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석사학위논문

프라보노이드와 유기음이온 수송체 (hOAT 과 hOAT3)간의 상호작용에 관한 연구

Interaction Characteristics of Flavonoids with Human Organic Anion Transporter 1(hOAT1)and 3 (hOAT3)

2008 년 2 월 25 일

조선대학교 대학원

약 학 과

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지도교수 한 효 경

이 논문을 약학 석사학위신청 논문으로 제출함

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서 기 수

지 도 교 수 : 한 효 경

조선대학교 대학원 약학과

본 연구의 목적은 천연 프라보노이드와 유기음이온 수송체(hOAT1 과 hOAT3)간의 상호작용에 관한 연구이다. hOAT1 과 hOAT3 가 과다 발현되어 있는 MDCK cell 을 사용하여 5 가지 종류의 프라보노이드(morin, silybin, naringin, naringenin, quercetin)를 선택하여 hOAT1 과 hOAT3 간의 상호작용에 대해 알아보고자 실험하였다. 실험에 선택된 프라보노이드 중에서 특히 morin 과 silybin 에서 [^{3}H]-para-aminohippuric acid([^{3}H]-PAH)의 MDCK hOAT1 세포 내 흡수를 억제 시키는 효과가 두드러지게 나타났으며 Ki 값은 각각 0.46 μM, 24 μM 이었다. 반면에, 이를

제외한 다른 프라보노이드들은 hOAT1 에 비해 hOAT3 에서는 비교적 약한 상호작용을 나타내었고 hOAT1 에서 [^{3}H]-PAH 의 세포 내 흡수 억제는 경쟁적으로 이루어진다는 것을 확인 할 수 있었다. 본 연구에서, 신장 배설과 관련 있는 hOAT1 의 활성에 따른 프라보노이드-약물 상호작용을 통해 새로운 hOAT1 억제제를 확인할 수 있었다.

Abstract

Interaction Characteristics of Flavonoids with Human Organic Anion Transporter 1 (hOAT1) and 3 (hOAT3)

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The present study aimed to investigate the interaction characteristics of flavonoids with human organic anion transporter 1 (hOAT1) and 3 (hOAT3). Five flavonoids (morin, silybin, naringin, naringenin and quercetin) were selected and their interaction characteristics with hOAT1 and hOAT3 were examined in MDCK cells overexpressing hOAT1 or hOAT3. Among tested flavonoids, morin and silybin exhibited significant inhibition effects on the cellular uptake of

[^{3}H]-para-aminohippuric acid ([^{3}H]-PAH) in MDCK-hOAT1 cells with Ki of 0.46 μM and 24 μM, respectively, while all the tested flavonoids appeared to be less interactive with hOAT3 compared to hOAT1. A kinetic study suggested that morin and silybin inhibited hOAT1 mediated-cellular uptake of [^{3}H]-PAH in a competitive manner. Furthermore, morin and silybin were translocated by hOAT1 across the cellular membrane. In conclusion**,** the present study identified some of flavonoids as a new class of hOAT1 inhibitors, suggesting a potential for flavonoid-drug interactions via the modulation of hOAT1 activity.

1.Introduction

As public interest in complementary and alternative medicines increases substantially, various herbal products are available over-the counter in the form of phytopharmaceuticals and the use of herbal products has become more popular for health maintenance or as an adjunct to the mainstream medical care [1, 2]. Accordingly, there is increasing risk of herbal ingredient-mediated pharmacokinetic interactions with conventional medications as indicated by numerous studies elsewhere [3-5]. Among plant polyphenolic compounds, flavonoids are widely present in fruits, vegetables, plant-derived foods and beverages and have shown various biological activities including anticancer and anti-oxidation [6-9]. Furthermore, flavonoids are known to affect drug absorption and disposition via the modulation of drug metabolizing enzymes as well as drug transporters [10, 11]. So far, numerous studies have been done for the interaction of flavonoids with ATP-binding cassette (ABC) efflux transporters such as P-gp, MRP1, MRP2 and ABCG2 [11, 12] but only few studies have been conducted to elucidate the interaction of flavonoids with uptake transporters. For examples, several studies have demonstrated that some of herbal extracts as well as citrus juices could inhibit the OATP-B mediated

cellular uptake of certain drugs during the intestinal absorption [13-15]. Also, Wang et al. [16, 17] have reported that flavonoids could modulate the activity of other uptake transporters such as MCT1 and OATP1B1 (OATP-C). However, little is yet known about the potential interaction of flavonoids with uptake transporters expressed in the kidney.

