use for the patient in a chronic regimen and more cost-effective because of the decreased hospitalization. The bioavailability of many orally administered anticancer drugs are low, which contribute to the first-pass extraction mediated by multidrug resistance (MDR) transporters and phase I and phase II metabolizing enzymes in the intestine and liver. MDR is a term used to describe the phenomena that tumor cells resistant a number of structurally and functionally unrelated chemotherapeutic agents. The transport-mediated MDR is caused by the ATP-binding cassette (ABC) family membrane transport ATPases. P-glycoprotein (P-gp), an important member of ABC family, expresses highly in solid tumours of epithelial origin, such as the colon (Cordon-Cardo et al., 1990), kidney (Fojo et al., 1987), and breast (Merkel et al., 1989) to efflux substrates out of cells. MRPs, another important member of the ABC family, has been described as a Vanadate-sensitive magnesium-dependent glutathione S-conjugate (GS-conjugate) ATPase (GS-X pump) capable of transporting organic anion drug conjugates as well as intact anticancer drugs (Grant et al., 1994; Borst et al., 1997). Several isoforms of MRPs have been identified and the mainly mentioned are MRP1 and MRP2 (cannalicular multispecific organic anion transporter, cMOAT), which have been identified as organic anion transporters (Borst et al., 1997).

P-gp and MRPs are present widely in many normal excretory organs, such as the liver, kidney and intestine, where they provide a barrier to eliminate the substrates out of the body. P-gp, and MRP2 co-localized to the apical membrane of the intestine, liver, kidney, and blood–brain barrier (Thiebaut et al., 1987; Buchler et al., 1996; Fromm et al., 2000; Schaub et al., 1999), and MRP1 is localized to the basolateral membranes of polarized epithelial cells of the intestinal crypt (Peng et al., 1999), renal distal and collecting tubules (Peng et al., 1999), and liver (Mayer et

al., 1995; Roelofsen et al., 1997).

P-gp and MRPs are co-localized with phase I and Phase II metabolizing enzymes CYP 3A4, UDP-glucuronosyltransferases and Glutathione-*S*-transferases in the liver, kidney and intestine (Sutherland et al., 1993; Turgeon et al., 2001). The CYP3A subfamily was reportedly involved in approximately 40–50% of Phase I metabolism of marketing drugs (Guengerich, 1995). Specifically, CYP3A4 accounts for 30% of hepatic CYP and 70% of small intestinal CYP (Schuetz et al., 1996). A substantial overlap in substrate specificity exists between CYP3A4 and P-gp (Wacher et al., 1995). Thus, a synergistic relationship exists between the transporters and metabolizing enzymes, such as CYP3A4 versus P-gp and conjugating enzymes versus MRP2, within excretory tissues to protect the body against invasion by foreign compounds, which also decrease the oral bioavailability of many drugs, especially anticancer drugs.

Since MDR is one of the main obstacles to successful chemotherapy of cancer, a number of biochemical, pharmacological and clinical strategies have been devised to overcome it. The first generation of MDR modulators including the calcium channel blockers, such as verapamil, felodipine and diltiazem), quinolines, cyclosporines (Cyclosporin A) (Ford and Hait, 1990; Ford and Hait, 1993) are therapeutic agents. They could reverse MDR at concentrations much higher than those required for their individual therapeutic activity, which resulted in unnecessary adverse effects and toxicities. The second generation of modulators is more potent and less toxic, such as the analogs of verapamil, dexverapamil (less cardiotoxic *R*-enantiomer of verapamil), the non-immunosuppressive analog of Cyclosporin A, PSC 833 and so on. Although these agents appear to be well tolerated in combination with anticancer drugs, it is need to be determined whether these compounds are suitable for clinical application in anticancer therapy.

Tamoxifen is a nonsteroidal antiestrogen and is the agent of choice for treating and preventing breast cancer (Powles, 1992; Stone, 1992; Jaiyesimi et al., 1995).

Tamoxifen has a relatively low toxicity and is less harmful than most chemotherapeutics. The main adverse effects of tamoxifen in humans are that there might be an increased risk of endometrial cancer and thromboembolic diseases (Fornander et al., 1993; Meier and Jick, 1998). Orally administered tamoxifen undergoes extensive hepatic metabolism and the subsequent biliary excretion of its metabolites (Buckley and Goa, 1989). Demethylation of the aminoethoxy side chain to *N*-desmethyltamoxifen is the main route of tamoxifen metabolism (Lonning et al., 1992; Stearns et al., 2003). Tamoxifen and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 (Desta et al., 2004). The major primary metabolite, *N*-desmethyltamoxifen, was catalyzed by CYP3A4/5, and the minor metabolite, 4-hydroxytamoxifen, catalyzed by CYP2D6 CYP2C9 and CYP3A (Mani et al., 1993; Crewe et al., 1997) (Figure 1). Other minor primary metabolites include α-, 3-, and 4'-hydroxytamoxifen and one unidentified metabolite (M-I) were primarily catalyzed by CYP3A4, CYP2B6/2C19, and CYP3A5, respectively. Tamoxifen secondary metabolism showed that *N*-desmethyltamoxifen was predominantly biotransformed to α-hydroxy *N*-desmethyl-, *N*-didesmethyl-, and 4-hydroxy *N*-desmethyl-tamoxifen (endoxifen), whereas 4-hydroxytamoxifen was converted to 3,4-dihydroxytamoxifen and endoxifen (Figure 2). Except for the biotransformation of *N*-desmethyltamoxifen to endoxifen, which was exclusively catalyzed by CYP2D6, other routes of *N*-desmethyl- and 4-hydroxytamoxifen biotransformation were catalyzed predominantly by the CYP3A subfamily. 4-hydroxytamoxifen is a relatively minor metabolite, but it has been studied by a number of investigators because it is a more potent antiestrogen than tamoxifen (Jordan et al., 1977; Borgna and Rochefort, 1981; Coezy et al., 1982; Robertson et al., 1982). Jordan et al. (1997; 1982) demonstrated that high first-pass metabolism of tamoxifen results in a significant increase in its activity and characterized the first active primary metabolite, 4-hydroxy-tamoxifen. Although the plasma and tumor concentrations of 4-hydroxytamoxifen are only about 2% of

those of the parent compound (Daniel et al., 1981), it has been shown to possess a high affinity for estrogen receptors and 30- to 100-fold more potency than tamoxifen in suppressing estrogen-dependent cell proliferation (Borgna and Rochefort, 1981; Robertson et al., 1982; Coezy et al., 1982; Jordan, 1982). A secondary metabolite of tamoxifen, endoxifen, exhibits potency similar to 4-hydroxytamoxifen with respect to estrogen receptor binding affinity, suppression of estrogen-dependent cell growth, and gene expression (Stearns et al., 2003; Johnson et al., 2004). So, tamoxifen has been referred to as a prodrug that requires activation to exert its effects.

Since tamoxifen and its metabolites, *N*-desmethyltamoxifen and 4-hydroxytamoxifen, are substrates for the efflux of P-gp and MRP2 (Rao et al., 1994; Gant et al., 1995, Sugimoto et al., 2003; Kauffmann et al., 1998), the MDR modulators might be able to improve the bioavailabilty of tamoxifen. As upper mentioned, although some P-gp modulators such as verapamil, cyclosporine A and PSC 833 have been proved to be the potent P-gp inhibitors *in vitro*, but their toxicities have hindered their use in clinical application (Bradshaw and Arceci, 1998). Some flavonoids as natural products were shown to be modulators of the MDR transporters (Castro and Altenberg, 1997; Scambia et al., 1994) and phase I and phase II metabolizing enzymes (Tsyrlov et al., 1994; Walle et al., 1995). They have many health promoting benefits and have no consistent side effects, it could be expected as one of the nature members to improve the bioavailability of the anticancer drugs.

Flavonoids are widely distributed in dietary supplements such as vegetables, fruit, tea and wine (Hertog et al., 1993b). Flavonoids have many beneficial effects including antioxidant, antibacterial, antiviral, antiinflammatory, antiallergic, and anticarcinogenic actions (Ross and Kasum, 2002; Hodek et al., 2002) though whether these effects can be attributed to the aglycone forms or their metabolites is not entirely clear. High-level consumption of flavonoids was found to associate

with an overall lower risk of getting a wide variety of cancers (Knekt et al., 1997). Epidemiological studies also support an association between dietary flavonoids and a reduced risk of certain cancers, such as stomach carcinoma and lung cancer (Dorant et al., 1996; Knekt et al., 1997). Proposed mechanisms for anticancer benefits include numerous effects on signal transduction pathways involved in cell proliferation (Weber et al., 1996; Lepley and Pelling, 1997) and angiogenesis (Fotsis et al., 1997), as well as inhibition of enzymes involved with procarcinogen bioactivation such as cytochrome P450 (Tsyrlov et al., 1994) and sulfotransferase enzymes (Walle et al., 1995). A high dietary intake of flavonols could reduce the incidence of cardiovascular disease (Hertog et al., 1993b; Knekt et al., 1996). Some flavonoids, such as quercetin, protect LDL cholesterol from oxidative damage. Others, such as the anthocyanidins from bilberry, purple cabbage, and grapes, may help protect the lens of the eye from cataracts. Animal research suggests that naringenin, found in grapefruit, may have anticancer activity (So et al., 1996). The total daily intake of flavonoids via the dietary supplements has been 23 mg/day in Dutch population (Hertog et al., 1993a).

Several studies have shown that flavonoids can modulate the activities of both P-gp and MRP1 (Zhang and Morris, 2003a; Bobrowska-Hagerstrand et al., 2003) affecting drug accumulation, cell viability following cytotoxic drug exposure, and the ATPase activity of P-gp (Bobrowska-Hagerstrand et al., 2003). Some flavonoids have been reported to interact with the intrinsic ATPase of P-gp, both inhibition and stimulation of P-gp ATPase activity have been observed for silymarin, morin, and biochanin A (Zhang and Morris, 2003b). Some flavonoids reduced the secretory flux of talinolol across Caco-2 cells, such as hesperetin, quercetin, kaempferol, spiraeoside, isoquercitrin and naringin, but none of the selected flavonoids was able to replace [^{3}H]talinolol from its binding to P-gp, which might be due to an interaction with P-gp without competition of the talinolol binding site of P-gp (Ofer et al., 2005). Several flavonoids, specifically

methoxylated flavonoids, are confirmed to be the good inhibitors of MRP1 and 2 (van Zanden et al., 2005).

The flavonol kaempferol (3,4',5,7-tetrahydroxyflavone), like its hydroxylated analog quercetin, is most widely distributed in onions, broccoli and black tea etc. The epidemiological study showed that ingestion of the high dose of kaempferol correlated to a reduced risk of cerebrovascular disease (Knekt et al, 2002). Everyday intake of kaempferol in humans in USA and Netherland estimated to be 6-10 mg, and it accounts for 25-33% of mean total flavonol intake in humans (Hertog et al, 1993a; Sampson et al, 2002). Due to the high degree of structural similarity between kaempferol glucosides and quercetin glucosides, kempferol glucosides are almost certainly absorbed from the small intestine as quercetin glucosides by active transport by sodium glucose cotransporter (SGLT1) (Gee et al, 2000) and deglycosylation processes catalyzed by lactase phlorizin hydrolase and cytosolic β-glucosidase (Day et al, 2000b, 2003; Nemeth et al, 2003). Kaempferol-3-glucoside is a substrate for lactase phlorizin hydrolase, whereas kaempferol-3-(6-malonyl)-glucoside and kaempferol-3-glucuronide are poor substrates for lactase phlorizin hydrolase (Nemeth et al, 2003). It is unlikely that kaempferol-3-glucuronide is absorbed directly from the jejunum, as the compound is relatively hydrophilic and will be charged at the pH of the small intestine, which reducing the ability of the compound to diffuse across the biological membrane. Endive is a rich source of kaempferol, containing up to 246 mg/kg in fresh weight (DuPont et al, 2000). Kaempferol mainly presents in endive as kaempferol-3-glucuronide rather than a glycoside. Administration of endive account for 9 mg of Kaempferol showed T_{max} at 5.8 h, suggests absorption occurs at the distal section of the small intestine and/or the colon (DuPont et al., 2004) where microbial degradation of kaempferol-3-glucuronide might occur. By administration of endive, a high level of nonconjugated kaempferol was detected in the plasma and urine. Kaempferol-3-glucuronide was shown to be a substrate for human recombinant β-glucuronidase

(O'Leary et al, 2001). The catalytic efficiency of the enzyme was higher for kaempferol-3-glucuronide compared with quercetin-3-glucuronide since no unconjugated quercetin was detected in plasma or urine of volunteers fed considerable amounts of quercetin from onions (Day et al, 2001). The chemical structures of some flavonols were shown in Figure 4.

