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Investigation of potential drug–drug interactions between peficitinib (ASP015K) and methotrexate in patients with rheumatoid arthritis

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Conflict of interest

TZ, UV, YC, and WZ are employees of Astellas Pharma Global Development Inc. SM is an employee of Astellas Research Institute of America. TS is an employee of Astellas Pharma Inc. BA was an employee of Astellas Pharma Global Development Inc. at the time of the study, and
is now an employee of Regeneron Pharmaceuticals; he reports no conflict of interest. VC is an employee of Pinnacle Research Group/Anniston Medical Clinic, which received funding from Astellas to conduct the study.
Supplementary material: *in vitro* transporter inhibition study

1 METHODS

Chemicals and materials

Peficitinib (synthesized at Astellas Pharma, Inc. Tokyo, Japan) was dissolved in 50% dimethyl sulfoxide (DMSO) to give stock solutions. $[^{3}H]$Estradiol-17β-D-glucuronide was obtained from PerkinElmer, Inc. (MA, USA). $[^{3}H]$methotrexate was obtained from Moravek Biochemicals, Inc. (CA, USA). *para*-Aminohippuric acid, *p*-[glycyl-2-$^{3}$H] and estrone sulfate ammonium salt [6,7-$^{3}$H(N)] were obtained from PerkinElmer, Inc. (MA, USA). To confirm the suitability of the assay, benzbromarone, sulfasalazine and probenecid, which are known inhibitors of MRP2/4, BCRP and OAT1/3, respectively, were obtained from Sigma-Aldrich. All other reagents and chemicals were of analytical grade and obtained from commercial sources.

Vesicles and Cells

Membrane vesicles prepared from Sf9 cells expressing human BCRP, MRP2 and MRP4 were purchased from Genomembrane, Inc. (Yokohama, Japan). S2 cells, the immortalized cell line that derived from the S2 segment of the renal proximal tubule of the temperature sensitive SV40 T antigen gene transgenic mouse, originally established by Fuji Bio Medix Co., Ltd. (Saitama, Japan) and now owned by ADME & Tox. Research Institute, Sekisui Medical Co., Ltd., was used. S2 cells expressing OAT1 and OAT3 were established by transfection of a pcDNA vector (Invitrogen, CA, USA) containing full-length human transporter cDNA. Control S2 cells were
established by transfection of the empty vector. The expression level of transporter mRNA in transfected cells was verified periodically by real-time polymerase chain reaction. The culture medium, RITC80-7 (Research Institute for the Functional Peptides, Yamagata, Japan) containing fetal bovine serum (FBS, final concentration: 4.7%), epidermal growth factor (EGF, final concentration: 9.5 µg/L), insulin (final concentration: 0.076 unit/mL) and transferrin (final concentration: 9.5 mg/L) was exchanged every 2 days.

1.1 MRP2-, MRP4- and BCRP-mediated transport assay

The potential for peficitinib to inhibit MRP2/4- and BCRP-mediated transport was assessed using a vesicular transport assay with \([^{3}H]\)estradiol-17ß-D-glucuronide or \([^{3}H]\)methotrexate as substrates, respectively. The MRP2/4 and BCRP membrane vesicles (0.4 mg protein/mL) were incubated with either 1.6 µCi/mL \([^{3}H]\)estradiol-17ß-D-glucuronide and 5.0 µmol/L estradiol-17ß-D-glucuronide (MRP2/4) or 26 µCi/mL \([^{3}H]\)methotrexate and 64 µmol/L methotrexate, at 37°C in the presence of 4 mmol/L ATP or AMP, in the incubation buffer consisting of 25–26 mmol/L 3-[N-morpholino]propanesulfonic acid (MOPS)-Tris (pH 7.0), 35-36 mmol/L KCl and 4 mmol/L MgCl₂. For MRP2, glutathione (final concentration: 2 mmol/L) was also added. The final assay concentrations of peficitinib were 0, 3, 10, 25, 50 and 100 µmol/L. Benzbromarone (100 µmol/L), a known inhibitor of MRP2/4, and sulfasalazine (100 µmol/L), a known inhibitor of BCRP, were used as positive controls. After incubation for 5 (MRP2/4) or 7.5 min (BCRP), the reactions were terminated by adding 0.2 mL of ice-cold 70 mmol/L KCl-containing 40 mmol/L MOPS-Tris (pH 7.0) buffer. Aliquots were filtered through a glass microfiber filter.
The filters were washed four times with 4 mL of ice-cold 70 mmol/L KCl-containing 40 mmol/L MOPS-Tris buffer, transferred to a vial and added 10 mL of scintillator (OPTI-Fluor (PerkinElmer Inc.)) and the radioactivity retained on the filter was determined by scintillation counting. The uptake clearance (μL/min/mg protein) at each incubation time was calculated as: radioactivity on the filter (dpm) divided by (radioactivity in the assay buffer (dpm) × protein concentration [mg protein/µL] × incubation time [min]). Inhibitory effects, as a percentage of the control in the absence of inhibitor, were calculated from the corrected uptake clearance (uptake clearance in the presence of ATP – uptake clearance in the presence of AMP). IC\textsubscript{50} values were calculated by the least squares method from the relationship between peficitinib concentration and the corrected uptake clearance of substrates as a percentage of the control.

