Microbiology and Infectious Disease / BK Virus Tyrosine Kinase Inhibitors

Viral Drug Sensitivity Testing Using Quantitative PCR

Effect of Tyrosine Kinase Inhibitors on Polyomavirus BK Replication

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Key Words: BK polyomavirus; Infection; Tyrosine kinase

Abstract

Our objective was to determine whether quantitative polymerase chain reaction (PCR) can be used to measure the effect of tyrosine kinase (TK) inhibition on polyomavirus BK (BKV) replication. The BKV was grown in a cell culture system. The rate of viral replication in the presence or absence of the drug being tested was assessed by amplifying the viral genome using primers directed against the viral capsid 1 protein.

Dasatinib, erlotinib, gefitinib, imatinib, sunitinib, and sorafenib all showed antiviral activity at micromolar concentrations. The 50% effective concentration for erlotinib and sorafenib was within blood concentrations readily achieved in human subjects.

Quantitative PCR is a convenient method for viral drug sensitivity testing for slow-growing viruses that do not readily produce cytopathic effect. TK inhibitors deserve further consideration as a potential therapeutic option for BKV-associated nephropathy and hemorrhagic cystitis.

Viral drug sensitivity assays often use traditional plaque reduction, inhibition of hemagglutination, or DNA synthesis measurements that are laborious and may require bulk quantities of cultured cells, Southern blots, and radioactive probes.1,2 These assays are not suitable for slow-growing viruses such as polyomavirus BK (BKV), which do not easily form plaques and take several weeks to produce a cytopathic effect in commercially available cell lines. We have, therefore, developed a more convenient in vitro drug sensitivity assay that is suitable for screening for anti-BKV drugs. Compared with conventional assays, this polymerase chain reaction (PCR)-based assay is less time-consuming and generates reproducible quantitative data that are less prone to subjective interpretation and relatively insensitive to the multiplicity of infection.3,4 In addition, unlike biochemical assays using viral recombinant proteins, this cell-based assay can readily weed out compounds that are not permeable to cells or are cytotoxic. Although the assay measures the viral DNA replication rate, it is designed to identify antiviral compounds independent of the mechanism of action. Assays are performed after preincubation of cells with the drug for 2 hours before viral infection. This allows receptor blocking compounds to inhibit viral entry during the preincubation phase. The subsequent 7-day incubation period exceeds the duration of the viral life cycle. Hence, compounds affecting early or late gene viral expression, or viral assembly, will produce fewer infectious viral particles in the second round of viral replication and will be successfully flagged.

Antiviral screening for BKV has become necessary because this virus is now an increasingly recognized important pathogen in immunosuppressed patients. It causes viremia in 5% to 30% and nephropathy in 1% to 10% of kidney transplant
recipients. In addition, it has been linked to hemorrhagic cystitis occurring after bone marrow transplantation. No routinely effective antiviral therapy is available. Cidofovir, leflunomide, ciprofloxacin, and intravenous immunoglobulins are frequently used empirically without actually investigating whether the clinical strain in question is actually susceptible to these drugs.

This study demonstrated the usefulness of quantitative PCR to investigate the susceptibility of BKV to tyrosine kinase inhibitors. Our rationale for testing this class of compounds is based on the observations that BKV replication in human cells primarily uses host metabolic machinery and that cellular replication creates a milieu favorable for viral multiplication.

Cellular replication is known to be dependent on several protein kinase–dependent pathways, of which tyrosine kinase–regulated pathways are of particular interest. Thus, binding of epidermal growth factor or transforming growth factor to epidermal growth factor receptor (EGFR) leads to activated tyrosine kinase activity, which in turn results in receptor autophosphorylation and activation of the ras/mitogen-activated protein kinase signaling pathway of cell proliferation. Tyrosine kinase phosphorylates transcription factors like ATF-2 and CREB, which have the potential to stimulate tyrosine kinase–dependent cell signaling pathways of cell proliferation, cell survival, and DNA repair. Experiments with the chemical inhibitor genistein indicate that tyrosine kinase is also involved in the entry of polyomaviruses into cells by the caveolar and clathrin pathways. Cell lines lacking the expression of Ab1 family tyrosine kinases show reduced ganglioside GD1a expression, with consequent interference in receptor-mediated entry of BKV and murine polyomavirus.

In the past 2 decades, several tyrosine kinase inhibitors have become approved for clinical use. We, therefore, explored the hypothesis that these compounds might have usefulness in treating BKV infection. Targeting the host kinome for therapy offers prospects of developing broad-spectrum antiviral compounds. Unlike compounds that target viral proteins, the action of kinase inhibitors is not likely to be compromised by the development of mutations in the viral genome following continuous exposure.

Materials and Methods

The following compounds approved by the US Food and Drug Administration for other clinical indications were tested for effect on BKV replication in an in vitro culture system: (1) Dasatinib monohydrate (Sprycel) is an inhibitor of the cellular form of the Abelson leukemia virus tyrosine kinase (c-ABL; including mutant BCR-ABL) and c-SRC tyrosine kinases. It is used in patients with chronic myeloid leukemia and Philadelphia chromosome–positive acute lymphoblastic leukemia refractory to imatinib. (2) Erlotinib hydrochloride (Tarceva) is an EGFR-targeted type 1 receptor tyrosine kinase inhibitor that is approved for the treatment of cancer of the pancreas, non–small cell cancer of the lung, squamous cell carcinoma of the head and neck, and glioblastoma multiforme. (3) Gefitinib (Iressa) is another EGFR-targeted type 1 receptor tyrosine kinase inhibitor that is approved for non–small cell cancer of the lung and squamous cell carcinoma of the head and neck. (4) Imatinib mesylate (Gleevec; STI571) an inhibitor of BCR-ABL, platelet-derived growth factor receptor (PDGFR), KIT, and ARG kinases, is used in the treatment of chronic myeloid leukemia, gastrointestinal stromal tumor, and dermatofibrosarcoma protuberans. (5) Sunitinib (Sutent), a multitargeted tyrosine kinase inhibitor active against vascular endothelial growth factor receptor, PDGFR, FLT3, and c-KIT receptor stimulated tyrosine kinases, has usefulness in the therapy of refractory gastrointestinal stromal tumor, non–small cell lung carcinoma, and renal cell carcinoma. (6) Sorafenib tosylate (Nexavar) is a multitargeted tyrosine kinase inhibitor similar to sunitinib that is available for clinical use in patients with renal cell carcinoma and unresectable liver carcinoma.

Imatinib was obtained from Me-too Pharma Tech, Shanghai, China, and all others were obtained from Changsha Huajia Chemical Sciences, Changsha Hunan, China. The in vitro drug sensitivity protocol used in the study has been published. Briefly, BKV, Gardner strain, was obtained from the American Type Culture Collection (ATCC No. VR837) and grown in human WI-38 cells (ATCC No. CCL-75). BKV grows very slowly in culture, and this virus-susceptible cell line was chosen to correspond to the BKV load in human kidney tissue with latent infection. All experiments began with a 2-hour preincubation of the cells with the drug of interest. Infection was then allowed to proceed for 2 hours, following which unabsorbed virus was washed off and replaced with 200 μL of fresh medium containing the appropriate concentration of protein kinase inhibitors. Cultures were incubated for 7 days in Dulbecco modified Eagle medium, supplemented by 10% fetal bovine serum, and L-glutamine, at 37°C under 5%
carbon dioxide. Cells were harvested on day 7 using 0.25% trypsin-1 mmol/L sodium-EDTA digestion at 37°C for 10 minutes. DNA extraction on the cell lysates was performed with a commercially available kit (QIAamp 96 DNA blood kit, catalog No. 51162, Qiagen, Valencia, CA) using the manufacturer’s instructions. BKV viral capsid protein VP-1 DNA was targeted by a TaqMan quantitative PCR reaction performed in an ABI Prism 7700 Sequence Detector (ABI, Foster City, CA).

Viral DNA yields in cells treated with different concentrations of the drug for 7 days were expressed as a percentage of the viral DNA yield in control cultures that received no drug treatment. The 50% effective drug concentration (EC50) was then determined as the drug concentration that reduced the day 7 viral DNA yield by 50%. As a measure of drug toxicity, the 50% cytotoxic concentration was calculated as the drug concentration that reduced the concentration of cellular DNA by 50%. Measurement of cellular DNA was effected by amplifying a housekeeping gene called aspartoacylase.

### Results

The results of testing are given in Table I, wherein in vitro EC50 values are placed in the context of therapeutically relevant plasma concentrations obtained during the use of these drugs in patients with cancer. All experiments were repeated 2 or 3 times: it is remarkable that all compounds generated an EC50 that was consistently of the same order of magnitude, ranging from 0.6 to 9.4 μmol/L. Individual discussion of the drugs follows.

Dasatinib has a mean EC50 of 0.6 μmol/L, or 303.6 ng/mL, which is an order of magnitude higher than the mean peak plasma concentration of 36.6 ng/mL obtained in healthy subjects given two 50-mg doses of this medication.17

<table>
<thead>
<tr>
<th>Name</th>
<th>Targeted Tyrosine Kinase</th>
<th>EC50* μmol/L</th>
<th>EC50* ng/mL</th>
<th>Peak Clinical Blood Level μmol/L</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dasatinib monohydrate</td>
<td>c-ABL, c-SRC</td>
<td>0.6 ± 0.2</td>
<td>303.6 ± 101.2</td>
<td>36.6</td>
<td>Eley et al17</td>
</tr>
<tr>
<td>Erlotinib hydrochloride</td>
<td>EGFR</td>
<td>1.1 ± 0.4</td>
<td>472.1 ± 171.9</td>
<td>966-1,200</td>
<td>Druker et al21</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>EGFR</td>
<td>7.6 ± 2.6</td>
<td>2,457.9 ± 357.5</td>
<td>33.9-97.5</td>
<td>Hidalgo et al20 and Thomas et al19</td>
</tr>
<tr>
<td>Imatinib mesylate</td>
<td>BCR-ABL, PDGFRα, c-KIT, ARG</td>
<td>9.4 ± 3.1</td>
<td>5,543.2 ± 1,828.1</td>
<td>2,300</td>
<td>Sweisland et al20</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>VEGFR, PDGFRα, FLT3, c-KIT, c-KIT, RET</td>
<td>2.7 ± 1.8</td>
<td>1,075.9 ± 717.3</td>
<td>919</td>
<td>Britten et al22</td>
</tr>
<tr>
<td>Sorafenib tosylate</td>
<td>Raf, VEGFR, PDGFRβ, FLT3, c-KIT, p38</td>
<td>1.1 ± 0.4</td>
<td>700.7 ± 269.2</td>
<td>9,350-82,700</td>
<td>Avada et al22 and Strumberg et al24</td>
</tr>
</tbody>
</table>

The mean EC50 of erlotinib (1.1 μmol/L, or 472.1 ng/mL) is lower than that reported in two different clinical studies. Patients with advanced solid malignancies receiving a dose of 150 mg/d achieved a mean ± SE minimum steady state plasma concentration of 1,200 ± 620 ng/mL and a peak plasma concentration of 2,120 ± 1,520 ng/mL.18 In a second study of patients with head and neck squamous cell carcinoma, the same dose resulted in a plasma concentration of 966 ± 409 ng/mL 2 hours after drug administration.19

Testing of gefitinib yielded a mean EC50 of 7.6 μmol/L, or 2,457 ng/mL. This is unacceptably high because a clinically relevant dose of 300 mg/d corresponds to a peak plasma concentration of 33.9 to 97.5 ng/mL.20

The mean EC50 of imatinib (9.4 μmol/L, or 5,543 ng/mL) is also unfavorable because it substantially exceeds the mean peak plasma level (2,300 ng/mL) and trough level (746 ng/mL) measured at steady state in patients with chronic myeloid leukemia receiving 400 mg/d of imatinib for 4 weeks.21 However, it is worth mentioning that imatinib has also been administered for short periods at twice the aforementioned dose to patients in the blast crisis phase.

Sunitinib has an EC50 (2.7 μmol/L, or 1,075.9 ng/mL) that is significantly higher than the mean plasma peak (91.9 ng/mL) and trough (79.9 ng/mL) levels observed in patients with refractory malignancy evaluated in the steady state after 14 days of therapy with 50 mg/d of sunitinib.22

The EC50 for sorafenib (1.1 μmol/L, or 700.7 ng/mL) is comparable to that for sunitinib, but 2 studies indicate that its pharmacokinetic properties are more favorable for potential anti-BKV use. Awada et al23 reported that patients with advanced refractory tumors can tolerate much higher plasma levels in the range of 39,600 to 201,000 ng/mL (mean, 82,700 ng/mL). Strumberg et al24 observed a mean peak plasma concentration of 9,350 ng/mL in patients with cancer treated with 400 mg/d of this drug.
Discussion

Our results indicate that the in vitro EC50 values for erlotinib and sorafenib are lower than blood levels considered to be acceptable when these Food and Drug Administration–approved drugs are used in patients with cancer. Hence, these 2 compounds may have usefulness in treating BKV nephropathy and hemorrhagic cystitis in immunosuppressed patients. In the absence of comparative information about actual tissue concentrations, it might be argued that further investigations should first be initiated with sorafenib because its beneficial effect in patients with renal cell carcinoma indicates that this drug can attain biologically significant concentrations within the urinary tract. However, it is possible that the actual measurement of drug levels within the renal tubules and bladder urothelium may reveal a different rank order of efficacy than suggested by available pharmacokinetic data in plasma. Most tyrosine kinase inhibitors are known to be metabolized primarily by the liver.

Any proposed use of tyrosine kinase inhibitors for BKV infection must take into account their cytotoxic properties. The cells remained viable as assessed by morphologic evaluation and trypan blue staining. The 50% cytotoxicity of these compounds in WI-38 cells, which consist of rapidly proliferating fetal fibroblasts, was comparable to the antiviral EC50 (data not shown). Toxicity studies in more differentiated cell lines were not considered necessary because significant clinical toxicology data on these compounds are already available. Erlotinib frequently causes diarrhea and skin rash, but, in one study, these adverse effects responded to conservative treatment if the dose did not exceed 150 mg/d.18 In a second study, 6 of 31 patients discontinued therapy owing to unacceptable diarrhea or skin rash, but the drug could be subsequently resumed at 100 mg/d.19 Patients receiving long-term erlotinib also need to be monitored for mucositis, headache, liver function, and pancytopenia. The toxicity profile of sorafenib is similar to that of erlotinib with the additional occurrence of severe fatigue in 6%, alopecia in 2%, and pancreatitis in 4% of patients.24

One approach to minimize the impact of these toxic reactions would be to reserve the use of erlotinib and sorafenib for patients with refractory BKV nephropathy progressing to graft loss. It may also be possible to use chemotherapy in short pulses that lower the viral load to a point at which the body’s immune system can successfully resolve the infection. Alternatively, one could consider the combined use of erlotinib and sorafenib to allow an additive effect with lower blood levels and a greater safety margin than possible with either drug alone. A very desirable long-term goal would be to develop safer tyrosine kinase inhibitors, which might make prophylactic drug administration feasible for patients with asymptomatic infection before significant virus-induced tissue damage has occurred.

References


