Communication to the Editor

Time-Dependent Inhibition of hOAT1 and hOAT3 by Anthraquinones

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We previously showed that anthraquinones (including rhein, emodin, aloe-emodin, chrysophanol and physcion) were inhibitors of human organic anion transporter 1 (hOAT1) and hOAT3, causing transporter-mediated drug–drug interactions in rats. In this study, the time-dependent inhibition (TDI) of hOAT1 and hOAT3 by anthraquinones was investigated. Madin–Darby canine kidney (MDCK)-hOAT1, HEK293-hOAT3 and their parental cells were used. Preincubation with chrysophanol or physcion for 30 min significantly increased the inhibition of hOAT1, but preincubation with rhein, emodin, aloe-emodin or probenecid had no effect on hOAT1 activity. By contrast, preincubation of hOAT3 with emodin, aloe-emodin, chrysophanol or physcion for 30 min significantly increased its inhibition, but preincubation with rhein or probenecid had no effect on activity. As the incubating time lengthened, from 0 to 60 min, both the inhibition of hOAT1 by chrysophanol and physcion and the inhibition of hOAT3 by emodin, aloe-emodin, chrysophanol and physcion were observed to increase in a time-dependent manner. In conclusion, our results suggest that some anthraquinones contribute to the TDI of hOAT1 and hOAT3. An inhibition study without the preincubation procedure may underestimate the inhibitory potential of anthraquinones against hOAT1 and hOAT3. The underlying mechanisms of TDI of hOAT1 and hOAT3 need to be further investigated.

Key words organic anion transporter; anthraquinone; time-dependent inhibition

In CYP450 enzyme-mediated metabolism, time-dependent inhibition (TDI) has attracted significant attention. TDI of CYP450 enzymes refers to a change in the inhibitory potency during an in vitro incubation or dosing period in vivo. However, does drug transporter-based TDI exist? Amundsen et al. reported that inhibition of organic anion transporting polypeptide 1B1 (OATP1B1) by cyclosporine A occurred in a time-dependent manner. Preincubation of OATP1B1 with cyclosporine A increased the inhibition of this protein; however, the underlying mechanism was not described in that report. Other researchers found that preincubation of OATP2B1 with apple juice or orange juice increased their inhibition effect. In general, there are very few articles that report on drug transporter-based TDI.

In our previous study, we found that anthraquinones (rhein, emodin, aloe-emodin, chrysophanol or physcion) inhibited human organic anion transporter 1 (hOAT1) and hOAT3. Anthraquinones were coincubated with substrates of hOAT1 or hOAT3 in that study. However, we observed that preincubation of hOAT1 and hOAT3 with some of the anthraquinones increased hOAT1 and hOAT3 inhibition. Currently, TDI of the OATs family has not been studied. Therefore, in this study, the inhibitory effect of preincubation anthraquinones with hOAT1 and hOAT3 was investigated. The results provide evidence for the complex inhibitory mechanisms of drug transporters.

MATERIALS AND METHODS

Drugs and Reagents Rhein, emodin, aloe-emodin, chrysophanol, physcion and probenecid were obtained from the National Institutes for Food and Drug control (Beijing, China). Six-carboxyl fluorescein (6-CFL) and fluorescein (Fluo) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (Gaithersburg, MD, U.S.A.). Geneticin (G418) was purchased from Calbiochem Co. (San Diego, CA, U.S.A.). Hygromycin was purchased from Roche Co. (Mannheim, Germany). The bicinchoninic acid (BCA) kit was purchased from Beyotime Biotechnology (Jiangsu, China). Other reagents used are analytical grade.

Cell Origins and Cell Culture Madin–Darby canine kidney (MDCK)-hOAT1 and MDCK-mock cell lines were established in our laboratory. HEK293-hOAT3 and HEK293-mock cell lines were a kind gift from Dr. Dafang Zhong (Shanghai Institute of Materia Medica, Chinese Academy of Science, China). MDCK cells were grown in DMEM medium containing 10% FBS, 350 µg/mL G418, 100 units/mL penicillin and 10 units/mL streptomycin. HEK293 cells were grown in DMEM medium containing 10% FBS, 100 µg/mL hygromycin, 100 units/mL penicillin and 100 units/mL streptomycin. All cells were cultured at 37°C and under 5% CO2 conditions.

Inhibition Study Cells were seeded in 48-well culture plates and incubated for 48 h for MDCK and 72 h for HEK293. Cells were washed twice with preheated Hank’s balanced salt solution (HBSS) and were preincubated with blank HBSS or HBSS containing a series of concentrations of inhibitors for 30 min. After the preincubation procedure, the preincubation buffer was removed and HBSS containing inhibitors (with the same concentrations as the preincubation procedure) and substrates were added to initiate cellular uptake. For hOAT1, 4 µM of 6-CFL and a 4 min incubation period were used, whereas for hOAT3, 5 µM of Fluo and a 3 min incubation period were used. Uptake was terminated by the addition of ice-cold phosphate-buffered saline (PBS) and washed thrice with ice-cold PBS rapidly. Cells were lysed with 200 µL of 0.5 M sodium hydroxide and 0.1% TritonX-100. An aliquot of 100 µL lysisate was used to determine the fluorescence by a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) and normalized to the total protein concentration in the lysate. The protein concentration in the cell lysate was determined by a BCA assay.

