ABC G2 Modulates Chlorothiazide Permeability

*In Vitro*–characterization of Its Interactions

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Summary: We are showing that chlorothiazide, a diuretic, is an ABCG2 substrate. It is a Biopharmaceutics Classification System/Biopharmaceutics Drug Distribution and Classification System (BCS/BDDCS) Class IV drug with low bioavailability. Therefore, we tested if chlorothiazide interacts with major apically located intestinal efflux transporters. Our data show that chlorothiazide is transported by ABCG2 with a Km value of 334.6 µM and does not interact with ABCB1 or ABCC2. The chlorothiazide–ABCG2 interaction results in a vectorial transport in MDCKII-BCRP and Caco-2 cells with efflux ratios of 36 and 8.1 respectively. Inhibition of ABCG2 in Caco-2 cells reduced the efflux ratio to 1.4, suggesting that ABCG2 plays a role in limiting chlorothiazide bioavailability in humans.

Keywords: chlorothiazide; absorption; ABCG2 substrate; vesicular transport; MDCKII-BCRP; Caco-2; probe for regulatory studies

Introduction

Chlorothiazide is a thiazide-type diuretic drug.1 It is highly H-bonded and partly ionized at a physiological pH, explaining the compound’s low passive permeability.2 Chlorothiazide has a low oral bioavailability.3 As chlorothiazide is basically non-metabolized4 this implicates a role as an efflux transporter. Chlorothiazide is classified as a Class IV drug,5 a class for which both influx and uptake transporters are suggested to play a role in the pharmacokinetics of member compounds.5

In the present work we tested if interaction of chlorothiazide with efflux transporters located in the apical membrane of enterocytes modulates chlorothiazide permeability. We are showing that chlorothiazide specifically interacts with ATP-binding cassette sub-family G member 2 (ABCG2/BCRP) and ABCG2 modulates permeability of chlorothiazide in human colon carcinoma cells (Caco-2) and Madin-Darby canine kidney type II (MDCKII) cells over-expressing the breast cancer resistance protein (MDCKII-BCRP). In addition, we characterized the chlorothiazide–ABCG2 interaction employing a vesicular transport assay.

Materials and Methods

Materials: [3H]-Methyl-quinidine was purchased from Dr. Csaba Tömböly (Biological Research Center, Hungary). [3H]-Estradiol-17-β-D-glucuronide (41.8 Ci/mmol) and [3H]-Estrone-3-sulfate (54.3 Ci/mmol) (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) were purchased from Medispect Kft. (Fót, Hungary). Ko143 was obtained from Professor Gerrit-Jan Koomen (U. Amsterdam, Amsterdam, The Netherlands).

Fetal bovine serum (FBS, Lonza, Basel, Switzerland), Eagle’s Minimum Essential Medium (EMEM, Lonza), Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza), and penicillin-streptomycin (Lonza) were purchased from Biocenter Kft. (Szeged, Hungary). Chlorothiazide and other chemicals were purchased from Sigma-Aldrich (Sigma Aldrich Kft, Budapest, Hungary) and were of analytical grade.

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[Note: The text continues with further details about the experimental methods, data analysis, and conclusions derived from the study.]
Membranes: Membrane vesicle preparations expressing the human ABCB1, ABCC2, and ABCG2 transporters were prepared by Solvo Biotechnology (Szeged, Hungary) from baculovirus-infected Sf9 cells (MDR1-Sf9, MRP2-Sp, and BCRP-HAM-S9, respectively) and from ABCG2 over-expressing mammalian cells (BCRP-M) and its control (M-Ctrl).

