Multidrug Resistance Protein 2 Implicates Anticancer Drug-Resistance to Sorafenib

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Sorafenib and sunitinib is a small molecule inhibitor of certain receptor tyrosine kinases, and have improved outcomes for patients with advanced renal cell carcinoma. Inhibitory concentration of 50% cell growth of sorafenib significantly rose to 6.4-fold in a multidrug resistance protein 2 (MRP2) transfected cell line versus control cell line. The concentration of sorafenib was significantly decreased to 74% of control cells after 3 h treatment. In contrast, a tyrosine kinase inhibitor sunitinib did not show alteration of inhibitory concentration of 50% cell growth and accumulation into the cells of MRP2 transfected cells. The present study suggest that sorafenib is a substrate for MRP2, suggesting that MRP2 may implicate drug resistance to sorafenib.

Key words sorafenib; sunitinib; multidrug resistance protein 2; inhibitory concentration

Sorafenib and sunitinib are developed as molecular target medicines for advanced renal cell carcinoma, and known to inhibit 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. Sorafenib is a multitikine inhibitor targeting the Raf serine/threonine kinases and the vascular endothelial growth factor (VEGF) receptor 1—3, platelet-derived growth factor (PDGF) receptor-β, c-Kit, Fli3 and p38 tyrosine kinases and, which block the VEGF and PDGF-dependent angiogenesis. Sunitinib malate targets receptor tyrosine kinases of VEGFR1-3, PDGFR-α, PDGFR-β, FMS-like tyrosine kinase 3, c-Kit, RET and colony-stimulating factor receptor type 1, some of which have been implicated in tumor growth angiogenesis and metastasis. Large clinical trials have been demonstrated that sorafenib and sunitinib have improved outcomes for patients with advanced renal cell carcinoma. The family of ATP binding cassette (ABC) transporters, for example, p-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2) have important roles in the detoxification and excretion of xenobiotics. MRP2, an ATP-dependent membrane transport protein (P-gp) and (ABC) transporters, for example, p-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2) have important roles in the detoxification and excretion of xenobiotics. MRP2 is important clinically as it modulates the terminal excretion of cytotoxic and carcinogenic substances. MRP2 is important clinically as it modulates the terminal excretion of cytotoxic and carcinogenic substances. Multidrug Resistance Protein 2 Implicates Anticancer Drug-Resistance to Sorafenib

Materials and Methods

Chemicals Sorafenib and sunitinib were purchased from Toronto Research Chemicals Inc. (North York, Canada). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Other reagents and acteinonitrile for high performance liquid chromatography analysis were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Cell Culture A pig transformed renal cell line, LLC-PK1 and LLC-PK1/MRP2 cell lines were kindly provided by Dr. Furukawa (Department of Molecular Oncology, Kagoshima University). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mm glutamine and 100 units/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B at 37 °C in a 5% CO2 humidified atmosphere. Cell cultures were maintained at exponential growth by replacing the media every 2—3 d. Culture medium was replaced with fresh medium the day before the experiment.

Assays Inhibition concentration of 50% cell growth (IC50) were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay. Cells were incubated in culture medium with various concentrations of the 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 was dissolved in 80 μl of 20% sodium dodecyl sulfate solution. The plates were shaken mechanically for 5 min and read immediately at 570 nm using a model 550 Micro Plate Reader (Bio-Rad, Richmond, CA, U.S.A.). Accumulation of sorafenib in the cells was evaluated with HPLC analysis. The HPLC system consisted of an LC-10AS HPLC pump, SPD-6A UV detector, and C-RSA recorder (Shimadzu Corp., Kyoto, Japan). Analysis condition for HPLC analysis was modified Affify et al. report. The column temperature was maintained at 30 °C in a CTO-6A column oven (Shimadzu Corp., Kyoto, Japan). Samples were injected onto a reverse-phase column (Puresil 5 μ C18, 4.6×150 mm, Waters). Oper-
RESULTS AND DISCUSSION