The effect of increasing the preincubation time on the inhibitory activity was also investigated. For hOAT1 inhibition, HBSS containing 1 µM of chrysophanol or physcion was preincubated with cells for 0, 10, 20, 30 and 60 min. For hOAT3 inhibition, HBSS containing 0.5 µM of emodin, 2 µM of aloe-emodin, 5 µM of chrysophanol or 5 µM of physcion was
preincubated with cells for 0, 10, 20, 30 and 60 min. After pre-
incubation, HBSS containing substrates and inhibitors (with 
the same concentrations as the preincubation procedure) was 
added to initiate cellular uptake. Other procedures were the 
same as described above.

RESULTS AND DISCUSSION

As shown in Fig. 1, shows that preincubation with chryso-
phanol or physcion for 30 min increased their inhibition poten-
cy towards hOAT1, whereas preincubation with rhein, emodin, 
alo-emodin or probenecid had no effect on the inhibition of 
hOAT1. In contrast, preincubation with emodin, alo-emodin, 
chrysophanol or physcion for 30 min increased their inhibi-

![Fig. 1. Inhibition of hOAT1-Mediated 6-CFL Transport by Five Anthraquinones and Probenecid after Preincubation with (Black Bars) or without 
These Inhibitors (White Bars) for 30 min 
The results shown are the mean±S.D. (n=3). *p<0.05, **p<0.01, ***p<0.001 vs. control group.]

![Fig. 2. Inhibition of hOAT3-Mediated Fluo Transport by Five Anthraquinones and Probenecid after Preincubation with (Black Bars) or without 
These Inhibitors (White Bars) for 30 min 
The results shown are the mean±S.D. (n=3). *p<0.05, **p<0.01, ***p<0.001 vs. control group.]

tion potency towards hOAT3, whereas preincubation with rhein or probenecid had no effect on the inhibition of hOAT3 (Fig. 2). The influence of preincubation with anthraquinones on hOAT1 and hOAT3 is not the same. Preincubation with emodin and aloe-emodin increased their inhibitory potency towards hOAT3 but not hOAT1. The effect of preincubation time on the inhibitory effect towards hOAT1 and hOAT3 was also investigated. Prolonging the preincubation time increased the inhibitory effect, as showed in Figs. 3 and 4. Preincubation for 60 min did not lead to a full inhibition.

Our previous study observed that chrysophanol and physcion were weak inhibitors of hOAT1 and hOAT3, with IC$_{50}$ values over 10 $\mu$M. However, preincubation with 1 $\mu$M chrysophanol or physcion for 60 min resulted in 62 and 49% inhibition of hOAT1, respectively. In addition, preincubation with 5 $\mu$M chrysophanol or physcion for 60 min resulted in 64 and 57% inhibition of hOAT3, respectively. The inhibition of emodin and aloe-emodin to hOAT3 were increased as well. IC$_{50}$ value of emodin and aloe-emodin were 1.22 and 5.37 $\mu$M to hOAT3 in our previous study.$^5$ In this study, preincubation with 0.5 $\mu$M emodin or 2 $\mu$M aloe-emodin for 60 min resulted in 76 and 70% inhibition of hOAT3. These results indicate that preincubation with some anthraquinones significantly increase their inhibition effects towards hOAT1 and hOAT3, and also suggest that an inhibition study without a preincubation procedure may underestimate the inhibition potency of anthraquinones towards OATs.

What are the underlying mechanisms of TDI by anthraquinones? In the inhibition of CYP450 enzymes, potential mechanisms for TDI include the formation of more potent

![Fig. 3. The Effect of the Preincubation Time on the Inhibition of hOAT1-Mediated 6-CFL Uptake by Chrysophanol (1 $\mu$M) and Physcion (1 $\mu$M). The results shown are the mean±S.D. (n=3).](image)

![Fig. 4. The Effect of the Preincubation Time on the Inhibition of hOAT3-Mediated Fluor Uptake by Emodin (0.5 $\mu$M), Aloe-Emodin (2 $\mu$M), Chrysophanol (5 $\mu$M) and Physcion (5 $\mu$M). The results shown are the mean±S.D. (n=3).](image)
inhibitory metabolites or mechanism-based inhibition. In mechanism-based inhibition, the inhibitor is metabolized initially by enzymes to form a reactive intermediate that can covalently bind to enzymes, leading to enzyme inactivation. However, there is no report indicating that OATs have the potency to metabolize OATs substrates. Such mechanisms may not apply to OATs.

Preincubation may increase the intracellular concentration of inhibitors and then increase their inhibitory activity. However, preincubation with probenecid could not increase its inhibitory activity to hOAT1 and hOAT3. In addition, not all anthraquinones showed TDI of hOAT1 and hOAT3, and preincubation with anthraquinones showed different effects between hOAT1 and hOAT3. We found in our previous study that five anthraquinones were not substrates of hOAT1 or hOAT3. Therefore, the point that TDI is caused by the increased intracellular concentration could not explain the selectivity of TDI by only some of the anthraquinones. Besides, hOAT1 and hOAT3 are expressed on the cell membrane and mediate the cellular uptake of substrates. Inhibitors may bind to transporters from inside or outside of the cell membrane. If inhibitors bind to transporters from outside, intracellular concentration may not be related to the binding of inhibitors to transporters. The mechanisms of TDI of OATs by some of the anthraquinones need to be investigated in the future.

In conclusion, preincubation of particular anthraquinones with hOAT1 and hOAT3 increased their inhibition potency against these proteins. This is the first study about TDI inhibition of hOAT1 and hOAT3. However, the underlying mechanism remains unknown and requires further investigation. Our study suggests that drug transporter inhibition may be caused by complex mechanisms.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES