Regorafenib Is Transported by the Organic Anion Transporter 1B1 and the Multidrug Resistance Protein 2

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Regorafenib is a small molecule inhibitor of tyrosine kinases, and has been shown to improve the outcomes of patients with advanced colorectal cancer and advanced gastrointestinal stromal tumors. The transport profiles of regorafenib by various transporters were evaluated. HEK293/organic anion transporting polypeptide 1B1 (OATP1B1) cells exhibited increased drug sensitivity to regorafenib. Regorafenib inhibited the uptake of $^3$H-estrone sulfate by HEK293/OATP1B1 cells in a dose-dependent manner, but did not affect its elimination by P-glycoproteins. The concentration of regorafenib was significantly lower in LLC-PK1/multidrug resistance protein 2 (MRP2) cells than in LLC-PK1 cells treated with the MRP2 inhibitor, MK571. MK571 abolished the inhibitory effects of regorafenib on intracellular accumulation in LLC-PK1/MRP2 cells. The uptake of regorafenib was significantly higher in HEK293/OATP1B1 cells than in OATP1B1-mock cells. Transport kinetics values were estimated to be $K_u=15.9\mu M$ and $V_{max}=1.24\mu mol/mg/min$. No significant difference was observed in regorafenib concentrations between HEK293/OATP1B3 and OATP1B3-mock cells. These results indicated that regorafenib is a substrate for MRP2 and OATP1B1, and also suggest that the substrate preference of regorafenib may implicate the pharmacokinetic profiles of regorafenib.

Key words regorafenib; IC$_{50}$; multidrug resistance protein 2; organic anion transporting polypeptide 1B1; vectorial drug transport

Regorafenib has been developed as a molecular targeted medicine for patients with metastatic colorectal cancer and advanced gastrointestinal stromal tumors, and inhibits certain tyrosine kinases. The biological effects of receptor tyrosine kinase activation are mediated by a complex cascade of intracellular signaling molecules that are potential targets for therapy, including the Raf, mitogen-activated protein extracellular kinase (MEK), and extracellular signal-regulated kinase (ERK) pathways. Regorafenib is a multikinase inhibitor that targets Raf/serine/threonine kinases and the vascular endothelial growth factor (VEGF) receptor, platelet-derived growth factor (PDGF) receptor-β, c-Kit, Flt3, and p38 tyrosine kinases, which blocks VEGF- and PDGF-dependent angiogenesis.

The family of ATP-binding cassette (ABC) transporters, including P-glycoprotein (P-gp, ABCB1) and multidrug resistance protein 2 (MRP2, ABCC2), plays important roles in the detoxification and excretion of xenobiotics. MRP2, an MRP isoform, is a clinically important transporter that has been shown to function in the terminal excretion of cytotoxic and carcinogenic substances. MRP2 is important clinically because it modulates the pharmacokinetics of many drugs, and its expression and activity are also altered by certain drugs and disease states. Organic anion transporting polypeptides (OATPs) belong to the superfAMILY OF SOLUTE CARRIER TRANSPORTERS AND ARE CLASSIFIED WITHIN THE SOLUTE CARRIER (SLCO) GENE FAMILY. OATPs FUNCTION IN THE UPTAKE OF A WIDE RANGE OF AMPHATHIC COMPOUNDS BY CELLS, INCLUDING NUMEROUS ENDO- AND XENOBIOTICS. OATPs ARE KNOWN TO BE CRITICALLY INVOLVED IN THE ABSORPTION, DISTRIBUTION, AND EXCRETION OF DRUGS AND OTHER XENOBIOTICS. TWO MEMBERS OF THE OATP1 FAMILY, OATP1B1 AND OATP1B3, ARE EXPRESSED IN THE BASOLATERAL MEMBRANE OF THE LIVER. THE EXPRESSION OF THESE TRANSPORTERS IS ALTERED IN MANY DIFFERENT TYPES OF CANCERS; THESE ALTERED EXPRESSION LEVELS HAVE BEEN CORRELATED WITH CANCER STAGE. OATPs ARE CAPABLE OF TRANSPORTING MULTIPLE COMPOUNDS THAT AFFECT CANCER CELL GROWTH AND SURVIVAL, INCLUDING HORMONES, HORMONE PRECURSORS, AND ANTICANCER DRUGS.