Cell viability was evaluated by MTT assay (Table 1). LLC-PK1/MRP2 was generated by transfection human MRP2 cDNA into parent cells LLC-PK1.\(^{11,12}\) Methotrexate (MTX) was employed as the positive control experiment for drug resistance against MRP2.\(^{13}\) LLC-PK1/MRP2 cells showed drug resistance to MTX (Table 1). LLC-PK1/MRP2 cells also showed drug resistance to sorafenib, but not sunitinib. To evaluate accumulation of sorafenib into LLC-PK1 and LLC-PK1/MRP2 cells, HPLC analysis was performed (Figs. 1, 2). The cells were incubated for 5, 30, 90 and 180 min at a dose of 4 μM of sorafenib. The concentration of sorafenib in the LLC-PK1/MRP2 cells was significantly decreased following 90 and 180 min incubation (90 min: LLC-PK1, 0.65±0.04, LLC-PK1/MRP2, 0.52±0.04; 180 min: LLC-PK1, 0.69±0.06, LLC-PK1/MRP2, 0.51±0.03 μmol/μg, mean ±S.E., Fig. 2). The concentration of sunitinib in the LLC-PK1/MRP2 cells showed no significant difference following 180 min incubation (Table 2). Accumulation of sorafenib in the cells were reduced to 74%, resistance ratio rose to 6.4-fold in a multidrug resistance protein 2 (MRP2) transfected cell line versus control cell line. Similarly, Tiwari et al. reported that accumulation of paclitaxel was reduced to 80%, resistance ratio rose to 26-fold in an ABCB1 (P-glycoprotein) overexpressed cell line (KB-C2) versus control cell line (KB-3-1).\(^{14}\) It was assumed that reduction of sorafenib accumulation in the cells may elevate resistance ratio.

Intracellular concentration of sorafenib or sunitinib after incubation at a dose of 4 μM of sorafenib or sunitinib for 180 min. Concentration of sorafenib and sunitinib is demonstrated as per amount of whole cell homogenate protein. Each value indicates mean±standard error of the mean (μmol/μg protein) from 5 times measurement. Differences between individual treatments and groups evaluated using the Williams test. Asterisk indicates significant difference from vehicle group (*p<0.05).

Table 1. Inhibition Concentration of 50% Cell Growth and Resistance Ratio

<table>
<thead>
<tr>
<th></th>
<th>LLC-PK1</th>
<th>LLC-PK1/MRP2</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>MTX</td>
<td>181±19</td>
<td>33154±2394***</td>
<td>183</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>3658±607</td>
<td>23376±1786***</td>
<td>6.39</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>2916±276</td>
<td>3761±480</td>
<td>1.29</td>
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Inhibition concentration of 50% cell growth was evaluated with MTT assay. Each value indicates mean±standard error of the mean (nM) from 6 independent experiments. Resistance ratio of LLC-PK1/MRP2 cell line to LLC-PK1 cell line is shown in the ratio column. LLC-PK1: pig kidney cell line, LLC-PK1/MRP2: human MRP2 gene transfected cell line. Statistical analyses for paired samples were performed by two-tailed Student’s t test. Asterisk indicates significant difference from vehicle group (+++p<0.001).

Table 2. Concentration of Sorafenib or Sunitinib in the Cells (μmol/μg Protein) after 180 min Incubation

<table>
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<tr>
<th></th>
<th>LLC-PK1</th>
<th>LLC-PK1/MRP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorafenib</td>
<td>0.69±0.06</td>
<td>0.51±0.03***</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>0.67±0.04</td>
<td>0.62±0.09</td>
</tr>
</tbody>
</table>

Intracellular concentration of sorafenib or sunitinib after incubation at a dose of 4 μM of sorafenib or sunitinib for 180 min. Concentration of sorafenib and sunitinib is demonstrated as per amount of whole cell homogenate protein. Each value indicates mean±standard error of the mean (μmol/μg protein) from 5 times measurement. Differences between the treatments were evaluated using the two-tailed Student’s t test. Asterisk indicates significant difference from vehicle group (*p<0.05).
blocks function of P-gp and BCRP. It is not clear whether sorafenib and sunitinib would be effective. Eichelberg chemotherapy, it was reported that sequential treatment with sorafenib for the second-line treatment. In clinical effective sunitinib treatment. Consequently, it might be effective to use sorafenib for the initial treatment, and then to use sunitinib for the second-line treatment. In clinical chemotherapy, it was reported that sequential treatment with sorafenib and sunitinib would be effective. Eichelberg et al. reported that sequential tyrosine kinase inhibitors therapy with the sorafenib followed by sunitinib has clinical validity in some patients with advanced renal cell carcinoma when progressive disease occurs under the initial tyrosine kinase inhibitors therapy.

In conclusion, this study suggests that sorafenib is a substrate for MRP2. In contrast, a tyrosine kinase inhibitor sunitinib is not substrate for MRP2, suggesting that differences of substrate preference sorafenib and sunitinib may implicate clinical drug resistance.

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REFERENCES