Organic anion transporters (OATs) located in the apical and basolateral membranes of tubular epithelial cells play a critical role in the distribution and elimination of structurally diverse organic anions including various therapeutics [18, 19]. Among OATs, hOAT1 and hOAT3 are localized in the basolateral membrane of the proximal tubular cells and their expression levels appeared to be much higher than those of other organic anion transporters in the human kidney cortex [20]. Furthermore, many previous studies have reported that hOAT1 and hOAT3 actively involved in the tubular uptake of various therapeutics [18, 19]. Therefore, the modulators of hOAT1 and hOAT3 activities may have high potential for drug interactions in the combination therapy and alter the pharmacokinetics of various xenobiotics particularly that are eliminated from the body mainly via urinary excretion. Indeed, the competition for those transporters could

cause the retention of certain drugs including acyclovir, zidovudine and penicillin, resulting in longer plasma half-lives of drugs [21-23].

Given that the activities of hOAT1 and hOAT3 are important determinants for the pharmacokinetics of various drugs, elucidation of the interaction characteristics of flavonoids with those drug transporters will help us to predict potential flavonoid-drug interactions via the modulation of renal excretion and may lead us to the certain therapeutic benefit in current medical practice. Therefore, the present study aimed to investigate the modulation of hOAT1 and hOAT3 activities by naturally occurring flavonoids. Five flavonoids with structural variation (Fig. 1) were selected and their interaction characteristics with hOAT1 and hOAT3 were examined by using MDCK cells overexpressing hOAT1 or hOAT3.

2.Materials and Methods

Materials: Naringenin, naringin, silybin, morin, quercetin, [^{3}H]-Para-aminohippuric acid (PAH), [^{3}H]-estrone sulfate and BCA protein assay kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal Bovine Serum (FBS), cell culture media, antibiotics and all other reagents used in cell culture studies were purchased from Seolin Science Co. (Seoul, Korea). Madin-Darby canine kidney cells stably transfected with hOAT1 (MDCK-hOAT1 cells) were kindly provided by Dr. John B. Pritchard (NIEHS, North Carolina, USA). MDCK cells overexpressing hOAT3 (MDCK-hOAT3 cells) have been generated as described in the previous report [24] and the functional expression of hOAT3 was confirmed by the uptake of [H^3]-estrone sulfate. MDCK cells were obtained from Korean Cell Line Bank (Seoul, Korea). All other chemicals were reagent grade and all solvents were HPLC grade.

Cell Cultures: MDCK cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% nonessential amino acids, 1 mM sodium pyruvate, 1% L-glutamine and penicillin (100 U/mL)/streptomycin (100 mg/mL). MDCK-hOAT1 and MDCK-hOAT3 cells were maintained in the same media

with G-418 (200 μg/mL) or Blasticidin S (2 μg/mL), respectively. All cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37˚C.

Uptake Studies of morin and silybin: Cells were seeded in 6-well culture plates at a density of 10^5 cells/cm^2. At 5 days post-seeding, the cells were washed twice with pH 7.4 uptake buffer containing 1 mM $CaCl_2$, 1mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose, and 5 mM HEPES. Cells were incubated with a drug solution containing 40 μM of morin or silybin. At the end of 10 min incubation, drug solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline. After the cell lysis, cells were harvested and sonicated for 1-2 min. Acetonitrile (1 mL) was added to the cell lysate, vortexed rigorously, and centrifuged for 5 min at 3000 rpm. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analyzed by HPLC. The protein amount of each sample was determined with BCA protein assay kit following the manufacturer's instruction (Sigma Chemical Co., St. Louis, MO, USA).

Inhibition studies of flavonoids in transfected MDCK cells: The studies were carried out in 12-well plates with confluent cells as

described in the uptake studies. MDCK-hOAT1 and MDCK-hOAT3 cells were incubated with a drug solution containing either 5 μM [^{3}H]-PAH (for hOAT1) or 50 nM [^{3}H]-estrone sulfate (for hOAT3) in the absence or presence of various concentrations of each flavonoid (0.1~400 μM). At the end of 10 min incubation, drug solution was removed and the cells were washed three times with ice-cold PBS. One milliliter of 1.5 % ice-cold Triton X solution was added to each well. After 15 min incubation, cells were harvested and the radioactivity in each sample was determined by a scintillation counter. IC_{50} is defined as the drug concentration to show the 50% inhibition on the uptake of [^{3}H]-PAH or [^{3}H]-estrone sulfate and was determined from the nonlinear regression of a dose-response curve by using the SigmaPlot® 9.0 (Systat Software Inc., Point Richmond, CA, USA).

Kinetic analysis: As described above in the inhibition studies, the concentration dependent uptake of [^{3}H]-PAH was determined in the absence or presence of flavonoids in MDCK-hOAT1 cells. Cells were incubated in a drug solution containing various concentrations of [^{3}H]-PAH in the absence or presence of either morin (50 μM) or silybin (50 μM) for 10 min. Based on the uptake of [^{3}H]-PAH under each condition, double reciprocal plot analyses were performed as shown in Fig. 4.

HPLC analysis: Concentrations of morin and silybin were determined by a HPLC assay slightly modified from the method reported by Hou et al. [25]. Naringenin was used as the internal standard for the assay. The chromatographic system was consisted of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Tokyo, Japan). An octadecylsilane column (Gemini C18, 4.6 × 250 mm, 5μm; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase consisting of acetonitrile: 0.1% ortho-phosphoric acid (30:70, v/v %) for morin or acetonitrile: 10 mM ammonium acetate (35:65, v/v %) for silybin. The flow rate was 1.0 mL/min with the detection wavelength set at 250 nm for morin or 280 nm for silybin. The calibration curve from the standard samples was linear over the concentration range of 0.01 μg/mL to 5 μg/mL. The limit of detection was 0.02 μg/mL.

Statistical analysis: All the means are presented with their standard deviation. The statistical significance of the difference in the parameters was determined using ANOVA followed by a Dunnett's post hoc test or by a Student's t-test. A P value < 0.05 was considered statistically significant.

3.Results

Inhibition effects of flavonoids on hOAT1 and hOAT3: The inhibitory effects of five flavonoids such as morin, naringin, naringenin, silybin and quercetin on the uptake of [^{3}H]-PAH or [^{3}H]-estrone sulfate were determined in MDCK-hOAT1 and MDCK-hOAT3 cells, respectively. Flavonoids were not cytotoxic over the tested concentrations (data not shown). As illustrated in Fig. 2A, all the tested flavonoids except naringin exhibited the significant ($p<0.05$) inhibition effect on the cellular uptake of [^{3}H]-PAH in MDCK-hOAT1 cells. Particularly, morin and silybin exhibited the strong inhibition effect on the cellular uptake of [^{3}H]-PAH with IC_{50} values of 0.5 and 25 μM, respectively (Fig. 3). Compared to the interaction with hOAT1, all the tested flavonoids appeared to be less interactive with hOAT3 and exhibited weak inhibition effects on the cellular uptake of [^{3}H]-estrone sulfate over the drug concentrations up to 400 μM (Fig. 2B) (IC_{50} was not determined due to the solubility limit). To gain insight into the mechanism underlying the inhibition of hOAT1-mediated uptake of [^{3}H]-PAH by flavonoids, [^{3}H]-PAH uptake kinetics in the absence and presence of morin or silybin was determined by Lineweaver-Burk plots. As shown in Fig. 4, both morin and silybin inhibited [^{3}H]-PAH uptake

in a competitive manner with the inhibition constant values (Ki) of 0.46 μM and 24 μM, respectively.

Cellular uptake of morin and silybin mediated by hOAT1: To examine whether hOAT1 recognizes morin and silybin as substrates, the cellular uptake of morin and silybin were evaluated in MDCK and MDCK-hOAT1 cells. As illustrated in Fig. 5, the cellular accumulation of morin and silybin was two to three fold higher in MDCK-hOAT1 cells than the uptake in MDCK cells.