Kaempferol was a substrate of P-gp (Wang et al., 2005) and it also inhibited P-gp activity (Romiti et al., 2004). Kaempferol and its hydroxylated analog quercetin exhibited a remarkable inhibition of P-gp-mediated efflux of ritonavir by increasing its cellular uptake, which were comparable with the inhibitory effect of quinidine, a well-known inhibitor of P-gp (Patel et al., 2004). Kaempferol and quercetin also caused substantial inhibition of CYP3A4-mediated cortisol metabolism with 89.7% and 90.1% of intact cortisol, respectively, compared with 45.9% in the control (Patel et al., 2004). Kaempferol reduced the efflux of talinolol across Caco-2 cells without replacement of talinolol from its binding to P-gp (Ofer et al., 2005).

Orally administered kaempferol, as the P-gp and CYP3A inhibitor, might affect the pharmacokinetics of tamoxifen. This study investigated the effect of kaempferol administration on the pharmacokinetics of tamoxifen and its metabolite, 4-hydroxytamoxifen, in rats.

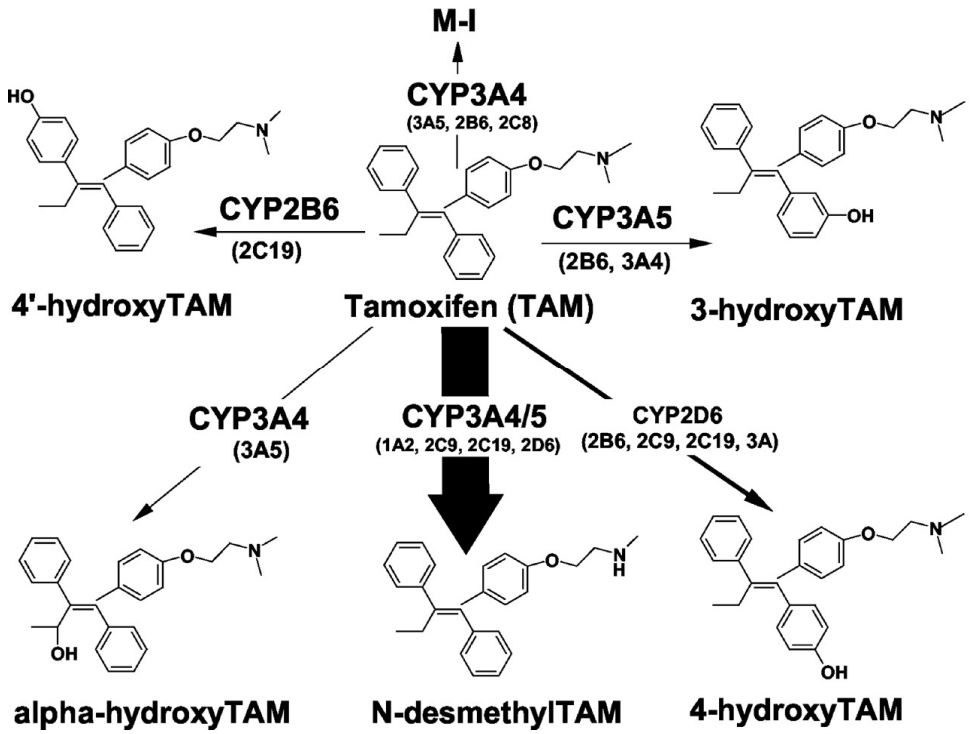


Figure 1. Proposed in vitro biotransformation pathways of tamoxifen to its primary metabolites and the P450s involved. The relative contribution of each pathway to the overall oxidation of tamoxifen is shown by the thickness of the arrow, and the principal P450 isoforms responsible are highlighted in larger fonts and in bold. M-I, unidentified primary metabolite (Cited from Desta et al., 2004).

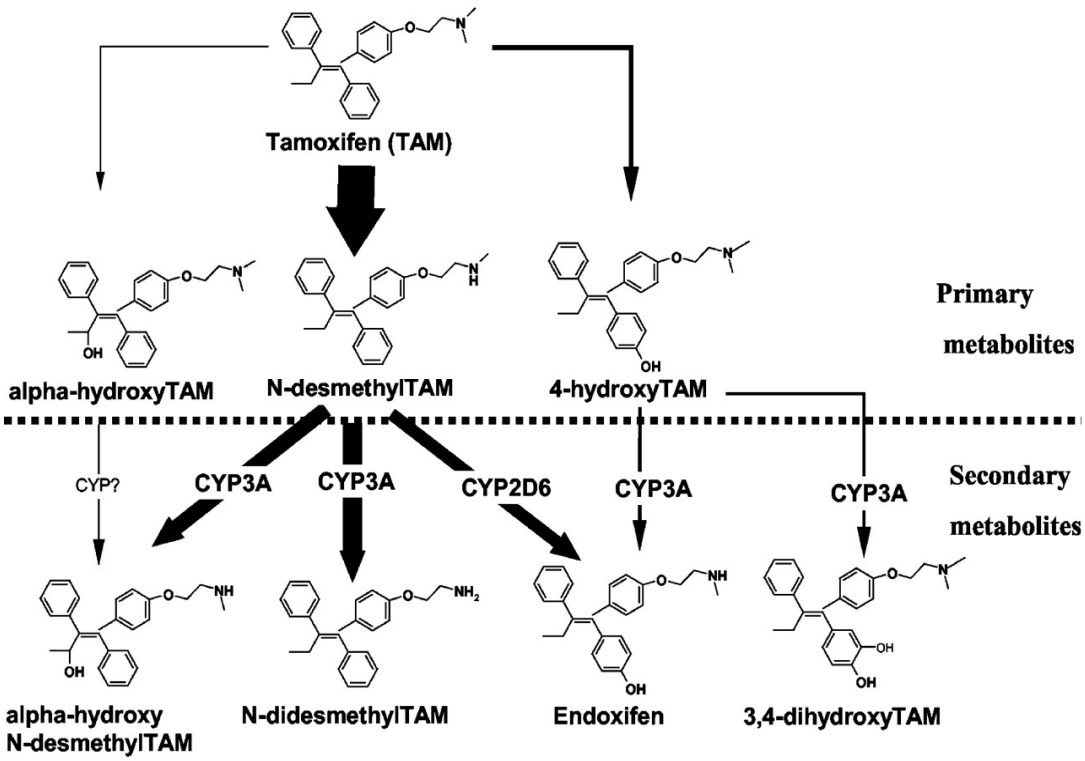
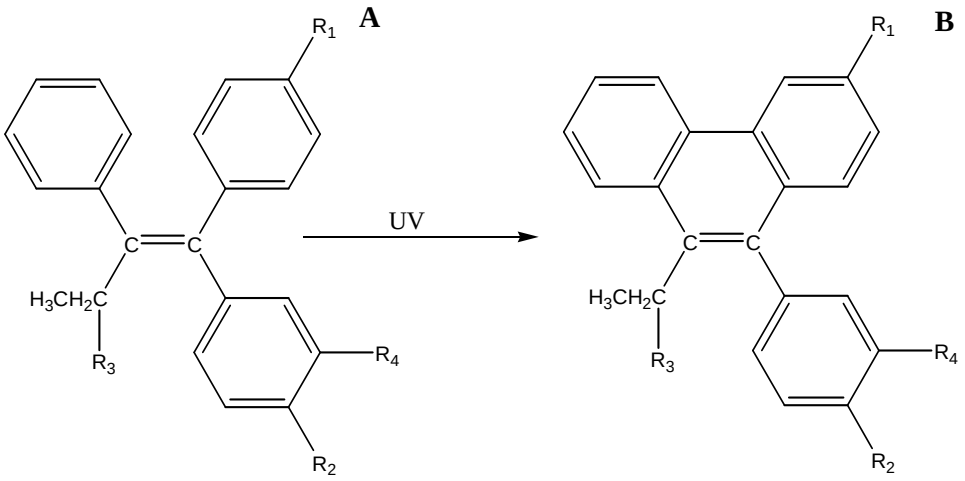


Figure 2. Proposed in vitro biotransformation pathways of tamoxifen to its secondary metabolites and the P450 isoforms involved. The thickness of each arrow indicates the relative contribution of each pathway to the formation of a specific metabolite. The principal enzymes responsible are highlighted in larger fonts and in bold (Cited from Desta et al., 2004).



	R_1	R_2	R_3	R_4
Tamoxifen	$OCH_2CH_2N(CH_3)_2$	H	H	H
Primary metabolites				
N-desmethyltamoxifen	$OCH_2CH_2NHCH_3$	H	H	H
4-hydroxytamoxifen	$OCH_2CH_2N(CH_3)_2$	OH	H	H
alpha-hydroxytamoxifen	$OCH_2CH_2N(CH_3)_2$	H	OH	H
4'-hydroxytamoxifen	$OCH_2CH_2N(CH_3)_2$	H	H	OH
Secondary metabolites				
N-desdimethyltamoxifen	$OCH_2CH_2NH_2$	H	H	H
Endoxifen	$OCH_2CH_2NHCH_3$	OH	H	H
alpha-hydroxy N-desmethyltamoxifen	$OCH_2CH_2NHCH_3$	H	OH	H
3,4-dihydroxytamoxifen	$OHCH_2CH_2N(CH_3)_2$	OH	H	H

Figure 3. Chemical structures of tamoxifen and its metabolites (A) along with their fluorescent phenanthrene derivatives (B).

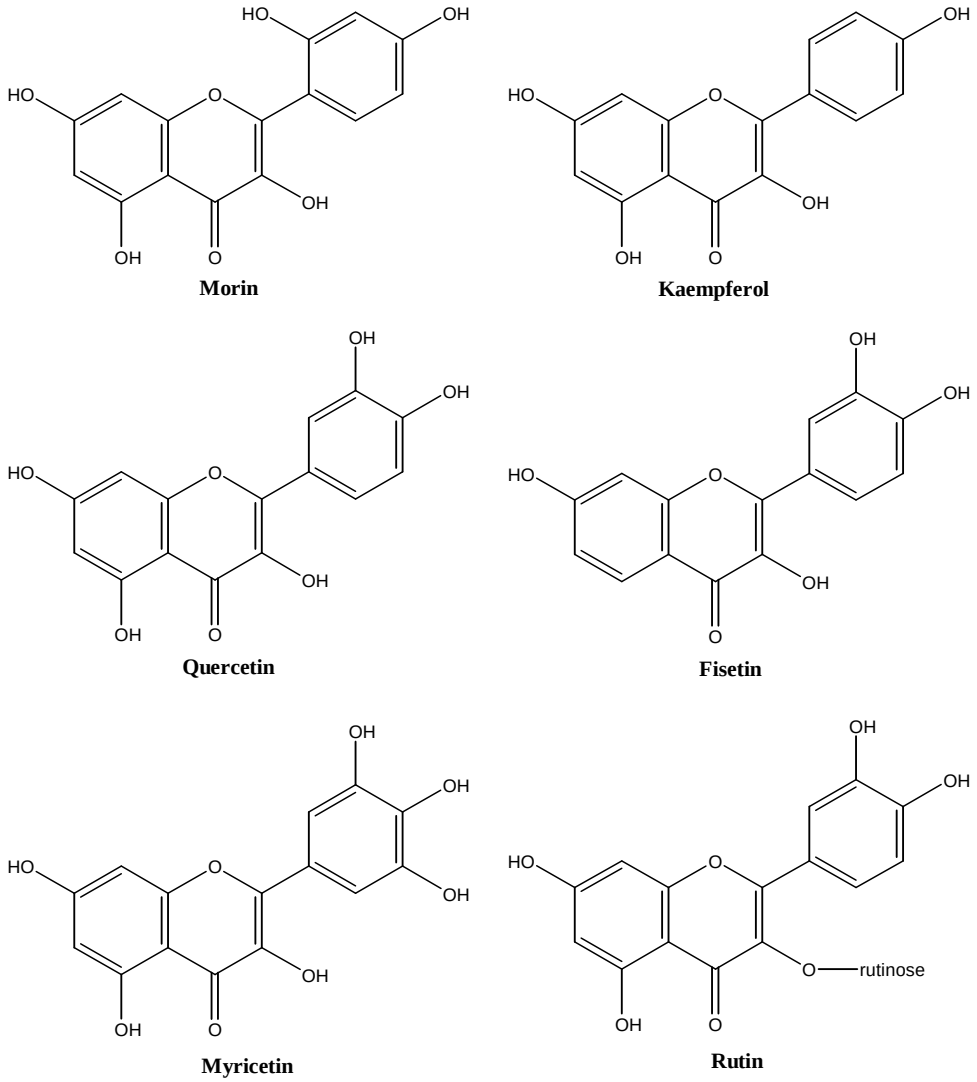


Figure 4. Chemical structures of the most common dietary polyhydroxyflavones, Morin, kaempferol, quercetin, fisetin, myricetin and rutin.

2. Materials and methods

2.1. Chemicals and apparatus

Tamoxifen, 4-hydroxytamoxifen, kaempferol and butyl paraben (p-hydroxybenzoic acid n-butyl ester) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were acquired from the Merck Co. (Darmstadt, Germany). All other chemicals for this study were of reagent grade and were used without further purification. Water was deionized and filtered through a Millipore Milli-Q system (Bedford, MA, USA).