Some tyrosine kinase inhibitors are substrates or inhibitors of drug transporters. It was recently reported that nilotinib and vandetanib were transported by OATP1B1 and OATP1B3. Sorafenib was identified as potent inhibitors of OATP1B1 in vitro. The substrate preferences of transporters can lead to differences in pharmacokinetic profiles and drug-interaction characteristics. However, detailed substrate preferences of regorafenib on P-gp, MRP2, OATP1B1 and OATP1B3 were not clear. In this study, we report the substrate preferences for the transporters about regorafenib.

**MATERIALS AND METHODS**

**Chemicals** Regorafenib was purchased from CHEMSCENE, LLC. (Monmouth Junction, NJ, U.S.A.). Sorafenib was purchased from Toronto Research Chemicals Inc. (North York, Canada). Dulbecco’s modified Eagle’s medium (DMEM) and collagen (C9791) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Tritium-labeled [6,7-$^3$H]-estrone sulfate, ammonium salt (2.12 TBq/mmol), and [3H]-taurocholic acid

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Inhibition Assay The inhibitory effects of regorafenib on OATP1B1 and OATP1B3 were evaluated. HEK293/OATP1B1 cells were incubated at doses of 1, 3, 5, 10, 30, and 50 µM of regorafenib. The concentration of regorafenib was significantly lowered in LLC-PK1/MRP2 cells than in LLC-PK1 cells those treated with MK571. MK571 returned to regorafenib concentration in the LLC-PK1/MRP2 cells. The P-gp inhibitor, cyclosporin A did not affect the elimination of regorafenib from KB-31 and KB-G2 cells (Table 2). HEK293/OATP1B1 cells were incubated at doses of 1, 3, 5, 10, 30, and 50 µM of regorafenib for 3 min. The concentration of regorafenib was significantly higher in HEK293/OATP1B1 cells than in OATP1B1-mock cells. Transport kinetics values were estimated to be \( K_m = 15.9 \mu M \) and \( V_{\text{max}} = 1.24 \text{nmol/mg/min} \) (Fig. 2b). No significant difference was observed in regorafenib concentrations between HEK293/OATP1B3 cells and OATP1B3-mock cells (Fig. 3).

Inhibitory Effect of Regorafenib on Cell Growth Cell viability was evaluated by the MTT assay. Etoposide was employed as the positive control for drug resistance against MDR1. KB-G2 cells exhibited drug resistance to regorafenib and etoposide. Methotrexate was also employed as the positive control for drug resistance against MRP2. LLC-PK1/MRP2 cells showed drug resistance to MTX. HEK293/OATP1B1 cells exhibited increased drug sensitivity to regorafenib, that of LLC-PK1/MRP2 cells was increased (Table 1).

Accumulation of Regorafenib in Cells The concentration of regorafenib in cells was determined by the UPLC analysis (Fig. 1). Cells were incubated for 60 min at a dose of 1 µM of regorafenib. The concentration of regorafenib was determined after overnight shaking the using the iMark Micro Plate Reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

**RESULTS**

**Inhibitory Effect of Regorafenib on Cell Growth** Cell viability was evaluated by the MTT assay. Etoposide was employed as the positive control for drug resistance against MDR1. KB-G2 cells exhibited drug resistance to regorafenib and etoposide. Methotrexate was also employed as the positive control for drug resistance against MRP2. LLC-PK1/MRP2 cells showed drug resistance to MTX. HEK293/OATP1B1 cells exhibited increased drug sensitivity to regorafenib, that of LLC-PK1/MRP2 cells was increased (Table 1).