4.Discussion

Among the tested flavonoids, morin appeared to be an effective inhibitor for hOAT1 with a K_i value of 0.46 μM. So far, the pharmacokinetic data of morin are scarce, particularly in humans and it is hard to discuss the clinical dose of morin at this moment. However, in animal studies, Hou et al. [25] reported that morin achieved C_{max} of 3.1±0.8 μM after an oral administration of 25 mg/kg to rats. Considering that Km values of hOAT1-mediated cellular uptake were in the range of 24 ~ 58 μM for adefovir, AZT and cidofovir and 554 μM for methotrexate [26-28], the inhibition potency of morin against hOAT1 activity as well as the rat pk data raises the awareness of potential flavonoid-drug interactions via the modulation of hOAT1 mediated renal excretion. This type of potential flavonoid-drug interactions may produce some therapeutic benefits to reduce or delay the renal toxicity of nephrotoxic agents. Previous studies indicated that hOAT1 should be directly involved in the mechanism of nephrotoxicity associated with cidofovir and thus probenecid, a hOAT1 inhibitor, could be served as nephroprotectants for cidofovir via the inhibition of active renal secretion [26, 29]. However, for nephroprotective effects, probenecid requires administration of a relatively high dose that is

often associated with gastrointestinal intolerance and other adverse effects [30]. On the other hand, Mulato et al. [31] have demonstrated that NSAIDs may reduce or delay the emergence of adefovir nephrotoxicity and /or other drugs that accumulate in proximal tubules primarily via hOAT1. However, chronic therapy with high doses of certain NSAIDs might be also associated with renal insufficiency and other renal effects [32]. Furthermore, hepatotoxicity and other adverse effects have been reported with certain NSAIDs [33]. Therefore, identification of effective hOAT1 inhibitors having superior safety profiles would be more beneficial to reduce or delay the hOAT1-mediated nephrotoxicity of certain drugs. Since there are no obvious safety issues reported so far for natural flavonoids, the results from the present study suggest that morin might be useful to reduce or delay the hOAT1-mediated nephrotoxicity. Considering the greatly increased interest in flavonoids as health supplements, the clinical importance of this finding should be further investigated in clinical studies. On the other hand, all of the tested flavonoids appeared to be less interactive with hOAT3 compared to hOAT1, implying that there should be distinct difference in the substrate specificity between hOAT1 and hOAT3 (Fig. 2).

Kinetic analysis indicated that the inhibition of hOAT1-mediated transport by morin and silybin was competitive in nature (Fig. 4). This result appeared to be comparable to the findings from previous studies using other hOAT1 inhibitors such as ibuprofen and salicylurate [34]. Competitive inhibition was also seen for the inhibitory effects of bumetanide, hydrochlorothiazide, furosemide, and acetazolamide against hOAT1-mediated [^{14}C]-PAH uptake [35]. A competitive type of inhibition of flavonoids against hOAT1 indicates that flavonoids could presumably bind into the transporter active site. Accordingly, the cellular accumulation of morin and silybin were significantly related to the enhancement of the hOAT1 activity in MDCK-hOAT1 cells, implying that morin and silybin should be translocated by hOAT1 across the cellular membrane. Therefore, morin and silybin appeared to be substrates as well as inhibitors of hOAT1.

In conclusion, the present study identified some of flavonoids as a new class of hOAT1 inhibitors, suggesting a potential for flavonoid-drug interactions via the modulation of hOAT1-mediated renal excretion.

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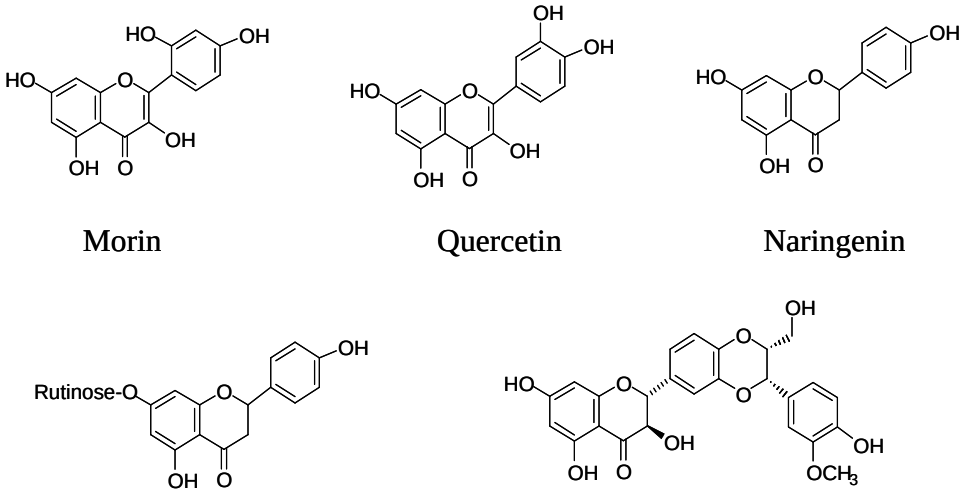
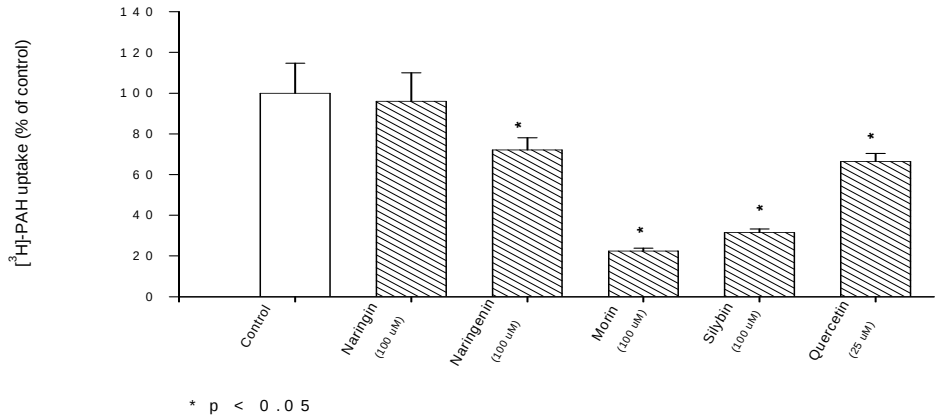