Apparatus used in this study were a high performance liquid chromatograph equipped with a Waters 1515 isocratic HPLC Pump, a Waters 717 plus autosampler and a Waters^{TM} 474 scanning fluorescence detector (Waters Co., Milford, MA, USA), a HPLC column temperature controller (Phenomenex Inc., CA, USA), a Bransonic® Ultrasonic Cleaner (Branson Ultrasonic Corporation, Danbury, CT, USA), a vortex-mixer (Scientific Industries Co., NY, USA) and a high-speed micro centrifuge (Hitachi Co., Tokyo, Japan).

2.2. Animal experiments

The male Sprague-Dawley rats weighing 270 to 300 g were purchased from the Dae Han Laboratory Animal Research Co. (Choongbuk, Korea), and were given access to a commercial rat chow diet (No. 322-7-1, Superfeed Co., Gangwon, Korea) and tap water *ad libitum*. The animals were housed, two per cage, maintained at 22 ± 2^{o}C, and 50-60% relative humidity, under a 12:12 h light-dark cycle. The experiments started after acclimation under these conditions for at least 1 week. The Animal Care Committee of Chosun University (Gwangju, Korea) approved the design and the conduct of this study.

The rats were fasted for at least 24 h prior to beginning the experiments and had access to tap water freely. Each animal was anaesthetized slightly with ether. The left femoral artery and vein were cannulated using polyethylene tubing (SP45, i.d. 0.58 mm, o.d. 0.96 mm; Natsume Seisakusho Co. LTD., Tokyo, Japan) for blood sampling and i.v. injection, respecitively.

2.3. Drug administration

2.3.1. Intravenous administration of tamoxifen

Tamoxifen solution for intravenous administration (2 mg/kg) was prepared by dissolving tamoxifen in saline containing 10% of tween 80 (1.5 ml/kg). It was injected through the femoral vein within 1 min (n = 6). A 0.45-aliquot of blood sample was collected into heparinized tubes from the femoral artery before and 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after the injection, respectively. The blood samples were centrifuged at 13,000 rpm for 5 min, and the plasma samples stored at -40˚C until HPLC analysis. A 0.4-ml aliquot of 0.9% NaCl-injectable solution was used to flush the cannula immediately after each blood sampling to replace the blood lose and a 0.2-aliquot of the heparinized 0.9% NaCl-injectable solution (20-units/ml) was used to prevent blood clotting.

2.3.2. Oral administration of tamoxifen

An tamoxifen solution for oral administration (10 mg/kg) was prepared by dissolving tamoxifen in the distilled water containing 10% of tween 80 (3.0 ml/kg). It was then intragastrically administered immidiatedly using feeding tube (n = 6). Blood samples (0.45 ml) were collected into heparinized tubes from the femoral artery before and 0.25, 0.5, 1, 2, 3, 4, 6, 8 12, 24 and 36 h after the administration. Other treatment procedure is same as i.v. injection.

2.3.3. Oral administration of kaempferol

Kaempferol suspentions (3 and 10 mg/kg) were prepared by mixing kaempferol in distilled water (3.0 ml/kg), respectively. They were orally administered by feeding tube 30 min prior to an oral administration of tamoxifen (n = 6, each). Other treatment procedure is same as oral administration of tamoxifen.

2.4. HPLC analysis

2.4.1. Sample preparation

The plasma concentrations of tamoxifen and its metabolite, 4-hydroxytamoxifen, were determined by a HPLC assay method reported by Fried et al. (1994) after a slight modification. Briefly, a 50-μl aliquot of 8-μg/ml butylparaben, an internal standard, and a 0.2-ml aliquot of acetonitrile were mixed with a 0.2-ml aliquot of the plasma sample in a 2.0-ml polypropylene microtube (Axygen Scientific Co., Calif., USA). The resulting mixture was then vortex-mixed vigorously for 2 min and centrifuged at 13,000 rpm for 10 min. A 50-μl aliquot of the supernatant was injected into the HPLC system.

2.4.2. HPLC condition

Chromatographic separations were achieved using a Symmetry® C_{18} column (4.6 mm i.d. × 150 mm long; particle size 5 μm, Waters Co.), and a μBondapak™ C_{18} HPLC Precolumn Inserts (Particle size 10 μm; Waters Co.) was used before the analytical column. The mobile phase consisted 20 mM dipotassium hydrogen phosphate (pH 3.0, adjusted with phosphoric acid)-acetonitrile (60: 40, v/v) was passed through a 0.45-μm membrane filter and degassed by an Ultrasonic Cleaner under vaccum before use. The flow-rate of the mobile phase was maintained at 1.0 ml/min. Chromatography was performed at a temperature of 30˚C that was set by a HPLC column temperature controller. The fluorescence detector was operated at an

excitation wavelength of 254 nm with an emission wavelenth of 360 nm. A homemade post-column photochemical reactor was supplied with a bactericidal ultraviolet lamp (Sankyo Denki Co, Japan), and a Teflon® tubing (i.d. 0.01", o.d.1/16", 2 m long) was crocheted and fixed horizontally with a stainless steel frame under the lamp at a 10 cm-distance in order to convert the tamoxifen and 4-hydroxytamoxifen to the fluorophors, since photochemical conversion of tamoxifen and its metabolites to highly fluorescent phenanthrenes could increase the lower limit of dectection of tamoxifen and 4-hydroxytamoxifen at HPLC (Figure 3). Data were acquired and processed with breeze™ Software (Version 3.2) (Waters Co.).

Standard calibration curves were prepared by adding tamoxifen, 4-hydroxytamoxifen and butylparaben into a 0.2-ml aliquot drug-free rat plasma to form the plasma concentration of 5, 10, 20, 50, 100, 200 and 500 ng/ml for tamoxifen, and 0.5, 1, 2, 5, 10, 20 and 50 ng/ml for 4-hydroxytamoxifen, and 2 μg/ml for butylparaben. These plasma samples were extracted as described above. Calibration curves of tamoxifen and 4-hydroxytamoxifen were computed using the ratio of the peak area of tamoxifen or 4-hydroxytamoxifen and that of butylparaben as a function of the tamoxifen or 4-hydroxytamoxifen concentrations in plasma. The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Intra-day variability was tested on five different rat's plasma samples using the same calibration curve in a same day. Inter-day variability was tested on five different days. The mean relative standard deviation (RSD) of the mean predicted concentration for the independently assayed standards provided the measure of precision. Accuracy was calculated by the percentage deviation of the mean predicted concentration of tamoxifen and 4-hydroxytamoxifen from the expected target value.

2.5. Pharmacokinetic analysis

The plasma concentration data were analyzed by noncompartmental method using WinNonlin software version 4.1 (Pharsight Corporation, Mountain View, CA, USA). The elimination rate constant (K_{el}) was calculated by log-linear regression of tamoxifen or 4-hydroxytamoxifen concentration data during the elimination phase and 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 tamoxifen or 4-hydroxytamoxifen 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 infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el}. Total body clearance (CL/F) was calculated by Dose/ $AUC_{0-\infty}$. The absolute bioavailability (AB) of tamoxifen was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral}$, and the relative bioavailability (RB) of tamoxifen was estimated by $AUC_{coadmin}/AUC_{control} \times 100$. The metabolite-parent ratio (MR) was estimated by $(AUC_{4\text{-hydroxytamoxifen}}/AUC_{\ tamoxifen}) \times (MW_{tamoxifen}/MW_{4\text{-hydroxytamoxifen}}) \times 100$.

2.6. Statistical analysis

Statistical analysis was conducted using a one-way ANOVA followed by *a posteriori* testing with the use of the Dunnett correction. Differences were considered to be significant at a level of $p < 0.05$. All mean values are presented with their standard deviation (Mean ± S.D.).

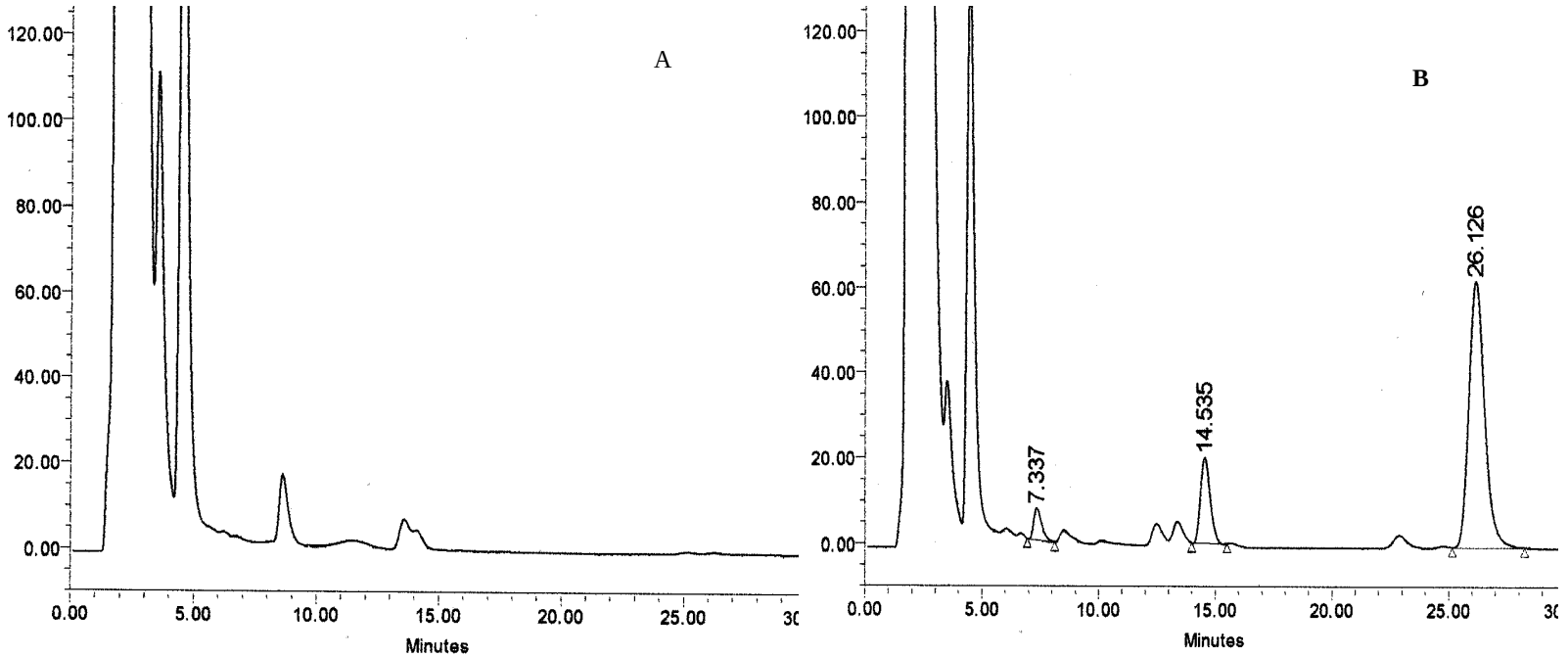


Figure 5. Chromatograms of the rat's blank plasma (A), and the plasma (B) spiked with tamoxifen (26.1 min), 4-hydroxytamoxifen (7.3 min) and butylparaben (Internal standard, 14.5 min).

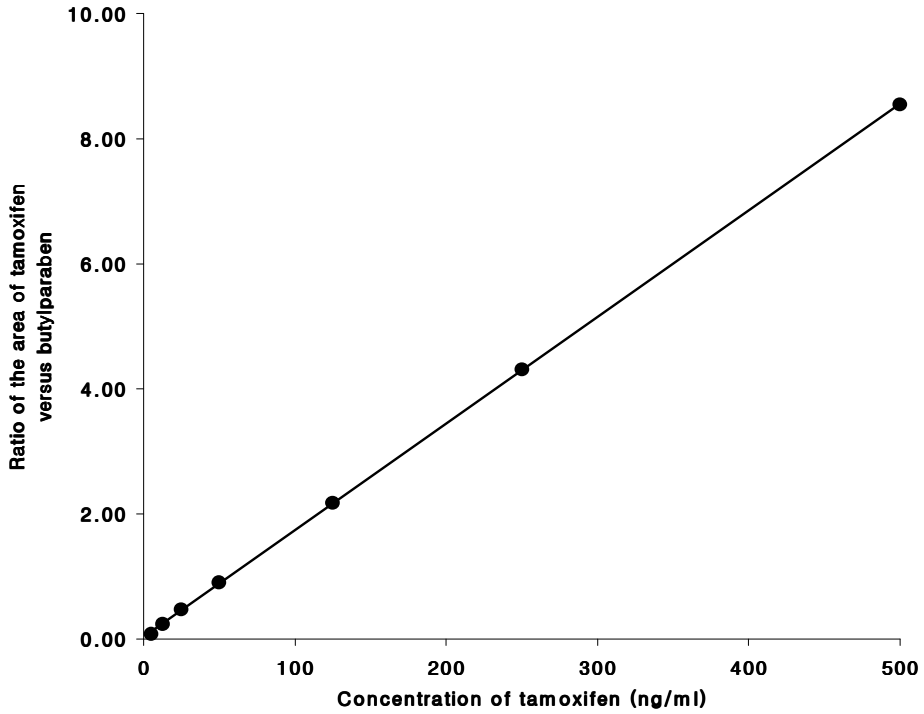


Figure 6. Calibration curve of tamoxifen when spiked in rat's blank plasma, where *y* is the peak area ratio of tamoxifen against butylparaben and *x* is the ratio of the area of tamoxien versus butylparaben, R^2=0.999.