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**Inhibition Assay** The inhibitory effects of regorafenib on OATP1B1 and OATP1B3 were evaluated. HEK293/OATP1B1 was generated by transfecting human MRP2 cDNA into parent LLC-PK1 cells. The OATP1B1/HEK293 and OATP1B3/HEK293 cell lines were kindly provided by Dr. Yamaguchi (Department of Pharmacy, Tohoku University, Japan).

Cells were grown in DMEM, supplemented with 10% fetal bovine serum and 2 mM glutamine, at 37°C in a 5% CO₂ humidified atmosphere. Cells were seeded at a concentration of 2 × 10⁵ cells/mL on the day before the regorafenib treatment.

**Cell Growth Assays** The inhibition concentration of 50% (IC₅₀) cell growth was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated in culture medium with various concentrations of drugs in a final volume of 100 µL. After 3 d, 20 µL of MTT (2.5 mg/mL) was added to each well and the plates were incubated for an additional 3 h. The resulting formazan solution was centrifuged at 12000 × g for 3 min at doses of 1, 3, 5, 10, 30, and 50 µM of regorafenib. The concentration of regorafenib and the internal standard were 2.65 min and 2.18 min, respectively.

The ultra pressure liquid chromatography (UPLC) analysis was conducted to evaluate the accumulation of regorafenib in cells. The UPLC system consisted of a QSM pump, TUV detector, CHA column heater and SM-FTN auto sampler (Waters, Milford, U.S.A.). The analysis conditions used for the UPLC analysis were modified from van Erp et al. The column temperature was maintained at 50°C in the column heater. Two microliter samples were injected onto a reverse-phase column (ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 1 mm × 50 mm, Waters). The operation conditions were as follows: Detector, ultraviolet absorption photometer wavelength of 254 nm; mobile phases, 0.1% formic acid and 2 mM ammonium acetate in water/0.1% formic acid and 2 mM ammonium acetate in methanol (35:65); flow rate of 0.4 mL/min. The retention times of regorafenib and the internal standard were 2.65 min and 2.18 min, respectively (Fig. 1). The plates were read at 590 nm after overnight shaking using the iMark Micro Plate Reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

**Drug Accumulation Assay** The cells were incubated in KH buffer with regorafenib for 3 min. They were then deproteinized with methanol and the mobile-phase solution. The retention times of regorafenib and the internal standard were 2.65 min and 2.18 min, respectively. No significant difference was observed in regorafenib concentrations between HEK293/OATP1B3 cells and OATP1B3-mock cells (Fig. 3).

**Drug Concentration Assay** The inhibition concentration of 50% (IC₅₀) cell growth was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated in culture medium with various concentrations of drugs in a final volume of 100 µL. After 3 d, 20 µL of MTT (2.5 mg/mL) was added to each well and the plates were incubated for an additional 3 h. The resulting formazan solution was centrifuged at 12000 × g for 15 min. Methanol was removed by evaporation from the supernatant, and residue was then deproteinized with the mobile-phase solution. The solution was filtrated through a 0.2-µm filter (Advantec Toyo Kaisha, Ltd., Hydrophobic PTFE Membrane Filter). Protein concentrations were determined using a protein assay (Bio-Rad Laboratories).

**Cell Culture** The inhibition concentration of 50% (IC₅₀) cell growth was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated in culture medium with various concentrations of drugs in a final volume of 100 µL. After 3 d, 20 µL of MTT (2.5 mg/mL) was added to each well and the plates were incubated for an additional 3 h. The resulting formazan solution was centrifuged at 12000 × g for 15 min. Methanol was removed by evaporation from the supernatant, and residue was then deproteinized with the mobile-phase solution. The solution was filtrated through a 0.2-µm filter (Advantec Toyo Kaisha, Ltd., Hydrophobic PTFE Membrane Filter). Protein concentrations were determined using a protein assay (Bio-Rad Laboratories).