Figure 1. Structures of flavonoids

(A)



(B)

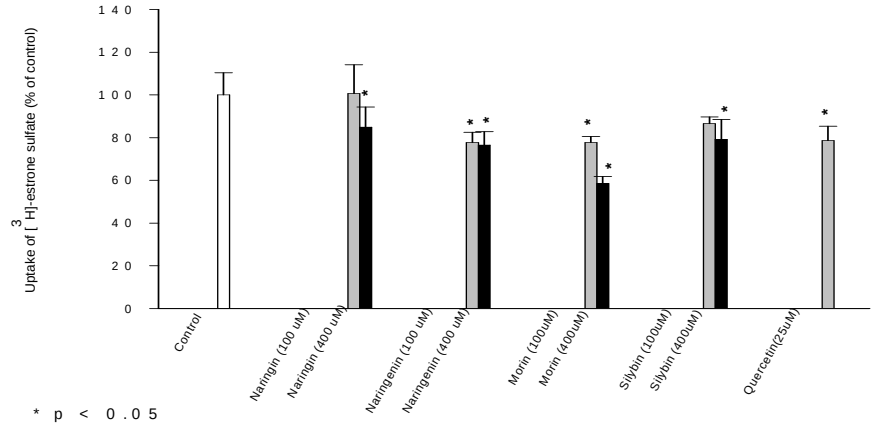
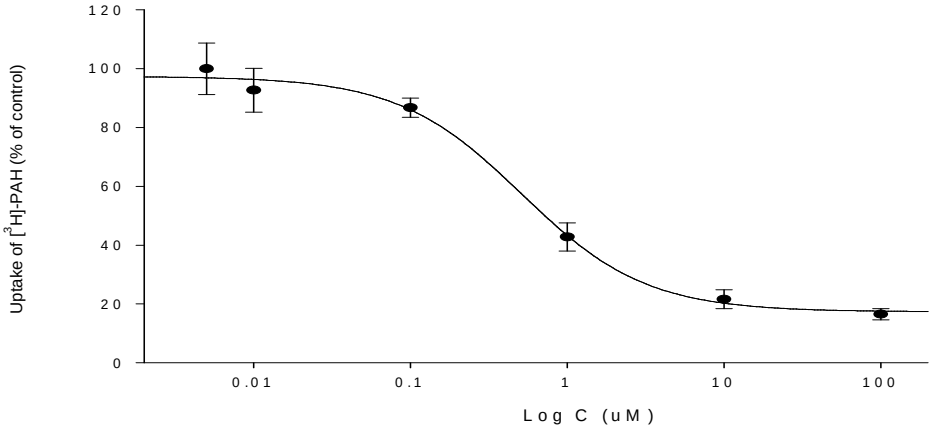


Figure 2. Inhibition effects of flavonoids on the cellular uptake of [^{3}H]-PAH (A) and [^{3}H]-estone sulfate (B) in the transfected MDCK cells (Mean±SD, n=6).