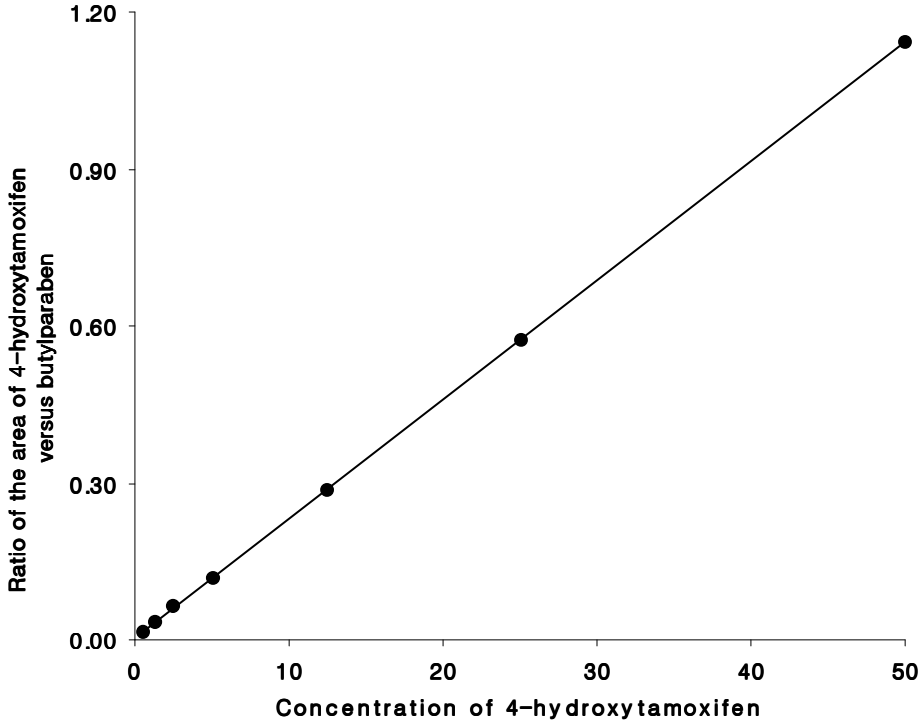


Figure 7. Calibration curve of 4-hydroxytamoxifen when spiked in rat's blank plasma, where *y* is the peak area ratio of 4-hydroxytamoxifen against butylparaben and *x* is the ratio of the area of 4-hydroxytamoxien versus butylparaben, R^2=0.999.

Table 1. Mean (+ S.D.) plasma concentration-time profiles of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) (n = 6, each).

Time (hour)	Control	Kaempferol		i.v. tamoxifen (2 mg/kg)
		3 mg/kg	10 mg/kg	
0	0	0	0	2140 ± 514
0.25	63.9 ± 15.3	120 ± 28.8	116 ± 27.8	411 ± 98.6
0.5	92.8 ± 22.3	181 ± 43.4	177 ± 42.5	280 ± 67.2
1	126 ± 30.2	195 ± 46.8	188 ± 45.1	191 ± 45.8
2	119 ± 28.6	183 ± 43.9	179 ± 43.0	146 ± 35.0
3	105 ± 25.2	167 ± 40.1	162 ± 38.9	–
4	97.1 ± 23.3	147 ± 35.3	143 ± 34.3	96.2 ± 23.1
6	88.8 ± 21.3	131 ± 31.4	127 ± 30.5	64.1 ± 15.4
8	81.9 ± 19.7	115 ± 27.6	109 ± 26.2	45.3 ± 10.9
12	61.1 ± 14.7	89.1 ± 21.4	85.2 ± 20.4	27.9 ± 6.70
24	30.8 ± 7.39	43.0 ± 10.3	40.3 ± 9.67	14.2 ± 3.41
36	15.0 ± 3.60	22.3 ± 5.35	20.1 ± 4.82	–

Table 2. Mean (+ S.D.) plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) (n = 6, each).

Time (hour)	Control	Kaempferol	
		3 mg/kg	10 mg/kg
0	0	0	0
0.25	2.39 ± 0.57	3.43 ± 0.82	3.02 ± 0.72
0.5	5.58 ± 1.34	7.60 ± 1.82	7.06 ± 1.69
1	9.81 ± 2.35	11.7 ± 2.81	11.1 ± 2.66
2	11.6 ± 2.78	13.6 ± 3.26	12.8 ± 3.07
3	11.2 ± 2.69	12.6 ± 3.02	11.9 ± 2.86
4	10.3 ± 2.47	12.0 ± 2.88	11.3 ± 2.71
6	9.47 ± 2.27	11.1 ± 2.66	10.6 ± 2.54
8	8.83 ± 2.12	10.3 ± 2.47	9.80 ± 2.35
12	7.41 ± 1.78	9.01 ± 2.16	8.50 ± 2.04
24	4.21 ± 1.01	5.50 ± 1.32	5.01 ± 1.20
36	2.43 ± 0.58	3.31 ± 0.79	3.03 ± 0.73

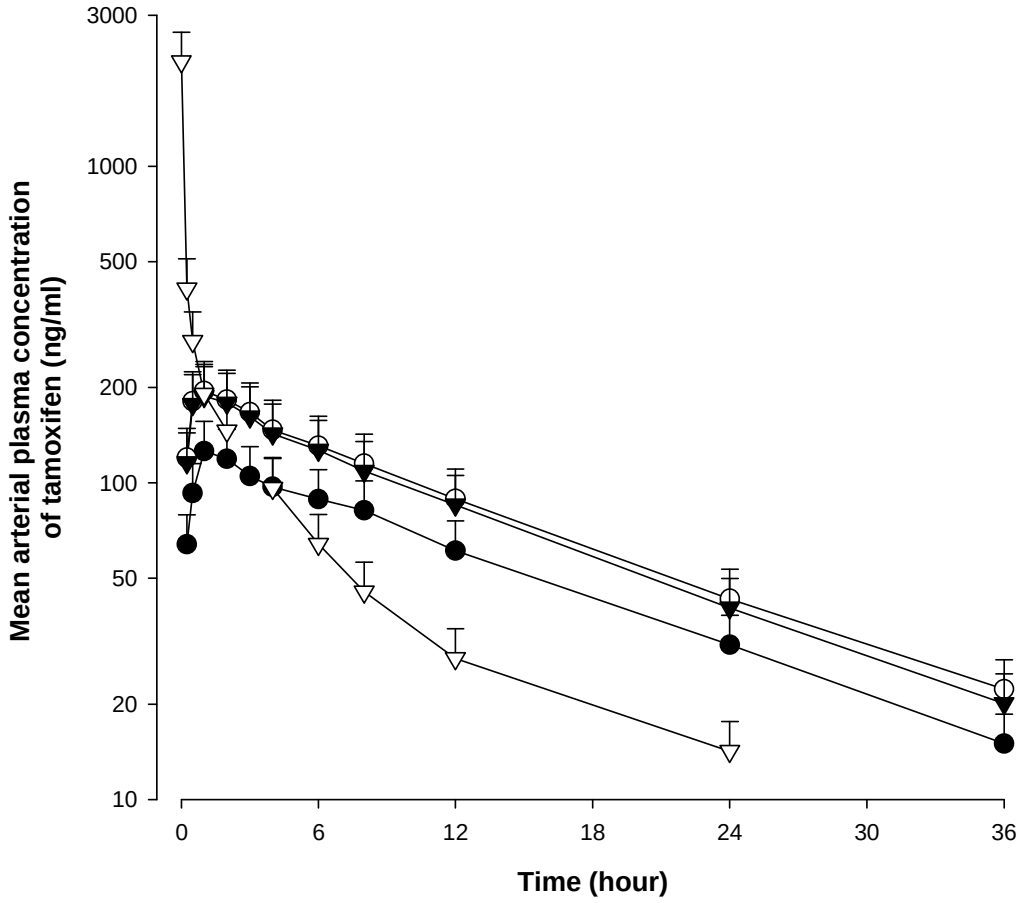


Figure 8. Mean plasma concentration-time profiles of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) (n = 6, each).

Bars represent the standard deviation, (▽) Intravenous administration of tamoxifen (2 mg/kg); (●) Oral administration of tamoxifen (10 mg/kg); (○) the presence of 3 mg/kg of kaempferol; (▼) the presence of 10 mg/kg of kaempferol.

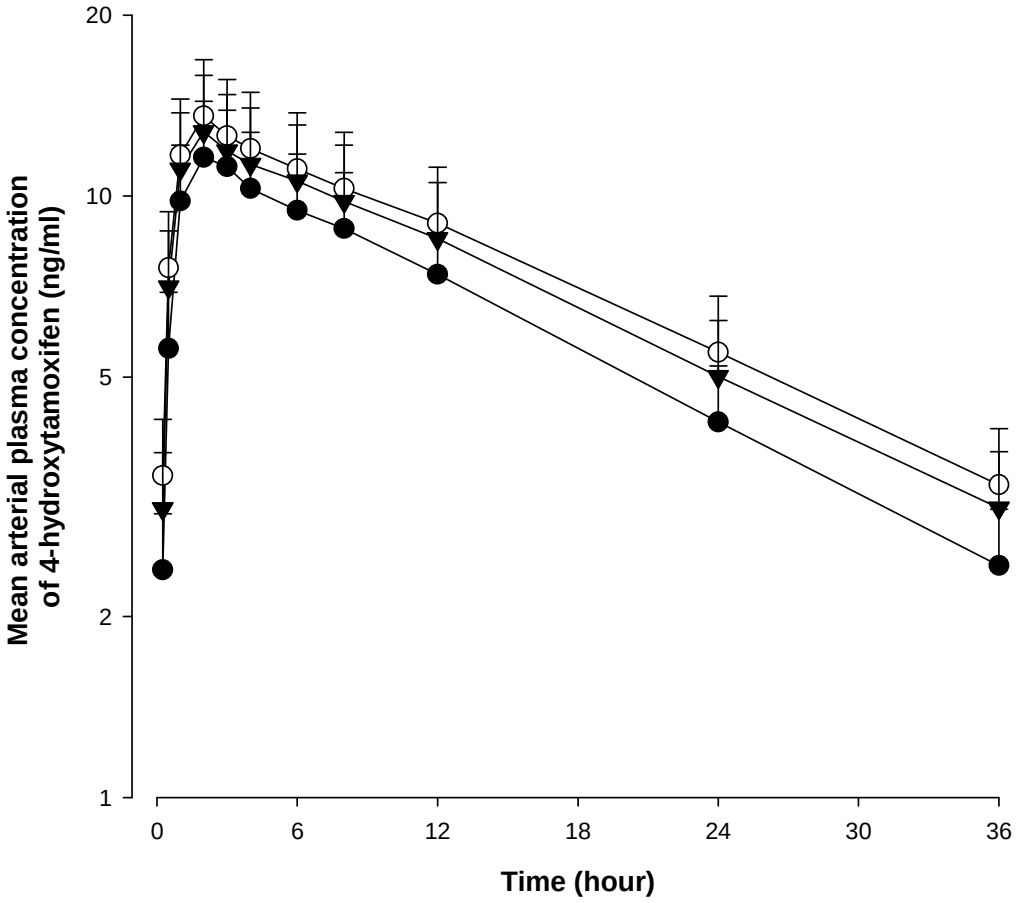


Figure 9. Mean plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) (n = 6, respecitively).

Bars represent the standard deviation, (●) Oral administration of tamoxifen (10 mg/kg); (○) the presence of 3 mg/kg of kaempferol; (▼) the presence of 10 mg/kg of kaempferol.

Table 3. Mean (+ S.D.) pharmacokinetic parameters of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 or 10 mg/kg) (n = 6, each).

Parameter	Control	Kaempferol		i.v. tamoxifen (2 mg/kg)
		3 mg/kg	10 mg/kg	
$AUC_{0\sim\infty}$ ($ng \cdot h \cdot ml^{-1}$)	2137 ± 513	3165 ± 760*	2991 ± 511*	1783 ± 428
C_{max} (ng/ml)	126 ± 30.2	195 ± 46.8*	188 ± 45.1*	
T_{max} (h)	1.00	1.00	1.00	
CL/F ($ml \cdot min^{-1} \cdot kg^{-1}$)	78.0 ± 18.7	52.7 ± 12.6*	55.7 ± 13.4*	18.7 ± 4.49
$t_{1/2}$ (h)	11.6 ± 2.78	11.8 ± 2.84	11.4 ± 2.75	8.80 ± 2.11
AB (%)	23.9 ± 5.74	35.5 ± 8.52*	33.5 ± 5.49*	100
RB (%)	100	148	139	

* $p < 0.05$ compared to control.

$AUC_{0\sim\infty}$: area under the plasma concentration-time curve from 0 h to infinity;

C_{max}: peak plasma concentration;

T_{max}: time to reach peak concentration;

CL/F: total clearance;

$t_{1/2}$: terminal half-life;

AB: absolute bioavailability;

RB: relative bioavailability.

Table 4. Mean (+ S.D.) pharmacokinetic parameters of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 or 10 mg/kg) (n = 6, each).

Parameter	Control	Kaempferol	
		3 mg/kg	10 mg/kg
$AUC_{0\sim\infty}$ ($ng \cdot h \cdot ml^{-1}$)	271 ± 65.0	349 ± 83.7	322 ± 77.2
C_{max} (ng/ml)	11.6 ± 2.78	13.6 ± 3.26	12.8 ± 3.07
T_{max} (h)	2	2	2
CL/F ($ml \cdot min^{-1} \cdot kg^{-1}$)	616 ± 148	478 ± 115	518 ± 124
$t_{1/2}$ (h)	15.0 ± 3.60	17.0 ± 4.07	16.4 ± 39.9
MR (%)	12.2 ± 2.90	10.6 ± 2.51	10.3 ± 2.49
RB (%)	100	128	119

$AUC_{0\sim\infty}$: area under the plasma concentration-time curve from 0 h to infinity;

C_{max}: peak plasma concentration;

T_{max}: time to reach peak concentration;

CL/F: total clearance;

$t_{1/2}$: terminal half-life;

MR: Ratio of the area of metabolite versus parent compound;

RB: relative bioavailability.

3. Results

3.1. Chromatographic separation

Figure 5 illustrates the chromatograms of blank rat plasma (A) and the plasma spiked with tamoxifen, 4-hydroxytamoxifen and the internal standard, butylparaben (B). The tamoxifen, 4-hydroxytamoxifen and butylparaben were eluted with retention times at 26.1, 7.3 and 14.5 min, respectively. Figure 6 and 7 showed the calibration curve of tamoxifen and 4-hydroxytamoxifen constructed by plotting the ratio of the peak area of tamoxifen and 4-hydroxytamoxifen and that of butylparaben as a function of the plasma tamoxifen and 4-hydroxytamoxifen concentrations. There is an excellent linearity over these concentration ranges. The typical equation describing the calibration curve in rat plasma for tamoxifen was y=0017x-0.0384, where y is the peak area ratio of tamoxifen against butylparaben and x is the concentration of tamoxifen, with a mean correlation coefficient of 0.999; for 4-hydroxytamoxifen was y=0.0227x+0.0055, where y is the peak area ratio of 4-hydroxytamoxifen against butylparaben and x is the concentration of 4-hydroxytamoxifen, with a mean correlation coefficient of 0.999. The lower limit of detection for tamoxifen and 4-hydroxytamoxifen in rat plasma defined at a minimum signal-to-noise of three was 5 ng/ml and 0.5 ng/ml, respectively. The lower limit of quantification for tamoxifen and 4-hydroxytamoxifen in rat's plasma was 5 ng/ml and 0.5 ng/ml with an acceptable precision and accuracy (RSD: 11.5%, Deviation: −4%, n = 5).

3.2. Effect of kaempferol on the pharmacokinetics of tamoxifen

Mean arterial plasma concentration-time profiles of tamoxifen following an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of

tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) were shown in Figure 8 and Table 1, corresponding pharacokinetic parameters were shown in Table 3. The presence of kaempferol significantly altered the pharmacokinetic parameters of tamoxifen. Compared to the control group (given oral tamoxifen alone), the presence of tamoxifen significantly reduced ($p < 0.05$, 28.6-32.4%) the total plasma clearance (CL/F), and significantly increased ($p < 0.05$, 39.9-48.1%) the area under the plasma concentration-time curve ($AUC_{0-\infty}$) of tamoxifen. The peak plasma concentration (C_{max}) of tamoxifen also increased significantly ($p < 0.05$, 49.2-54.7%). The absolute bioavailability (AB) of tamoxifen in oral control group was 23.9%, which was elevated significantly ($p < 0.05$) to 33.5-35.5%. Relative bioavailability (RB) of tamoxifen in the presence of kaempferol was 1.39- to 1.48-fold greater. The time to reach peak concentration (T_{max}) and the terminal half-life ($t_{1/2}$) of tamoxifen were not altered significantly in the presence of kaempferol.

3.3. Effect of kaempferol on the pharmacokinetics of 4-hydroxytamoxifen

Mean arterial plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of kaempferol (3 and 10 mg/kg) were shown in Figure 9 and Table 2, correlated pharacokinetic parameters were shown in Table 4. Compared to the control group, although the presence of kaempferol reduced the CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, they were not statistically different. The metabolite-parent ratios (MR) of 4-hydroxytamoxifen also decreased insignificantly, suggesting the production of 4-hydroxytamoxifen was not affected cosiderably by kaempferol. Pretreatment of kaempferol has not significantly altered other parameters such as C_{max}, $t_{1/2}$ and T_{max} of 4-hydroxytamoxifen.

4. Discussion

Using the modified HPLC method, the excellent separation of tamoxifen, 4-hydroxytamoxifen and the internal standard was acquired in the chromatograms (Figure 5). No apparent interfering peaks from the endogenous substances were observed in any of the plasma samples. The method was linear over the concentration range of 5–500 ng/ml for tamoxifen and 0.5-50 ng/ml for 4-hydroxytamoxifen (Figure 6, 7).

Tamoxifen and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 (Desta et al., 2004). Tamoxifen and its metabolites, *N*-desmethyltamoxifen and 4-hydroxytamoxifen, are substrates for the efflux of P-gp and MRP2 as well (Rao et al., 1994; Gant et al., 1995, Sugimoto et al., 2003; Kauffmann et al., 1998). CYP3A and P-gp inhibitors might interact with tamoxifen and its metabolites and could contribute to substantial alteration of their pharmacokinetic fate.

As shown in Table 3, the prsence of kaempferol significantly reduced the CL/F of oral tamoxifen and significantly increased the $AUC_{0-\infty}$ and C_{max} of tamoxifen. Kaempferol is a substrate for P-gp (Wang et al., 2005) and exhibits a remarkable inhibition of P-gp-mediated efflux of ritonavir and CYP3A4-mediated cortisol metabolism (Patel et al., 2004), and oral tamoxifen is a substrate for CYP3A-catalyzed metabolism and P-gp-mediated efflux in the intestine and liver, pretreated kaempferol might be effective to obstruct this pathway. This result is consistent with the result acquired by Shin et al. (2006) that coadministration of the hydroxylated kaempferol analogue quercetin at doses of 2.5 and 7.5 mg/kg to rats significantly increased the $AUC_{0-\infty}$ and C_{max} of tamoxifen in rats. Interestingly, 3 mg/kg of kaempferol altered the parameters more than 10 mg/kg although they were not significantly different. The report of Shin et al. (2006) showed that quercetin at the higher dose (15 mg/kg) did not alter the pharmacokinetic

parameters significantly, and in the report of Hsiu et al. (2002) also showed that 50 mg/kg of quercetin significantly decreased the AUC of cyclosporin in rats and pigs, which needs to be clarified in the future. In regarding of the pharmacokinetic fate of 4-hydroxytamoxifen, although the presence of kaempferol reduced the CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, they were not significant statistically. MR of 4-hydroxytamoxifen also decreased insignificantly (Table 4). Since the main enzyme to produce 4-hydroxytamoxifen is CYP2D6, and CYP3A is only a minor one (Mani et al., 1993; Crewe et al., 1997), kaempferol might not affect this biotransformation pathway considerably. Many reports suggested that 4-hydroxytamoxfen was much more effective in suppressing estrogen-dependent cell proliferation. Since kaempferol has not inhibited its formation, the anticancer activity of tamoxifen should be more potent via combination of kaempferol. On the other hand, it migh make the toxicity of tamoxifen occure more frequently.

The presence of kaempferol enhanced the oral bioavailability of tamoxifen. The adjustment of the dose of tamoxifen should be taken into consideration in the presence of kaempferol and the dietary complements full of kaempferol in patients to avoid the toxicity of tamoxifen.

Part II. The effect of morin on the bioavailability of tamoxifen in rats

Abstract

The effect of morin on the pharmacokinetics of tamoxifen and its metabolite, 4-hydroxytamoxifen, was investigated in rats. A single dose of tamoxifen was administered intravenously (2 mg/kg) to the male Sprague-Dawley rats, and orally (10 mg/kg) with or without morin (3 or 10 mg/kg). Plasma concentration of tamoxifen was determined by HPLC equipped with fluorescence detector.

When tamoxifen was administered orally, the presence of morin significantly altered the pharmacokinetics of tamoxifen. Compared to the oral control group (given tamoxifen alone), the CL/F of tamoxifen decreased significantly ($p < 0.01$, 35.9-40.8%) in the presence of morin. The $AUC_{0-\infty}$ and C_{max} of tamoxifen significantly ($p < 0.05$ for 3 mg/kg morin, $p < 0.01$ for 10 mg/kg morin) increased 50.6-68.9% and 65.5-80.9%, respectively. Consequently, AB of tamoxifen in the presence of morin was 37.4-40.5%, which was enhanced significantly ($p < 0.05$) compared to the oral control group (23.9%). RB of tamoxifen was 1.56- to 1.68-fold greater than the control group. The increase in the bioavailability of tamoxifen is likely to be due to the decrease in the first-pass extraction by the intestine and liver.

When morin was coadministered at a dose of 10 mg/kg, significant reduction in the CL/F ($p < 0.05$, 33.8) and consequent increase in the $AUC_{0-\infty}$ ($p < 0.05$, 50.9%) were observed for 4-hydroxytamoxifen, but the metabolite-parent ratio (MR) of 4-hydroxytamoxifen was not altered significantly, implying that the formation of 4-hydroxytamoxifen was not affected considerably but the elimination of tamoxifen and the metabolite was decreased parallelly and significantly by administration of

morin.

The increase in the oral bioavailability of tamoxifen by the coadministration of morin should be taken into consideration in the clinical setting in order to avoid potential toxic reactions of tamoxifen.

Key words: tamoxifen, morin, P-glycoprotein (P-gp), CYP3A, pharmacokinetics, bioavailability, rats.

1. Introduction

Oral administration of drugs has many advantages over intravenous injection because it is less invasive, easier to use for the patient in a chronic regimen and more cost-effective because of the decreased hospitalization. The bioavailability of many orally administered anticancer drugs are low, which contribute to the first-pass extraction mediated by multidrug resistance (MDR) transporters and phase I and phase II metabolizing enzymes in the intestine and liver. MDR is a term used to describe the phenomena that tumor cells resistant a number of structurally and functionally unrelated chemotherapeutic agents. The transport-mediated MDR is caused by the ATP-binding cassette (ABC) family membrane transport ATPases. P-glycoprotein (P-gp), an important member of ABC family, expresses highly in solid tumours of epithelial origin, such as the colon (Cordon-Cardo et al., 1990), kidney (Fojo et al., 1987), and breast (Merkel et al., 1989) to efflux substrates out of cells. MRPs, another important member of the ABC family, has been described as a Vanadate-sensitive magnesium-dependent glutathione S-conjugate (GS-conjugate) ATPase (GS-X pump) capable of transporting organic anion drug conjugates as well as intact anticancer drugs (Grant et al., 1994; Borst et al., 1997). Several isoforms of MRPs have been identified and the mainly mentioned are MRP1 and MRP2 (cannalicular multispecific organic anion transporter, cMOAT), which have been identified as organic anion transporters (Borst et al., 1997).

P-gp and MRPs are present widely in many normal excretory organs, such as the liver, kidney and intestine, where they provide a barrier to eliminate the substrates out of the body. P-gp, and MRP2 co-localized to the apical membrane of the intestine, liver, kidney, and blood–brain barrier (Thiebaut et al., 1987; Buchler et al., 1996; Fromm et al., 2000; Schaub et al., 1999), and MRP1 is localized to the basolateral membranes of polarized epithelial cells of the intestinal crypt (Peng et al., 1999), renal distal and collecting tubules (Peng et al., 1999), and liver (Mayer et

al., 1995; Roelofsen et al., 1997).

P-gp and MRPs are co-localized with phase I and Phase II metabolizing enzymes CYP 3A4, UDP-glucuronosyltransferases and Glutathione-*S*-transferases in the liver, kidney and intestine (Sutherland et al., 1993; Turgeon et al., 2001). The CYP3A subfamily was reportedly involved in approximately 40–50% of Phase I metabolism of marketing drugs (Guengerich, 1995). Specifically, CYP3A4 accounts for 30% of hepatic CYP and 70% of small intestinal CYP (Schuetz et al., 1996). A substantial overlap in substrate specificity exists between CYP3A4 and P-gp (Wacher et al., 1995). Thus, a synergistic relationship exists between the transporters and metabolizing enzymes, such as CYP3A4 versus P-gp and conjugating enzymes versus MRP2, within excretory tissues to protect the body against invasion by foreign compounds, which also decrease the oral bioavailability of many drugs, especially anticancer drugs.

Since MDR is one of the main obstacles to successful chemotherapy of cancer, a number of biochemical, pharmacological and clinical strategies have been devised to overcome it. The first generation of MDR modulators including the calcium channel blockers, such as verapamil, felodipine and diltiazem), quinolines, cyclosporines (Cyclosporin A) (Ford and Hait, 1990; Ford and Hait, 1993) are therapeutic agents. They could reverse MDR at concentrations much higher than those required for their individual therapeutic activity, which resulted in unnecessary adverse effects and toxicities. The second generation of modulators is more potent and less toxic, such as the analogs of verapamil, dexverapamil (less cardiotoxic *R*-enantiomer of verapamil), the non-immunosuppressive analog of Cyclosporin A, PSC 833 and so on. Although these agents appear to be well tolerated in combination with anticancer drugs, it is need to be determined whether these compounds are suitable for clinical application in anticancer therapy.

Tamoxifen is a nonsteroidal antiestrogen and is the agent of choice for treating and preventing breast cancer (Powles, 1992; Stone, 1992; Jaiyesimi et al., 1995).

Tamoxifen has a relatively low toxicity and is less harmful than most chemotherapeutics. The main adverse effects of tamoxifen in humans are that there might be an increased risk of endometrial cancer and thromboembolic diseases (Fornander et al., 1993; Meier and Jick, 1998). Orally administered tamoxifen undergoes extensive hepatic metabolism and the subsequent biliary excretion of its metabolites (Buckley and Goa, 1989). Demethylation of the aminoethoxy side chain to *N*-desmethyltamoxifen is the main route of tamoxifen metabolism (Lonning et al., 1992; Stearns et al., 2003). Tamoxifen and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 (Desta et al., 2004). The major primary metabolite, *N*-desmethyltamoxifen, was catalyzed by CYP3A4/5, and the minor metabolite, 4-hydroxytamoxifen, catalyzed by CYP2D6 CYP2C9 and CYP3A (Mani et al., 1993; Crewe et al., 1997) (Figure 1). Other minor primary metabolites include α-, 3-, and 4'-hydroxytamoxifen and one unidentified metabolite (M-I) were primarily catalyzed by CYP3A4, CYP3A5, CYP2B6/2C19, and CYP3A4, respectively. Tamoxifen secondary metabolism showed that *N*-desmethyltamoxifen was predominantly biotransformed to α-hydroxy *N*-desmethyl-, *N*-didesmethyl-, and 4-hydroxy *N*-desmethyl-tamoxifen (endoxifen), whereas 4-hydroxytamoxifen was converted to 3,4-dihydroxytamoxifen and endoxifen (Figure 2). Except for the biotransformation of *N*-desmethyltamoxifen to endoxifen, which was exclusively catalyzed by CYP2D6, all other routes of *N*-desmethyl- and 4-hydroxytamoxifen biotransformation were catalyzed predominantly by the CYP3A subfamily. 4-hydroxytamoxifen is a relatively minor metabolite, but it has been studied by a number of investigators because it is a more potent antiestrogen than tamoxifen (Jordan et al., 1977; Borgna and Rochefort, 1981; Coezy et al., 1982; Robertson et al., 1982). Jordan et al. (1997; 1982) demonstrated that high first-pass metabolism of tamoxifen results in a significant increase in its activity and characterized the first active primary metabolite, 4-hydroxy-tamoxifen. Although the plasma and tumor concentrations of

4-hydroxytamoxifen are only about 2% of those of the parent compound (Daniel et al., 1981), it has been shown to possess a high affinity for estrogen receptors and 30- to 100-fold more potency than tamoxifen in suppressing estrogen-dependent cell proliferation (Borgna and Rochefort, 1981; Robertson et al., 1982; Coezy et al., 1982; Jordan, 1982). A secondary metabolite of tamoxifen, endoxifen, exhibits potency similar to 4-hydroxytamoxifen with respect to estrogen receptor binding affinity, suppression of estrogen-dependent cell growth, and gene expression (Stearns et al., 2003; Johnson et al., 2004). So, tamoxifen has been referred to as a prodrug that requires activation to exert its effects.

Since tamoxifen and its metabolites, *N*-desmethyltamoxifen and 4-hydroxytamoxifen, are substrates for the efflux of P-gp and MRP2 (Rao et al., 1994; Gant et al., 1995, Sugimoto et al., 2003; Kauffmann et al., 1998), the MDR modulators might be able to improve the bioavailabilty of tamoxifen. As upper mentioned, although some P-gp modulators such as verapamil, cyclosporine A and PSC 833 have been proved to be the potent P-gp inhibitors *in vitro*, but their toxicities have hindered their use in clinical application (Bradshaw and Arceci, 1998). Some flavonoids as natural products were shown to be modulators of the MDR transporters (Castro and Altenberg, 1997; Scambia et al., 1994) and phase I and phase II metabolizing enzymes (Tsyrlov et al., 1994; Walle et al., 1995). They have many health promoting benefits and have no consistent side effects, it could be expected as one of the nature members to improve the bioavailability of the anticancer drugs.

Flavonoids are widely distributed in dietary supplements such as vegetables, fruit, tea and wine (Hertog et al., 1993b). Flavonoids have many beneficial effects including antioxidant, antibacterial, antiviral, antiinflammatory, antiallergic, and anticarcinogenic actions (Ross and Kasum, 2002; Hodek et al., 2002) though whether these effects can be attributed to the aglycone forms or their metabolites is not entirely clear. High-level consumption of flavonoids was found to associate

with an overall lower risk of getting a wide variety of cancers (Knekt et al., 1997). Epidemiological studies also support an association between dietary flavonoids and a reduced risk of certain cancers, such as stomach carcinoma and lung cancer (Dorant et al., 1996; Knekt et al., 1997). Proposed mechanisms for anticancer benefits include numerous effects on signal transduction pathways involved in cell proliferation (Weber et al., 1996; Lepley and Pelling, 1997) and angiogenesis (Fotsis et al., 1997), as well as inhibition of enzymes involved with procarcinogen bioactivation such as cytochrome P450 (Tsyrlov et al., 1994) and sulfotransferase enzymes (Walle et al., 1995). A high dietary intake of flavonols could reduce the incidence of cardiovascular disease (Hertog et al., 1993b; Knekt et al., 1996). Some flavonoids, such as quercetin, protect LDL cholesterol from oxidative damage. Others, such as the anthocyanidins from bilberry, purple cabbage, and grapes, may help protect the lens of the eye from cataracts. Animal research suggests that naringenin, found in grapefruit, may have anticancer activity (So et al., 1996). The total daily intake of flavonoids via the dietary supplements has been 23 mg/day in Dutch population (Hertog et al., 1993a).

Several studies have shown that flavonoids can modulate the activities of both P-gp and MRP1 (Zhang and Morris, 2003a; Bobrowska-Hagerstrand et al., 2003) affecting drug accumulation, cell viability following cytotoxic drug exposure, and the ATPase activity of P-gp (Bobrowska-Hagerstrand et al., 2003). Some flavonoids have been reported to interact with the intrinsic ATPase of P-gp, both inhibition and stimulation of P-gp ATPase activity have been observed for silymarin, morin, and biochanin A (Zhang and Morris, 2003b). Some flavonoids reduced the secretory flux of talinolol across Caco-2 cells, such as hesperetin, quercetin, kaempferol, spiraeoside, isoquercitrin and naringin, but none of the selected flavonoids was able to replace [^{3}H]talinolol from its binding to P-gp, which might be due to an interaction with P-gp without competition of the talinolol binding site of P-gp (Ofer et al., 2005). Several flavonoids, specifically

methoxylated flavonoids, are confirmed to be the good inhibitors of MRP1 and 2 (van Zanden et al., 2005).

Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) is a flavonoid constituent of many herbs and fruits. Morin, like quercetin, kaempferol, fisetin, rutin, myricetin, is known as the most common dietary polyhydroxyflavone (Figure 3). Based on in vitro studies, morin has been reported to show various beneficial activities, including antioxidation (Hanasaki et al., 1994; Kok et al., 2000; Ramanathan et al., 1994), anti-mutagenesis (Bhattacharya and Firozi, 1988; Francis et al., 1989) and anti-inflammation (Kim et al., 1999; Raso et al., 2001; Fang et al., 2003). Zhang and Morris (2003a) reported that morin was able to increase daunomycin accumulation in P-gp positive cell-lines concentration-dependently and P-gp expression level-dependently, which suggests that morin inhibited P-gp mediated cellular efflux. The increase of daunomycin accumulation by morin in all the P-gp positive cells is comparable with that with verapamil, a potent P-gp inhibitor. In human liver microsomes, the formation of 6α-hydroxypaclitaxel (formed by CYP2C8) was inhibited by morin, but C_3'-hydroxypaclitaxel and C_2-hydroxypaclitaxel (formed by CYP3A4) was less affected (Vaclavikova et al., 2003). Buening et al. (1981) also reported that morin could inhibit cytochrome P-450 reductase in human liver microsomes.

Orally administered morin, as the P-gp inhibitor, might improve the oral bioavailability of tamoxifen. This study investigated the effect of morin administration on the pharmacokinetics of the tamoxifen and 4-hydroxytamoxifen in rats.

2. Materials and methods

2.1. Chemicals and apparatus

Tamoxifen, 4-hydroxytamoxifen, morin and butylparaben (p-hydroxybenzoic acid n-butyl ester) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were acquired from the Merck Co. (Darmstadt, Germany). All other chemicals for this study were of reagent grade and were used without further purification. Water was deionized and filtered through a Millipore Milli-Q system (Bedford, MA, USA).

Apparatus used in this study were a high performance liquid chromatograph equipped with a Waters 1515 isocratic HPLC Pump, a Waters 717 plus autosampler and a Waters™ 474 scanning fluorescence detector (Waters Co., Milford, MA, USA), a HPLC column temperature controller (Phenomenex Inc., CA, USA), a Bransonic® Ultrasonic Cleaner (Branson Ultrasonic Corporation, Danbury, CT, USA), a vortex-mixer (Scientific Industries Co., NY, USA) and a high-speed micro centrifuge (Hitachi Co., Tokyo, Japan).

2.2. Animal experiments

The male Sprague-Dawley rats weighing 270 to 300 g were purchased from the Dae Han Laboratory Animal Research Co. (Choongbuk, Korea), and were given access to a commercial rat chow diet (No. 322-7-1, Superfeed Co., Gangwon, Korea) and tap water *ad libitum*. The animals were housed, two per cage, maintained at 22 ± 2^{o}C, and 50-60% relative humidity, under a 12:12 h light-dark cycle. The experiments started after acclimation under these conditions for at least 1 week. The animal care committee of Chosun University (Gwangju, Republic of Korea) approved the design and the conduct of this study.

The rats were fasted for at least 24 h prior to beginning the experiments and had access to tap water freely. Each animal was anaesthetized lightly with ether. The left femoral artery and vein were cannulated using polyethylene tubing (SP45, i.d. 0.58 mm, o.d. 0.96 mm; Natsume Seisakusho Co. LTD., Tokyo, Japan) for blood sampling and i.v. injection, respecitively.

2.3. Drug administration

2.3.1. Intravenous administration of tamoxifen

Just before administration, 2 mg/kg of tamoxifen solution for i.v. administration was prepared by dissolving tamoxifen in saline containing 10% of tween 80 (1.5 ml/kg). It was injected through the femoral vein within 1 min (n = 6). A 0.45-aliquot of blood sample was collected into heparinized tubes from the femoral artery before and 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after the injection, respectively. The blood samples were centrifuged at 13,000 rpm for 5 min, and the plasmas stored at -40˚C until HPLC analysis. A 0.4-ml aliquot of 0.9% NaCl-injectable solution was used to flush the cannula immediately after each blood sampling to replace the blood lose and a 0.2-aliquot of the heparinized 0.9% NaCl-injectable solution (20-units/ml) was used to prevent blood clotting.

2.3.2. Oral administration of tamoxifen

Tamoxifen solution for oral administration (10 mg/kg) was prepared by dissolving tamoxifen in the distilled water containing 10% of tween 80 (3.0 ml/kg). It was then intragastrically administered immidiatedly using feeding tube (n = 6). Blood samples (0.45 ml) were collected into heparinized tubes from the femoral artery before and 0.25, 0.5, 1, 2, 3, 4, 6, 8 12, 24 and 36 h after the administration. Other treatment procedure is the same as i.v. injection.

