Interaction of baicalin with transporters

PhD thesis

Kalaposné Kovács Bernadett

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Supervisor: Dr. Imre Klebovich, D.Sc
External Consultant: Dr. Péter Krajcsi, D.Sc

Official reviewers: Dr. Éva Szökő, D.Sc
Dr. Györgyi Horváth, Ph.D

Head of the Final Examination Committee:
Dr. Tamás Török, D.Sc

Members of the Final Examination Committee:
Dr. László Tóthfalusi, Ph.D
Dr. Gábor Halmos, Ph.D

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Introduction

Radix Scutellariae (RS), officially listed in the Chinese Pharmacopoeia, is the dried root of the medicinal plant Scutellariae baicalensis, known as Huang Qin in Chinese traditional medicine. More than 50 flavonoids have been purified and identified from RS. The major components are baicalin (baicalein-7-O-glucuronide, BG), and its aglycone baicalein (5, 6, 7 -trihydroxyfavone, B). Due to their relatively low toxicity and high abundance in RS, baicalin and baicalein became the most widely researched components in recent years. Numerous in vivo and in vitro studies carried out in the last decade demonstrated that baicalin and its aglycone baicalein were important medical agents with a variety of pharmacological activities such as chemopreventive, hepatoprotective, antioxidant, anti-allergic, anti-microbial, anti-inflammatory, neuroprotective, as well as anti-tumor effects.

Upon oral intake, baicalin is either directly absorbed from the upper intestinal tract or undergoes hydrolysis to release its aglycone B, which will then be absorbed via passive diffusion.

Concomitantly upon oral intake of baicalein, baicalein is absorbed via passive diffusion. Absorbed baicalein undergoes extensive first-pass intestinal Phase II metabolism in enterocytes, including glucuronidation (>90%), resulting in its conjugated glucuronide metabolite, baicalin. Although baicalein demonstrates good permeability due to its good lipophilicity, its glucuronide baicalin formed inside the intestinal epithelial cells is too polar to cross the lipid bilayer by passive diffusion. Several animal studies showed that baicalin, instead of baicalein, was the predominant form in the general blood circulation after oral administration of baicalein or baicalin.

In recent years, a wealth of evidence has been generated from *in vitro* and *in vivo* studies showing that baicalin could interact extensively with drug transporters and might play critical roles in multidrug resistance reversal and drug disposition.

Recent study using rat intestine perfusion model and Caco-2 monolayer model uncovered that baicalein was rapidly converted to baicalin, before being transported to

the mesenteric system. In addition, significant biliary as well as sinusoidal transport of baicalin from hepatocytes was shown. An increase of the sinusoidal transport was seen in Mrp2-deficient rats. Thus, the effective enterohepatic transport of intracellularly formed glucuronides of baicalein likely depends on a carrier-mediated transport. ATP-binding cassette (ABC) transporters and organic anion transporting polypeptides (OATP) transport a wide variety of substrates including metabolic products. The members of multidrug resistance associated proteins (MRPs), especially MRP2 (ABCC2) and MRP3 (ABCC3), possess similar substrate selectivity and prefer to transport organic anions and phase II metabolites, including glutathione, glucuronide and sulfate conjugates. It also seems likely that MRP2 and/or breast cancer resistance protein (BCRP; ABCG2) contribute to the efflux of flavonoid conjugates across the intestinal apical membrane. In recent studies, baicalin was shown to inhibit very efficiently BCRP-, MRP2- and MRP3-mediated vesicular transport and to activate the ATPase activity of BCRP, MRP2 and MRP3. This indicates that MRP3 could play a role in the basolateral transport of baicalin from intestinal cells while MRP2 and BCRP might be the transporters effluxing baicalin on the apical side of enterocytes. Another interaction of baicalin with candidate uptake transporters organic anion transporting polypeptide 2B1 (OATP2B1) and OATP1B3 has been shown in inhibition assays. In contrast, OATP1B1 and OATP1A2, the other hepatically and/or intestinally localized uptake transporters were not inhibited by baicalin.

Moreover, by inhibiting the activity of ABC transporters, baicalin could modulate absorption and disposition of drugs, increasing the risk of therapeutic failure, adverse effects and toxicity. Altered drug disposition due to pharmacokinetic interactions may result in clinically relevant changes in drug ADME properties and therefore drug efficacy or toxicity. These effects are crucial and must be considered while developing clinical regimens.

Aims

As explained in the introduction, in recent years, a wealth of evidence has been generated from *in vitro* and *in vivo* studies showing that baicalin could interact extensively with drug transporters and might play critical roles in multidrug resistance reversal and drug disposition. Altered drug disposition due to pharmacokinetic interactions may result in clinically relevant changes in drug ADME properties and therefore drug efficacy or toxicity. Moreover, since baicalin and baicalein have such enormous therapeutic potentials, a better understanding of their pharmacokinetics and bioavailability is necessary for extrapolating the data from pharmacological assays to clinical effects and developing clinical regimens.

The objectives of this thesis were therefore:

- to investigate the inhibitory effect of baicalin on ABC transporters
- to identify the transporters responsible for the efflux of baicalin from enterocytes and hepatocytes
- to identify transporters responsible for the uptake of baicalin into hepatocytes

The information obtained from these studies will help us better understand and predict the potential in vivo baicalin -drug interactions mediated by these drug transporters and to elucidate pharmacokinetics of baicalin.

Methods

Efflux transport studies

It has been demonstrated that the vesicular transport assay is a useful tool to investigate the contribution of transporters in the permeability of flavonoids. Current FDA and EMA guidance for drug transporter interactions is dependent on IC_{50} measurements as these are utilized in determining whether a clinical interaction study is warranted. These guidances contain decision trees on whether a clinical drug-drug interaction study is warranted which are based on the IC_{50} value in combination with clinical drug concentrations

Vesicular transport inhibition studies

First, inhibitory and direct vesicular transport studies were performed, using membrane vesicles from cells overexpressing the transporter of interest. The vesicular transport assay relies on membrane preparations enriched in inside-out vesicles. In this orientation, the binding sites of the transporter are facing the solvent; thus substrate molecules will be pumped into the membrane vesicles in an ATP dependent manner. Amount of transported substrate into vesicles was investigated using rapid filtration techniques.

Inhibitory effects of baicalin:

- on transport of N-methyl-quinidine in membrane vesicles from human myelogenous leukemia cells (K562) overexpressing multidrug resistance protein 1(MDR1),
- on transport of bimane-glutathion conjugate (BAICALINS) in membrane vesicles from *Spodoptera frugiperda* ovarian cells (Sf9) overexpressing MRP1,
- on transport of estradiol-17-β-D-glucuronide (E217βG) in membrane vesicles from Sf9, then human embryonic kidney cells (HEK293) overexpressing human MRP2,

- on transport of E217βG in membrane vesicles from Sf9, then HEK293 cells overexpressing human MRP3,
- on transport of DHEAS in membrane vesicles from HEK 293 cells overexpressing human MRP4 and
- on transport of estrone-3-O-sulfate (E3S) in vesicles from Michigan Cancer Foundation-7 (MCF7) cells expressing high levels of BCRP were investigated in the presence and absence of ATP, by adding increasing concentrations of baicalin, with rapid filtration techniques.

Inside-out membrane vesicles were preincubated. The ^{3}H labeled transporter specific substrate was added to the mixture. The reaction volume was 75 µl in each well, with 50 µg protein/well. 0.75 µl of a baicalin dilution series was added to each well. The dilution series was diluted 150-fold when added to the wells. Transport was initiated with the addition of 4 mM ATP or AMP in the appropriate well. Transport was carried out under specific incubation temperature and time conditions.

The transport was stopped by the addition of cold wash buffer, after specific incubation time. The samples were transferred to class B glass fiber filters, 1-µm pore size (Millipore, Billerica, MA). Filters were washed with 5 x 200 µL of ice-cold wash buffer and dried. After adding 100 µL of scintillation cocktail to each well, radioactivity retained on the filter was measured by liquid scintillation counting (Perkin Elmer 1450 LSC, Luminescence counter, Microbeta Trilux).

Results were obtained in cpm.

ATP-dependent transport of the radiolabeled substrate was calculated. The IC_{50} is defined as the concentration needed to inhibit transport of the reporter substrate by 50%. The IC_{50} parameters were derived from the equation of a one-binding site, dose-response curve fitted onto the relative activity against the concentration of baicalin, plotted by non-linear regression using GraphPad Prism 5 (San Diego, CA, USA).

Calculations:

ATP dependent transport (cpm): the mean cpm values measured in the absence of ATP were substracted from the mean cpm values measured in the presence of ATP.

ATP dependent transport (pmol/mg/min): *Total activity (cpm)* was calculated by multiplying the cpms measured in the designated well. The rate of transport in pmol/mg membrane protein/min was calculated using the following formula:

$$\frac{ATP\ \ dependent\ \ \ transport\ \ \ (cpm)}{Total\ \ activity\ \ (cpm)} * \frac{Substrate\ \ \ concentrat\ \ \ ion\ \ (nM) * Volume\ \ (ml)}{membrane\ \ \ \ protein\ \ (mg) * tim\ e\ (min)}$$

ATP dependent transport (%): the percent activation or inhibition of the test drug. In this representation the ATP dependent transport determined in the *drug fee control* were taken as 100% and all other values were represented on this relative scale.

All experiment were performed 3 times in duplicates.

Vesicular transport subtstrate studies

Direct vesicular transport feasibility experiments were performed to evaluate whether ATP-dependent accumulation of baicalin could be detected directly in transporter-overexpressing membrane vesicles, with rapid filtration techniques.

Accumulation of baicalin:

- in membrane vesicles from K562 cells overexpressing MDR1,
- in membrane vesicles from Sf9 cells overexpressing MRP1,
- in membrane vesicles from HEK293 cells overexpressing human MRP2,

- in membrane vesicles from HEK293 cells overexpressing human MRP3,
- in membrane vesicles from HEK293 cells overexpressing human MRP4 and
- in vesicles from MCF7 cells expressing high levels of BCRP were investigated in the presence and absence of ATP with rapid filtration techniques.

All experiments were also performed on functionally defective membranes (HEK293 control, control K, control M). The effectiveness of the membranes was controlled with a specific substrate for each transporter.

The reaction was performed at two baicalin concentrations (low and high) - adjusted to the previous IC_{50} data for each transporter, and two time points. Experiments were repeated 2 more times with criteria were the accumulation rate was maximal.

Inside-out membrane vesicles were preincubated. Baicalin was added to the mixture. The mixture was incubated in the presence of 4 mM ATP of AMP in the appropriate assay buffer and temperature. The reaction volume was 75 µl in each well, with 50 µg protein/well. The transport was stopped by the addition of the appropriate cold wash buffer. The samples were transferred to class B glass fiber filters, 1-µm pore size (Millipore, Billerica, MA). Filters were washed with 5 x 200 µL of ice-cold wash buffer. The vesicles were lysed with 2 x 150 µl methanol and the eluted volumes, containing baicalin, were collected, the organic solvent dried with a speedvac concentrator (Thermo DNA 120) and the baicalin on the plates were subjected to bioanalysis.

The amount of transported baicalin was determined by LC-MS/MS analysis.

An LC-MS/MS method was developed to quantify baicalin in membrane vesicles. The analytical method was developed in collaboration with the Institute of Organic Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences. The chromatographic separation used was an "inverse gradient elution" on a reversed phase column. Quantification of baicalin was determined by multiple reaction monitoring mode using electrospray ionization.

2 mode using electrospray ionization (ESI). ATP-dependent transport was calculated.

All experiment were performed 3 times in triplicates.

Uptake transport studies

Uptake transporter studies were performed on:

- OATP2B1-MDCKII,
- wild type MDCKII.
- OATP1B3-HEK293 and
- HEK293 mock cells to figure out whether baicalin is a substrate of these transporters.

First, feasibility screening, experiments were performed by adding baicalin at two different concentrations and the cells were incubated at 37ºC for 2 selected incubation times.

Time course experiments were performed to determine ideal incubation time. Cells were incubated at 37ºC for 8 indicated periods (1-45 minutes) after adding 5 μM baicalin to the transport medium in transfected and control cells.