(A) Morin



(B) Silybin

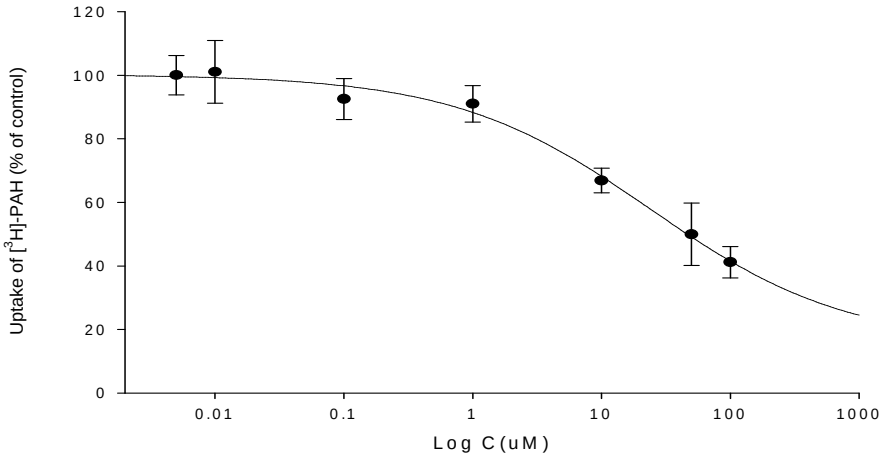
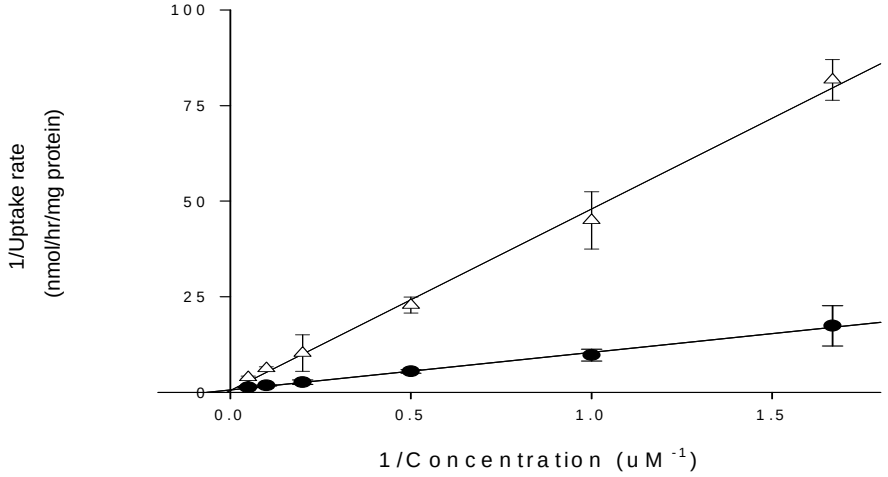


Figure 3. Concentration dependent inhibition of flavonoids on the cellular uptake of [^{3}H]-PAH in MDCK-hOAT1 cells (Mean±SD, n=6).

(A) Morin



(B) Silybin

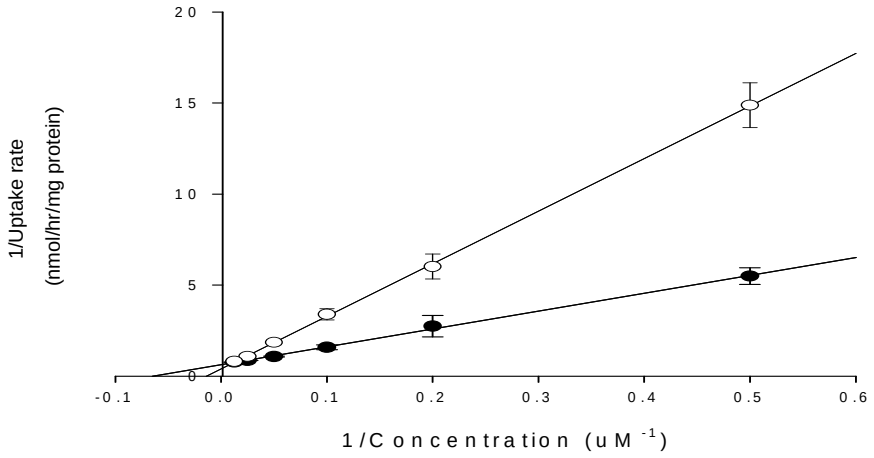


Figure 4. Lineweaver-Burk Plots for the cellular uptake of [^{3}H]-PAH in MDCK-hOAT1 cells (Mean±SD, n = 6). The uptake was measured in the absence (●) and presence of 50 μM morin (Δ) or 50 μM silybin (○).