2.3.3. Oral administration of morin

Morin suspentions (3 and 10 mg/kg) were prepared by mixing morin in distilled water (3.0 ml/kg), respectively. They were orally administered by feeding tube 30 min prior to an oral administration of tamoxifen (n = 6, each).

2.4. HPLC analysis

2.4.1. Sample preparation

The plasma concentrations of tamoxifen and 4-hydroxytamoxifen were determined by a HPLC assay method reported by Fried et al. (1994) after a slight modification. Briefly, a 50-μl aliquot of 8-μg/ml butylparaben, as an internal standard, and a 0.2-ml aliquot of acetonitrile were mixed with a 0.2-ml aliquot of the plasma sample in a 2.0-ml polypropylene microtube (Axygen Scientific Co., Calif., U.S.A.). The resulting mixture was then vortex-mixed vigorously for 2 min and centrifuged at 13,000 rpm for 10 min. A 50-μl aliquot of the supernatant was injected into the HPLC system.

2.4.2. HPLC condition

Chromatographic separations were achieved using a Symmetry® C_{18} column (4.6 mm i.d. × 150 mm long, particle size 5 μm, Waters Co.), and a μBondapak™ C_{18} HPLC Precolumn Inserts (particles size 10 μm, Waters Co.) was used before the analytical column. The mobile phase consisted 20 mM dipotassium hydrogen phosphate (pH 3.0, adjusted with phosphoric acid)-acetonitrile (60: 40, v/v) was passed through a 0.45-μm membrane filter and degassed by a Ultrasonic Cleaner under vaccum before use. The flow-rate of the mobile phase was maintained at 1.0 ml/min. Chromatography was performed at a temperature of 30˚C that was set by a HPLC column temperature controller. The fluorescence detector was operated at an excitation wavelength of 254 nm with an emission wavelength of 360 nm. A

homemade post-column photochemical reactor was supplied with a bactericidal ultraviolet lamp (Sankyo Denki Co, Japan), and a Teflon® tubing (i.d. 0.01", o.d. 1/16", 2 m long) was crocheted and fixed horizontally with a stainless steel frame under the lamp at a 10 cm-distance in order to convert the tamoxifen and 4-hydroxytamoxifen to the fluorophors, since photochemical conversion of tamoxifen and its metabolites to highly fluorescent phenanthrenes could increase the sensitivity of the detction of tamoxifen and 4-hydroxytamoxifen in the fluorescence detector (Figure 3). Data were acquired and processed with breeze™ Software (Version 3.2) (Waters Co., CA, USA).

Standard calibration curves were prepared by adding a 50-µl aliquot of tamoxifen solution at the concentrations of 20, 40, 80, 200, 400, 800 and 2,000 ng/ml, respectively, a 50-µl aliquot of 4-hydroxytamoxifen solution at the concentrations of 2, 4, 8, 20, 40, 80 and 200 ng/ml, respectively, and a 50-µl aliquot of butylparaben at a concentration of 8 µg/ml into a 0.2-ml aliquot drug-free rat plasma. Thus, the corresponding plasma calibration standards were 5, 10, 20, 50, 100, 200 and 500 ng/ml of tamoxifen in plasma, and 0.5, 1, 2, 5, 10, 20 and 50 ng/ml of 4-droxytamoxifen in plasma, and 2 µg/ml for butylparaben. These plasma samples were extracted as described above. Calibration curves of tamoxifen and 4-hydroxytamoxifen were computed using the ratio of the peak area of tamoxifen or 4-hydroxytamoxifen and that of butylparaben as a function of the tamoxifen or 4-hydroxytamoxifen concentrations in plasma. The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Intra-day variability was tested on five different rat's plasma samples using the same calibration curve in a same day. Inter-day variability was tested on five different days and each day a new calibration curve was constructed. The mean relative standard deviation (RSD) of the mean predicted concentration for the independently assayed standards provided the measure of precision. Accuracy was calculated by the percentage deviation of the mean predicted concentration of

tamoxifen and 4-hydroxytamoxifen from the expected target value.

2.5. Pharmacokinetic analysis

The plasma concentration data were analyzed by noncompartmental method using WinNonlin software version 4.1 (Pharsight Corporation, Mountain View, CA, USA). The elimination rate constant (K_{el}) was calculated by log-linear regression of tamoxifen or 4-hydroxytamoxifen 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 time to reach peak concentration (t_{max}) of tamoxifen or 4-hydroxytamoxifen 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 infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el}. Total plasma clearance (CL/F) was calculated by Dose/ $AUC_{0-\infty}$. The absolute bioavailability (AB) of tamoxifen was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral}$, and the relative bioavailability (RB) of tamoxifen was estimated by $AUC_{coadmin}/AUC_{control} \times 100$. The metabolite-parent ratio (MR) was estimated by $(AUC_{4\text{-hydroxytamoxifen}}/AUC_{\ tamoxifen}) \times (MW_{tamoxifen}/MW_{4\text{-hydroxytamoxifen}}) \times 100$.

2.6. Statistical analysis

Statistical analysis was conducted using a one-way ANOVA followed by *a posteriori* testing with the use of the Dunnett correction. Differences were considered to be significant at a level of $p < 0.05$. All mean values are presented with their standard deviation (Mean ± SD).

Table 5. Mean (+ S.D.) plasma concentration-time profiles of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 and 10 mg/kg) (n = 6, each).

Time (hour)	Control	Morin		i.v. tamoxifen (2 mg/kg)
		3 mg/kg	10 mg/kg	
0	0	0	0	2140 ± 514
0.25	63.9 ± 15.3	125 ± 30.0	132 ± 31.7	411 ± 98.6
0.5	92.8 ± 22.3	191 ± 45.8	209 ± 50.2	280 ± 67.2
1	126 ± 30.2	208 ± 49.9	228 ± 54.7	191 ± 45.8
2	119 ± 28.6	194 ± 46.6	213 ± 51.1	146 ± 35.0
3	105 ± 25.2	174 ± 41.8	192 ± 46.1	–
4	97.1 ± 23.3	154 ± 37.0	168 ± 40.3	96.2 ± 23.1
6	88.8 ± 21.3	139 ± 33.4	152 ± 36.5	64.1 ± 15.4
8	81.9 ± 19.7	122 ± 29.3	132 ± 31.7	45.3 ± 10.9
12	61.1 ± 14.7	94.0 ± 22.6	101 ± 24.2	27.9 ± 6.70
24	30.8 ± 7.39	45.0 ± 10.8	49.0 ± 11.8	14.2 ± 3.41
36	15.0 ± 3.60	23.5 ± 5.64	25.1 ± 6.02	–

Table 6. Mean (+ S.D.) plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 and 10 mg/kg) (n = 6, each).

Time (hour)	Control	Morin	
		3 mg/kg	10 mg/kg
0	0	0	0
0.25	2.39 ± 0.57	3.51 ± 0.84	3.92 ± 0.94
0.5	5.58 ± 1.34	7.60 ± 1.82	8.51 ± 2.04
1	9.81 ± 2.35	11.7 ± 2.81	13.1 ± 3.15
2	11.6 ± 2.78	13.3 ± 3.19	14.5 ± 3.48
3	11.2 ± 2.69	12.6 ± 3.02	14.1 ± 3.38
4	10.3 ± 2.47	12.1 ± 2.90	13.5 ± 3.24
6	9.47 ± 2.27	11.3 ± 2.71	12.6 ± 3.02
8	8.83 ± 2.12	10.6 ± 2.54	11.9 ± 2.86
12	7.41 ± 1.78	9.10 ± 2.18	10.2 ± 2.45
24	4.21 ± 1.01	5.71 ± 1.37	6.40 ± 1.54
36	2.43 ± 0.58	3.49 ± 0.84	4.00 ± 0.96

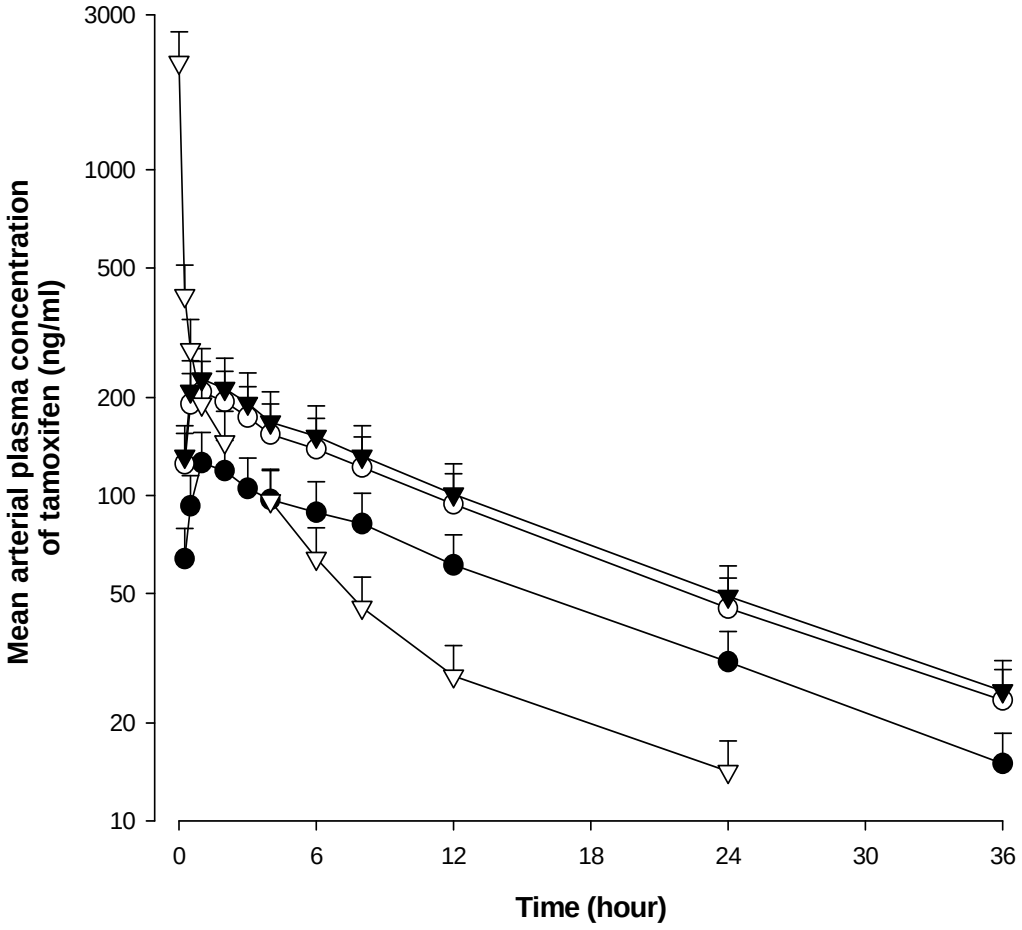


Figure 10. Mean plasma concentration-time profiles of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 and 10 mg/kg) (n = 6, each).

Bars represent the standard deviation, (▽) Intravenous administration of tamoxifen (2 mg/kg); (●) Oral administration of tamoxifen (10 mg/kg); (○) the presence of 3 mg/kg of morin; (▼) the presence of 10 mg/kg of morin.

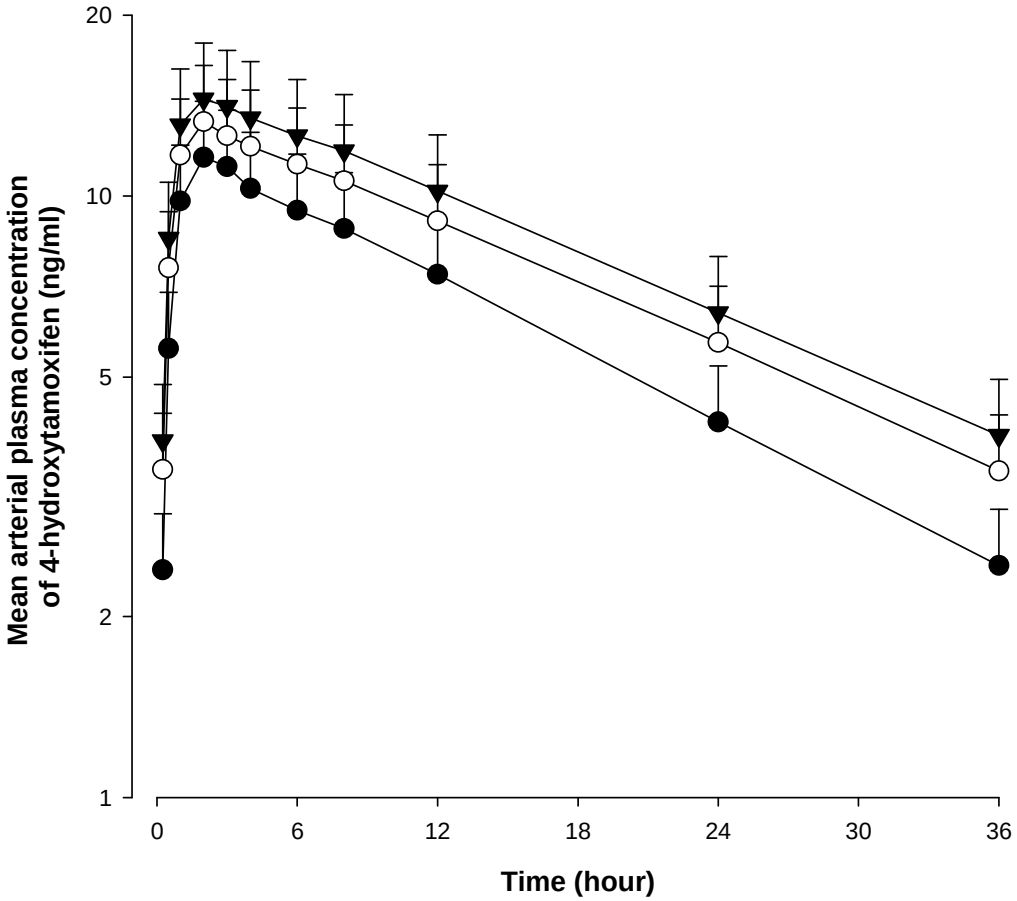


Figure 11. Mean plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 and 10 mg/kg).