Cell lines: MDCKII cells were kindly provided by Professor Kai Simons at Max Planck Institute (Dresden, Germany) and the MDCKII-BCRP cells originally obtained from Professor Heyo Klaus Kroemer at University of Greifswald (Greifswald, Germany) were from Solvo Biotechnology. The Caco-2 cell line was obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM (MDCKII) and EMEM (Caco-2) containing 10% (v/v) heat-inactivated FBS, 2 mM l-glutamine and 100 µg/ml penicillin-streptomycin, supplemented with 1% non-essential amino acids. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Vesicular transport assay: Inhibition experiments using vesicular transport employing ABCB1, ABCC2 and ABCG2 over-expressing Sf9 membranes were performed according to the manufacturer's suggestions, using 4 mM ATP and [³H]N-methylquinidine, [³H]Estradiol-17-β-d-glucuronide, and [³H]Estrone-3-sulfate as probe substrates for ABCB1, ABCC2, and ABCG2, respectively. The transport was stopped by addition of ice cold washing buffer and consecutive rapid filtration through class B 1 µm pore size glass fiber filters, 0.65 µm pore size Durapore membrane in a 96-well filter plate (MSFBN6B10, Millipore Corporation, Billerica, MA, USA). After washing the membranes with 200 µl ice-cold wash buffer five times, filters were dried and radioactivity was measured in a scintillation cocktail (Packard UltimaGold; Perkin-Elmer, Waltham, MA, USA) using a Wallac MicroBeta TriLux (Perkin-Elmer) liquid scintillation counter. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP.

Determination of chlorothiazide transport was carried out as described above using cold chlorothiazide. Quantitation of chlorothiazide was done by LC/MS as described below.

Vesicular transport assays were run in duplicate. Data are presented as mean ± S.D.

Bioanalytical quantitation of chlorothiazide: Concentration of chlorothiazide in the samples was determined on an Agilent 1100-Series HPLC System equipped with a mass selective detector Quad VL System (Agilent, Santa Clara, CA, USA) purchased from Kromat Kft. (Budapest, Hungary). The analytical column was a Synergy 2.5 μ Fusion-RP 100 angstrom Mercury, 20 × 4.0 mm (Phenomenex, Torrance, CA, USA) from GenLab Kft (Budapest, Hungary) in combination with a Zorbax Eclipse Plus-C8 Narrow Bore Guard Column, 2.1 × 12.5 mm, 5-Micron (Agilent) purchased from Kromat Kft (Budapest, Hungary). The mobile phases were 0.05% (v/v) acetic acid (A) and acetonitrile (B) with an isocratic elution (B = 10%). The flow rate was 0.5 ml/min and the column oven temperature was 25°C. Single ion monitoring was used for data acquisition (negative mode, m/z = 293.7).

ATPase assay: ATPase activity was measured using the PREDEASY ATPase kit for ABCG2 from Solvo Biotechnology according to the manufacturer’s instructions. In brief, in the activation mode the membrane vesicles (4 µg/well) were incubated with various concentrations of the test drug with or without 1.2 mM sodium orthovanadate for 10 min at 37°C. ATPase activities were determined as the difference in inorganic phosphate liberation measured in the absence and presence of 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). In the inhibition mode, the transporter was turned on by addition of sulfasalazine (10 µM), a reference substrate/activator, and inhibition was monitored in the presence of increasing concentrations of the test drug. The ATPase assay experiments were run in duplicate. Results are presented as vanadate-sensitive ATPase activities.

Monolayer assays: MDCKII and MDCKII-BCRP cells (5 × 10⁴ cells/well) were plated onto polycarbonate permeable supports with 0.7 cm² filter area and 0.4 µm pore size (Millipore Millicell, Millipore Hungary, Budapest, Hungary). Media were replaced with fresh media on day three, and transport experiments were conducted on day four. When using Caco-2 cells, 6 × 10⁴ cells per well were plated as described above. Media were replaced three times per week, and transport experiments were conducted on day 21–25.

All reaction mixtures were prepared in Hank’s Balanced Saline Solution (HBSS), pH 7.4. Compounds were diluted from dimethyl sulfoxide (DMSO) stocks to 100 µM (chlorothiazide) and 1 µM (Ko143) final concentration. DMSO content was leveled below 0.5% (v/v) in all wells. Ko143 was applied to both the donor and the receiver compartments.