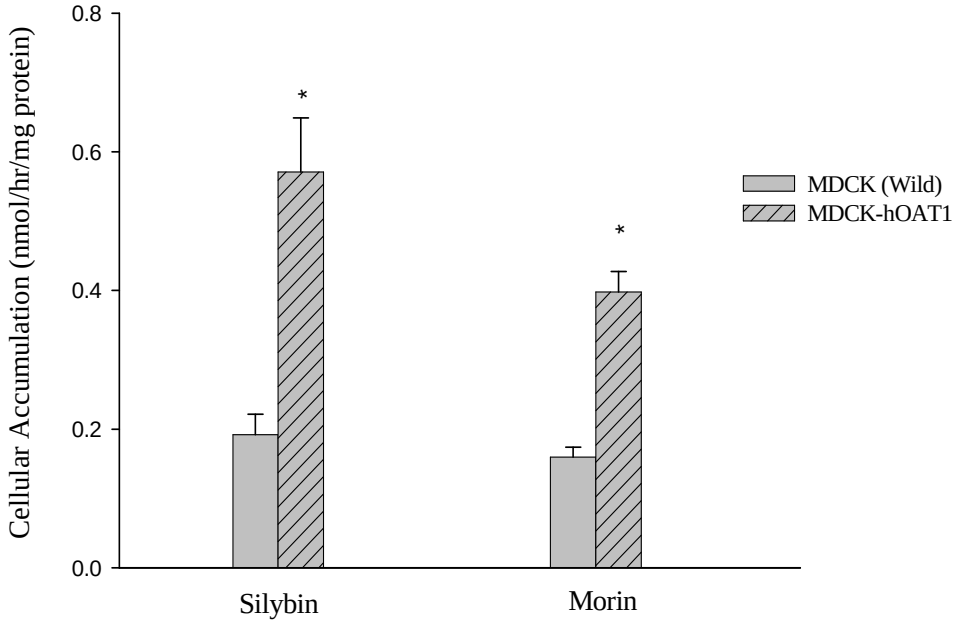


Figure 5. Cellular uptake of morin (40μM) and silybin (40μM) in MDCK and MDCK-hOAT1 cells (Mean±SD, n = 6). *: $p<0.05$, statistically significant difference compared to the uptake in MDCK cells (wild type).

저작물 이용 허락서

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논문제목	한글 : 프라보노이드와 유이음이온 수송체(hOAT1 과 hOAT3)간의 상호작용에 관한 연구 영어 : Interaction Characteristics of Flavonoids with Human Organic Anion Transporter 1 (hOAT1) and 3 (hOAT3)				

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( )

2008 년 2 월 25 일

저작자: 서 기 수 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

2 년간의 대학원 생활에서 부족한 저를 이끌어주시고, 힘들 때마다 격려해 주시며 때론, 따끔한 충고를 아끼지 않으신 저의 지도 교수님, 한효경 교수님께 무한한 감사와 사랑을 드립니다.

또한, 항상 뒤에서 저를 지켜봐 주신 최준식 교수님과 최후균 교수님께 감사드립니다. 저의 대학원 생활을 보다 더 즐겁고 행복하게 만들어준 나의 선배, 김명길, 오정현, 오요한, 천은파, 그리고 후배 임영빈, 임진영 에게도 깊은 감사를 드립니다.

자주 만나지 못해서 항상 아쉬웠던 동규, 동희, 재만, 오랜 시간 알아온 만큼 많은 것을 이해해 주었던 창한, 멋진 대한민국 육군 장교 진혁, 고민이 있을 때 마다 많은 도움을 받았던 창엽, 모두 다 적지 못하는 점이 아쉽지만 저를 응원해준 많은 친구들에게 감사하다고 전하고 싶습니다.

끝으로 사랑하는 부모님과 누나 현지에게, 언제나 걱정 끼쳐서 미안하고 지금까지 가족들이 내게 힘이 되어준 만큼 앞으로는 내가

가족들에게 큰 힘이 되어 줄 것이라고, 언제나 감사하다고 전하고 싶습니다.