Bars represent the standard deviation (n = 6), (●) Oral administration of tamoxifen (10 mg/kg); (○) the presence of 3 mg/kg of morin; (▼) the presence of 10 mg/kg of morin.

Table 7. Mean (+ S.D.) pharmacokinetic parameters of tamoxifen after an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 or 10 mg/kg) (n = 6, each).

Parameter	Control	Morin		i.v. tamoxifen (2 mg/kg)
		3 mg/kg	10 mg/kg	
$AUC_{0\sim\infty}$ ($ng \cdot h \cdot ml^{-1}$)	2137 ± 513	3334 ± 802*	3610 ± 867**	1783 ± 428
C_{max} (ng/ml)	126 ± 30.2	208 ± 49.9*	228 ± 54.7**	
T_{max} (h)	1	1	1	
CL/F ($ml \cdot min^{-1} \cdot kg^{-1}$)	78.0 ± 18.7	50.0 ± 12.1**	46.2 ± 11.1**	18.7 ± 4.49
$t_{1/2}$ (h)	11.6 ± 2.78	11.8 ± 2.83	11.7 ± 2.81	8.80 ± 2.11
AB (%)	23.9 ± 5.74	37.4 ± 8.98*	40.5 ± 9.72*	100
RB (%)	100	156	168	

* $p < 0.05$,** $p < 0.01$ compared to control.

$AUC_{0\sim\infty}$: area under the plasma concentration-time curve from 0 h to infinity;

C_{max}: peak plasma concentration;

T_{max}: time to reach peak concentration;

CL/F: total clearance;

$t_{1/2}$: terminal half-life;

AB: absolute bioavailability;

RB: relative bioavailability.

Table 8. Mean (+ S.D.) pharmacokinetic parameters of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 or 10 mg/kg) (n = 6, each).

Parameter	Control	Morin	
		3 mg/kg	10 mg/kg
$AUC_{0\sim\infty}$ ($ng \cdot h \cdot ml^{-1}$)	271 ± 65.0	361 ± 86.7	409 ± 98.1*
C_{max} (ng/ml)	11.6 ± 2.78	13.3 ± 3.19	14.5 ± 3.48
T_{max} (h)	2	2	2
CL/F ($ml \cdot min^{-1} \cdot kg^{-1}$)	616 ± 148	461 ± 111	408 ± 97.8*
$t_{1/2}$ (h)	15.0 ± 3.60	17.5 ± 4.20	17.8 ± 4.27
MR (%)	12.2 ± 0.290	10.4 ± 2.51	11.0 ± 2.83
RB (%)	100	128	149

* $p < 0.05$ compared to control.

$AUC_{0\sim\infty}$: area under the plasma concentration-time curve from 0 h to infinity;

C_{max}: peak plasma concentration;

T_{max}: time to reach peak concentration;

CL/F: total clearance;

$t_{1/2}$: terminal half-life;

MR: Ratio of the area of metabolite versus parent compound;

RB: relative bioavailability.

3. Results

3.1. Chromatographic separation

Figure 5 illustrates the chromatograms of blank rat plasma (A) and the plasma spiked with tamoxifen, 4-hydroxytamoxifen and the internal standard, butylparaben (B). The tamoxifen, 4-hydroxytamoxifen and butylparaben were eluted with retention times at 26.1, 7.3 and 14.5 min, respectively. Figure 6 and 7 showed the calibration curve of tamoxifen and 4-hydroxytamoxifen constructed by plotting the ratio of the peak area of tamoxifen and 4-hydroxytamoxifen and that of butylparaben as a function of the plasma tamoxifen and 4-hydroxytamoxifen concentrations. There is an excellent linearity over these concentration ranges. The typical equation describing the calibration curve in rat plasma for tamoxifen was y=0017x-0.0384, where y is the peak area ratio of tamoxifen against butylparaben and x is the concentration of tamoxifen, with a mean correlation coefficient of 0.999; for 4-hydroxytamoxifen was y=0.0227x+0.0055, where y is the peak area ratio of 4-hydroxytamoxifen against butylparaben and x is the concentration of 4-hydroxytamoxifen, with a mean correlation coefficient of 0.999. The lower limit of detection for tamoxifen and 4-hydroxytamoxifen in rat plasma defined at a minimum signal-to-noise of three was 5 ng/ml and 0.5 ng/ml. The lower limit of quantification for tamoxifen and 4-hydroxytamoxifen in rat's plasma was 5 ng/ml and 0.5 ng/ml with an acceptable precision and accuracy (RSD: 11.5%, Deviation: −4%, n = 5).

3.2. Effect of morin on the pharmacokinetics of tamoxifen

Mean arterial plasma concentration-time profiles of tamoxifen following an intravenous administration of tamoxifen (2 mg/kg), and an oral administration of

tamoxifen (10 mg/kg) to rats in the presence or absence of morin were shown in Figure 10 and Table 3, corresponding pharacokinetic parameters were shown in Table 5. The presence of morin significantly altered the pharmacokinetic parameters of tamoxifen. Compared to the control group (given oral tamoxifen alone), the presence of tamoxifen significantly reduced the total plasma clearance (CL/F) of tamoxifen ($p < 0.01$, 35.9-40.8%), and significantly ($p < 0.05$ at 3 mg/kg of morin, $p < 0.01$ at 10 mg/kg of morin) increased area under the plasma concentration-time curve ($AUC_{0-\infty}$) and the peak plasma concentration (C_{max}) of tamoxifen 56.0-68.9% and 65.1-80.9%, respectively. The absolute bioavailability (AB) of tamoxifen was 23.9% in oral control group, which was elevated significantly ($p < 0.05$) by 37.4-40.5%. Relative bioavailability (RB) of tamoxifen in the presence of morin was 1.56- to 1.68-fold greater. There are not any significant difference of the time to reach peak plasma concentration (T_{max}) and the terminal half-life ($t_{1/2}$) of tamoxifen in the presence of morin.

3.3. Effect of morin on the pharmacokinetics of 4-hydroxytamoxifen

Mean arterial plasma concentration-time profiles of 4-hydroxytamoxifen after an oral administration of tamoxifen (10 mg/kg) to rats in the presence or absence of morin (3 and 10 mg/kg) were shown in Figure 11 and Table 4, correlated pharacokinetic parameters were shown in Table 6. Compared to the control group, the presence of morin at a dose of 10 mg/kg significantly ($p < 0.05$) reduced the CL/F (33.8%) and increased the $AUC_{0-\infty}$ (50.9%) of 4-hydroxytamoxifen. Although the metabolite-parent ratio (MR) of 4-hydroxytamoxifen decreased in the presence of morin, it was not significant. These results suggest the production of 4-hydroxytamoxifen is not affected by addition of morin. The C_{max}, $t_{1/2}$ and T_{max} of 4-hydroxytamoxifen did not changed significantly by morin.

4. Discussion

Tamoxifen and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 (Desta et al., 2004). Tamoxifen and its metabolites, N-desmethyltamoxifen and 4-hydroxytamoxifen, are substrates for the efflux of P-gp and MRP2 as well (Rao et al., 1994; Gant et al., 1995, Sugimoto et al., 2003; Kauffmann et al., 1998). CYP3A and P-gp inhibitors might interact with tamoxifen and its metabolites and contribute to substantial alteration of their pharmacokinetic fate. Morin was supposed to inhibit P-gp mediated efflux of daunomycin, which was comparable with the potent P-gp inhibitor (Zhang and Morris, 2003a; Buening et al., 1981), and impeded, at least partly, CYP3A mediated C_3'-hydroxylation of paclitaxel in human liver microsomes (Vaclavikova et al., 2003). It is possible that concomitant administration of morin might affect the pharmacokinetics of orally administered tamoxifen.

As shown in Table 7, the prsence of morin significantly reduced the CL/F and increased the $AUC_{0-\infty}$ and C_{max} of tamoxifen. Since orally administered tamoxifen is substrate for CYP3A-catalyzed metabolism and P-gp-mediated efflux in the intestine and liver, pretreatment of morin might be effective to obstruct this pathway. These results are coincident with the report of Choi and Han (2005) in that the presence of morin significantly increased the $AUC_{0-\infty}$ and C_{max} of diltiazem, the P-gp and CYP 3A4 substrate, in rats, and the report of Choi et al. (2006) in that pretreatment of morin significantly increased the $AUC_{0-\infty}$ of paclitaxel in rats. Shin et al. (2006) also reported that coadministration of the morin analogue quercetin at doses of 2.5 and 7.5 mg/kg to rats significantly increased the $AUC_{0-\infty}$ and C_{max} of tamoxifen significantly. The presence of morin although significantly reduced the CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, the MR of 4-hydroxytamoxifen was not altered significantly (Table 4), which suggests morin can not affect the production of 4-hydroxytamoxifen, which was mainly formed by

CYP2D6 (Mani et al., 1993; Crewe et al., 1997). The unaltered MR of 4-hydroxytamoxifen suggests morin, like kaempferol, can not affect CYP2D6-mediated metabolism. The presence of morin could elevated the $AUC_{0-\infty}$ of tamoxifen and 4-hydroxytamoxifen more than its analogue, kaempferol, at the same dosage, which indicates that morin might be more potent than kaempferol in promoting anticancer activity of tamoxifen and the emergence of its toxicity as well.

The presence of the morin enhanced the oral bioavailability of tamoxifen. The dose of tamoxifen should be taken into consideration in the presence of morin and the dietary complements full of morin in patients to avoid the toxicity of tamoxifen.

CONCLUSION

The presence of kaempferol and morin affect the pharmacokinetic parameters of tamoxifen and 4-hydroxytamoxifen as follows:

(1) The total clearance (CL/F) of tamoxifen reduced significantly ($p < 0.05$);

(2) The area under the plasma concentration-time curve ($AUC_{0-\infty}$) increased significantly ($p < 0.05$);

(3) The peak plasma concentration (C_{max}) of tamoxifen increased significantly ($p < 0.05$);

(4) The absolute bioavailability (AB) of tamoxifen in the presence of flavonoids elevated significantly more than the control group.

(5) The relative bioavailability (RB) of tamoxifen increased approximately 1.5-fold.

(6) The two flavonoids reduced CL/F and increased the $AUC_{0-\infty}$ of 4-hydroxytamoxifen, but not significantly except for morin at a dose of 10 mg/kg ($p < 0.05$).

(7) The metabolite-parent ratios (MR) of 4-hydroxytamoxifen did not shown any significant difference.

The presence of the flavonoids, kaempferol and morin, enhanced the oral bioavailability of tamoxifen. Mean while, the systemic exposure of the potential metabolite, 4-hydroxytamoxifen, was elevated. Since kaempferol and morin have many health-improving benefits and have no consistent side effects, they might be provide the therapeutic benefits to improve the oral bioavailability and the anticancer potency of tamoxifen in humans, which need to be confirmed in the clinical setting. Meanwhile, the dosage regimen of tamoxifen in anticancer therapy may take into account of the potential toxic reaction when tamoxifen is used concomitantly with kaempferol and morin or the dietary supplements containing these flavonoids.

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논문제목	한글: 켐페롤과 모린이 타목시펜의 생체이용율에 미치는 영향 영문: THE EFFECT OF KAEMPFEROL AND MORIN ON THE BIOAVAILABILITY OF TAMOXIFEN IN RATS				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의 (√) 반대 ( )

2007 년 02 월

저작자: 설효찬 (서명 또는 인)

조선대학교 총장 귀하