Media were removed, and filters were rinsed twice with prewarmed (37°C) HBSS. After a 15-min incubation period, HBSS was removed and the reaction was started with addition of the appropriate reaction mixtures. Transport was carried out for 120 min. Samples from donor compartments were taken at 0 min, samples from receptor compartments were taken at 15, 30, 60 and 120 min.

Determination of cold chlorothiazide is described above.

Data analysis: Data analysis for membrane and monolayer assays employing Prism 4.0 (GraphPad Software, San Diego, CA, USA) was carried out as described earlier.⁷,⁸

Results

To screen for interaction of chlorothiazide with secretory intestinal ABC transporters we used vesicular transport inhibition assays (Fig. 1). Chlorothiazide efficaciously inhibited ABCG2-mediated estrone-3-sulfate uptake (Fig. 1A) with an IC₅₀ of 212.3 ± 210.8 µM. No interaction with ABCB1 or ABCC2 was detected (Figs. 1B and 1C).
To characterize the interaction of chlorothiazide with ABCG2, ATPase and vesicular transport assays were used (Fig. 2). Chlorothiazide activated ABCG2 ATPase to positive control levels (Fig. 2A), indicating that it is an efficiently transported substrate of ABCG2 (EC_{50} = 327.2 ± 9.4 µM). The observed K_m value in the vesicular transport experiment (Fig. 2B) was 334.6 ± 325.0 µM, which correlated well with the IC_{50} in the inhibition experiment (212.3 ± 210.8 µM) as well as the ATPase EC_{50} data of 327.2 ± 9.4 µM.

To confirm that this interaction may modulate absorption-distribution-metabolism-excretion (ADME) properties of the drug, we carried out monolayer efflux experiments (Fig. 3). In the MCKII-BCRP cells the corrected efflux ratio of around 36 was observed for the compound (Fig. 3A). This polarized transport in the B-A direction was inhibited down to the value observed in the parental cells (Fig. 3B)

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an ABCG2 substrate (Fig. 2). The ATPase EC₅₀ and the vesicular transport Kₘ values (327.2 ± 9.4 µM and 334.6 ± 325.0 µM, respectively) correlated well. Since in membrane assays kinetic values are not confounded by low permeability of tested drugs, this Kₘ value likely reflects the affinity of chlorothiazide for ABCG2.

Monolayer efflux experiments in MDCKII-BCRP as well as in Caco-2 cells confirmed that ABCG2 modulates chlorothiazide permeability (Fig. 3). As Caco-2 cells are the most relevant in vitro human intestinal absorption models our data strongly suggest that ABCG2 is in part behind the low bioavailability of the drug. It is of note that chlorothiazide has low permeability when ABCG2 is inhibited (Fig. 3) and that certainly contributes to the low bioavailability of the drug.

Interestingly, hydrochlorothiazide, which is an ABCG2 substrate,¹⁴ did not activate the ABCG2 ATPase to any significant extent (data not shown). It is likely that hydrochlorothiazide is transported at a lower rate by ABCG2 than chlorothiazide and that may explain the higher bioavailability (ca. 80%) of the former drug. The physicochemical properties of the two drugs are clearly different with chlorothiazide being significantly more acidic (pKₐ = 6.5) than hydrochlorothiazide (pKₐ = 7.9).¹⁵ This suggests that ABCG2 may play a significant role in determining the fraction absorbed value and bioavailability of this group of drugs.

In sum, ABCG2 modulates the permeability of chlorothiazide in all three experimental systems tested. The experimental systems employed in this study comply with current regulatory guidance and suggest that chlorothiazide is a legitimate ABCG2 probe candidate for regulatory studies. Clinical studies to test if the ABCG2 c.421C>A polymorphism affects chlorothiazide pharmacokinetics are warranted.

References


15) www.drugbank.ca/drugs/DB00999.