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cells significantly accumulated $^3$H-estrone sulfate, a substrate for OATP1B1 (HEK293/OATP1B1, 0.183±0.011 pmol/mg protein; HEK293/MOCK, 0.031±0.005 pmol/mg protein; means±standard error (S.E.), p=3×10^{-11}, Student’s t-test). Regorafenib inhibited the uptake of $^3$H-estrone sulfate by HEK293/OATP1B1 cells in a dose-dependent manner (Fig. 2c). HEK293/OATP1B3 cells significantly accumulated $^3$H-taurocholic acid, a substrate for OATP1B3 (HEK293/OATP1B3, 0.253±0.026 pmol/mg protein; HEK293/MOCK, 0.145±0.005 pmol/mg protein; means±S.E., p=0.002, Student’s t-test). Regorafenib did not inhibit the transport of $^3$H-taurocholic acid in HEK293/OATP1B3 cells (data not shown).

DISCUSSION

We here in demonstrated that regorafenib was transported by MRP2 and OATP1B1. Evidences to suggest that tyrosine kinase inhibitors (TKIs) are transported by the ATP-binding cassette (ABC) transporters is increasing. P-gp, MRP2, MRP1 and the breast cancer resistance protein (BCRP, ABCG2) have been shown to transport TKIs, such as imatinib, gefitinib, and sorafenib. Khurana et al. recently reported that nilotinib and vandetanib were transported by OATP1B1 and OATP1B3. Cells were incubated at doses of 1, 3, 5, 10, 30, and 50 µM of regorafenib for 60 min. Regorafenib concentrations were shown as per the amount of whole cell homogenate protein. Each value indicates means±S.E. of the mean (nmol/µg protein) from triplicate measurements. Differences between the treatments were evaluated using the Tukey–Kramer test; *p<0.05, significantly different from LLC-PK1 cells or those treated with MK571.

The present study is the first report to show that the tyrosine kinase inhibitor, regorafarinib was a substrate for MRP2 and OATP1B1. A summary of the pharmacokinetics of regorafenib (Stivarga tablets®, BAY73-4506) showed the efflux ratios of 1–50 µM; $K_M$=15.9 µM, $V_{max}$=1.24 nmol/mg/min.

Intracellular concentrations of regorafenib after the incubation of cells at a dose of 1 µM of regorafenib for 60 min. Regorafenib concentrations were shown as per the amount of whole cell homogenate protein. Each value indicates means±S.E. of the mean (nmol/µg protein) from triplicate measurements. Differences between the treatments were evaluated using the Student’s t-test. **p<0.01, ***p<0.001.

The concentration of regorafenib needed to inhibit cell growth by 50% was evaluated by the MTT assay. Each value indicates means±S.E. of the mean (pmol) from 3 independent experiments. Resistant ratios are shown in the ratio column. KB-G2 is a human MDR1 gene-transfected cell line with the human epidermoid carcinoma cell line KB-31. Statistical analyses for paired samples were performed by the two-tailed Student’s t-test. **p<0.01, ***p<0.001.

Table 1. Concentration of Regorafenib Required to Inhibit Cell Growth by 50% and Resistance Ratios

<table>
<thead>
<tr>
<th></th>
<th>KB-31</th>
<th>KB-G2</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regorafenib</td>
<td>5.5±0.3</td>
<td>9.1±0.1**</td>
<td>1.65</td>
</tr>
<tr>
<td>Etoposide</td>
<td>3.9±0.2</td>
<td>219.5±3.6****</td>
<td>56.2</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLC-PK1/MRP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regorafenib</td>
<td>42.0±3.2</td>
<td>82.4±2.7****</td>
<td>1.96</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>181±19</td>
<td>33154±2394****</td>
<td>183.2</td>
</tr>
<tr>
<td>HEK293</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293/OATP1B1</td>
<td>11.0±1.2</td>
<td>6.2±0.3**</td>
<td>0.56</td>
</tr>
</tbody>
</table>

The concentration of regorafenib needed to inhibit cell growth by 50% was evaluated by the MTT assay. Each value indicates means±S.E. of the mean (nmol/µg protein) from 3 independent experiments. Resistant ratios are shown in the ratio column. KB-G2 is a human MDR1 gene-transfected cell line with the human epidermoid carcinoma cell line KB-31. Statistical analyses for paired samples were performed by the two-tailed Student’s t-test. **p<0.01, ***p<0.001.