1.2 OAT1/3-mediated inhibition assay

For the OAT1/3 transport assays, S2 cells expressing OAT1 or OAT3, and control cells, were seeded in 24-well plates (Corning, NY, USA) at a density of 2.4–4.0 × 10\textsuperscript{5} cells/well, incubated in a CO\textsubscript{2} incubator (33°C; CO\textsubscript{2}, 5%) for 2 days. Medium in all plates was then removed by aspiration and cells were washed with Dulbecco's phosphate buffered saline (D-PBS) before preincubation with D-PBS at 37°C for 15 min. After preincubation, the D-PBS was replaced with test solution containing inhibitor and substrates (1 μmol/L para-aminohippuric acid, p-[glycyl-2-\textsuperscript{3}H] for OAT1 or 0.05 μmol/L estrone sulfate ammonium salt [6,7-\textsuperscript{3}H(N)] for OAT3), and the plate was incubated at 37°C for 2 min. The final assay concentrations of peficitinib were 0, 1, 3, 10, 30 and 100 μmol/L. The final assay concentrations of known inhibitor probenecid was 100 μmol/L. The final DMSO concentration was 0.2% for all assays. After incubation, the
test solution was removed, and the cells were washed three times with ice-cold D-PBS. The cells were lysed using 0.5 mL of 0.1 mol/L aqueous sodium hydroxide solution. After the cell lysate was mixed by pipetting, 0.3 mL of the lysate was collected into a glass vial and mixed with 10 mL of the scintillator Hionic-fluor (PerkinElmer, Inc.) to measure the radioactivity by scintillation counting.

Protein content was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., MA, USA, or Pierce, IL, USA) in accordance with the manufacture’s recommendations. The cleared volume (µL/mg protein) at each incubation time was calculated as: uptake amount (dpm/well) divided by (initial concentration [dpm/µL] × protein amount [mg/well]). Inhibitory effects, as a percentage of the control in the absence of inhibitor, were calculated from the corrected cleared volumes (cleared volume of transporter-expressing cells – cleared volume of control cells). IC<sub>50</sub> values were calculated by the least squares method from the relationship between peficitinib concentration and substrates uptake as a percentage of the control.

2 RESULTS

There were no significant inhibitory effects of peficitinib on ATP-dependent estradiol-17β-D-glucuronide transport via MRP2, and although inhibition was observed for MRP4 with increasing concentrations of peficitinib, the rate was less than 50% at the highest concentration tested (100 µmol/L). ATP-dependent methotrexate transport via BCRP was inhibited by increasing concentrations of peficitinib, and the IC<sub>50</sub> value of peficitinib for this transporter was calculated to be 13.5 µmol/L.
There was a slight reduction in the uptake of para-aminohippuric acid, \( p-[\text{glycyl}-2^{3}\text{H}] \) into OAT1-expressing cells in the presence of peficitinib, but the IC\(_{50}\) value was estimated to be >100 \( \mu\text{mol/L} \). In contrast, the uptake of estrone sulfate ammonium salt \( [6,7^{3}\text{H}(\text{N})] \) into OAT3-expressing cells was inhibited by peficitinib in a concentration-dependent manner, with an IC\(_{50}\) value of 5.01 \( \mu\text{mol/L} \).