For concentration dependence, experiments were performed by adding 8 different concentrations (1 μM - 100 μM) of baicalin to the transport medium in transfected and control cells, for the above determined incubation time. Kinetic parameters were determined using GraphPad Prism.

The amount of transported baicalin was determined by the above LC-MS/MS analysis. All experiment were performed 3 times in triplicates.

Bicinchoninic acid kit was used to check the total protein concentrations in cells. Positive control experiments with specific substrates of the transporters were performed to control the activity of the cells.

Results

- Concentration-dependent, pharmacologically relevant inhibition of BCRP-, MDR1-, MRP2-, MRP3- and MRP4-mediated transport by baicalin was observed after performing vesicular transport inhibition studies. Transport by BCRP was inhibited by baicalin with an IC_{50} of 3.41 ± 1.83 μM. Inhibition of MRP3 and MRP4 was also potent (IC_{50} values of 14.01 ± 2.51 μM and 14.39 ± 5.69 μM respectively). Inhibition of MDR1 (IC_{50} = 94.84 ± 31.10 μM) and MRP2 (IC_{50} = 210.13 ± 110.49 μM) was less potent.
- Vesicular transport study results clearly identified MRP3 and MRP4 as the transporters implicated in basolateral efflux of baicalin, and MRP2 and BCRP as the transporters involved in the apical efflux of baicalin.
- Uptake feasibility screen data showed that baicalin exhibited an about 7.89 – 21.51 -fold greater uptake in OATP2B1 expressing cells compared to wild type MDCKII control cells under identical conditions. In contrast, baicalin uptake was 1.08 – 1.91 -fold greater in OATP1B3-expressing cells compared to HEK293-Mock control cells. According to generally accepted regulatory guidelines a 2-fold uptake over control is considered pharmacologically significant. Therefore, further characterization was only carried out for the OATP2B1 – baicalin interaction.
- Time dependence of baicalin uptake by OATP2B1 was determined. Curve showed linear kinetics up to 10 minutes. Ideal incubation time was determined by fitting a line at 3 minutes.
- Concentration dependence of OATP2B1-mediated uptake of baicalin was assessed at 3 minutes incubation time. baicalin transport by OATP2B1 was saturable and displayed transporter specific K_m, V_{max} and intrinsic clearance (Cl_{int}) values of 9.71 ± 3.56 μM, 1575 ± 674.58 pmol/mg protein min and 162.13 μl/min mg protein respectively.

Conclusion

Since baicalin and baicalein have extensive therapeutic effects, a better understanding of their pharmacokinetics and bioavailability is necessary to specify clinical effects, developing clinical regimens and elucidating potential drug interactions.

Vesicular transport assay using vesicles overexpressing the transporter of interest were used to explore potential drug-herb interaction by investigating the inhibitory effect of baicalin on the transport of reporter substrates by efflux transporters and to identify the transporters responsible efflux of baicalin from enterocytes and hepatocytes. Uptake studies were performed on cells overexpressing the transporter of interest to identify transporters responsible for the uptake of baicalin into these cells.

According to the obtained results, it is probable that after passive diffusion of baicalein into the enterocytes, an intensive glucuronidation to baicalin takes place in the small intestinal wall. Baicalin will be effluxed to the mesenteric blood mainly by MRP3 and MRP4, located on the basolateral side. OATP2B1 cooperates with MRP3 and MRP4 to enhance absorption of baicalin in the upper intestinal tract. Part of baicalin will be pumped back to the intestinal lumen by apically located MRP2 and BCRP. The circulating baicalin would be expected to re-enter the gastrointestinal tract via the biliary excretion mechanism. In the liver, OATP2B1 mediates hepatic uptake from the blood, and MRP2 or BCRP mediate biliary excretion, whereas MRP3 and MRP4 are partly responsible for the sinusoidal efflux of baicalin back to the blood. Moreover, via interaction with transporters at a physiologically relevant concentration, baicalin could interfere with the absorption and disposition of drugs, e.g. methotrexate, increasing the risk of therapeutic failure, adverse effects and toxicity.

Author's publications

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