Table 2. Concentration of Regorafenib in Cells after a 60-min Incubation

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Cyclosporin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-31</td>
<td>1.26±0.08</td>
<td>0.93±0.06</td>
</tr>
<tr>
<td>KB-G2</td>
<td>1.27±0.08</td>
<td>1.09±0.09</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>1.40±0.06</td>
<td>1.36±0.09</td>
</tr>
<tr>
<td>LLC-PK1/MRP2</td>
<td>0.97±0.02*</td>
<td>1.39±0.05</td>
</tr>
</tbody>
</table>

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regorafenib to be 0.30 and 0.093, for LLC-PK1/MDR1 and LLC/PK1, respectively and also that regorafenib did not inhibit the basal to apical transport of dipyridamole (1 μM) when used at a dose of 10 μM. This summary also reported that 10 μM of regorafenib did not inhibit the uptake of pravastatin, a substrate for OATP, by HEK293/OATP1B1 cell, while 0.1 μM of regorafenib did not inhibit the uptake of pravastatin by HEK293/OATP1B3 cells.18) These findings indicated that regorafenib was not a substrate for P-gp, OATP1B1, or OATP1B3. In the present study, KB-G2 cells showed negligible drug resistance to regorafenib, and the inhibitory treatment with cyclosporine A for P-gp did not affect the accumulation of resistance to regorafenib, and the inhibitory treatment with cyclosporine A for P-gp did not affect the accumulation of regorafenib, and the IC50 of regorafenib (Table 2). The results of the present study also suggested that regorafenib was not a substrate for P-gp. MK571 inhibited the accumulation of regorafenib, and the IC50 of regorafenib was increased in LLC-PK1/MDR2 cells (Tables 1, 2). These results indicated that regorafenib was transported by MRP2. We also demonstrated that regorafenib inhibited the transport of 1H-estrone sulfate and accumulation of regorafenib in HEK293/OATP1B1 cells; therefore, regorafenib may be transported by OATP1B1. However, the summary of the pharmacokinetics of BAY73-4506 reported that 10 μM of regorafenib did not inhibit the uptake of pravastatin, suggesting that regorafenib was not a substrate for OATP1B1. The present study showed that the Km value was 15.9 μM, indicating that 10 μM of regorafenib could not inhibit the uptake of pravastatin.

Polarized tissues directly involved in drug disposition (the intestines, kidney, and liver) asymmetrically express various drug transporters on their apical and basolateral sides to allow vectorial drug transport. The OATPs family on the basolateral membrane and MRP2 on the apical membrane of hepatocytes have been shown to take up and excrete organic anionic compounds.19) Vectorial drug transport plays a crucial role in the elimination of organic anionic compounds from the blood to bile. For example, the markedly higher transcellular flux of enalapril and estradiol-17β-d-glucuronide from the basolateral to apical membrane was observed in double-transfected OATP1B1/MDR2/Madin–Darby canine kidney (MDCK) cells than in single-transfected OATP1B1/MDCK cells.20) A phase I study also reported that the pharmacokinetics of regorafenib showed a bimodal pattern for its concentrations in the plasma (tmax, 3.6 and 48 h); suggesting the disposition of regorafenib for enterohepatic circulation.21) The vectorial drug transport of OATP1B1 on the basolateral membrane and MRP2 on the apical membrane may play a role in the enterohepatic circulation of regorafenib.

In conclusion, the results of the present study suggests that regorafenib is a substrate for MRP2 and OATP1B1, and also that the substrate preference of regorafenib may affect the pharmacokinetic profiles and drug resistance to anticancer drugs.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


